The Measurement of DNA Damage in Spermatozoa and its Biological Consequences

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Manchester for the degree of Doctor of Philosophy in the
Faculty of Science

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Abstract.

Studies have demonstrated that a significant proportion of spermatozoa in the human ejaculate are genetically abnormal. There is also a high incidence of spontaneous abortions in human embryos and it has been suggested that DNA damage in the gametes may be responsible. The aims of this thesis were therefore to investigate the significance of DNA damage in spermatozoa and its consequences to fertility and embryo development. In order to study DNA damage in spermatozoa, ionising radiation was used as a model to induce DNA strand breaks in testicular germ cells and sperm from laboratory mice.

A new technique "the sperm comet assay" was developed in order to detect and measure DNA damage directly in spermatozoa. This was validated by exposing spermatozoa from mice and humans to doses of ^{60}Co γ-rays *in vitro*. Exposure of sperm *in vitro* to radiation doses from 0-100Gy produced linear dose-dependent increases in DNA strand breaks as measured by sperm comet assay. However spermatozoa were extremely resistant to the DNA damaging effect of radiation as indicated by the high radiation doses used in this experiment and also when compared to lymphocytes, which were used as an example of somatic cells. This may be related to differences in chromatin structure between somatic and germ cells.

Testicular germ cells are more sensitive to the damaging effects of radiation than spermatozoa. Therefore mice were irradiated *in vivo* with 4Gy testicular X-rays to investigate whether DNA damage could be transmitted through the spermatogenic process. Irradiation of different germ cell stages within the testis produced differing levels of DNA damage in spermatozoa. Spermatid stages were the most radioresistant whilst irradiation of spermatogonia produced the most damage in spermatozoa. These results mirror the radiosensitivity of germ-cells stages within the testis and are similar to a previous study where spermatogonia were identified as the most sensitive stage for the transmission of germline mutations. Examination of spermatozoa, 120 days post-irradiation demonstrated that DNA damage levels in spermatozoa were still significantly higher than controls suggesting permanent effects to the testis as a result of damage to stem cell spermatogonia. Further studies, using irradiation doses from 0-4Gy demonstrated that dose-dependent levels of DNA damage could be induced in spermatozoa after irradiation of spermatogonial cells. A dose of 0.5Gy X-rays was the minimum at which significant increases in DNA damage levels in spermatozoa could be detected using the comet assay. Using the dose response data, it was calculated that the doubling-dose required for the induction of DNA damage in sperm after spermatogonial irradiation was 0.35Gy. This is in agreement with results studies using other methodologies who have calculated doubling doses between 0.17 and 0.56Gy.

In order to investigate the consequences of genetic damage in spermatozoa upon reproduction, male animals were irradiated with 4Gy X-rays and then mated with females at timepoints post-irradiation corresponding to the exposure of spermatid, spermatocyte and spermatogonial germ cell stages. Effects upon fertilisation, embryo development, morphology and apoptosis in the preimplantation embryo were examined. Paternal irradiation resulted in decrease fertility as observed by reduced sperm counts and higher incidences of unfertilised oocytes and embryos with abnormal (fragmented) morphology. Effects were greatest after exposure of spermatogonial stages. Exposure to

4Gy X-rays also produced impaired embryonic development and an increased incidence of embryos containing apoptotic nuclei compared to unirradiated controls. These results suggest that DNA damage in spermatozoa can be transmitted via fertilisation producing detectable effects upon the preimplantation embryo. We hypothesise that apoptosis may occur in the preimplantation embryo as a result of transmitted genetic damage at fertilisation. It may function to eliminate DNA-damage cells and protect the genetic integrity of the embryo.

As well as external radiation sources, radionuclides have been shown to be toxic to organs including the testis after internal ingestion. Indium-114m emits high energy auger electrons during its decay and may therefore be more damaging than external radiation sources. Exposure of the testis to either X-rays of ^{114m}In result in decrease in testis weights and sperm counts. As observed previously with X-rays, exposure of testicular germ cells to ^{114m}In resulted in the transmission of DNA damage through the spermatogenic process resulting in genetically damaged spermatozoa. DNA damage in spermatozoa was equivalent after exposure to X-rays or ^{114m}In. These results demonstrate that exposure of testicular germ cells to chronic low dose irradiation can lead to potentially toxic and mutagenic consequences. Further experiments are required to investigate and compare relative biological effectiveness of auger emitting radionuclides at inducing toxic and mutagenic effects on the testes compared to non-auger emitting isotopes and external radiation sources.

DNA damage was also investigated using the comet assay in spermatozoa from transgenic mice defunct in apoptotic and DNA repair genes. It had been hypothesised that since apoptosis may play a critical role in protecting the genetic integrity of the germline that these mice may have higher levels of DNA damage in their spermatozoa. However, using the comet assay, no evidence was found for increased levels of endogenous DNA strand breaks when spermatozoa from knockout and wild-type animals were compared.

These experiments demonstrate that DNA damage induced in germ cells can be transmitted through the spermatogenic process resulting in the production of sperm with damaged DNA. It is suggested that genetic damage in spermatozoa can persist after fertilisation and that the developmental potential of the embryo may be related to spermatozoal integrity. Therefore abnormalities in the genetic integrity of spermatozoa may contribute to the high incidence of "poor quality" embryos observed in *in vitro* fertilisation clinics.

Declaration.

No portion of the work referred to in this thesis has been submitted in support of an application of another degree or qualification of this or any other university or institute of learning.

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Finally, a big thumbs up to everyone from The Isle of Man !!!

Preface.

I graduated from the University of Manchester in 1995 with the degree of B.Sc. (Hons) Pharmacology. Subsequently in October 1995, I enrolled for a Ph.D at the University of Manchester receiving a BBSRC and Westlakes Science and Technology CASE award. Further research has also been gained in an industrial environment; as an undergraduate a sandwich year was spent working in the Toxicology Department, Glaxo-Wellcome and a three-month period in the Department of Genetics, Westlakes Science and Technology Park, as part of my Ph.D programme.

Publications.

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List of Abbreviations.

The following abbreviations have been used in this thesis:

%	Percentage
α	Alpha
β	Beta
γ	Gamma
$\pm$	Plus or Minus
μCi	Micro Curie (Unit of Radioactivity)
μl	Microlitre
μm	Micrometer
<	Less than
^{137}Cs	Caesium-137
^{1252}Cf	Californium-252
^{60}Co	Cobalt-60
^{55}Fe	Iron-55
^{59}Fe	Iron-59
^{3}H	Tritium
^{125}I	Iodine-125
^{131}I	Iodine-131
^{111}In	Indium-111
^{114m}In	Indium-114m
^{239}Pu	Plutonium-239
^{90}Sr	Strontium-90
^{201}Tl	Thallium-201
^{204}Tl	Thallium-204
^{235}U	Uranium-235
A	Ampere
AO	Acridine Orange
ATPase	Adenosine 5'-triphosphatase
C	Carbon
Ca^{2+}	Calcium ion
cAMP	Cyclic Adenosine Monophosphate
CCD	Closed Circuit Device
cm	Centimetre
d.d. H_2O	Double Distilled Water
DAB	3,3'-diaminobenzidene
DAPI	4',6-diamidino-2-phenylindole
DBCP	1,2-dibromo-3-chloropropane
DNA	Deoxyribonucleic Acid
DNase I	Deoxyribonuclease I
dsb	Double-Strand Break
DTT	Dithiothreitol
dUTP	Uridine 5'-triphosphate nucleotide
e.g.	For example
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
EtO	Ethylene oxide

F_1	First Generation Offspring
F_2	Second Generation Offspring
FISH	Fluorescence In Situ Hybridisation
FITC	Fluorescein-5-isothiocyanate
g	Gram
Gy	Gray (Unit of Radiation Dose)
$H^\bullet$	Free Hydrogen Radical
H_2O_2	Hydrogen Peroxide
hCG	Human Chorionic Gonadotrophin
HCl	Hydrochloric Acid
$HO_2^\bullet$	Hydroperoxy Radical
HTO	Tritiated Water
i.p	Intraperitoneal
I.U	International Unit
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
ISEL	In Situ End Labelling
KCl	Potassium Chloride
KeV	Kiloelectronvolts
kg	Kilogram
KH_2PO_4	Potassium Dihydrogen Phosphate
KVp	Peak Kilovoltage
LD_{50}	Lethal Dose-50
LET	Linear Energy Transmission
$LiCO_3$	Lithium Carbonate
M	Molar
MBq	Mega Bequerel
mCi	Milli Curie (Unit of Radioactivity)
Mg^{2+}	Magnesium
MHz	Megahertz
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMS	Methyl Methanesulfonate
mRNA	Messenger Ribonucleic Acid
MSv	Millisievert (Unit of Radiation Dose
Na_2HPO_4	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometre
°C	Degrees Centigrade
$OH^\bullet$	Hydroxyl Radical
P	Probability
PBS	Phosphate Buffered Saline
PC	Personal Computer
PFA	Paraformaldehyde
PI	Propidium Iodide
PMSG	Pregnant Mares Serum Gonadotrophin
PVP	Polyvinylperolidone
RBE	Relative Biological Effectiveness

RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per Minute
RQ	Reaction Quality
S.E.M	Standard Error of the Mean
SCID	Severe Combined Immuno-Deficiency
SCSA	Sperm Chromatin Structure Assay
ssb	Single-Strand Break
$t_{1/2}$	Half-Life
TBE	Tris-Borate-Ethtlenediaminetetraacetic Acid Buffer
TdT	Terminal Deoxynucleotidyl Transferase
TE	Trophectoderm
Tris-Cl	Tris(hydroxymethyl)aminomethane Chloride
TUNEL	Terminal Deoxynucleotidyl Transferase Mediated End Labelling
U.V.	Ultra Violet
V	Volt
v/v	Volume by Volume
W	Watt
w/v	Weight by Volume
WHO	World Health Organisation
x g	times the Unit of Gravity

Chapter 1

General Introduction

1. INTRODUCTION.

Reproduction is a vital process that is fundamental for the existence of any species. However, the production of offspring is a complex procedure dependent on both the male and female of the species and it is becoming increasingly evident that many factors may be detrimental to this process and affect reproductive capacity. Human semen contains high numbers of both morphologically abnormal and immotile sperm which are often cited as the main causes of male infertility (World Health Organisation, 1992). However, a significant proportion of spermatozoa in the human ejaculate have also been shown to be genetically abnormal (Martin, 1983, 1987; Brandriff *et al.,* 1985; Kamiguchi & Mikamo, 1986) and it is thought that this may play a role in male infertility and be a large contributory factor to the high incidence of spontaneous abortions in human embryos. Since this data suggests a much greater frequency of chromosome abnormalities in human sperm compared with somatic cells, it might also be expected that DNA damage levels in sperm are also higher which may impair reproduction and contribute to infertility. However, at present there are still very few studies that have examined the relationship between DNA damage in sperm and infertility (Ballachey *et al.,* 1987; Sailer *et al.,* 1995b; Sun *et al.,* 1997; Lopes *et al.,* 1998).

It is well known that DNA lesions in germ cells can lead to abnormal reproductive outcomes such as spontaneous abortions, malformations, genetic diseases and increased incidences of cancer (Hassold *et al.,* 1984; Jacobs *et al.,* 1989; Lord *et al.,* 1998). Over the past fifty years the pharmaceutical industry has produced many thousands of chemicals with implications for use in both medicine and agriculture. Most of these have been evaluated toxicologically and many have been found to have adverse effects on male reproduction (reduction in semen quality, disruption of spermatogenesis and the occurrence of infertility in exposed men). In particular, the effects of changes in environmental factors (both physical and chemical) on male fertility has come to light recently following reports that male sperm counts have fallen by up to 50% over the last fifty years (Carlsen *et al.,* 1992). Increasing environmental pollution has been blamed for this observation and at the moment there is increasing evidence that in particular environmental estrogens (pesticides and herbicides) may be to blame (Sharpe, 1993). Many other agents e.g. radiation and cytotoxic chemicals have also been shown to produce harmful effects upon reproductive organs. There is increasing concern that the effects of these agents may not just be detrimental to the father but that his offspring may also be at risk as a result of the transmission of induced genetic defects via spermatozoa at fertilisation. Molecular studies have identified cytogenetic abnormalities in both the number and structure of paternal chromosomes in human offspring *in utero* and at birth (Hassold *et al.,* 1984; Jacobs *et al.,* 1989) and laboratory animal studies have demonstrated that exposure of male rodents to mutagens before mating can induce embryonic death (Ehling, 1980; Dobrzynska *et al.,* 1990; Dobrzynska & Gajewski, 1994) and transmissible genetic abnormalities in the offspring (Nomura, 1988; Lord *et al.,* 1998). In particular there has been much concern and controversy over recent reports suggesting that there may be a link between occupational exposure to ionising radiation of male workers at nuclear installations and an increased incidence of childhood leukaemia in their offspring (Gardner *et al.,* 1990; Roman *et al.,* 1993). Studies have also demonstrated genetic abnormalities in workers, offspring and wildlife exposed to radiation as a result of the Chernobyl nuclear accident (Shevchenko *et al.,* 1992; Dubrova *et al.,* 1996; Astaknova *et al.,* 1998). At the moment however, evidence is limited and as yet no clear link has been demonstrated between paternal exposure to exogenous agents, mechanism of damage transmission and abnormal effects in offspring.

This introduction will discuss genetic damage in spermatozoa and its possible effects. Firstly a description of the spermatozoon and the processes of spermatogenesis, fertilisation and early embryogenesis is given, followed by a review of the evidence for the presence of DNA damage in human spermatozoa. Finally the effects of radiation (a known genotoxin and good model for DNA damage) upon both spermatozoa and the testes are discussed.

1.1 The Spermatozoon.

The spermatozoon is the end product of spermatogenesis which occurs within the seminiferous tubules of the testis (Russell *et al.,* 1990). It is highly differentiated in structure in order to achieve its principle function, which is to reach and fertilise the female ovum. The mammalian spermatozoon can be divided in to two main components, the *head* and the *flagellum* or tail (Figure 1.1.1). The head consists of the acrosome, the nucleus, a small amount of cytoplasm and cytoskeletal structures. Its main function is to penetrate the oocyte and deliver its genetic material. The flagellum can be further divided into three parts, the *midpiece*, which consists of a mitochondrial sheath which is wrapped around the outside of the anterior part of the flagellum and provides the energy required for propulsion and the *principle* and *end pieces* of the flagellum, which make up the remainder of the tail and provide the mechanical machinery which enable the spermatozoon to achieve motility.

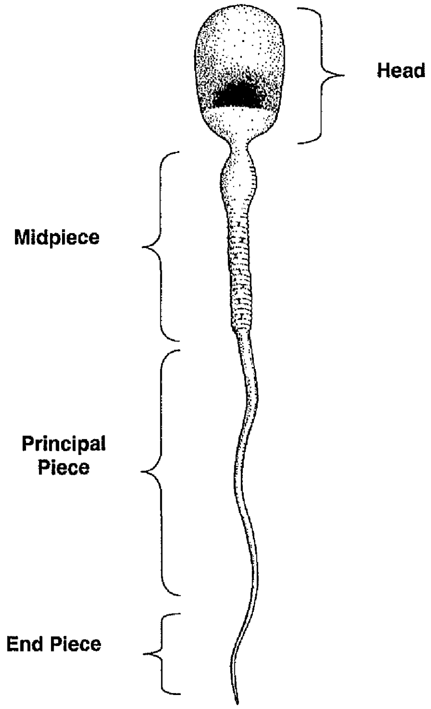

Figure 1.1 1 *: The mammalian spermatozoon. (Reproduced from Eddy & O'Brien, 1994).*

1.1.1 The Sperm Head.

The mammalian sperm head contains the nucleus and acrosome surrounded by a small amount of cytoplasm and a basic cytoskeleton. The surface of the sperm head is covered by a thin plasma membrane which can be subdivided into well delineated regional domains (Figure 1.1.1.1) which differ in composition and function (Friend, 1982; Primakoff & Myles, 1983; Holt, 1984). The acrosome is situated at the anterior end of the head close to the underside of the plasma membrane and cytoskeletal components lie between it and the nucleus (Eddy & O'Brien, 1994). Most mammals usually have a spatulate-shaped sperm head (human, sheep, and rabbit) although rodent sperm often have a falciform-shaped head (mouse, rat, and hamster). This is due to protrusion of the acrosome perpendicular to the flattened plane of the sperm head in these species.

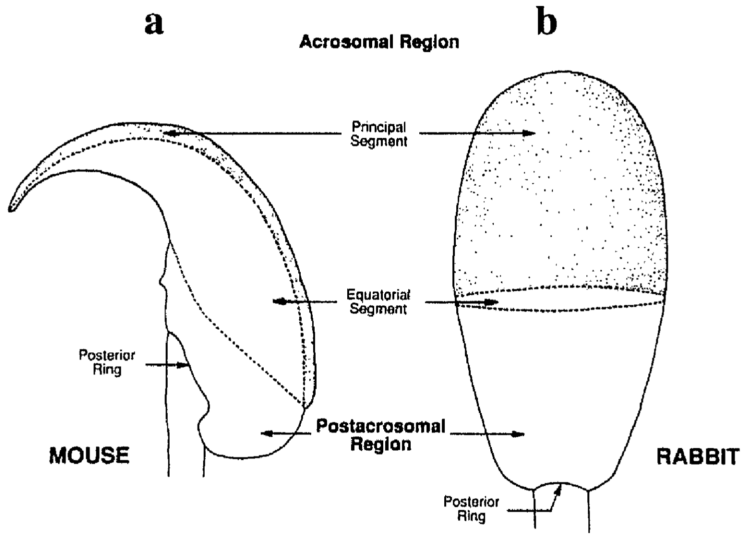

***Figure 1.1.1.1** : Falciform (a) and Spatulate (b) sperm head shapes showing different surface regions of the sperm head. (Reproduced from Eddy & O'Brien, 1994).*

1.1.1.1 Sperm Nucleus.

The chromatin in the sperm nucleus is highly condensed with a considerably smaller nuclear volume compared to somatic cells (Balhorn, 1982; Ward & Coffey, 1991). This reflects organisation of the nucleoproteins and DNA, which is unique to sperm nuclei. The major nuclear proteins found in mammalian sperm heads are protamines which are small sized, highly basic proteins due to their rich arginine and cysteine content (Bower *et al.,* 1987). The sperm nucleus is enclosed by a nuclear envelope which differs from that of somatic cell counterparts due to the facts that the majority of the envelope is lacking in nuclear pores and that the inner and outer membranes of the nuclear envelope lie in extreme proximity to one another (Fawcett, 1975a). Lining the inner surface of the nuclear envelope is a protein meshwork termed the *inner lamina.* This forms part of the nuclear skeleton and is thought to function by anchoring the sperm chromatin, helping to maintain the rigid structure of sperm nuclear DNA (Ward *et al.,* 1989).

1.1.1.2 Cytoskeleton of the Sperm Head.

There are two main components of the sperm head cytoskeleton namely the subacrosomal- and postacrosomal cytoskeletons. These structures can be isolated together and are referred to collectively as the *perinuclear theca* (Bellve *et al.,* 1992). The subacrosomal cytoskeleton is situated between the inner acrosomal membrane and the outer membrane of the nuclear envelope. It forms a prominent feature of rodent sperm, which primarily have falciform heads where it is also called the *perforatorium.* It is thought in these species that the perforatorium might have a mechanical role in oocyte penetration, although it may also serve as a structural reinforcement to the falciform sperm head shape (Eddy & O'Brien, 1994). The postacrosomal skeleton, also referred to as the postacrosomal sheath lies between the nuclear envelope and plasma membrane of the postacrosomal segment of the sperm head. It is more prominent in sperm with a spatulate head shape where it may function to provide support and prevent flexure of the sperm head during motion, or it may also act to link and stabilise the association of the acrosome with the nucleus and post-acrosomal plasma membrane.

1.1.1.3 The Acrosome.

The acrosome is a unique sperm organelle that is required for fertilisation in mammals (Fawcett, 1975b). It is formed from the Golgi complex in the spermatid (Lalli & Clermont, 1981) and is situated like a cap over the nucleus on the anterior surface of the sperm head. It forms a sack-like structure that is made up of inner- and outer acrosomal membranes and can be subdivided into two segments, the acrosomal cap (anterior acrosome) and the equatorial segment (posterior acrosome). During fertilisation the outer acrosomal membrane fuses with the plasma membrane of the sperm head (the acrosome reaction) releasing the contents of the acrosome which consists primarily of numerous enzymes (e.g. acrosin, hyaluronidase) which function to aid penetration of the sperm through the investments of the oocyte (Yanagimachi, 1994).

1.1.2 The Flagellum.

The flagellum is joined to the sperm head at the implantation fossa, which forms a basal plate at the posterior end of the sperm head (Eddy & O'Brien, 1994). A structure termed the *capitulum* is fused with both the basal plate and the outer dense fibres of the flagellum achieving a true link between tail and sperm head (Fawcett, 1975a). The main structural components that make up the flagellum of mammalian sperm are the axoneme, mitochondrial sheath, outer dense fibres and the fibrous sheath.

1.1.2.1 Axoneme.

The axoneme of the sperm flagellum has the same principal structure as cilia and flagella of plants and animals and provides the machinery responsible for flagellar motion (Figure 1.1.2.1.1). It consists of two central microtubules surrounded by a circular ring of nine microtubule doublets (Fawcett, 1975a; Eddy & O'Brien, 1994). Each microtubule double consists of an inner A-type microtubule joined to an outer B-type microtubule. Two arms extend from the A-type microtubule of one doublet towards the B-microtubule of the next doublet. Inner spokes also radiate helically from the central microtubule pair towards the individual outer doublets.

1.1.2.2 Mitochondrial Sheath.

The mitochondrial sheath makes up the midpiece of the flagellum, which is concerned with energy production for flagellar motion. Mitochondria are wrapped helically around the outer dense fibres at the midpiece the sperm tail (Woolley, 1971). They

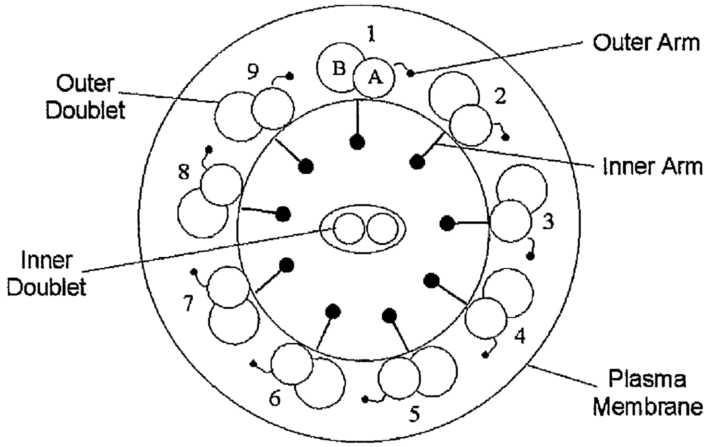

***Figure 1.1.2.1.1.** : Structure of the axoneme. (Reproduced from Eddy & O'Brien, 1994).*

are arranged end to end, although the numbers of mitochondrial, parallel helices and length of midpiece varies between species (Eddy & O'Brien, 1994).

1.1.2.3 Outer Dense Fibres.

Nine outer dense fibres surround the axoneme giving a 9+9+2 structure. They are usually teardrop-shaped in cross-section although there are variations in shape and size amongst individual fibres with some being larger than others (Fawcett, 1975a). Individual outer dense fibres are situated adjacent to microtubule doublets of the same numbering making up the axoneme. It has been speculated that these fibres are contractile and associated with an ATPase (Nelson, 1958). However, other investigators have not been able to find evidence supporting this and therefore it is unlikely that they are involved with flagellar motion. Despite this they may have passive elastic properties that may help provide recoil for the sperm tail (Fawcett, 1975a).

1.1.2.4 Fibrous Sheath.

The fibrous sheath is a tapering cylinder composed of two longitudinal columns connected to each other by circumferential ribs (Eddy & O'Brien, 1994). The individual columns are made up of longitudinally orientated filamentous structures and run peripheral to microtubule doublets 3 and 8 of the axoneme. They are also attached to outer dense fibres 3 and 8. It has been suggested that the fibrous sheath may function to modulate the plane of the flagellar beat. The attachment of the longitudinal columns to microtubule doublets 3 and 8 in the axoneme may limit their involvement in flagellar motion and the columns themselves may also limit bending of the tail in this plane (Fawcett, 1975a).

1.2 Organisation of the Testis.

The mammalian testis can be divided into two major compartments : 1) the **interstitial** compartment which contains Leydig cells and is the source of androgen production and 2) the **tubular** compartment (seminiferous tubules) in which spermatogenesis occurs in close association with Sertoli cells. These two compartments are structurally separated from each other forming a unique microenvironment in which spermatozoa can develop. Around the outside of the tubule a layer of peritubular myoid cells form a structural wall to the tubule (Clermont, 1958; Dym & Fawcett, 1970) whilst gap and tight junctional complexes between peritubular and Sertoli cells form an effective blood-tubule barrier (Setchell & Waites, 1975).

1.3 Spermatogenesis.

Spermatogenesis is the process that results in the production of mature spermatozoa (Figure 1.3.1). It occurs within the seminiferous tubules and is regulated by the secretion of androgens (mainly testosterone) from Leydig cells within the interstitium. Spermatogenesis may be divided into three distinct phases. These are : 1) the **proliferative** phase (where spermatogonia undergo numerous mitotic divisions in order to rapidly increase cell numbers). 2) the **meiotic** phase (during which spermatocytes halve their genetic material to become haploid cells) and 3) the **spermiogenic** stage (where spermatids undergo structural changes providing them with the equipment to reach and fertilise the female oocyte).

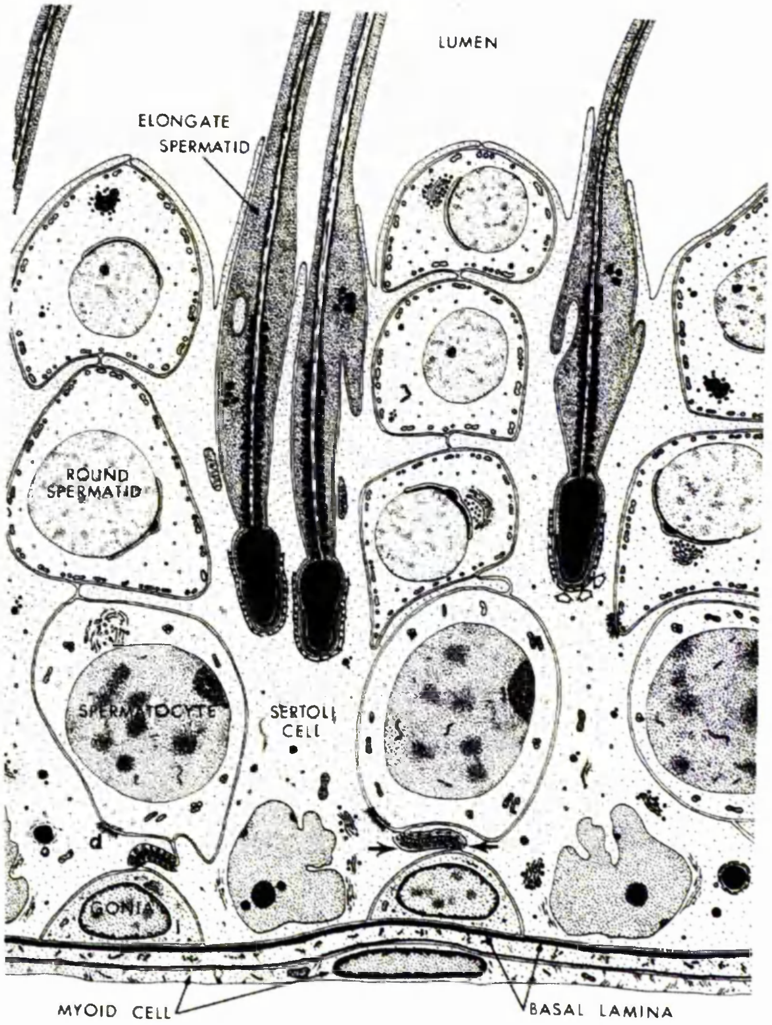
LUMEN
ELONGATE SPERMATID
ROUND SPERMATID
SPERMATOCYTE
SERTOLI CELL
d
GONIA
MYOID CELL
BASAL LAMINA

***Figure 1.3.1** : Spermatogenesis. (Reproduced from Russell et al., 1990).*

1.3.1 Proliferative Phase.

The process of spermatogenesis begins at puberty when spermatogonial stem cells are reactivated by hormonal cues to begin rounds of mitosis. Spermatogonial cells fulfil the need to rapidly increase cell number by undergoing numerous successive cell divisions producing large numbers of cells that ultimately undergo meiosis and differentiate into millions of mature spermatozoa. This proliferative phase of spermatogenesis takes place in the basal intratubular compartment of the testis with spermatogonia residing with one surface flattened on the basal membrane of the tubule and the other in contact with the Sertoli cell (Russell, 1977).

There are three types of spermatogonia : 1) **stem cell** spermatogonia, 2) **proliferative** spermatogonia and 3) **differentiating** spermatogonia (Figure 1.3.1.1). Stem cell spermatogonia act as a source of all germ cells. They are relatively resistant to a variety of toxic insults compared to other germ cell types (due to their infrequent divisions) and will often survive when other germ cells have been depleted allowing the complete repopulation of the seminiferous tubule (Dym & Fawcett, 1970; Huckins & Oakberg, 1978). It therefore follows that complete destruction of the spermatogonial stem cell population will result in the irreversible loss of spermatogenic capacity and therefore permanent infertility. Compared to stem cell spermatogonia, proliferative and differentiating spermatogonia have a much higher mitotic rate and are regarded as being much more sensitive to toxic insult.

Spermatogonial Types

Stem	***Proliferating***	***Differentiating***
$A_{isolated}$ (A_{is})	A_{paired} (A_{pr})	A_1, A_2, A_3, A_4
	A_{ali} ***Proliferating***	Intermediate (In)
	A_{paired} (A_{pr})	B
	$A_{aligned}$ (A_{al})	

***Figure 1.3.1.1** : Types of Spermatogonia. (Reproduced from Russell et al, 1990).*

The cells thought to be spermatogonial stem cells are referred to as $A_{isolated}$ spermatogonia (Huckins, 1971), whereas there are A_{paired} and $A_{aligned}$ types of proliferating spermatogonia (Figure 1.3.1.1). These two types of spermatogonia are so called because of the connections between cells by open cytoplasmic bridges (Fawcett *et al.*, 1959; Weber & Russell, 1987). These intracellular bridges are thought to promote synchronous development of germ cell types (Huckins, 1978b) and remain intact until the final stages of spermatogenesis when rupturing of the linkages occurs and mature spermatozoa are released into the lumen of the tubule. The remaining type A spermatogonia are classified as differentiating spermatogonia, however in addition there are also intermediate and type B forms (Clermont, 1972).

The following schematic represents the sequence of spermatogonial cell divisions that occurs during the proliferative stage of spermatogenesis :

$$A_{is} \rightarrow A_{pr} \rightarrow A_{al} \rightarrow A_{al} \rightarrow A_{al}$$

$$A_1 \rightarrow A_2 \rightarrow A_3 \rightarrow A_4 \rightarrow In \rightarrow B$$

***Figure 1.3.1.2** : Cell divisions during the proliferative phase of spermatogenesis. (Reproduced from Russell et al, 1990)*

Spermatogonia divide mitotically at regular intervals to produce "clones" of cells. During each division the number of cells theoretically doubles although there may be fewer divisions contributing to the pool of A_1 spermatogonia than shown in the diagram above. As many as sixteen A_1 spermatogonia have been seen connected by intracellular bridges (indicating them as the progeny of a single stem cell), (Huckins, 1978b) although this is not the norm and extensive cell death is also known to occur during these spermatogonial cell divisions (Roosen-Runge, 1973; Huckins, 1978a). There is no division of mature A_{al} cells to form A_1 spermatogonia although it is widely accepted that morphological changes occur which allow A_{al} and A_1 spermatogonia to be classified separately.

1.3.2 Meiotic Phase.

Following the end of the differentiating phase of spermatogenesis, type B spermatogonia divide to form primary spermatocytes also known as preleptotene spermatocytes (Pl). This is the last cell stage of the spermatogenic cycle to undergo DNA replication before meiotic prophase is entered (Russell *et al.,* 1990). During the two meiotic divisions chromosomes are recombined and genetic material halved resulting in the production of haploid spermatids. In addition germ cell numbers are quadrupled after the completion of the first and second meiotic divisions.

Meiotic prophase is extremely long lasting and takes around three weeks for completion. During this period cells undergo gradual morphological changes (cell size, nuclear changes) during the transition from one cell type to the next (Russell & Frank, 1978). Spermatocytes at different steps in meiotic prophase can be distinguished by the characteristic morphologies of their nuclei reflecting the state of their chromatin (Bruce *et al.,* 1974). The steps in the morphological development of primary spermatocytes during meiotic prophase are shown in Figure 1.3.2.1.

Preleptotene → Leptotene → Zygotene → Pachytene → Diplotene
(Pl) (L) (Z) (P) (Di)

***Figure 1.3.2.1** : Morphological development of primary spermatocytes. (Reproduced from Russell et al, 1990).*

The transition of preleptotene to leptotene spermatocytes signals the initiation of meiotic prophase. The cells take on a rounded form and move away from the base of the tubule to become isolated within the Sertoli cells (Russell, 1977; Russell & Frank, 1978). During the zygotene stage of prophase, homologous chromosomes pair up and synaptonemal complexes can be seen within the nucleus (Russell *et al.,* 1990). In pachytene cells, the chromosomes have become fully paired and this step accounts for the majority of time spent in meiotic prophase as the chromosomes remain paired for 1½-2 weeks to allow genetic recombination (crossing over) to occur. Whilst the pachytene stage of prophase is very long, the diplotene stage is in contrast extremely brief. The chromosome pairs separate and move to opposite poles of the cell allowing cytokinesis to take place resulting in the formation of secondary spermatocytes (Figure 1.3.2.2). This cell type is very short lived in the testis and rarely seen in histological sections as they rapidly undergo the second meiotic division to produce spermatids (Russell *et al.,* 1990).

Diplotene → Secondary spermatocytes → Spermatids
(Di) ↑ (2°) ↑
Meiosis-I **Meiosis-II**

***Figure 1.3.2.2** : Position of the two divisions during the meiotic stage of spermatogenesis. (Reproduced from Russell et al, 1990)*

1.3.3 Spermiogenic Phase.

Following the proliferative and meiotic stages of spermatogenesis large numbers of cells have been formed that are haploid in their genetic material. These cells must now undergo extensive structural modifications which will enable them to reach and fertilise the female oocyte when release into the female reproductive tract.

Although several events occur simultaneously in spermatids during spermiogenesis it is easier to consider these changes individually. The major structural changes that occur in this last phase of spermatogenesis are : 1) **Development of a flagellum**. This imparts motility to the cell, which allows it to swim to the oocyte though the cervical mucus. Although mature spermatozoa released into the lumen of the tubule possess a tail they do not achieve motile capacity until they pass through the epididymis (Russell *et al.,* 1990). 2) **Mid-piece formation**. Numerous mitochondria are recruited from the cytoplasm of the cell and are arranged round the mid-piece of the flagellum (Fawcett, 1958, 1975a). These mitochondria provide the energy required for whiplash-like movement of the flagellum resulting in the forward propulsion of the spermatozoa. 3) **Acrosome development**. The Golgi apparatus secretes small vesicles which coalesce to form one large acrosomal vesicle (Lalli & Clermont, 1981) containing proteolytic enzymes required by the spermatozoa for penetrating the egg vestments. 4) **Nuclear elongation and shaping**. Up to the spermatid stage of spermatogenesis the nucleus is roughly spherical in shape. However, following acrosomal formation, the acrosome may flatten or indent the nucleus. Following this cue nuclear elongation and condensation occurs resulting in the formation of a characteristic sperm head shape dependent upon the species (Fawcett, 1958; Dym & Fawcett, 1970; Fawcett *et al.,* 1971). The decrease in nuclear material is as a result of increased chromatin condensation and DNA packaging caused by changes in histones and other DNA-associated proteins. There is also a decrease in nuclear volume due to the elimination of fluid from the nucleus (Sprando & Russell, 1987). 5) **Elimination of cytoplasm**. During spermiogenesis the spermatid is reduced in volume (Sprando & Russell, 1987) to approximately 25% of its original size (the small size of the mature spermatozoon is thought to aid propulsion through the fluid environment of the cervical mucus). The main reduction in volume is caused by the elimination of water from the nucleus and cytoplasm during spermatid elongation. There is also some separation of the cytoplasm resulting in the formation of a cytoplasmic package (also known as the residual body) containing RNA and organelles no longer required by the spermatid. This residual body is pinched off by the Sertoli cell during sperm release into the tubular lumen where after it is phagocytosed and digested by the Sertoli cell (Fawcett & Phillips, 1969; Russell, 1984).

Following these extensive modifications the newly developed spermatozoa possess all the structural apparatus required to reach and fertilise the egg. The cytoplasmic bridges linking individual cells rupture and the mature spermatozoa are released into the lumen of the tubule in a process known as spermiation (Russell, 1984). The spermatozoa are then transported through the rete testis and epididymis to the vas deferens where they await release at ejaculation.

1.4 Fertilisation.

During natural fertilisation, sperm ascend the female reproductive tract to reach the ovulated egg. However, freshly ejaculated spermatozoa do not possess the capacity to fertilise the oocyte and must therefore undergo a series of biological changes during transit to the egg to attain fertilising ability (Yanagimachi, 1994). This maturation process is known as "CAPACITATION".

1.4.1 Capacitation.

The exact mechanisms involved in the capacitation of mature spermatozoa within the female reproductive tract are unclear. However, capacitation has two main elements : 1) **changes in the movement characteristics of spermatozoa** - the spermatozoa attains what is termed a "hyperactivated motility pattern" (Yanagimachi, 1981) with the flagellar beat increasing in both strength and amplitude, resulting in the forward propulsion of the spermatozoon in vigorous lurches. It is thought this hyperactivated motility aids penetration of the sperm through the egg vestments (i.e. cumulus matrix and zona pellucida) (Fraser & Quinn, 1981; Boatman & Robbins, 1991); 2) **changes in the surface membrane properties of the spermatozoon** - which prime the spermatozoon ready to undergo the acrosome reaction when reaching the oocyte (Weinmann & Williams, 1961).

The two key elements of capacitation described above are thought to be due to a number of biochemical changes that occur within the spermatozoa as they ascend the female reproductive tract. Firstly, there is a reduction in the stability of the sperm's surface membrane. This thought to be due to the loss of cholesterol from the sperm head membrane (Go & Wolf, 1983; Suzuki & Yanagimachi, 1989) and this step is thought to be important for the subsequent acrosome reaction. Secondly, possibly due to the increased permeability of the surface membrane there is an increase in the calcium permeability of the sperm membrane leading to an increase in intracellular Ca^{2+} (Fraser, 1990; Perry et al., 1997a, 1997b). Additionally calmodulin binding proteins are stripped from the sperm surface (Koehler, 1981; Oliphant *et al.,* 1985) and this may also make the sperm more responsive to the effects of calcium (it has been suggested that hyperactivated motility is a result of increased intracellular Ca^{2+} levels). As a result of the increase in intracellular Ca^{2+} there is also an increase in adenylate cyclase activity and a resulting rise in cyclic AMP levels and cAMP-dependent phosphorylation of spermatozoal proteins (Huacuja *et al.,* 1977; Berger & Clegg, 1983; Stein & Fraser, 1984; Duncan & Fraser, 1993). Finally, there is also increased activity of protein tyrosine kinases during capacitation leading to the phosphorylation and autophosphorylation of tyrosines in intracellular proteins (Leyton & Sailing, 1989; Duncan & Fraser, 1993). Again this is thought to be important for the acrosome reaction.

Following capacitation spermatozoa are fully prepared to penetrate the oocyte and undergo the acrosome reaction enabling fusion of both gametes and ensuring fertilisation.

1.4.2 Sperm-Oocyte Interactions.

Mammalian oocytes are surrounded by extracellular investments that act as selective barriers to spermatozoa and also as protective layers (Figure 1.4.2.1).

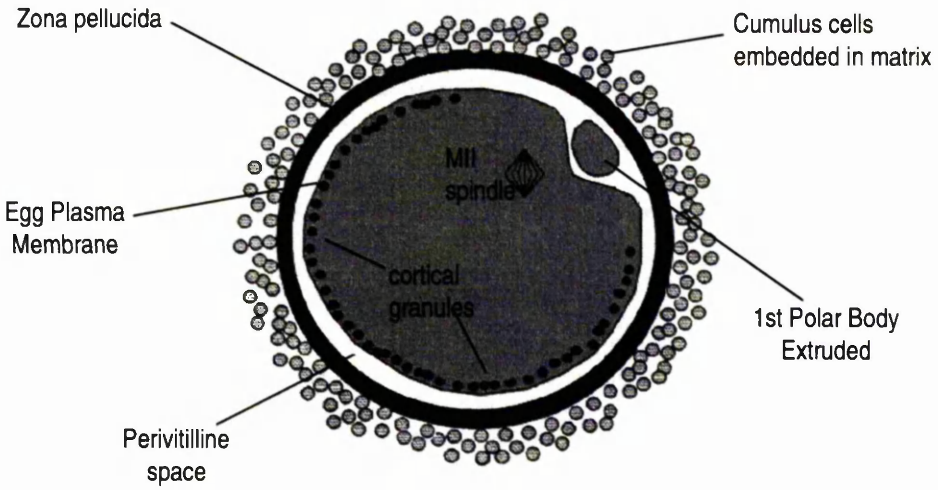

***Figure 1.4.2.1** : Structure of the ovulated oocyte. (Reproduced from Adashi et al, 1996).*

1.4.2.1 Cumulus Matrix Penetration.

The outermost layer consists of large numbers of small somatic cells that secrete an extracellular matrix consisting mainly of hyaluronic acid (Piko, 1979). This layer is known as the cumulus oophorus. The cumulus oophorus acts as a selective barrier to spermatozoa. Capacitated spermatozoa with hyperactivated motility possess sufficient momentum to force their way through the cumulus matrix (Talbot *et al.,* 1985; Green, 1988; Suarez & Dai, 1992). In contrast, both non-capacitated spermatozoa and sperm that have prematurely undergone the acrosome reaction adhere to the cumulus cells and are prevented from further penetration (Leyton & Sailing, 1989).

Recently another mechanism has been identified that may aid the spermatozoon's passage through the cumulus matrix. A sperm plasma membrane protein, PH-20 has been cloned and the amino terminus has been found to be highly homologous to that of the bee venom, hyaluronidase (Gmachl & Kreil, 1993). Purified PH-20 protein has been shown to degrade hyaluronic acid, which is a large constituent of the cumulus matrix (Lin *et al.,* 1994). Therefore it is possible that sperm PH-20 may help to disperse the cumulus matrix and aid sperm passage through the cumulus oophorus.

1.4.2.2 Zona Pellucida Penetration.

The second external layer consists of an acellular matrix known as the zona pellucida, which is secreted by the oocyte (Nichols & Gardner, 1989). This coat consists principally of three heavily glycosylated proteins: ZP1, ZP2 and ZP3 (Liang & Dean, 1993; Wassarman, 1994). ZP2 and ZP3 proteins form repeating ZP2-ZP3 heterodimers, which are crosslinked by ZP1 homodimers (Greve & Wassarman, 1985). This gives the zona pellucida a porous, highly organised 3D-structure. These proteins also play a part in sperm penetration of the zona. ZP3 and ZP2 have been shown to be involved in sperm recognition and binding whilst ZP1 appears to perform a purely structural role (Wassarman, 1988; Wassarman & Mortilo, 1991).

In mice, only acrosome-intact sperm are able to penetrate the cumulus oophorus and bind to the zona pellucida (Sailing & Storey, 1979; Bleil & Wassarman, 1980). It has been shown that ZP3 protein acts as the primary ligand for sperm binding and that this triggers the acrosome reaction (Moller *et al.,* 1990; Wassarman, 1994; Brewis *et al.,* 1996). Following the completion of the acrosome reaction the sperm remains in contact with the zona pellucida and penetrates the matrix. Therefore there appears to be two parts to sperm binding: Primary binding mediated by the ZP3 protein and secondary binding following the acrosome reaction (Bleil *et al.,* 1988; Sailing, 1989). Several proteins ($p95^{sperm}$, galactosyltransferase, sp56 and ZRK) have been put forward as candidate sperm receptors (Bleil & Wassarman, 1980, 1986; Leyton & Sailing, 1989; Shur, 1993) although the relationships between these binding proteins and their functions are not well defined.

1.4.2.3 Acrosome Reaction.

Binding of the sperm to ZP3 protein on the surface of the zona pellucida results in the triggering of the acrosome reaction (Wassarman, 1994). The exact mechanism by which this signal is transduced intracellularly is not understood although it is thought that the sperm protein, ZRK is probably involved in transmembrane signalling (Adashi *et al.,* 1996). ZRK may act as a protein tyrosine kinase and in response to ZP3 binding undergo autophosphorylation, initiating the exocytotic signalling cascade (Leyton & Sailing, 1989; Leyton *et al.,* 1992). A G-protein linked mechanism then stimulates the opening of voltage-dependent Ca^{2+} channels in the sperm head membrane (Florman *et al.,* 1992) resulting in a rise in intracellular Ca^{2+} and cAMP levels and also a rise in intracellular pH (Yanagimachi, 1994). The acrosome then swells and the acrosomal membrane fuses with the overlying sperm plasma membrane at multiple sites (Yanagimachi, 1981) resulting in the exteriorisation of both the contents of the acrosomal vesicle and inner acrosomal membrane by exocytosis. Following the acrosome reaction, the inner acrosomal membrane is exposed allowing secondary binding to occur (Bleil *et al.,* 1988; Mortillo & Wassarman, 1991). This involves the zona glycoprotein, ZP2 which binds to specific sperm proteins (proacrosin and PH-20 have been implicated), (Jones, 1991; Myles & Primakoff, 1991). Following membrane fusion during the acrosome reaction the acid environment of the acrosome is alkalinised resulting in the autoactivation of proacrosin to its active enzymatic form, acrosin (Topfer-Peterson *et al.,* 1990). Acrosin proteolytically digests a narrow penetration slit in the zona which combined with the hyperactivated tail movements of the spermatozoa allows it to penetrate through the zona and into the perivitilline space (Yanagimachi, 1994).

1.4.3 Sperm-Oocyte Fusion.

Following zona penetration the sperm comes to lie tangentially to the oocyte membrane within the perivitilline space. Adherence of the sperm head to the egg occurs rapidly (Yanagimachi, 1978). Following adherence fusion is initiated at the equatorial segment of the sperm head by the sperm fusion protein, fertilin and an integrin-like molecule in the oolemma (Myles, 1993). Only capacitated spermatozoa that have undergone the acrosome reaction are capable of fusion and it has been suggested that the acrosome reaction may activate fusion proteins located on the equatorial segment of the sperm head (Takeno *et al.*, 1993).

1.5 Early Embryo Development.

Immediately after fusion of the oocyte with the spermatozoon there is a dramatic increase in intracellular Ca^{2+} levels (Fulton & Whittingham, 1978; Jaffe, 1983; Lorca *et al.*, 1993). This increase in calcium levels has two main actions. 1) it promotes the fusion of the cortical granules with the plasma membrane of the oocyte causing the release of cortical enzymes into the perivitilline space (Gulyas, 1980). These enzymes modify ZP3 and ZP2 proteins within the zona pellucida preventing further sperm binding and acrosomal digestion of the zona therefore preventing polyspermy (Gulyas, 1980; Adashi *et al.*, 1996); and 2) it causes the release of the MII metaphase arrest that has been in place since the oocyte was ovulated, allowing the resumption of differentiation (Jaffe, 1983; Lorca *et al.*, 1993). One of the first events that takes place following fertilisation, is the extrusion of the second polar body of the oocyte. This is a useful criterion for assessing fertilisation.

The contents of the sperm cell (now fused with the oocyte membrane) move into the oocyte cytoplasm. The sperm nuclear membrane breaks down and decondensation of the sperm chromatin occurs as the protamines that enabled the sperm to highly pack its chromatin are released and replaced by normal histones (Zirkin *et al.*, 1989; Perrault, 1990). Between 4 and 7 hours after fertilisation the two sets of haploid chromosomes (one set from the sperm, and the other from the oocyte) become surrounded by distinct membranes to form pronuclei. The two pronuclei then move into the centre of the cytoplasm to lie adjacent to one another and both sets of haploid chromosomes synthesise DNA in preparation for the first mitotic division (Johnson & Everitt, 1996). The pronuclear membranes break down and a mitotic spindle is formed with the male and female chromosomes coming together in a process known as syngamy (Yanagimachi, 1994). Approximately 18-24 hours after fertilisation a cleavage furrow is formed and cytokinesis occurs resulting in the formation of a two-cell embryo. The conceptus remains at the oviductal site of fertilisation for a further few days and during this time it undergoes further cell divisions at regular intervals. In the mouse, embryos from most strains undergo their first and second cleavages at intervals of 20-26 hours (Molls *et al.,* 1983). Later cleavage stages occur much more frequently approximately every 10-12 hours (Streffer *et al.,* 1980; Harlow & Quinn, 1982).

It is know understood that the earliest events that take place after fertilisation occur without any mRNA synthesis by the embryos own chromosomes are therefore regulated by maternal genes transcribed during oogenesis and oocyte maturation (Pedersen & Burdsal, 1994). Embryonic DNA is not transcribed until the two-cell stage in mice and the third cleavage division (8-cells) in humans with the complete destruction of all maternally derived mRNA at these points (Braude *et al.,* 1988).

At around the eight- to 16-cell stage in most species there is a change in the morphology of the conceptus as it develops into a morula (Pratt, 1987). It becomes impossible to recognise individual blastomeres due to a process known as compaction when the peripheral blastomeres become polarised. Following polarisation there are two distinct populations of cells in the conceptus, those polarised outer cells which develop into a trophectoderm and the inner cells which develop into the inner cell mass during fluid accumulation and the formation of the blastocyst (Pedersen & Burdsal, 1994). At around this time the conceptus moves into the uterus and the blastocyst hatches out of the zona pellucida in preparation for implantation.

1.6 Incidence of Genetic Damage in Spermatozoa.

In humans, 50% of conceptions do not lead to a fetus (Boue *et al.,* 1985; Gilbert, 1991). This observation is consistent with the presence of genetic damage in human gametes. Estimation of the frequency of chromosome abnormalities in human spermatozoa has been a topic of great interest and discussion but has proved very difficult to accurately assess. Information on liveborn offspring and frequency of spontaneous abortions were previously used to estimate the frequency of chromosome abnormalities in the absence of an experimental protocol (Boue *et al.,* 1975). In 1978 however, Rudak *et al.* demonstrated that human spermatozoa could actually penetrate zona-free hamster oocytes and that this technique allowed the analysis of human sperm chromosomes. This important development has since allowed a number of groups to determine the actual frequency of chromosome abnormalities in human spermatozoa.

Martin *et al.,* (1987) conducted a large study, which analysed the chromosomes from 1582 sperm obtained from 30 normal men in order to assess the frequency and types of chromosomal aberrations present in the normal ejaculate. It was found that the mean frequency of sperm with chromosome abnormalities was 10.4%. This was similar to the results of a previous smaller study, 8.5% (Martin, 1983) and the results of two other large studies, 9.4% (Brandriff *et al.,* 1985) and 13.9% (Kamiguchi & Mikamo, 1986). Taken together these results suggest that an estimate of the frequency of chromosome abnormalities in human sperm is approximately 10%. The mean frequency of numerical abnormalities (hyperhaploid, hypohaploid and multiple aneuploidy) was 2.8% with structural abnormalities accounting for 7.6% of the total chromosome abnormalities observed.

The most common types of structural aberration found in these studies were chromosome breaks: 36% (Martin *et al.,* 1987), 55% (Brandriff *et al.,* 1985) and 45% (Kamiguchi & Mikamo, 1986). Other types of chromosome aberrations however were also observed (chromatid breaks and gaps, dicentrics and other exchanges). It was also shown that there was a relationship between the frequency of sperm chromosomal abnormalities and the age of the donor. With increasing age there was a decreased risk of aneuploid sperm and an increased incidence of sperm with structural chromosomal abnormalities (Martin *et al.,* 1987).

Mikamo *et al.,* (1990) compared the frequency of chromosome abnormalities in human spermatozoa with frequencies in other species (mouse and Chinese hamster). It was found that human spermatozoa had much higher frequencies of chromosome aberrations than those of both the mouse (1.5%) and Chinese hamster (2.1%). Although from the study it was not possible to determine the exact causes of the higher incidence in humans it was suggested that environmental and daily life-associated mutagens might be responsible for this observation.

Genetic damage in spermatozoa is being implicated as a possible contributory factor in male infertility. A number of laboratories have utilised new techniques to analyse DNA integrity of mature spermatozoa in the human ejaculate directly. The terminal deoxynucleotidyl transferase (TUNEL) assay has been used to examine the incidence of spermatozoa in the human ejaculate containing fragmented DNA. A study has suggested that oligozoospermic men may have a higher incidence of sperm with fragmented DNA in their ejaculate compared to men with normal semen parameters (Unpublished information). Furthermore Sun *et al.,* (1997) demonstrated a negative correlation between the percentage of sperm with fragmented DNA in the ejaculate and sperm concentration, motility and morphology, suggesting that sperm with damaged DNA are more prevalent in semen samples of poor quality. Additionally it was also reported that ejaculates from men who smoked contained higher numbers of TUNEL-labelled sperm and that semen samples with increased levels of sperm with fragmented DNA had lower fertilisation rates and were less likely to result in embryo cleavage. Studies using a different technique, the *in situ* nick translation assay which measures endogenous nicks in DNA have also suggested a correlation between the percentage of spermatozoa with endogenous nicks in the ejaculates of men and reduced fertility (Sakkas *et al.,* 1999).

There is increasing concern over the use and selection of sperm in *in vitro* fertilisation procedures. This has particularly focused on the procedure of intracytoplasmic sperm injection (ICSI). Reports have suggested that children born as a result of *in vitro* fertilisation do not have an increased incidence of chromosome abnormalities (FIVNAT, 1995). However, some reports have suggested that children born as a result of ICSI procedures have an elevated frequency of sex chromosome aberrations (In't Veld *et al.,* 1995). Sperm are selected for ICSI on the basis of their morphological and motility characteristics and as described above some studies have shown that there is a negative correlation between sperm with DNA fragmentation and sperm motility and morphology (Sun *et al.,* 1997; Lopes *et al.,* 1998). Therefore this selection procedure may reduce the risk of selecting a genetically abnormal sperm. However, studies have shown that 10-15% of morphologically normal sperm are genetically abnormal (Martin, 1983; Brandriff *et al.,* 1985; Kamiguchi & Mikamo, 1986; Martin *et al.,* 1987) and the fertilisation rate in ICSI cases is only about 65% which may be as a result of selection of sperm with genetic abnormalities. Studies on human spermatozoa selected for ICSI have suggested that there is a negative correlation between sperm DNA fragmentation and fertilisation rates after ICSI (Lopes *et al.,* 1998).

Two other techniques, namely the sperm chromatin structure assay (SCSA) and the comet assay have been used to examine the genetic integrity of spermatozoa. Animal studies using SCSA have demonstrated chromatin abnormalities in spermatozoa after treatment with cytotoxic chemicals and radiation (Evenson *et al.,* 1993; Evenson & Jost, 1993, 1994; Sailer *et al.,* 1995a). A correlation has also been demonstrated using SCSA between chromatin structure and fertility in bulls, stallions and rams (Ballachey *et al.,* 1987; Sailer *et al.,* 1995b). The comet assay has been used to examine the effects of various substances on spermatozoa *in vitro* (Hughes *et al.,* 1996; McKelvey-Martin *et al.,* 1997; Anderson *et al.,* 1997b, 1997d; Singh & Stephens, 1998). Recently a study has examined the genetic integrity of human spermatozoa in 28 ejaculates by all three methods and reported a significant relationship between the results for SCSA, TUNEL and comet assay on human spermatozoa (Aravindan *et al.,* 1997).

The reason for the considerable genetic variety in the quality of semen is unknown. The reports above suggest that a significant proportion of "normal ejaculates" contain genetically damaged sperm, however it appears from the evidence above it appears that their may be a relationship between poor semen parameters (as defined by World Health Organisation, 1992) and increased incidences of genetically abnormal spermatozoa. This may reflect problems occurring during spermatogenesis in these patients and two hypotheses have been suggested to explain the origin of DNA damage in the human ejaculate.

The structure of sperm chromatin is highly unique and it is packaged in a highly condensed manner because of the small volume of the sperm nucleus and to render it functionally inert (Ward & Coffey, 1991). Studies in rats and mice have shown that endogenous nicks are present in sperm DNA at specific stages of spermatogenesis (during the transition from round to late spermatids) but are no longer observed after the process of chromatin packaging is complete (McPherson & Longo, 1993a; Sakkas *et al.,* 1995; Chen & Longo, 1996). This suggests that these nicks may have a functional role during chromatin remodelling that occurs in spermiogenesis and it has been suggested that endogenous nuclease activity is necessary within the sperm head during spermiogenesis in order to complete protamination (Sakkas *et al.,* 1995). It has been proposed that the enzyme, topoisomerase II may both create and ligate nicks during spermiogenesis in order to aid chromatin rearrangement and facilitate the replacement of histones with protamines (McPherson & Longo, 1993a, 1993b; Chen & Longo, 1996). Therefore the presence of endogenous strand breaks in sperm DNA may reflect incomplete chromatin remodelling and several studies have demonstrated that the presence of DNA damage in spermatozoa in correlated with abnormal sperm chromatin structure as a result of under protamination (Evenson *et al.,* 1989a; Gorczyca *et al.,* 1993c; Evenson & Jost, 1994; Sailer *et al.,* 1995b; Manicardi *et al.,* 1995).

The second hypothesis suggests that the endogenous nicks present in human spermatozoa are characteristic of DNA damage that occurs during programmed cell death (apoptosis) in somatic cells, and may serve as a means of eliminating defective germ cells (Gorczyca *et al.,* 1993b, 1993c).

1.6.1 Fluorescent In Situ Hybridisation and the Measurement of Aneuplody in Spermatozoa.

Human sperm karyotyping using the hamster-oocyte technique allows the direct analysis of both structural and numerical chromosome abnormalities. However, this technique is extremely time consuming and labour intensive with only relative small numbers of spermatozoa able to be examined. Recent advances in fluorescent in-situ hybridisation (FISH) using chromosome-specific DNA probes permits large scale, direct assessment of aneuploidy frequencies in human spermatozoa (Martin *et al.,* 1996; Spriggs *et al.,* 1996). Studies with spermatozoa from men of normal fertility have estimated disomy frequencies of approximately 0.1% per chromosome with an overall aneuploidy frequency in human sperm of approximately 5-6% (Spriggs *et al.,* 1996; Martin, 1998). Using specific probes it has been demonstrated that all chromosomes are susceptible to meiotic nondisjunction. However, it appears that higher frequencies of aneuploidy are observed for chromosome 21 and the sex chromosomes. Martin (1998) examined aneuploidy in 306,035 sperm from infertile patients. Compared to control donors, sperm from infertile patients showed increased disomy for chromosomes 1, 13, 21 and the sex chromosomes. This is consistent with other studies using multicolour FISH analysis which have also reported increased frequencies of aneuploidy in spermatozoa from infertile men (Bernadini *et al.,* 1997; Lahdetie *et al.,* 1997).

1.6.2 Intracytoplasmic Sperm Injection (ICSI) and Chromosome Abnormalities.

Intracytoplasmic sperm injection (ICSI) is now widely applied in the treatment of male factor infertility. The issue of possible genetic risks in offspring derived from this new procedure has attracted considerable attention. Int'Veld *et al.,* (1995) reported five cases of sex chromosome abnormalities in a small study among 15 fetuses conceived by ICSI prompting suggestions that children born as a result of ICSI may be at risk from increased incidences of genetic abnormalities. More recent studies have not detected such a high rate of chromosome abnormalities amongst ICSI offspring but have suggested that the frequency of sex chromosome aneuploidy in ICSI fetuses is approximately 1% (Tournaye *et al.,* 1995; Mau *et al.,* 1997; Van Steirteghem *et al.,* 1997) which is 3-4 times higher than the frequency (0.3%) observed in newborns from the general population (Jacobs, 1992). Some of the genetic abnormalities in these cases may arise *de novo* without a predisposing parental chromosomal aberration (Van Opstal *et al.,* 1997). However studies have suggested increased frequencies of chromosome abnormalities in couples undergoing ICSI treatment and a high incidences of sex chromosome aneuploidy has previously been reported amongst infertile men (Bernadini *et al.,* 1997; Lahdetie *et al.,* 1997; Martin, 1998). Therefore this may contribute to the observed increased incidence after ICSI. Further studies are currently in progress but these results suggest the need for genetic screening and counselling of all patients (both male and female) undergoing ICSI treatment.

1.7 Effects of Genotoxic Agents upon Reproduction.

Spermatogenesis is a lengthy process, which lasts many weeks. During spermatogenesis there are many rounds of cellular multiplication (mitosis and meiosis) and differentiation (Russell *et al.,* 1990). Due to the diverse nature of this process and the variety of different cell types within the testis there are many potential targets for disruption of spermatogenesis and subsequent effects upon reproduction. Many chemicals and other agents (e.g. radiation) are known to interfere with spermatogenesis (Morris *et al.,* 1996). Some may target Sertoli cells and impair the ability of the testis to make functional sperm, whilst others may target Leydig cells resulting in altered hormonal support of spermatogenesis. Toxicants working through these mechanisms whilst affecting male fertility do not create a risk to the next generation. Considering the considerable amount of DNA replication that takes place during spermatogenesis it is not surprising that the major germ cell toxins are drugs used for cancer chemotherapy (Meistrich, 1993). These compounds induce damage in DNA and produce genotoxic effects as well as inhibiting cell division and cell death. Cyclophosphamide, a potent alkylating agent has profound effects upon the testis (Morris *et al.,* 1996). Cyclophosphamide can form inter- and intrastrand crosslinks preventing normal cell replication. Chromosomal proteins may also be alkylated causing DNA strand breaks as a result of increased stress in the chromatin structure (Neidle & Waring, 1983). Therefore treatment with cyclophosphamide causes extensive death of germ cells within the testis resulting in a decrease in sperm counts and either temporary or permanent infertility depending on the dose (Lu & Meistrich, 1979; Watson *et al.,* 1985). As alkylated DNA bases can be excised and inefficiently repaired, cyclophosphamide is genotoxic and has been shown to induce dominant lethal mutations and specific-locus mutations in male germ cells (Trasler *et al.,* 1987; Bentley & Working, 1988).

Radiation is another agent that has profound effects upon the testis. Treatment with radiation induces DNA strand breaks and causes cell death in the testis probably as a result of induced DNA damage (Meistrich, 1986a, 1993). The effects of radiation have been extensively studied and it has been used as a model to study infertility and the response of the testis to cytotoxic insult.

1.8 Radiation and DNA Damage.

1.8.1 Ionizing Radiations.

Ionizing radiation's have the capacity to produce excitations and ionizations during the absorption of energy in biological material. If the radiation has sufficient energy to eject one or more orbital electrons from the atom or molecule then an ionization is said to have taken place. An important characteristic of ionizing radiation is the localised release of large amounts of energy, typically enough energy to disrupt strong chemical bonds (e.g. covalent C-C interactions). Ionizing radiations can be divided into two categories. These are electromagnetic and particulate radiation's (Hall & Cox, 1994). Examples of electromagnetic radiations are X-rays and γ-rays. These radiations are indirectly ionizing in that they themselves do not produce chemical and biological damage, but when absorbed in the medium through which they pass, they give up energy to produce fast moving electrons. Examples of particulate radiations are alpha-particles, electrons, neutrons and protons. These radiations are classed as directly ionizing because they can produce chemical and biological damage through direct interaction and ionization of target material.

1.8.2 Radiation Quality and Linear Energy Transfer (LET).

Radiations of different ionization densities have both quantitatively and qualitatively different biological effects. The energy deposited in biological materials by radiation is in the form of ionizations and excitations. These tend to be distributed along the tracks of individually charged particles as they pass through the biological material. The pattern of energy deposition differs depending on the type of ionizing radiation involved (Hall & Cox, 1994). Electromagnetic photons give rise to fast electrons, which have a negative charge and very small mass. The tracks of these energetic electrons produce events (ionizations and excitations) that are well separated and for this reason X-rays and γ-rays are described as sparsely ionizing (Berry & Denekamp, 1988). Alpha particles on the other hand carry one or two units of positive charge and have a mass approximately 8000 times that of an electron. When alpha particles pass through biological material this gives rise to individual ionizing events that occur close together, resulting in tracks that consist of a column of ionizations. Alpha particles are therefore described as densely ionizing.

The quality of radiation can be described quantitatively in terms of linear energy transfer (LET). Linear energy transfer is defined as the average energy imparted locally to the absorbing medium per unit length of track for a given type of radiation (Hall & Cox, 1994). Therefore sparsely ionising radiations have low LET values (e.g. X-rays- 2keV/μm) whereas densely ionising radiations are referred to as high LET radiations (α-particles 2000keV/μm). Simplified, the higher the density of ionization, the more biological damage the radiation can potentially induce and the higher the LET value of the radiation.

1.8.2.1 X-rays and γ-rays.

X-rays are probably the form of ionizing radiation that most people are familiar with. They are used widely in the medical field for diagnostic purposes and the treatment of cancer. They are also used industrially for the testing of welds and the determination of crystal structures. X-rays can be produced by accelerating electrons towards a metal target and are produced if the electrons hit the target with sufficient energy (Nias, 1990). Therefore the characteristics of the X-rays depends on the energy of the electrons and the atomic number of the metal target.

Gamma rays, like X-rays are also examples of electromagnetic radiation. However, gamma rays are produced intranuclearly usually as result of radioactive decay of radioisotopes (Berry & Denekamp, 1988). They represent excess energy that is given off in the form of electromagnetic waves as an unstable nucleus breaks up and decays in order to reach a stable form. X-rays and γ-rays have similar properties when they interact with atomic matter and it is therefore simpler to consider them together. Transfer of energies by X-rays and γ-rays occurs by a number of processes. These are the photoelectric effect, Compton scattering and pair production (Hall & Cox, 1994). Absorption of photon energy by all these methods results in the ejection of an electron from the target atom leaving an unstable condition (ionization).

1.8.2.2 β-particles.

In addition to the electrons produced by the absorption of X-ray and γ-ray photons, external electron sources are also used in the treatment of cancer. Emitted electrons are also known as β-particles. They are negatively charged, have very small mass and are easily deflected by other electrons. Therefore their penetration into a tissue will be much less than their range in air. However there will be greater ionization at the end of their track due to their decreased velocity and the increased probability if interaction between the electron and target material when it is travelling slowly (Hall & Cox, 1994).

1.8.2.3 α-particles.

Whereas X-rays, γ-rays and β-particles are considered to be of low LET, α-particles are an example of high LET radiation. They are positively charged (two protons and two neutrons - helium nuclei) and because of their large size (8000 times heavier than an electron) they move slowly through a tissue and penetrate only a short distance (a few hundred μm). By themselves they do not pose much of a danger. However, the fact that many radionuclides emit α-particles (e.g. radon) makes them important if such radionuclides are deposited internally. As with electrons the greatest density of ionization will occur at the end of the track, but this will be short and straight in contrast to β-particles. Because of the low velocity and double charge of α-particles they are very densely ionizing and can create large amounts of biological damage (Nias, 1990).

1.8.2.4 Auger Electrons.

Radionuclides which decay by orbital electron capture or internal conversion emit low energy electrons known as Auger electrons (Baverstock & Charlton, 1988). These electrons have a very low range (up to only several hundred nanometers) but show highly localised energy deposition leading to the formation of free radical species in the immediate area of the decay site. They therefore have very similar biological properties to α-particles and may cause severe damage if the site of decay is close to radiosensitive sites within the cell (e.g. the nucleus).

1.8.2.5 Radioactive Isotopes.

These are unstable atoms that disintegrate randomly with the emission of ionizing radiation (Table 1.8.2.5). The radioactivity of isotopes decays exponentially and each isotope has a specific half-life (time taken for amount of radioactivity to fall by half). Nuclear power stations utilise the disintegration of ^{235}U which yields both neutrons and other radioactive isotopes e.g. ^{90}Sr (β-emitter, $t_{1/2}$=28 years), ^{137}Cs (γ-emitter, $t_{1/2}$=30 years) and ^{131}I (γ-emitter, $t_{1/2}$=8days). The long half lives of these waste products presents a problem as these must be stored or reprocessed. This can be done but the consequences of the release of such long enduring isotopes into the environment or food chain as occurred at Chernobyl in 1986, may have extremely serious consequences for human health.

***Table 1.8.2.5** : Commonly encountered Radioisotopes.*

Radioisotope	**Half-Life**	**Radiation**
^{137}Cs	30 years	Gamma
^{60}Co	5.3 years	Gamma
^{3}H (Tritium)	12.3 years	Beta
^{125}I	60 days	Gamma
^{235}U	4.5×10^{9} years	Gamma
^{239}Pu	2.4×10^{5} years	Alpha
^{114m}In	50 days	Beta & Auger

The Chernobyl incident demonstrates that humans may be exposed to radiation from external sources. However, radioisotopes are also commonly used internally for diagnostic purposes in medicine. It has recently been discovered that some of these medical isotopes decay with the emission of highly damaging Auger electrons (Baverstock & Charlton, 1988). Also worrying are the observations that some radioisotopes can selectively accumulate in critical organs (e.g. ^{131}I-Thyroid, ^{90}Sr-Bone, ^{114m}In - Testis).

1.8.3 Ionization and Free Radical Formation.

Ionizing radiation results in the formation of pairs of abnormal ions (free radical ions) which are not in equilibrium with one another because of the displacement of electrons and are therefore very reactive (Berry & Denekamp, 1988; Nias, 1990). The majority of free radical formation following irradiation is as a result of the ionization of water within cells. This results in the formation of both free hydrogen ($H^\bullet$) and hydroxyl ($OH^\bullet$) radicals and is of great importance since most biological materials consist of 10-90% water. If oxygen is present in the irradiated tissue then there will be an increased yield of free radicals that are biologically damaging. The reaction of oxygen with free radicals results in the formation of the relatively stable hydroperoxy radicals ($HO_2^\bullet$) and hydrogen peroxide (H_2O_2) which are very toxic to biological structures. Indirect ionization of water probably accounts for the majority of free radicals from after irradiation, however organic molecules present in the cell may also generate free radicals although this is thought to occur as a result of direct ionization by radiation (Berry & Denekamp, 1988).

1.8.4 DNA Strand Breaks.

Ionizing radiation can damage cells and there is strong evidence that DNA is the principal target for its biological effects (including cell killing, mutation and carcinogenesis) although it is also likely that disruption of the nuclear membrane is also important. Damage to DNA can create disastrous consequences for the cell since DNA is used as a template for mRNA transcription and ultimately protein translation. Therefore any radiation-induced damage in DNA may result in an altered cell phenotype.

Ionizing radiation generates a wide spectrum of DNA damage. When cells are irradiated with X-rays many single strand breaks are generated and these can be measured as a function of dose if the DNA is denatured and the sugar-phosphate backbone stripped away (Hall & Cox, 1994). In intact DNA, single strand breaks are probably not important as far as cell killing is concerned as they are easily repaired using the intact complementary strand as a template. However the radiation-induced single strand breaks may result in mutations if incorrect repair takes place and it has been suggested that the incidence of ssb's increases the probability of misrepair and mutation.

Double strand breaks also occur as a result of irradiation albeit less frequently than single-stranded DNA breaks. However, double strand breaks are believed to be the most important lesion produced in chromosomes by radiation and are directly related to cell killing (Olive, 1998).

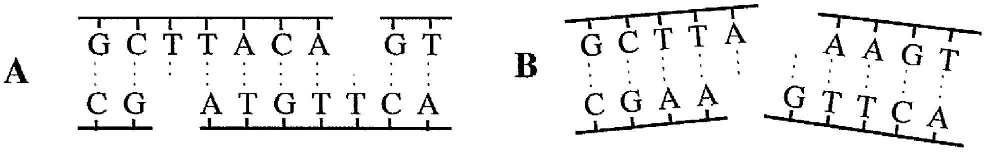

***Figure 1.8.4.1** : DNA double strand breaks. (Reproduced from Hall & Cox, 1994)*

There are at least two forms of double strand breaks (Figure 1.8.4.1). If both strands of DNA are broken but well separated (Figure 1.8.4.1 A) then as with single-strand breaks this type of damage is again easily repaired by using the complementary strand as a template. However if the breaks in the two strands are opposite one another, or separated by only a few base pairs (Figure 1.8.4.1 B) this may result in the physical separation of the two pieces of DNA. There may also be considerable damage produced in the region of the break as a result of the passage of the charged particle through the chromatin fibres. Unlike single-strand breaks this type of damage is usually unrepairable and thought to be the type of lesion primarily responsible for radiation-induced cell killing.

DNA strand breaks however are not the only type of damage induced in DNA by radiation. This is because both direct ionizations and free radicals may be involved. Therefore each radiation-induced strand break (single and double) is likely to be part of a wide variety of complex lesions (e.g. base damage) that could spread out up to 20 base pairs from the initial site of interaction. The term *locally multiplied damaged site* has been coined to describe this phenomenon.

1.8.5 Chromosome Aberrations.

One of the traditional methods of investigating DNA damage following radiation exposure is by measuring the frequency of chromosome aberrations (Hall & Cox, 1994).

Usually lymphocyte preparations that have been irradiated and arrested at metaphase are used for this purpose.

When cells are irradiated, breaks may be produced in the chromosomes. These breaks and the resulting fragments may behave in a number of ways: 1) The breaks may rejoin in their original configuration. This will lead to no visible aberrations at the next mitosis; 2) The breaks may fail to rejoin resulting in an aberration which can be seen as a deletion (part of chromosome missing) at the next mitosis; 3) Broken ends may reassort and rejoin other broken ends to give rise to distorted and unusually shaped chromosomes at the next mitosis.

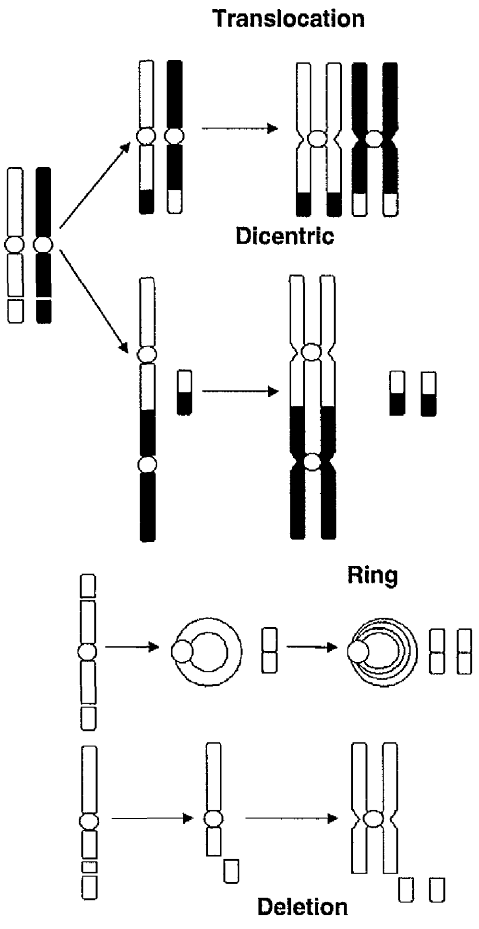

Figure 1.8.5.1. *: Structural chromosome aberrations. (Reproduced from Hall & Cox, 1994)*

1.9 Effects of Ionising Radiation on Male Reproduction.

Effects of ionising radiation on reproduction were first observed almost a century ago (Albers-Schonberg, 1903). It is now well known that radiation (both whole body exposure and localised testicular irradiation) can produce temporary or permanent sterility to males (Meistrich, 1993). Testicular irradiation has a direct toxic effect upon the cells of the testis whilst whole body irradiation may also indirectly affect testicular function due to disruption of the hypothalamic-pituitary axis resulting in gonadotrophin deficiency and impaired sex steroid production (Ogilvy-Stuart & Shalet, 1993).

It has been reported that radiation preferentially affects actively growing cells such as those undergoing mitosis or meiosis (Goldman & Johnson, 1993). Within the seminiferous tubules of the testes the process of spermatogenesis takes place. During this process an enormous number of cells are undergoing division (both mitotically and meiotically) and differentiation (Russell *et al.,* 1990). Therefore it is not surprising that radiation can produce dramatic disruption of spermatogenesis.

Testicular damage after local or whole body irradiation is well documented in both animals and humans. The testis is one of the most radiosensitive tissues in the human body with a single testicular dose of 0.15Gy resulting in significant decreases in sperm counts and doses as low as 0.3Gy producing temporary azoospermia (Rowley *et al.,* 1974).

Studies have shown that proliferating spermatogonia are the most radiosensitive cell type within the testis (Meistrich, 1986a, 1993). Experiments *in vivo* have shown that following testicular irradiation with X-rays there is a higher incidence of preimplantation embryo loss following mating in the 6th and 7th week irradiation, coinciding with a decrease in epididymal sperm counts to a minimum level and a significant increase in the number of morphologically abnormal sperm at this time point (Bateman, 1958; Ehling, 1971; Searle & Beechey, 1974; Bruce *et al.,* 1974). More recently, Matsuda *et al.,* (1985a) investigated these observations using an *in vitro* method. Male mice were subjected to testicular X-irradiation (2Gy) and sperm recovered weekly following radiation exposure. Collected sperm was then used to fertilise oocytes *in vitro*. It was found that the oocyte fertilisation rate was lowest in the 7th week following irradiation. This corresponds with the time it takes for proliferating spermatogonia to develop into spermatozoa and therefore confirms the results of the earlier *in vivo* studies that they are the most sensitive of the germ cell stages to radiation.

In humans, doses of radiation as low as 1Gy are enough to significantly reduce proliferating spermatogonia numbers with a significant decrease in their daughter cells, preleptotene spermatocytes also observed (Rowley *et al.,* 1974). The high radiosensitivity of proliferating spermatogonia is probably due to their high mitotic rate and therefore higher susceptibility to DNA damage (Goldman & Johnson, 1993). A higher dose of radiation (2-3Gy) is required to kill spermatocytes and results in an inability to fully complete meiotic division and therefore a significant decrease in spermatid numbers. Spermatids however, are quite radioresistant, a dose of 4-6Gy results in no overt damage but a significant decrease in spermatozoa numbers signifying some spermatid damage (Meistrich, 1993).

Due the differing radiosensitivities of the differing germ cell types following testicular irradiation spermatogonia disappear from the testis first followed by spermatocytes and then spermatids. A cycle of the seminiferous epithelium takes about 68 days in a human testis and transport of sperm through the epididymis and vas deferens a further 4-12 days (Meistrich, 1993). Following acute low dose irradiation (1.5-2Gy) sperm production and sperm count is only slightly reduced for the first 50-60 days following radiation exposure but then falls dramatically with resultant temporary olio- or azoospermia (Meistrich, 1993; Ogilvy-Stuart & Shalet, 1993). Higher doses of radiation result in a more rapid onset of sterility due to a direct effect on the later stages (spermatocytes, spermatids) of spermatogenesis as well as the spermatogonia.

Following irradiation and temporary oligo-, azoospermia, repopulation of the seminiferous epithelium takes place due to the proliferation of surviving spermatogonial stem cells which are relatively radioresistant (Meistrich, 1986a, 1986b). With acute irradiation, recovery is dose dependent, taking 9-18 months following exposures of up to 1Gy, 30 months for 2-3Gy and 5 or more years after 4-6Gy (Heller *et al.,* 1966; Rowley *et al.,* 1974).

Cancer patients commonly experience problems with fertility and permanent sterility following chemotherapeutic and radiotherapeutic treatments. Treatment of cancer usually involves repeated low dose exposures of radiation. It has been suggested that fractionated radiation doses cause more stem cell killing than acute single dose regimes (Rowley *et al.,* 1974; Ogilvy-Stuart & Shalet, 1993; Meistrich, 1993). After chronic fractionated courses of irradiation, azoospermia has been induced with testicular doses of radiation as low as 0.35Gy (Sandeman, 1966). Doses of between 2 and 3Gy result in almost total ablation of the germinal epithelium with recovery of spermatogenesis taking up to 10 years. The sensitising effect of fractionation is thought to be due to the long cell cycle time of primitive stem cells. This results in a significant population of cells that are relatively radioresistant because of their position in the cell cycle. Fractionated radiation is thought to be more effective in eradicating these cells than single acute doses as they are able to progress into more sensitive phases of the cell cycle between dose fractions.

The above observations suggest that the main site of action for radiation within the testis is the germinal epithelium. There is however evidence that other cell types within the testis (Leydig cells and Sertoli cells) can be damaged by radiation. These cells however are post-mitotic and therefore much less sensitive to the toxic effects if radiation. A dose of 25Gy causes almost complete destruction of the germinal epithelium and Leydig cell function is affected in most patients (decreased serum testosterone levels), (Brauner *et al.,* 1983; Tsatsoulis *et al.,* 1990). However at lower doses, irradiation studies in rodents have shown no long term reductions in the weights of the seminal vesicle (an androgen dependent tissue) indicating that a reduction in testosterone levels probably does not play a part in radiation-induced germ cell death (Meistrich, 1986a).

The above observations describe the toxic cell killing effects of radiation on the testis. However, as well as causing the destruction of the spermatogenic epithelium at sub-lethal doses radiation can induce DNA damage without killing the cell. This may cause potentially more serious consequences than temporary sterility caused by high doses of radiation because such DNA damage may be carried through the spermatogenic process resulting in the formation of spermatozoa with mutated DNA. If DNA damage occurs in spermatogonial stem cells then it will cause a germline mutation and therefore a proportion of the sperm produced by this testis will contain damaged DNA and this has potentially disastrous consequences if fertilisation of an oocyte occurs with such a damaged sperm.

Recent experiments by Sailer *et al.*, (1995a) demonstrate the potential for radiation-induced DNA damage to be carried through the process of spermatogenesis. Adult mice were testicularly irradiated using X-rays (0-4Gy) and cauda epididymal sperm collected weekly following radiation exposure. Sperm DNA was analysed by flow cytometry using the recently developed Sperm Chromatin Structure Assay (SCSA). The results of this study showed that irradiation with X-rays increased the susceptibility of the sperm DNA to *in situ* denaturation, in particular the highest susceptibility to *in situ* denaturation was found amongst sperm present in the cauda epididymis 40 days after exposure to X-irradiation. This corresponds with the collected sperm being spermatogonial cells at the time of irradiation. Furthermore the results of this study also demonstrate that damage to spermatogonial cells is not completely repaired, but is expressed or carried through the spermatogenic process since an increased susceptibility of the sperm DNA to *in situ* denaturation was observed a period of weeks following testicular irradiation. It was also shown that increasing doses of radiation led to a higher degree of DNA denaturation susceptibility, indicating that radiation-induced damage was relatively proportional to the dose of X-rays.

There are also studies reporting increased morphological abnormalities of sperm in the weeks following an acute dose of radiation (Bruce *et al.*, 1974) and increased incidences of sperm chromosome aberrations (Matsuda *et al.*, 1983, 1985c; Mikamo *et al.*, 1990). Increased frequencies of dominant lethal mutations in the offspring have also been reported following acute testicular radiation (Ehling, 1971; Searle & Beechey, 1974; Russell *et al.*, 1998).

The above evidence demonstrates that radiation may cause both toxic and mutagenic effects following external radiation exposure. However, nowadays in medicine there is the frequent use of radioactive isotopes internally and there is also the hazard of ingesting radionuclides due to nuclear accidents (e.g. Chernobyl) and occupational exposure (nuclear workers). There are many reports that some radionuclides may accumulate within certain tissues in the body thereby delivering a constant (albeit decreasing) dose of radiation (Miller & Bowman, 1983; Raabe & Parks, 1993; Hoyes *et al.,* 1996a, 1996b). Particularly concerning is data demonstrating that the widely used medical isotope, Indium and the nuclear waste product plutonium may accumulate in the testis (Miller & Bowman, 1983; Hoyes *et al.,* 1996a, 1996b). This is due to an active uptake mechanism that enables them to be transported from the bloodstream across the blood-testis barrier. Experiments have demonstrated that within the testis these radioisotopes become localised within the Sertoli cells. This is potentially disturbing as it demonstrates that contamination with these radionuclides may result in the direct irradiation of germinal cells which could result in mutations in both spermatogonial stem cells and other germ cells which may be propagated as the cells divide and differentiate into spermatozoa.

This may have serious consequences as spermatozoa themselves are reported to have no mechanism to repair damage to their DNA (Ono & Okada, 1977). Therefore the offspring of a male exposed to a damaging dose of radiation may be at risk if fertilisation occurs with a genetically damaged spermatozoa and if the damage is transmitted to the zygote and/or ineffectively repaired.

1.10 Paternal Transmission of Radiation-Induced Genetic Damage.

Using recent cytogenetic techniques, paternal chromosome analysis can be performed post-fertilisation after *in vitro* exposure of spermatozoa to various types and doses of radiation (Matsuda *et al.,* 1985c; Mikamo *et al.,* 1990).

The effects of radiation on spermatozoa of mice have been examined (Matsuda *et al.,* 1983, 1985c). Capacitated sperm were exposed to a variety of doses of X-rays before the fertilisation of oocytes *in vitro*. It was found that irradiation of mature sperm with X-rays prior to fertilisation had no effect on *in vitro* fertilisation rate (99.1% control, 96-100% X-rays). Following cytogenetic analysis of paternal chromosomes in the fertilised oocytes it was observed that following irradiation, the frequencies of eggs with structural chromosome aberrations increased with increasing dose of X-rays (0.9% control, 4.4-38.5% X-rays). The relationship between the frequency of chromosome aberrations per egg and dose of X-irradiation was exponential. These observations demonstrate that although irradiation was able to produce genetic damage within the sperm, their ability to fertilise oocytes was not affected.

The effects of X-irradiation on unfertilised eggs and zygotes have also been investigated. When fertilised eggs were exposed to acute irradiation (0.4Gy) it was found that many of the eggs were retarded in their further development (Matsuda *et al.,* 1985b). In the control group approximately 93% of fertilised oocytes progressed to metaphase stage at 15-17 hours after insemination. However, in the group exposed to X-rays, 38% of zygotes were still at the pronuclear stage at this time point. The frequency of radiation-induced chromosomal aberrations was 2.9% in spermatozoa which was slightly higher than the control, whilst the frequencies of aberrations in mature oocytes and early pronuclear zygotes exposed to radiation was 11.0% and 20.6% respectively. This suggests that that the comparative sensitivity to radiation is : mature spermatozoa < mature oocytes < early pronuclear zygotes (Matsuda *et al.,* 1983, 1985a, 1985b). Therefore zygotes at the early pronuclear stage are the most sensitive to radiation and there is a close relationship between embryonic death and chromosomal aberrations induced by X-rays.

It has also been shown that the frequency of chromosome aberration is increased following the exposure of mouse sperm to ultra-violet (U.V) irradiation and some mutagenic chemicals, methyl- and ethylmethanesulphonate (Matsuda & Tobari, 1988).

The effects of different types of radiation on the induction of chromosome abnormalities has been performed with human spermatozoa using the hamster oocyte-fusion technique (Mikamo *et al.,* 1990; Kamiguchi *et al.,* 1990a, 1990b). Human spermatozoa were capacitated in culture medium containing varying amounts of tritiated water (HTO) and were therefore exposed to various doses of β-radiation (0.13 - 3.74 Gy). Following exposure to tritium β-rays it was found that the incidence of structural chromosome aberrations was significantly higher in irradiated groups when compared to the control (Kamiguchi *et al.,* 1990a). The incidence of spermatozoa with chromosome abnormalities was related to the amount of exposure to β-rays and increased linearly with increasing radiation dose.

Similar observations have been recorded in experiments investigating the effects of X-irradiation and γ-radiation on human spermatozoa (Tateno *et al.,* 1989; Mikamo *et al.,* 1990; Kamiguchi *et al.,* 1990b). Semen samples from five healthy men were exposed to doses of ^{137}Cs-γ-rays (0.27, 0.54 and 1.08Gy). As with β-irradiation the incidence of radiation-induced structural chromosomal aberrations increased linearly with increasing radiation dose (13.1% - 0.27Gy, 26.8% - 0.54Gy and 40.1% - 1.08Gy). Sperm samples exposed to X-irradiation also showed a dose dependent linear increase in the incidence of structural chromosome aberrations.

These studies have demonstrated the susceptibility of human sperm chromosomes to radiation and that exposure of spermatozoa to different types of radiation (X-, β-, γ-radiation) produces an increase in the incidence of structural chromosomal aberrations. When compared, it has been found that the relative biological effectiveness (RBE) of these different types of radiation are in fact very similar. However, more recently the induction of chromosome aberrations in human spermatozoa was examined after bombardment *in vitro* with ^{252}Cf fission neutrons and it was demonstrated that these particles were more effective at inducing aberrations than ^{60}Co γ-rays (Tateno *et al.,* 1996).

Surprisingly these experiments showed that radiation treatment did not decrease fertilisation rates even at the highest dose exposures. This suggests that spermatozoa with chromosomal damage are not selected out at fertilisation and are quite capable of fertilising oocytes. A high fertilising ability of irradiated spermatozoa (0.5 - 4.0 Gy) has also been observed in the mouse (Matsuda *et al.,* 1985c).

It has been reported that mammalian spermatozoa have no capacity to repair DNA damage induced by radiation or chemicals (Ono & Okada, 1977). This suggests that spermatozoa exposed to mutagenic chemicals and/or radiation repeatedly, or over a long period of time may accumulate DNA damage and that this may be transmitted to the next generation without being selected out at fertilisation. It has also been shown that spermatozoa with radiation-induced chromosome damage are capable of fertilisation *in vitro* (Matsuda *et al.,* 1985c; Mikamo *et al.,* 1990).

Although spermatozoa are unable to repair DNA damage themselves, some DNA lesions may be repaired after fertilisation within the oocyte. In the studies where human spermatozoa were exposed to X-rays or β-radiation, it was found that approximately 10% of the radiation-induced chromosome aberrations were exchange-type (Kamiguchi *et al.,* 1990a, 1990b; Mikamo *et al.,* 1990). This suggests that DNA repair systems present in hamster oocytes are to some extent capable of repairing radiation-induced DNA lesions. It has also been demonstrated in the mouse that oocyte DNA repair systems are also capable of partially repairing radiation-induced spermatozoal DNA damage (Matsuda & Tobari, 1989). An inhibitor of DNA excision repair (3-aminobenzamide) potentiated the induction of chromosome-aberrations in sperm-derived chromosomes. It has also been suggested that there may also be species differences in the ability of fertilised oocytes to repair radiation-induced sperm DNA damage, although further investigations are required.

The above observations demonstrate that DNA-damaged sperm are still capable of fertilisation and that a real potential for the transmission of radiation-induced mutations exists. It is also worrying that human sperm are apparently much more sensitive to the mutagenic effects of radiation than laboratory rodents (Mikamo *et al.,* 1990).

As well as increased incidences of chromosome aberrations in pronuclear embryos following irradiation of spermatozoa other investigators have reported increased incidences of developmental abnormalities and cancers in the F_1 generation following paternal exposure to radiation and mutagenic chemicals (Nomura, 1988; Lord *et al.,* 1998).

Nomura (1988) found evidence for increases in prenatal anomalies of 18 day old mouse fetuses following paternal exposure of spermatozoa (0.12Gy) and spermatogonia (0.27Gy) following X-irradiation. The increase in phenotypical abnormalities following irradiation was 4-40 times higher than the ordinary mutation rate in mice. Paternal irradiation of germ cells (spermatozoa and spermatids) with X-rays (0.36-5.04Gy) resulted in 2-3 fold and 5-7 fold increases in the incidences of adult tumours and leukemia incidence in ICR and LT strains of mice respectively. However, similar experiments in another strain of mice (BALB/c) which have a propensity for lung tumour formation was unable to confirm these findings and suggested that cyclic and seasonal variations in tumour incidence may account for the results (Cattanach *et al.*, 1995). Despite this a newer study has suggested that preconceptional exposure to paternal irradiation (^{239}Pu) may render the offspring more vulnerable to tumour induction after secondary exposure to an unrelated carcinogen (Lord *et al.*, 1998). Heritable mutations have also been demonstrated following paternal exposure to ^{239}Pu α-particles (Pomerantseva *et al.*, 1989; Generoso *et al.*, 1989).

Hoyes *et al.*, (1994) investigated the potential of the widely used medicinal radionuclide, Indium (^{114m}In) to induce defects in the F_1 generation following paternal exposure. ^{114m}In and other medicinal radionuclides (Gallium) have been shown to accumulate and be retained by the testis (Jackson *et al.*, 1991). Following a single i.p injection of 14.8mbq/kg ^{114m}In, 0.25% of the total dose had localised to the testis after 24 hours and the level of radionuclide in the testis remained constant for the duration of the experiment (200 days). This dose of ^{114m}In (2.295Gy total dose at 200 days) resulted in both cytotoxic and mutagenic effects to the testis with a maximum 21% increase in dominant lethal mutations observed following matings 66-84 days (1.9-2.1Gy exposure) after dosing. A lower dose of 3.7mbq/kq ^{114m}In resulted in only mutagenic effects with a 12% increase in dominant lethal mutations at the same mating period. F_1 males from the high dose group had decreased testis weights when compared to control animals. F_1 mating trials with male progeny from the 14.8MBq/Kg group also provoked a higher incidence of dominant lethal mutations in F_2 progeny indicting the presence of a germline mutation.

So far much of the evidence for paternal transmission of radiation-induced mutations has come from animal studies with there being very little evidence available for humans. Three epidemiological studies have suggested that their may be a link between the paternal radiation exposure of nuclear workers and the apparent increased incidences of leukemias in childhood cancers in their children and children living near of around nuclear installations (Gardner *et al.*, 1990; Roman *et al.*, 1993; Sorahan & Roberts, 1993). In particular, one study in 1990, centred on the controversial Sellafield Nuclear Reprocessing plant and the much publicised high incidence of leukemia and non-Hodgkin's lymphoma among children living in the neighbouring village of Seascale (Gardner *et al.,* 1990). The study concluded that the raised incidences of cancers amongst children near Sellafield was associated with paternal employment at the nuclear installation and recorded external dose of whole body penetrating radiation before conception. Furthermore children born to fathers exposed to a preconceptual dose of greater than 100mSv had a 6½ times greater chance of developing cancer than normal children. This report promoted much concern, discussion and controversy amongst both radiation experts and the British Government and there is still much debate as to whether the conclusion from the data is valid. Other epidemiological studies conducted both at British nuclear installations and abroad suggested there was no evidence to link paternal radiation exposure to increased risk of cancers in offspring (Urquart *et al.,* 1991; McLaughlin *et al.,* 1992). However the debate was complicated further in 1993 by the publication of another report, this time concentrating on the area surrounding the Aldermaston and Burghfield atomic weapons establishment (Roman *et al.,* 1993). Again this study suggested a link between preconceptual dose of paternal radiation and increased leukemic risk in children. It was also suggested that paternal exposure to radionuclides is a more likely risk factor for childhood cancer than exposure to external radiation (Roman *et al.,* 1993; Sorahan & Roberts, 1993). More recently still, a study conducted following the nuclear accident at Chernobyl suggests an increased mutation rate in children born to parents contaminated by the accident (Dubrova *et al.,* 1996). However a similar study in Japan investigating the offspring of people exposed to radiation as a result of the Hiroshima and Nagasaki bombing suggested there is no evidence for increased mutation rates and cancer risks (Kodaira *et al.,* 1995). Therefore the data for humans seems to be split with evidence both for and against a link between paternal radiation exposure and increased incidence of cancer and other genetically-linked aberrations in offspring available.

1.11 Aims and Objectives.

It was the aim of this project to investigate the significance of genetic damage in spermatozoa and it biological consequences. Experiments were performed using radiation as a model for DNA damage induction in both mouse and human spermatozoa. A new technique for measuring DNA damage in sperm directly has been developed and assessed both *in vitro* and *in vivo*. Also the effects of paternal DNA damage on the embryo have been investigated and DNA damage levels examined in transgenic mice defunct in apoptotic and DNA repair pathways, which normally protect the testis.

Chapter 2

Materials and Methods.

2. MATERIALS AND METHODS.

2.1 Animals.

Male MF1 mice (Harlan Olac) were obtained from the BSU, Stopford Building, University of Manchester. Male and Female BDF1 mice and knockout mice p53$^{+/+\&-/-}$ (C57BL/6 background - CRC Beatson Institute, Glasgow); Bcl2$^{+/+\&-/-}$, Bax$^{+/+\&-/-}$ (C57BL/6 Background - Jackson Laboratories, Bar Harbour, Maine, U.S.A) and SCID (BALB/cBJY Background - Jackson Laboratories, Bar Harbour, Maine, U.S.A) were obtained from the Animal Unit at the Paterson Institute for Cancer Research, Wilmslow Road, Manchester. All animals were housed under standard animal house conditions (lights on 0700-1900) and allowed access to food and water *ad libitum*.

2.2 Scientific Procedures.

All licensed procedures involving the use of animals were performed within the jurisdiction of the Animals Scientific Procedures Act (1988) under Project Licence, PPL 50/01154.

2.3 Chemicals.

Unless otherwise stated all reagents were purchased from the Sigma Chemical Company, Fancy Road, Poole, Dorset.

2.4 Collection of Sperm Samples.

2.4.1 Mouse Sperm Samples.

Animals were killed by cervical dislocation. Both cauda-vas deferens were dissected out and placed into a petri dish containing 1ml of PBS (*in vivo* samples and ^{114m}In samples) or 1ml of T6 medium at 34°C (samples for *in vitro* irradiation). Sperm were expelled from the vas deferens using watchmaker's forceps and aspirated gently using a 200µl Gilson pipette to aid dispersion.

2.4.2 Human Sperm Samples.

Ejaculates (obtained by masturbation) were provided by 3 normozoospermic donors as assessed by WHO criteria, 1992 (concentration $\geq 20 \times 10^6$ cells per ml, motility > 50%, normal morphology > 30%) who were routinely used by the Department of Assisted Reproduction, St Mary's Hospital Manchester. Semen samples were allowed to liquefy at room temperature for 30 minutes and then washed by resuspending in 10mls BWW medium (Biggers *et al.,* 1971) followed by centrifugation (10 minutes, 1600rpm). This process was repeated twice.

2.5 Collection of Whole Blood and Preparation of Lymphocytes.

2.5.1 Mouse Blood.

Whole blood (approximately 0.8ml) was obtained by cardiac puncture under terminal anaesthesia (Halothane, Zeneca Laboratories) into heparinised tubes.

2.5.2 Human Blood.

Whole blood (approximately 10ml) was obtained by venupuncture from 3 male subjects (age range 22-48 years) into heparinised tubes.

2.5.3 Lymphocyte Preparation.

Whole blood was carefully layered on top of Ficoll Hypaque solution (Organon-Technika Ltd.) and centrifuged at 3000g for 15 minutes. Following centrifugation the lymphocyte layers were removed and mixed with phosphate buffered saline (PBS, Mg^{2+} and Ca^{2+} free). Cells were centrifuged once more at 3000g for 5 minutes and the pellet resuspended after cell counting on a hemocytomer (Neubauer Ltd) at a concentration of 1×10^6 cells per ml in PBS.

2.6 ^{60}Co γ-Irradiation of *In vitro* Samples.

2ml aliquots of cellular suspension (both sperm and lymphocytes) were transferred into a number of sterile plastic bijou tubes. These were loaded into a metal rack and irradiated on ice using a ^{60}Co γ-ray source (Dose Rate 3.1 Gy.min^{-1}). Sperm samples were exposed to doses of 0, 25, 50, 75 and 100Gy whilst lymphocytes were exposed to doses of 0, 10, 25, 50 and 75Gy.

2.7 *In vivo* Testicular X-irradiation.

Animals were placed in plastic restraining tubes and placed in the rack underneath the source. The front two thirds of the animals were covered with lead sheeting leaving the final third and testes exposed. The X-ray source was adjusted to a height of 510mm above the animals and a dose of 4Gy using 300 kVp X-rays (880 monitor units, dose rate 0.5 Gy.min^{-1}) delivered. Immediately after irradiation animals were placed back in their cages and allowed access to food and water *ad libitum.*

2.8 Comet Assay.

2.8.1 Solutions.

Regular Agarose
1g Agarose (1% w/v)
100mls d.d.H_2O
Mixture heated in microwave until dissolved.

Low Melting Point Agarose
1g Low melting point Agarose - Type VII (1% w/v)
100mls d.d.H_2O
Mixture heated in microwave until dissolved.

Lysis Buffer
292.2g NaCl (2.5M)
200ml 0.5M EDTA solution (100mM)

3.152g Tris-HCl (10mM)
Made up to 2 litres with d.d.H_20. pH 10.0-10.5

0.5M EDTA Solution

292.2g EDTA (0.5M)

1800ml d.d.H_20 added and the mixture alkalinised with NaOH pellets and placed on magnetic stirrer until dissolved.

pH adjusted to 8.0 and final volume made up to 2 litres with d.d. H_20.

Neutral Electrophoresis Buffer

5X Stock Solution

108g Tris Base
55g Boric Acid
40ml 0.5M EDTA solution Made up to 2 litres with d.d. H_20.

1X Working Strength Solution

200mls 5X stock solution
Made up to 1 litre with d.d.H_2O.

Alkaline Electrophoresis Buffer

100ml 0.5M NaOH solution (0.05M)
2ml 0.5M EDTA solution (1mM)
Made up to 1 litre with d.d.H_2O Final pH 13.0

0.5M NaOH solution

40g NaOH pellets
Made up to 2 litres with d.d.H_2O.

0.4M Tris-Cl (Neutralisation Solution)

31.52g Tris-HCl
Made up to 500ml with d.d.H_2O Final pH 7.0

SYBR Green Nucleic Acid Stain (1:10,000 dilution)

5µl SYBR Green (Molecular Probes Ltd)

Made up to 50ml with d.d.H_2O Stored at 4°C

2.8.2 Preparation of Comet Slides.

Single frost microscope slides (Superfrost, BDH Laboratories, Poole Dorset) were cleaned with 70% (v/v) Ethanol and allowed to air dry. 1% (w/v) regular agarose was melted by heating in a microwave and 500µl of agarose applied to the slide and thinly spread with the pipette tip to form a thin layer. The gels were allowed to set at room temperature and then dried overnight in an oven at 50°C. Dried slides were stored in slide boxes until required.

2.8.3 Preparation of Microgels.

Slides were labelled in pencil on the frosted end. The 1% (w/v) low melting point agarose was melted by microwave heating and maintained at a temperature of 40°C in a water bath. Cell samples were diluted in 1% (w/v) low melting point agarose to give a concentration of $1x10^4$ $cells.ml^{-1}$. 1ml of this cellular suspension was applied to the surface of a precoated microscope slide and spread using the pipette tip to form an even layer. Slides were then allowed to gel for 5 minutes at 4°C.

2.8.4 Lysis.

500mls of lysis buffer was placed into plastic containers. To this 5ml (1%v/v) of Triton X-100 (BDH Laboratories, Poole Dorset), 3.09g DTT (40mM) and Proteinase K (Boehringher Mannheim) final concentration $100µg.ml^{-1}$ were added (Lymphocyte samples were lysed in lysis buffer alone). Microgels were loaded into perspex racks and immersed in lysis buffer so that it covered the surface of the slides by approximately 0.5cm. The containers were sealed with airtight lids and covered with foil to prevent entry of light. Lysis was carried out for an initial period of 1 hour at room temperature followed by 2½ hours in a 37°C incubator. After the lysis period was complete slide racks were removed from lysis solution and washed three times at 20 minute intervals in d.d.H_2O to remove all traces of salt and detergent.

2.8.5 Neutral Electrophoresis.

After lysis and washing, slides were carefully removed from racks and placed on an electrophoresis tray. This tray was gently lowered into an electrophoresis tank containing 1 litre of neutral electrophoresis buffer (TBE) which covered the surface of the slides by approximately 0.5cm. The lid was placed on the tank to protect the slides from light and slides allowed to equilibrate with the buffer for 20 minutes. After this equilibration period slides were electrophoresed at 25V, 0.01A for 20 minutes at room temperature. When electrophoresis was complete, the buffer was gently drained from the tank and the tray holding the slides removed. Slides were rinsed with d.d.H_2O to remove traces of buffer, covered with foil and air dried overnight.

2.8.6 Alkaline Electrophoresis.

After lysis and washing slides were carefully removed from racks and placed on an electrophoresis tray. This tray was gently lowered into an electrophoresis tank containing 1 litre of alkaline electrophoresis buffer which covered the surface of the slides by approximately 0.5cm. The lid was placed on the tank to protect the slides from light and then microgel samples were allowed to unwind in buffer for 20 minutes to permit the alkali to convert double-stranded DNA into single-strands. Following unwinding, slides were electrophoresed at 25V, 0.08A for 20 minutes at room temperature. When electrophoresis was complete, the buffer was gently drained from the tank and the tray holding the slides removed. Slides were rinsed three times at five minute intervals with 0.4M Tris-Cl (pH 7.0) to neutralise traces of alkali and then slides covered with foil and allowed to air-dry overnight.

2.8.7 Staining of Slides with SYBR Green.

Microgels were rehydrated for 30 minutes by covering the surface of the slide with d.d.H_2O from a Pasteur pipette. The d.d.H_2O was then drained from the slides and 1ml of SYBR Green stain (1:10,000 dilution) applied to the surface of the slides for 1hour. After staining slides were rinsed with d.d.H_2O and coverslips applied.

2.8.8 Analysis and Scoring of Comet Slides.

Image analysis of Comet Slides was performed using a x20 objective (PlanFluor, Nikon U.K.) attached to a Nikon Optiphot-2 epifluorescence microscope (100W). Individual cells or "comets" were viewed at x200 magnification through a FITC filter and analysis performed using an intensified solid state CCD camera (Sony CCD-IRIS) attached to the microscope and linked to Comet Assay II image analysis software (Perceptive Instruments, U.K.) running on a Pentium 133MHz PC. Samples were run in duplicate and 50 cells analysed per slide and scored for comet tail length, comet tail moment and % tail DNA

2.9 Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin End-labeling (TUNEL) of Embryos.

2.9.1 Solutions.

Paraformaldehyde

1.85g (3.7% w/v) Paraformaldehye (BDH laboratories)
40mls PBS

The above ingredients were placed in a 50ml polystyrene tube (Falcon Laboratories) and 10 drops of 1M NaOH solution added. The tube was placed on a horizontal tray shaker and agitated until dissolved. The pH of the solution was adjusted to 7.4 using 1M HCl and the final volume made up to 50ml with PBS. The solution was filtered through a 0.22μm filter (Millipore) before use.

Phosphate Buffered Saline(PBS) Solution

40g NaCl
7.2g Na_2HPO_4
1.0g KCl
1.2g KH_2PO_4
5 litres d.d.H_2O Final pH 7.4.

PBS/PVP solution

150mg PVP (3mg/ml)
50 mls PBS solution

The above solution was filtered through a 0.22μm filter (Millipore) before use.

Triton X-100 in PBS

250µl (0.5% v/v)Triton X-100 (BDH Laboratories)

49.75ml PBS solution

2.9.2 Superovulation and Mating Procedure.

21 day old female BDF1 mice were injected with 5 I.U PMSG i.p at approximately 12:00hrs and then 48-52 hours later with 5 I.U hCG i.p. After hCG injection 2 females were housed in a cage with 1 adult male overnight. In the morning, the vaginas of female animals were checked for the presence of copulation plugs (used as a positive marker that mating had taken place) and the male removed to a separate cage. Female animals without copulation plugs were removed from the experiment.

2.9.3 Embryo Collection.

Approximately 100hrs after hCG injection, mated females were killed by cervical dislocation. The uterine horns were dissected out into a petri dish containing flush medium at 37°C. Individual uteri were placed in welled petri dishes (Falcon laboratories) and cut in two through the midline at the base of the uterus. Using a dissecting microscope (SMZ-2T, Nikon U.K; magnification x10 - x240) a blunt 30-gauge needle attached to a 1ml syringe was inserted into the oviductal end of the uterine horn and the contents flushed out with approximately 0.5ml of flushing medium. Petri dishes containing embryos were then placed on ice.

2.9.4 Fixation of Embryos.

Embryos flushed from individual animals were collected using a mouth-operated, hand-pulled glass micropipette and washed twice in filtered PBS. Embryos were then transferred to a 96-well U-bottomed cell culture plate containing 250µl 3.7% PFA per well. Embryos collected from individual animals were kept in separate wells. Culture plates were placed in the fridge and embryos allowed to fix overnight.

2.9.5 Protocol for the TUNEL labeling of Embryos (Brison & Schultz, 1997).

All manipulation of embryos was performed using hand-pulled glass micropipettes and 96-well U-bottomed cell culture plates viewed using a Nikon SMZ-2T

Stereomicroscope (magnification range x10 - x240). TUNEL reagents (dUTP-FITC label and TdT enzyme) are the active ingredients of the *In situ* cell death detection kit (Boehringer Mannheim).

Fixed embryos were removed from 3.7% PFA and washed through four separate changes of 200μl PBS/PVP. After the final wash embryos were placed in 200μl 0.5% Triton X-100 in PBS and allowed to permeabilise for 1 hour at room temperature. Following permeabilisation, the embryos were washed once more through 2 changes of 200μl PBS/PVP and then placed into 20μl of dUTP-FITC label (Boehringher Mannheim) and allowed to stand at room temperature for 10 minutes. After pre-incubation with label 70μl of TUNEL mix (10μl TdT enzyme + 60μl dUTP-FITC label) was added to each well and the culture plate incubated at 37°C for 1 hour. Following TUNEL labelling, embryos were washed three times in 200μl PBS/PVP. Embryos were then transferred to 150μl PI/RNase solution for 30 minutes. After staining with PI embryos were washed three times at 15 minute intervals in PBS/PVP and then washed gently in sequence through separate drops of 25%, 50%, 75% vectashield (Vector Laboratories) in PBS and then finally 100% vectashield. Embryos were mounted on microscope slides in 18μl of vectashield and a coverslip mounted on vaseline pegs. The coverslip was gently pressed down to trap the embryos and then the edges sealed with clear nail varnish. Slides were wrapped in foil and stored at 4°C until analysis.

Positive Controls - Embryos were incubated in 50μl of 50I.U.ml^{-1} RQ DNase I (Promega) for 20 minutes at 37°C and washed twice in PBS/PVP before the TUNEL labelling steps.

Negative Controls - The TdT enzyme was omitted from the TUNEL mixture and replaced with d.d.H_2O.

2.9.6 Analysis of Embryos.

Slides containing stained embryos were viewed at x200 and x400 magnification (Nikon PlanFluor objectives) using a Nikon Optiphot-2 (100W) epifluorescent microscope equipped with a Dichromatic PI/FITC filter. PI stains the nuclei of all blastomeres red whilst apoptotic nuclei are stained green/yellow by the FITC-conjugated TUNEL reagents.

2.9.7 Confocal Microscopy.

Confocal microscopy of TUNEL labelled embryos was performed with the help of Steve Bagley at the University of Manchester, Confocal Microscopy Unit using a Bio-Rad MRC-600 Laser Scanning Confocal Microscope equipped with a Krypton/Argon laser. Excitation was performed at 488nm and fluorescent emissions collected at 630nm (Propidium Iodide - Red Channel) and 530nm (FITC - Green Channel). Z series image sections were collected at 3μm sections through the embryos and then images processed using the Confocal Assistant, Version 4.02 software package.

2.10 Histology.

In all experiments the right testis from each animal was retained for histological examination.

2.10.1 Solutions.

Bouin's Solution

50ml Saturated Aqueous Picric Acid
10ml 40% Formaldehyde
5ml Glacial Acetic acid
35ml d.d.H_2O

2.10.2 Fixation of Testes.

Following dissection and weighing, testes were immediately submerged in Bouin's solution. After immersion for 3-4 hours the apical tips of the testes were removed using a scalpel blade to allow penetration of Bouin's to the centre of the tissue. Testes were allowed to fix for a 24 hour period and then the Bouin's solution was decanted and replaced by 70% ethanol with a few drops of $LiCO_3$. The tissues were gradually dehydrated by a graded series of alcohol's (70%, 1 hour; 90%, 1 hour; 95%, 1hour) and finally stored in 100% ethanol for at least 18 hours prior to embedding.

The tissue was embedded in paraffin wax in cassettes and 5µm transverse sections cut and attached to microscope slides. Some sections were stained with haematoxylin and eosin, whilst others were left unstained for subsequent ISEL. Sectioning, staining and mounting of testes sections were carried out with the technical assistance of the histology technician, Histology unit, Paterson Institute for Cancer Research, Manchester.

2.10.3 In Situ End-Labeling (ISEL) of Testis Sections.

Slides were placed in racks and de-waxed through 2 changes of xylene, 5 minutes per wash. Slides were then washed twice for 5 minutes each in 100% ethanol. Tissues were gradually rehydrated through a graded series of alcohols (95% Ethanol, 3 minutes; 70% ethanol, 3 minutes; 40% ethanol, 3 minutes) and finally placed in PBS for 5 minutes. Slides were then removed from racks and placed flat in a humidifed chamber. On each slide the boundary of the testis sections was drawn on with a Dako Pen (Dako Ltd) to aid the containment of reagents. Protein digestion was performed by applying 150µl of 10µg.ml^{-1} proteinase K (Boehringer Mannheim) and incubating at room temperature for 15 minutes in the humidifed chamber. Following protein digestion tissue sections were washed through 4 changes of d.d.H_2O at 2 minute intervals. After washing slides were gently blotted dry and 75µl of Equilibration buffer (Oncor) applied to each section for up to 30 minutes at room temperature. Equilibration buffer was then carefully drained from the slides and 54µl of Working Strength TdT Enzyme (Oncor) applied to each section. Slides were incubated for 1 hour at 37°C in a humidified atmosphere. Following 37°C incubation slides were placed into a coplin jar containing Stop/Wash Buffer (Oncor) and left at room temperature for 10 minutes to halt the enzymatic reaction and then washed three times in PBS at 5 minute intervals. Slides were gently blotted dry and 55µl of Anti-Digoxigenin-Peroxidase applied to each section. Slides were then allowed to incubate for 30 minutes at room temperature. Following incubation with antibody slides were again washed in PBS (4 x 5 minute washes) to remove excess antibody. 125µl of DAB substrate was applied to each section and slides allowed to stand for 15 minutes at room temperature to allow colour development. Slides were then loaded back into racks and counterstained with methyl green for 10 minutes. They were then washed through 3 changes of d.d. H_2O (30 seconds per wash) followed by 3 changes of butanol (BDH laboratories) at 30 seconds per wash. Finally slides were washed through two changes of xylene (3 minutes per wash) and coverslips mounted using Depex medium (BDH Laboratories). Slides were allowed to dry overnight and then stored at room temperature in slide boxes until analysis.

2.10.4 Scoring of ISEL labeled Slides.

Testis sections were scored at x200 magnification. Fields of view were randomly selected and both the numbers of tubules containing positively labelled (brown) cells and the number of positively labelled cells recorded. At least 50 tubules were examined for each testis section and two sections scored for each experimental animal.

2.10.5 Photography and Graphics.

Slides were photographed using a Nikon Optiphot-2 equipped with a Nikon UFX-DXII photographic system onto 35mm Kodak E160T colour slide film. Images were scanned directly from 35mm slide film into Adobe Photoshop, Version 5.0 using a Nikon LS-1000 35mm film scanner with the help of Ian Miller and Tony Bentley; Electron Microscopy, Photography and Graphics Unit, School of Biological Sciences, University of Manchester.

2.11 Indium 114m Radionuclide.

1mCi of ^{114m}In (0.1ml solution in 0.5M HCl) was obtained from NEN Life Sciences. The calculated activity of the radioisotope on the day of injection was 10mCi per ml stock solution.

2.11.1 Preparation of 100μCi per ml ^{114m}In Injection Solution.

10mCi per ml ^{114m}In stock solution was diluted 100 fold as shown below 30 minutes before injection of experimental animals.

100μCi.ml^{-1} ^{114m}In.

40μl 1mCi per ml ^{114m}In stock solution
3.96mls sterile 0.9% saline (w/v)

2.11.2 Treatment of Animals with ^{114m}In.

Animals were injected intraperitoneally with 50µCi ^{114m}In (dose volume 0.5ml). Control animals were injected intraperitoneally with 0.5ml 0.9% saline containing 1% 0.5M HCl (v/v).

2.11.3 Measurement of Radioactivity.

All radioactivity measurements were performed using a Miniaxi-γ Autogamma 5000 series Gamma Counter (United Technologies Packard). 10µl of the injection solution of ^{114m}In was placed in a plastic counting tube and the total radioactivity measured on the day of injection. 45 days post-injection of ^{114m}In, animals were killed and tissues removed for analysis (liver, spleen, testes, epididymis and a portion of gastrocnemis muscle). Tissue were weighed and placed into separate plastic counting tubes. Radioactivity counts (cpm) were determined for each tissue and activity was also calculated per gram of tissue.

2.12 Statistical Analyses.

All statistical analyses were performed using the SPSS software package. Details of statistical tests performed on sets of data are described in the table and figure legends accompanying the results in each chapter. Asterisks have been used to denote results statistically significant from controls. * = $P<0.05$, ** = $P<0.01$.

Chapter 3

Detection and Measurement of DNA Damage in Spermatozoa after Irradiation *in vitro*.

3.1 INTRODUCTION.

The maintenance of the genetic integrity of the cell is of uppermost importance. Damage to DNA can lead to cell death and transmissible genetic defects such as mutations and cancer (Evan & Littlewood, 1998). Many agents have the ability to cause DNA damage; therefore the success of any technique used in genotoxic screening is its ability to measure damage induced in DNA. A number of such techniques have been developed over the years, which measure both double-, and single-stranded DNA breaks (Ahnstrom, 1988; Radford, 1988). Some of these different techniques and the principles behind them are explained below.

3.1.1 Techniques to Measure DNA Strand Breaks.

3.1.1.1 Velocity Sedimentation.

When extracted DNA is placed on top of a sucrose gradient and centrifuged at a given speed, different sizes of DNA strands will migrate through the gradient at different rates. Therefore the number of strand breaks can be calculated from the size distribution of the fragmented DNA molecules (Lett, 1981). This technique which was first described in 1966, was soon applied to mammalian cells and used to investigate the induction of strand breaks by a number of physical agents (e.g. chemicals and radiation) (McGrath & Williams, 1966). However it soon became apparent that this method had a number of drawbacks. Firstly, because long DNA molecules sediment very slowly at high speeds, they could only be examined using very low rotor speeds and long centrifugation times (Zimm, 1974; Hutchinson, 1975). As well, any DNA molecules that came into contact with the walls of the gradient container tended to be lost leading to a major source of error (Lett, 1981). It also became apparent that if the DNA consisted of too many large molecules then they could also interact with each other which would then influence sedimentation rate (Ahnstrom, 1988).

3.1.1.2 DNA Unwinding.

Double stranded DNA molecules can be converted into single-strand DNA or unwound through exposure to alkaline pH (pH>12.3). It was discovered that the presence of DNA strand breaks increases the rate of alkaline-induced denaturation of the DNA double helix (Davison, 1966; Rydberg, 1975). This principle was used by a number of investigators to develop techniques for the measurement of single strand DNA breaks (ssb's). Cells are lysed in an alkaline solution for a period of time and then neutralised. Ultrasonic treatment fragments the DNA reducing the renaturation of DNA molecules that contain both single-and double-stranded regions. The relative amounts of single- and double-stranded DNA are then determined using hydroxyapatite chromatography (Ahnstrom & Erixon, 1973; Ahnstrom & Edvardsson, 1974). Rydberg & Johanson, (1978) also modified the technique in order to study ssb's in single cells. Cells were embedded in agarose and subjected to alkaline treatment. After neutralisation the cells were stained with acridine orange which stains double stranded DNA green and single stranded DNA red. Therefore the ratio of red to green fluorescence could be measured to give a measure of the number of strand breaks.

3.1.1.3 Filter Elution.

Elution techniques use filters to discriminate between DNA of different sizes. The filters act mechanically to impede the passage of DNA. Filter elution techniques can be used to measure single- and double-strand breaks, alkali-labile sites, DNA-protein crosslinks and DNA interstrand crosslinks (Kohn *et al.,* 1981; Kohn, 1986). Cellular DNA is normally radiolabelled with ^{3}H-thymidine before the assay. After treatment or when required for analysis the labelled cells are applied to polyvinyl chloride or polycarbonate filters. A lysing solution is then allowed to drip through by gravity and after protein digestion, the filters are connected to a peristaltic pump and a solution slowly passed through the filters and fractions collected. DNA double strand breaks can be measured by using a neutral pH elution solution and single strand breaks by using a solution of higher alkaline pH. The fractions of DNA remaining on the filters, and that in each collected fraction can then be determined with time and used as an indication of the number of strand breaks present.

3.1.1.4 Nucleoid sedimentation

Many of the techniques discussed above utilise alkaline treatment to denature DNA into single strands exposing the single strand breaks. However, many DNA damaging agents do not induce only strand breaks but also produce lesions which are converted to strand breaks on alkaline treatment (so called alkali labile sites). Often alkaline techniques which report the measurement of single-strand DNA breaks are in fact measuring both single strand breaks and alkali labile sites (Ahnstrom, 1988). Therefore in order to detect just single strand breaks it is useful to have techniques that avoid the use of alkali. Mammalian DNA is supercoiled which creates a tension in the DNA (Vogelstein *et al.,* 1980). This tension can be partially relaxed by the presence of a single strand break and when centrifuged, relaxed DNA sediments more slowly than supercoiled DNA. Therefore in order to measure strand breaks; cells are lysed in neutral solution on top of a neutral sucrose gradient. The so-called nucleoids sediment more slowly with increasing dose of radiation. After centrifugation the gradients can be fractionated and the profile of the sedimented DNA measured and used as a measure of the number of strand breaks (Ahnstrom, 1988).

3.1.1.5 DNA Precipitation.

When mammalian cells are lysed in 2 per cent sodium dodecyl sulphate (SDS) followed by the addition of potassium chloride, a precipitate is formed containing proteins and nucleic acids. If DNA is damaged by strand breaking agents then some of the DNA is separated from the protein and is not precipitated by the KCl but instead remains in the supernatant after centrifugation. Therefore the percentage of DNA remaining in the supernatant can be measured as an indicator of the number of strand breaks. Both single- and double-stranded DNA breaks can be measured as the cells can be lysed under either neutral or alkaline conditions (Olive, 1988).

3.1.1.6 Pulsed-Field Gel Electrophoresis.

The mobility of large DNA molecules is highly correlated to the size of the DNA molecule. This is related to the end-on migration of the DNA molecules through agarose gels. Cells are placed in agarose plugs and using inverse or transverse pulsed electric field through electrophoresis gels very large DNA molecules can be separated according to size. This allows large DNA fragments produced as a result of DNA strand breaks to be separated. The migration pattern of the DNA fragments can be used to measure levels of DNA strand breaks (Carle *et al.,* 1986; Radford, 1988).

3.1.2 Single Cell Gel Electrophoresis or Comet Assay.

In 1984, Ostling & Johanson developed a technique that allowed the direct visualisation of DNA damage in individual cells. Irradiated single cells were suspended in a thin agarose layer attached to a microscope slide and then lysed and electrophoresed. The electric current was able to pull the charged DNA away from the nucleus so that relaxed and broken DNA fragments were able to migrate further than the intact DNA. By staining with a fluorescent DNA dye, these images could be seen using a fluorescent microscope and were subsequently referred to as comets giving the assay its name. Ostling and Johnson measured the size of these comets and determined that the extent of DNA that migrated from the head of the comet during electrophoresis was directly related to the dose of radiation used. Since this first publication, the assay has been used by many laboratories to examine DNA strand-breaks in cells (Fairbairn *et al.,* 1995). The assay has also been adapted and modified to increase both its sensitivity and specificity.

The original method of Ostling and Johnson used mild lysis conditions that probably did not remove all the DNA-associated proteins and the major response after irradiation was the appearance of a halo around the nucleus. This halo is probably the result of single strand-breaks, which disrupt supercoiling allowing the migration of DNA. More stringent lysis conditions have now been developed which allow the removal of >95% of DNA-associated proteins and these lysis condition together with neutral electrophoresis form the basis of the modern "neutral comet assay" which is used to detect DNA double strand breaks (Olive *et al.,* 1991). Neutral electrophoresis conditions allow specificity for double-stranded DNA breaks as at neutral pH's the DNA helix remains double stranded and therefore only duplex DNA is examined. By applying alkaline denaturing conditions to this assay it has further been developed to measure single strand DNA strand breaks. Two laboratories have independently developed the single-strand assay over the years (Singh *et al.,* 1988; Olive, 1989). One group has concentrated on maximising the sensitivity of the assay for the detection of low levels of strand breaks (Singh *et al.,* 1994, 1995) whilst the other group has concentrated on examining cellular heterogeneity within a population to drug or radiation sensitivity (Olive, 1989; Olive *et al.,* 1990, 1993a).

3.1.2.1 Assay Principle.

DNA is arranged within the nucleus in a highly ordered structure. Proteins are associated with the DNA which enable it to be packaged into the nucleus. In somatic cells DNA is wound twice round histone octomers every 200 base pairs, forming nucleosome structures (McGhee & Felsenfield, 1980). These nucleosomes then come together forming solenoids (Finch & Klug, 1976). Because of the way DNA is wound round histones it is negatively supercoiled (Vogelstein *et al.,* 1980; Liu, 1983). In order to measure DNA breaks the DNA-associated proteins must first be removed. This is achieved using lysis buffers, which normally contain detergents to disrupt membranes and allow access of the buffer to the nucleus and high concentrations of salt which facilitate the displacement of the proteins from DNA. Enzymes such as proteinase K may also be included in the lysis buffer to aid protein digestion (Fairbairn *et al.,* 1995). Once the DNA-associated proteins have been removed the DNA must be denatured if the measurement of single-strand breaks is intended. A high pH (> 12.3) is generally used to aid unwinding of the DNA and denature it into single strands. Electrophoresis of lysed DNA allows the detection of DNA breaks. Comets are formed when broken ends of the negatively charged DNA molecule are free to migrate towards the anode. There are two main factors, which are thought to determine comet formation. Firstly the ability of DNA to migrate is related to both the size of the DNA and the number of broken ends. Although these broken ends may be from larger pieces of DNA which are attached to the nuclear matrix they are still able to migrate a short distance from the comet head or nucleus. Tail length increases proportionally to the amount of damage but will reach a maximum that is defined by the electrophoresis conditions and not the size of the fragments. At low levels of DNA damage it is thought that stretching of attached DNA strands occurs rather than migration of individual pieces. At higher levels of damage, pieces of DNA are free to migrate, forming a distinct tail of the comet. Therefore by measurement of the relative fluorescent intensities of the head and tail of the comet and distance of migration information regarding the relative numbers of strand breaks can be calculated.

3.1.2.2 Methodology.

The comet assay is designed to measure DNA damage in single cells. Therefore samples need to be in form of single cell suspensions. Virtually any eukaryotic cell can be used in the comet assay as long as a single cell suspension can be prepared (Fairbairn *et al.,* 1995). However recently there have been reports of DNA damage in an isolated structure (intestinal crypts) being analysed by comet assay although it was still able to distinguish the individual cells that made up the tissue (Brooks & Winton, 1996). The most important concern when isolating cells or preparing single cell suspensions is that samples be prepared without inducing additional damage, or allowing repair of induced DNA breaks. Therefore most samples are collected and prepared on ice to minimise these factors.

Single cells are suspended in low melting point agarose (concentration 0.5-1%) at 35-45°C and spread onto a microscope slide to form a minigel. Some laboratories advocate the use of fully frosted slides to aid gel adherence but this has the disadvantage that the frosting may lead to an increase in fluorescent noise after staining, making quantitative analysis difficult. Other laboratories promote the precoating of microscope slides with a thin layer of regular agarose to promote adherence. There are basically two types of gels used in the comet assay. The single layer gel and the sandwich gels (where the cells are contained in the middle layer of three distinct layers of agarose).

The prepared gels are then immersed in lysis buffer, which facilitates the removal of DNA-associated proteins from the embedded cells. Neutral and alkaline lysis conditions are used for double- and single-strand break detection respectively. The slides are then equilibrated with electrophoresis solution prior to electrophoresis. In the alkaline form of the assay the gels are exposed to buffer containing NaOH with a pH >12.3, which unwinds the DNA and also converts alkali labile sites within the DNA into single-strand breaks. The neutral assay utilises a buffer of a neutral pH, commonly TBE where the DNA remains double-stranded. Slides are then electrophoresed at low voltage for a short period of time. After electrophoresis the slides are generally neutralised to remove traces of alkali and the DNA stained using a fluorescent dye (commonly used stains are propidium iodide and ethidium bromide). Using a fluorescent microscope the comets can be visualised and a number of computerised image analysis systems are available to quantify and measure the comet images.

The main measurements used to quantify and compare DNA damage in comet samples are "Comet Tail Length" (Singh *et al.,* 1988) and "Comet Tail Moment" (Olive *et al.,* 1990). Comet tail length is simply the distance from the centre of origin to the furthest point of DNA migration. Comet tail moment is defined as the distance between the centres of DNA mass multiplied by the percentage of DNA in the comet tail (Figure 3.1.2.2.1)

3.1.2.3 Factors, which Influence the Comet Assay.

3.1.2.3.1 Variations in Methodology.

One difficulty in comparing results produced by comet assay is the variety in the technique and its application in different laboratories. Many different protocols are in use (reviewed by Fairbairn *et al.,* 1995) which, although they share a great deal of similarities, differ enough to maybe affect the results in such a way that direct comparison is not possible. Lysis conditions (i.e. salt concentration, pH and lysis time/temperature) can affect the ability to detect DNA damage as the extent of cell lysis may depend upon the technique. Also the washing of slides before electrophoresis may also influence the outcome. Residual salt left over in the gels from lysis can be inhibitory to DNA migration (Olive *et al.,* 1992). This is because salt partially neutralises the charge of the DNA phosphates. If the slides have not been washed to remove the salt or equilibrated with electrophoresis buffer then gradients of salt will rise out of the slide during electrophoresis. If there is more rapid loss of salt from the top of the gel the comets will display longer tails than those at the bottom of the gel and this will result in heterogeneity within the sample making accurate determination of results impossible. This problem can be overcome in a number of ways i) by using the sandwich gel method, ii) by recirculating the buffer during electrophoresis and iii) by simply equilibrating the slide with the running buffer prior to electrophoresis.

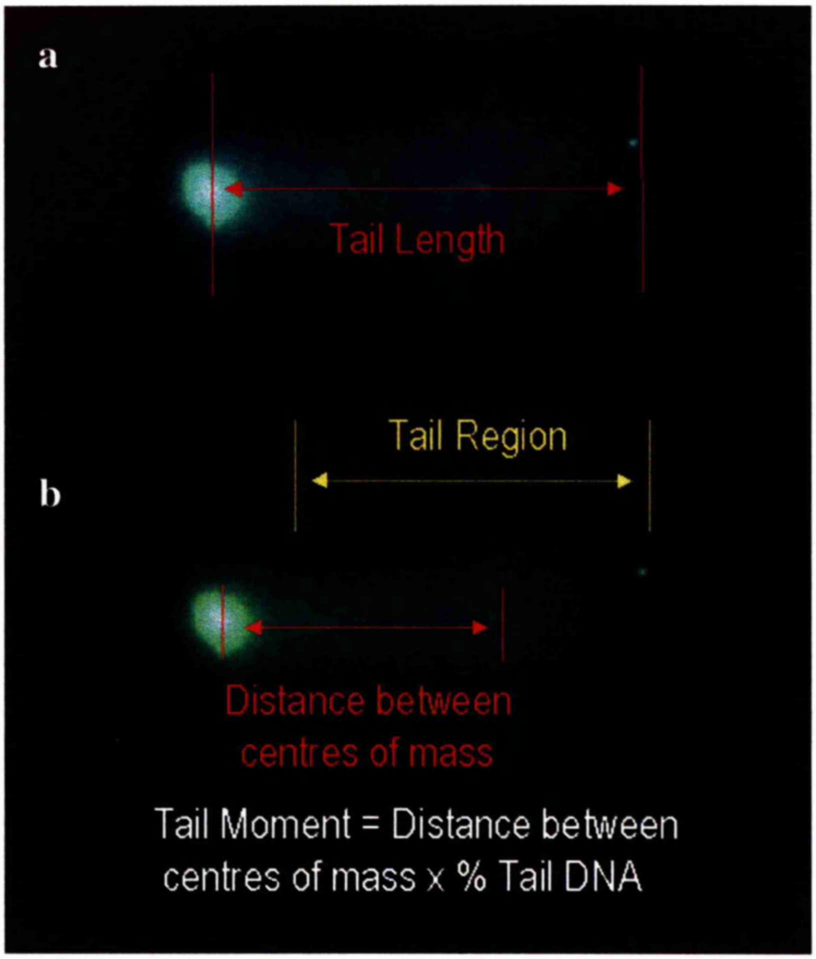
a
Tail Length
b
Tail Region
Distance between centres of mass
Tail Moment = Distance between centres of mass x % Tail DNA

***Figure 3.1.2.2.1** : Methods used to quantify DNA damage in the comet assay. a) Comet Tail Length and b) Calculation of Comet Tail Moment.*

3.1.2.3.2 Electrophoresis.

Since the measurement of the comet is carried out using a microscope the DNA is required only to move a very small distance before differences can be detected. Therefore electrophoresis voltages and running times are a lot lower than those used for conventional nucleic acid electrophoresis. Typically voltages in the range of 0.5-5.0 V/cm are used in the comet assays with electrophoresis times of 5-30 minutes (Fairbairn *et al.,* 1995). However it is in the electrophoresis step that there appears to be considerable variation in technique between laboratories.

3.1.2.3.3 DNA Staining.

Following electrophoresis slides are washed and stained with fluorescent DNA binding dyes. For many years propidium iodide (Olive *et al.,* 1990, 1991) and ethidium bromide (Singh *et al.,* 1988, 1990) have proved popular although these dyes may often show high non-specific background fluorescence which may compromise the detection of small changes in comet length. More recently a number of highly sensitive and specific DNA stains have been developed such as YOYO-1 (Singh *et al.,* 1994, 1995) and SYBR-GREEN (Kiltie & Ryan, 1997). The advantage of these new dyes is that their fluorescence is many times brighter than that of PI and EtBr and therefore they can detect much smaller amounts of DNA. As they are very specific they produce much lower background staining which also contributes to an increase in sensitivity of the assay. However, disadvantages of these new generation dyes are that they are expensive and that their binding may be affected by pH therefore careful neutralisation of slides after electrophoresis is required.

3.1.2.3.4 Cell Cycle Position.

It as been shown that the position of the cell in the cell cycle can influence the outcome of both neutral and alkaline comet assays(Olive *et al.,* 1988, 1991; Olive & Banath, 1993). This is due to the complications produced by S-phase DNA. In the alkaline assay, DNA replication forks are interpreted as strand breaks when denatured therefore resulting in apparently higher levels of DNA damage in S-phase cells (Olive *et al.,* 1988). Sites of active DNA replication also results in DNA tangling which retards the migration of DNA during electrophoresis and decreases the ability of the neutral assay to detect strand breaks in these cells (Olive *et al.,* 1990; Olive & Banath, 1993).

3.1.2.4 Applications of the Comet Assay.

3.1.2.4.1 Radiation Biology.

Many DNA strand breaks assays were developed to measure the damage induced by ionizing radiation (Ahnstrom, 1988; Radford, 1988; Fairbairn *et al.,* 1995). The comet assay is no exception and allows rapid and sensitive measurement of DNA strand breaks (both single and double-) in a population of cells exposed to radiation (Singh *et al.,* 1988; Olive *et al.,* 1990, 1991). However, what is unique about the comet assay which has made it very useful in this field is that it can be used to measure heterogeneity in response of cells to ionizing radiation (Olive *et al.,* 1990). By examining the repair kinetics of irradiated cells it has been possible using the comet assay to detect subpopulations of cells that repair single-strand breaks rapidly and slowly after irradiation. Differences in the radiation response of tumours of similar type, size and grade have been ascribed to tumour heterogeneity. There has been an increasing interest recently in using the comet assay as a predictive test to determine whether a tumour will benefit from a particular therapy. The comet assay can provide information on sensitivity to a particular treatment, proportion of actively growing tumour cells, and the presence of tumour cells lacking oxygen, all of which are known to affect their response to treatment (Olive *et al.,* 1990, 1993a). Also since the comet assay requires only a few thousand cells, a sample can be obtained simply and conveniently using a fine needle aspiration biopsy.

3.1.2.4.2 Excisable DNA Damage.

Agents such as U.V irradiation form lesions which cannot be measured directly as DNA strand breaks (Gedik *et al.,* 1992; Green *et al.,* 1992). This is because these lesions are converted into strand breaks as the cell tries to repair the lesion. The strand breaks arise due to the incision step of the excision repair system (Sancar, 1996). Using the comet assay it is possible to detect these lesions (Gedik *et al.,* 1992). Irradiated cells allowed no repair time show no detectable comet tail formation. With repair time comets are seen to appear and then disappear in accordance of the appearance of strand breaks from these lesions which are in turn repaired by rejoining. The comet assay has been shown to be effective in detecting the syndrome Xeroderma pigmentosum as lymphocytes from these patients are characteristically lacking in excision repair enzymes (Green *et al.,* 1992). These experiments have suggested that the assay may be useful as diagnostic tool for xeroderma pigmentosum and other syndromes characterised by defects in excision repair.

3.1.2.4.3 DNA Crosslinks.

DNA interstrand crosslinks are produced by many chemotherapeutic agents (e.g. cisplatin, mitomycin C, nitrogen mustard) and are probably responsible for the cytotoxicity of these drugs (Neidle & Waring, 1983). Existing chromatographic and electrophoretic detection methods are based on the principle that crosslinks will prevent denaturation of DNA produced by alkali addition or heating. However crosslinking of DNA produced by cisplatin and nitrogen mustard has also been measured using the comet assay (Olive *et al.*, 1990, 1992). Instead of looking at the retardation of denaturation, the investigators found that crosslinks prevented the migration of DNA produced during alkaline comet assay of X-irradiated cells. Therefore by comparing the retardation of migration of cells both X-irradiated and treated with a cross-linking agent with those cells receiving X-irradiation alone it is possible to get a relative measure of the amount of cross-linking produced by the drug.

3.1.2.4.4 Oxidative Damage.

Oxygen radicals can cause chromosome aberrations and have also been implicated in the aetiology of cancer (Cerutti, 1985; Guyton & Kensler, 1993). As reactive oxygen species are generated as intermediates in many biological systems including the testes, they are fundamentally important and their DNA damaging potential has been examined by many investigators (Imlay & Linn, 1988; Moraes *et al.*, 1990; Meyers *et al.*, 1993). Hydrogen peroxide (H_20_2) is a common intermediate in a variety of oxidative stresses and has been demonstrated to produced DNA damage leading to mutagenesis (Imlay & Linn, 1988). Although H_20_2 does not interact directly with DNA it produces radicals such as the hydroxyl radical (OH•) which can produce strand breaks when it interacts with DNA. H_2O_2 has frequently been used as a positive control for DNA damage in the comet assay in many laboratories. However one problem with H_2O_2 is the heterogeneity in cellular response to damage which may be as a result of the wide variety of lesions produced by different oxygen radicals. A system has also been devised to detect endogenous oxidative DNA damage (Collins *et al.*, 1993). Purified endonuclease III is added to slides post lysis to allow the measurement of oxidised bases. Endonuclease III converts oxidised bases (specifically pyrimidine) into strand breaks which can then be detected using the alkaline comet assay. Hydrogen peroxide induced damage levels were shown to be greater after endonuclease III treatment, demonstrating the persistence of oxidised base damage and the differential expression of lesions following repair. Other enzymes are now being investigated in order to determine whether they can be used in conjunction with the comet assay allowing the detection of specific lesions.

3.1.2.4.5 Genetic Toxicology.

The comet assay may prove very useful as a genotoxic screen as it has the ability to detect DNA damage induced by a variety of agents. In theory, the assay is applicable to virtually any eukaryotic cell type and it can be used in both animal and human systems (Fairbairn *et al.,* 1995). As well as giving information about agents that cause DNA damage it can also be used to detect compounds that may interfere with DNA repair mechanisms which in the long term could prove equally as damaging. Therefore the possibilities appear endless with the only major limitation being that a single cell suspension can be generated form the target tissue. Many *in vitro* genotoxicity and mutagenicity screens are already available (e.g. AMES test, cytogenetics, unscheduled liver DNA synthesis; Purves *et al.,* 1995). However, it is the ability of the comet assay to detect DNA damage from samples exposed *in vivo* that may underlie its success in genotoxic screening in the future. Compounds, which affect specific organs, can have genotoxicity evaluations carried out in that tissue, which has not been possible in the past, therefore as more evaluations are carried out using the *in vivo* comet assay then its use will become more widespread in genotoxicity testing.

3.1.2.4.6 Apoptosis.

Apoptosis is a process of active cell death which can be initiated by a wide range of stimuli both physical (e.g. drugs, radiation) and physiological. It is distinguished from necrotic cell death, which occurs passively by a number of characteristic morphological features e.g. nuclear and cytoplasmic condensation and nuclear fragmentation (Kerr *et al.,* 1972). One of the most clearly defined events in apoptosis is nucleosomal fragmentation. This occurs due to the activation of endogenous endonucleases within the cell, which actively digest the DNA into nucleosomal fragments (Arends *et al.,* 1990). Because apoptotic DNA fragmentation is characterised by the generation of double-stranded DNA breaks, both the neutral and alkaline assays can be used with equal efficiency for the detection of apoptosis. The extent of DNA damage in apoptotic cells is so great that they are easily distinguishable from undamaged cells (Olive *et al.,* 1993b). Most of the DNA is extensively degraded that it is able to migrate from the head into the tail of the comet and migrates several times the length of the original undamaged head. Therefore the comet assay represent a simple system for the determination of the apoptotic fraction of a population of cells.

3.1.3 Detection of Strand Breaks in Spermatozoa.

Spermatozoa have no DNA repair mechanisms (Ono & Okada, 1977), therefore any DNA damage present in spermatozoa is a potential cause of mutagenicity if transferred at fertilisation. Many chemicals have been shown to have adverse effects on fertility and lead to dominant lethal mutations and increased frequency of developmental abnormalities in the offspring (Ehling, 1971; Nomura, 1988; Dobrzynska & Gajewski, 1994). Although there are toxicological screens available to detect paternally-mediated genotoxic effects there are very few reported techniques available to directly assess and measure DNA damage in spermatozoa after exposure to toxic agents. Most of the data regarding the reproductive and genetic toxicology of these agents has come about through investigation of their effects on the testis and sperm counts, fertilisation and development of the offspring. However with advances in scientific knowledge and technology there is considerable pressure and responsibility to develop new more practical and effective tests.

As has been described earlier in this chapter their are numerous techniques that have been widely used to measure DNA damage in mammalian cells (Ahnstrom, 1988; Radford, 1988). Therefore it is interesting to note that these techniques have not been widely applied for reproductive toxicology purposes. It is widely accepted that chromosome breakage is correlated with dominant lethality in the offspring (Goldstein *et al.,* 1978). Cytogenetic techniques are available to examine chromosome aberrations induced in spermatozoa and a number of physical agents and their ability to produce chromosome aberrations when exposed to mouse spermatozoa *in vitro* have been evaluated (Matsuda *et al.,* 1985c, 1989). With the advent of the human-hamster oocytes penetration test it is now possible to examine chromosome aberrations in human sperm as well as rodents after *in vitro* exposure to toxic agents (Mikamo *et al.,* 1990, 1991). However, these techniques are very difficult and time-consuming and therefore impractical for use on a large scale.

Alkaline elution has been widely used to measure single-strand breaks induced by radiation and other agents in mammalian cells (Kohn *et al.,* 1976, 1981; Kohn, 1986). Some groups have also applied this technique to measure DNA damage in sperm after exposure to toxic agents. Sega *et al.,* (1986) used alkaline elution to measure DNA damage in spermiogenic stages of mice exposed to the alkylating agent, methyl methanesulfonate (MMS). MMS is a genotoxic chemical which when administered to males causes dominant lethal mutations in the offspring (Partington & Bateman, 1964; Ehling *et al.,* 1968). The highest number of dominant lethals are observed in the offspring of animals mated 6-12 days after injection of MMS. This corresponded to the most sensitive germ cell stages to the effects of MMS being early spermatozoa to late spermatids. These germ-cell stages are also the most sensitive to the induction of specific locus mutations by MMS (Ehling, 1980). Sega and Generoso (1986) treated male mice with a single 50mg/kg dose of MMS injected intraperitoneally. Sperm were then recovered daily from the vas deferens of animals and then processed for alkaline elution. It was found that as early as 1 day after treatment with MMS, increased elution of DNA was detectable (6.5% control, 10.6% MMS treated) whilst 9 days after injection of MMS, when dominant lethals are at their highest, DNA elution had increased to 29.7%. 19 days after injection of MMS the eluted fraction of DNA had decreased to 15.2% and this is because sperm at this point are derived from germ-cell stages that are capable of DNA repair. Therefore this study showed that DNA damage produced by MMS could be measured in sperm by alkaline elution and that the elution profile correlated with biological damage (e.g. dominant lethals, specific locus mutations) assessed by other methods. The same group also demonstrated that using higher doses of MMS resulted in the elution of more of the total DNA indicative of higher levels of DNA damage. As well as MMS, Sega and Generoso have also demonstrated similar results with the chemical ethylene oxide, which also targets early spermatozoa and late spermatids (Sega & Generoso, 1988).

Van Loon *et al.,* (1993) used alkaline elution to study the induction and repair of single strand breaks induced by *in vitro* exposure of spermatocytes, round spermatids and elongate spermatids to ionizing radiation. It was found that with doses from 0-8Gy alkaline elution could detect an increase in single strand breaks and that spermatocytes and round spermatids were able to repair these breaks if the cells were incubated under culture conditions after irradiation. Elongate spermatids however were much less sensitive the induction of strand breaks by irradiation and were unable to repair these breaks in culture.

As well as the above examples alkaline elution has been used to look at strand breaks induced by chronic cyclophosphamide exposure on rat spermatozoa (Qiu *et al.,* 1995a, 1995b) and on rat and human sperm exposed *in vitro* to the reprotoxin dibromochloropropane (DBCP), (Bjorge *et al.,* 1996). Van Loon *et al.,* (1991) have also used an immunochemical detection method to look at single strand breaks after DNA unwinding induced by ionizing radiation in spermatogenic cells of the hamster.

Therefore although it appears possible in some cases to use techniques such as alkaline elution to measure DNA damage in sperm, this technique has not become widespread. This may reflect problems that some groups have encountered with high background levels of DNA damage in spermatozoa compared to other cell types and this may be attributed as an artefact due to assay conditions.

With the advent of the comet assay, there are a number of publications, which have examined the damaging effects of chemicals on spermatozoa using this new technique. Singh *et al.,* (1989) used an alkaline comet assay to examine background levels of DNA damage in mouse and human sperm. When spermatozoa were lysed and electrophoresed comets were produced that were characteristic of cells that had been irradiated with high doses of X-rays. It was concluded that this high level of background damage in spermatozoa was due to high levels of endogenous alkali labile sites present in spermatozoal DNA.

Hughes *et al.,* (1996, 1998) have examined the effects of hydrogen peroxide and antioxidants supplement upon human spermatozoa using a modified alkaline comet assay. Treatment of spermatozoa *in vitro* with 100μm H_2O_2 for 1 hour produced sperm with increased DNA migration (Hughes *et al.,* 1996). This group also noted that spermatozoa obtained from infertile patients were more susceptible to DNA damage induced by H_2O_2 treatment and X-rays (30Gy). More recent studies have also demonstrated that antioxidant supplements (ascorbic acid and alpha tocopherol) included in the culture medium help to protect against damage caused by *in vitro* exposure to H_2O_2 or X-rays (Hughes *et al.,* 1998).

Another group has also used the method of Hughes *et al.,* (1996) to look at the *in vitro* effects of estrogenic chemicals (diethylstilbestrol, β-estradiol and nonylphenol) upon spermatozoal DNA (Anderson *et al.,* 1997b). This group demonstrated variable but positive damaging effects of these compounds although extremely high and possibly unrealistic doses were required *in vitro.* This group have also demonstrated that some mutagens present in food can damage sperm DNA, albeit also at high concentrations *in vitro* (Anderson *et al.,* 1997a). Certain flavanoids appeared to show antigenotoxic effects in modulating the response of sperm to food mutagens similar to the results shown with antioxidants by Hughes *et al.,* (1998)

3.1.3.1 Reactive Oxygen Species and Damage to Spermatozoa.

Reactive oxygen species (ROS) are routinely detected in 25% of semen samples in infertility clinics (Iwasaki & Gagnon, 1992). Oxidative stress has been suggested to be detrimental to sperm function (Macleod, 1943) and studies have shown that ROS are generated by human spermatozoa (Alvarez *et al.,* 1987; Aitken & Clarkson, 1988). It has been reported that ROS activity is significantly increased in ejaculates from infertile men (Aitken *et al.,* 1989, 1992; Sharma & Agarwal, 1996). Additionally leukocytes present in semen are also a source of ROS and the level of oxygen metabolites in semen has been correlated with leukocyte concentrations (Aitken, 1995). Oxidative stress may disrupt human sperm function by a number of mechanisms. Firstly, ROS may cause peroxidative damage to unsaturated fatty acids in the sperm plasma membrane (Jones *et al.,* 1979; Aitken *et al.,* 1993). Additionally ROS are also known to attack DNA inducing DNA strand breaks, chromatin cross-linking and oxidative base damage in spermatozoa. Studies *in vitro* have demonstrated how exogenous ROS can produce both lipid peroxidation and DNA damage in human spermatozoa which result in reductions in both sperm motility and sperm-oocyte fusion (Aitken *et al.,* 1998; Twigg *et al.,* 1988). However, despite these harmful effects it has been demonstrated that exposure of spermatozoa to low-levels of ROS may actually reduce genetic damage and enhance the ability of human spermatozoa to fuse with the oocyte (Aitken *et al.,* 1998). These data have lead to suggestions of a physiological role for ROS exposure during spermatogenesis in sperm chromatin packaging and also involvement in sperm capacitation.

3.1.4 Aims and Objectives.

It appears from the experiments described above that the comet assay may be useful as a tool to examine DNA damage in spermatozoa. The aim of the experiments in this chapter was to evaluate whether the comet assay could be used to detect DNA strand breaks (both single and double stranded) induced in mouse and human spermatozoa by *in vitro* irradiation with ^{60}Co γ-rays. Experiments were also performed with human and mouse lymphocytes (representative of somatic cells) for comparison with results from spermatozoa.

3.2 RESULTS

3.2.1 Sperm Comet Assay Methodology

Mouse spermatozoa were found to be resistant to lysis protocols described in many previous comet assay papers. As it is known that the arrangement of spermatozoal DNA is highly condensed, proteinase K was added to the lysis buffer and an additional lysis step (2½ hours) at 37°C was performed to aid displacement and removal of DNA-associated proteins. Although "halos" were now evident around sperm nuclei (evidence for some degree of lysis) no increases in comet tail length and moment were observed at damaging doses of radiation (50Gy). Therefore the reducing agent dithiothreitol (DTT) was added to the lysis buffer to help reduce disulphide links between protamine molecules to ensure more efficient removal. With the inclusion of DTT in the lysis protocol, the appearance of characteristic comets were seen after electrophoresis of irradiated cells (Figure 3.2.2.1) and therefore this protocol was used in future.

3.2.2 *In vitro* Irradiation of Human and Mouse Spermatozoa - Neutral Comet Assay.

Irradiation of mouse and human sperm (0-100Gy) resulted in the formation of comets of varying sizes (Figure 3.2.2.2). Using an image analysis system it is possible to gain quantitative measurements from these comet images. For each sample 2 duplicate slides were run and 50 cells scored per slide. Samples were obtained from three individual subjects for each species examined. A dose-dependent increase in comet tail length was observed for both human and mouse spermatozoa. Mean DNA migration was 25.4μm (Mouse) and 32.8μm (Human) in control spermatozoa. Following *in vitro* irradiation with 100Gy of ^{60}Co-γ-rays mean values for comet tail length had increased to 311.0μm (Mouse) and 161.7μm (Human). These effects were consistent between individual samples scored on separate slides and different subjects within each species.

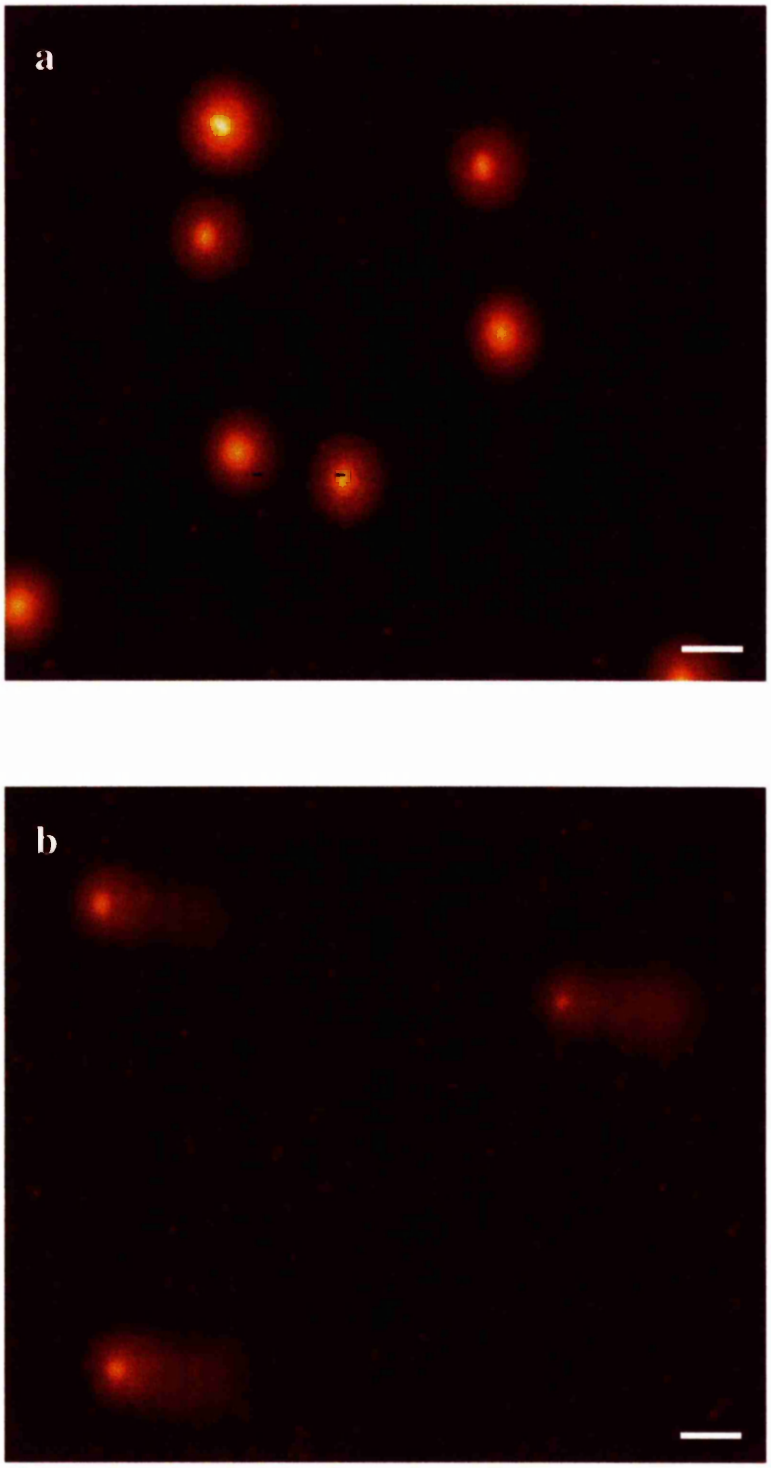

Figure 3.2.2.1 *: Effects of DTT in the comet lysis buffer. Panel a - mouse spermatozoa exposed in vitro to 50Gy* ^{60}*Co* γ*-rays and then lysed without DTT in the lysis buffer and electrophoresed. Panel b - mouse spermatozoa exposed in vitro to 50Gy* ^{60}*Co* γ*-rays and then lysed with 40mM DTT included in the lysis buffer and electrophoresed. Magnification x 160. Scale Bar = 50*μ*m.*

Figure 3.2.2.2b shows a graph of comet tail length vs. radiation dose for both mouse and human spermatozoa. It can be seen that *in vitro* irradiation of spermatozoa produced increases in comet tail length for both species. The relationship between DNA damage and comet tail length is linear as shown by the graph. However it is evident that at the same dose of radiation higher comet tail lengths were recorded for mouse sperm compared to human. Tail length is the simplest way of quantifying comets although most investigators use the comet tail moment. This measurement takes in account both the distance the DNA has migrated and the percentage of DNA that has migrated from the nucleus. Figure 3.2.2.3 shows a graph of comet tail moment vs. radiation dose. As with the graph for comet tail length a linear relationship is observed between comet tail moment and radiation dose. Again at similar doses of radiation, higher values for comet tail moment were recorded for mouse spermatozoa compared to human spermatozoa.

Table 3.2.2.1 shows the effect of *in vitro* irradiation upon the amount of DNA migrating from the nucleus (% Tail DNA). Even in unirradiated controls from both species some migration of DNA was observed from the nucleus. The control % Tail DNA values were higher for human (33.3%) than mouse (10.4%) spermatozoa. Irradiation of spermatozoa resulted in more DNA being able to migrate out of the nucleus to form the tail of a comet as shown by a dose-dependent increase in % Tail DNA with increasing radiation dose. Although at corresponding doses of radiation higher tail length and tail moment values were recorded for mouse sperm than human sperm, this was not reflected in the percentage of DNA in the tail of the comets. Apart from in unirradiated spermatozoa the percentage of DNA in the comet tails at each radiation dose is quite similar between the two species.

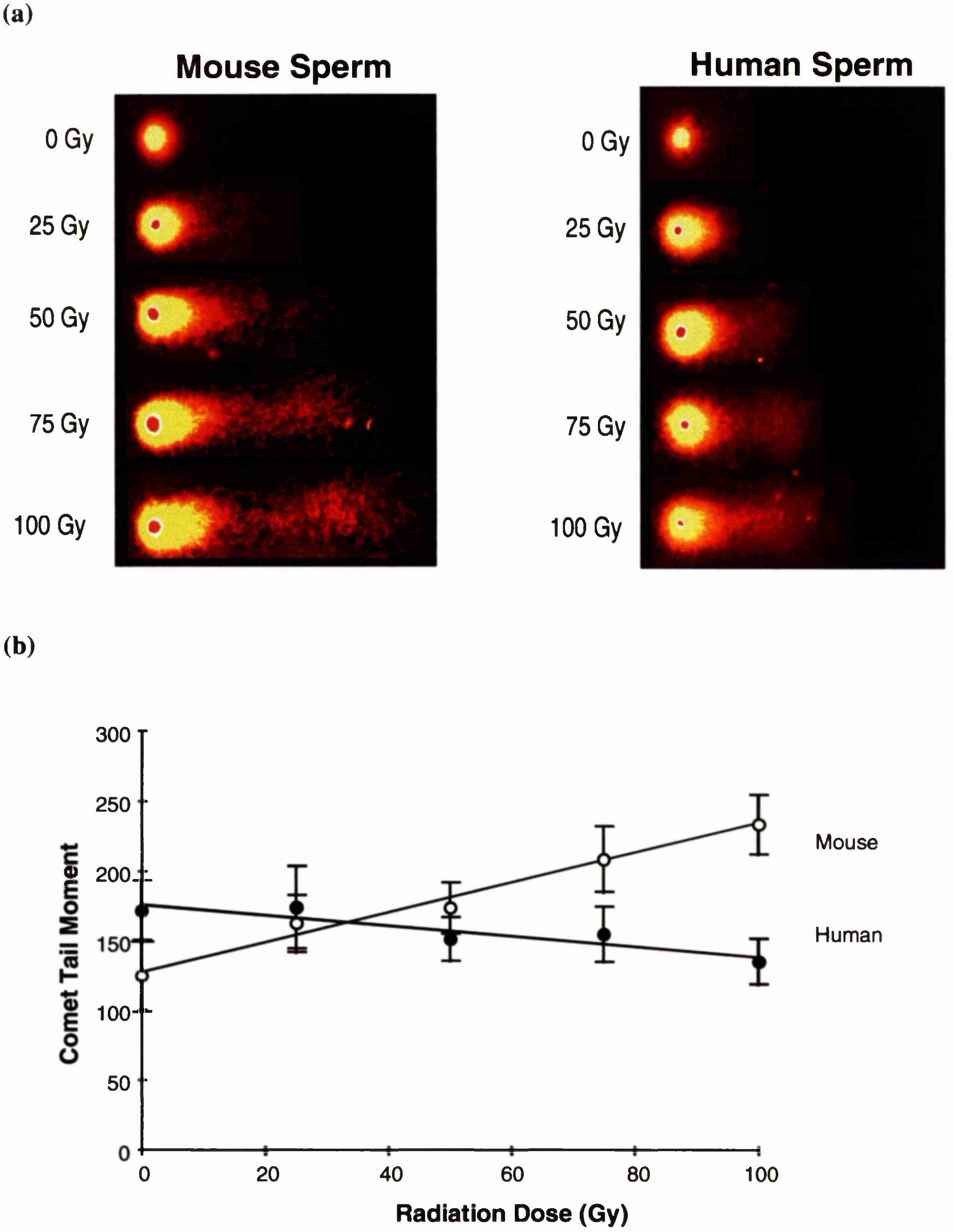

***Figure** 3.2.2.2 : DNA damage in mouse and human spermatozoa after in vitro irradiation with ^{60}Co γ-rays as measured by neutral sperm comet assay. (a) Appearance of comets after neutral electrophoresis of irradiated sperm cells and (b) the relationship between radiation dose and comet tail length. Each data point represents the mean results from 3 experimental subjects ± S.E.M, 100 cells scored per subject.*

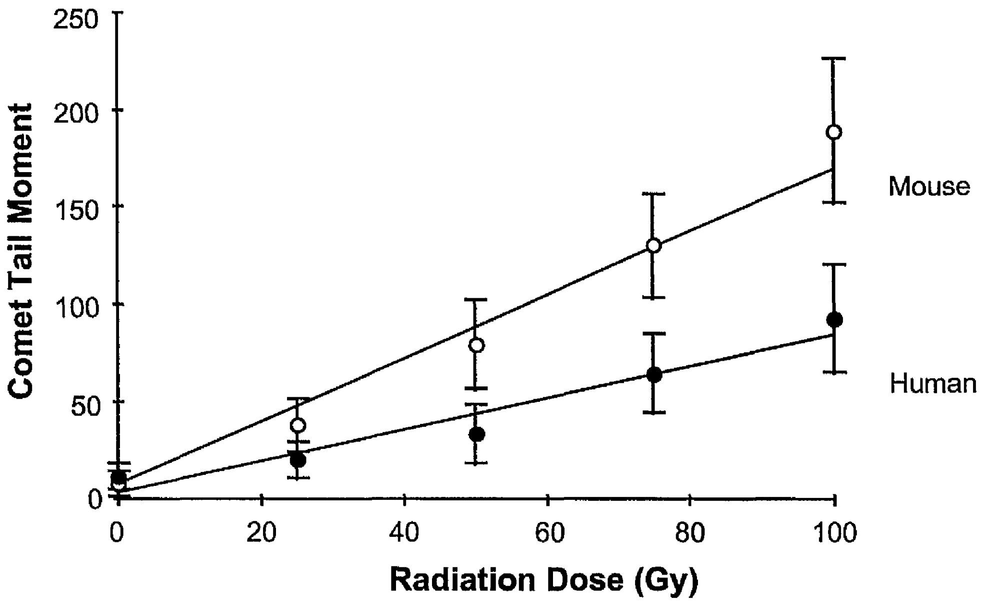

***Figure 3.2.2.3** : Effects of In vitro irradiation of mouse and human spermatozoa with* ^{60}Co *γ-rays upon neutral comet tail moment. Each data point represents the mean results from 3 experimental subjects ± S.E.M, 100 cells analysed per subject.*

***Table 3.2.2.1**: Effects of in vitro irradiation of mouse and human spermatozoa with* ^{60}Co *γ-rays upon percentage of the total DNA found in the tail of the comet as assessed by neutral sperm comet assay.*

	TAIL DNA (%)	
Dose (Gy)	*Mouse*	*Human*
0	10.4 ± 3.2	33.3 ± 6.5
25	33.1 ± 5.8**	38.3 ± 6.0
50	44.9 ± 5.9**	45.5 ± 5.8**
75	55.2 ± 5.3**	48.2 ± 5.9**
100	60.4 ± 5.6**	56.6 ± 6.4**

Results for each dose represent the mean ± S.E.M from 3 experimental subjects, 100 cells analysed per subject. Asterisks indicate values significantly different from the controls, * = P<0.05, ** = P<0.01. (One way ANOVA followed by Bonferonni's post-hoc test)

One advantage of the comet assay over other DNA damage assays is the ability to look at data from individual cells rather than just a large population. The previous results in this chapter have been presented as mean results of all the cells analysed. However using the comet assay it is possible to see if these mean results reflect a similar degree of damage in every cell or whether there is heterogeneity of response with just some cells being damaged by irradiation and skewing the means. Figures 3.2.2.4 and 3.2.2.5 are frequency distribution for DNA migration (comet tail length) for both mouse and human sperm. The x-axis show the length of the comet tails whilst the y-axis shows the percentage of cells that have tails of that length. It is therefore possible to determine how many cells in total had a tail length above a certain size or whether there maybe two distinct separate populations (i.e. undamaged and damaged cells). As previously discussed it can be seen that irradiation causes a shift in the frequency distributions with all cells showing increased tail lengths at higher doses of radiation. However, what is evident from these frequency distributions is that all the cells in the population have similar responses to irradiation (i.e. they show similar degrees of damage as measured by comet tail length). Each population is represented by a histogram representative of a normal distribution with an increase in median with increasing radiation dose. However it appears that after irradiation all the cells show some degree of damage and therefore move rightwards along the axis. With increasing doses of radiation we do not see two separate populations i.e.) an undamaged population at the left of the x-axis and a damaged population further rightwards. It appears that the response of the cells to *in vitro* irradiation is homogenous across the population although at larger doses the range and standard deviation of the population does increase.

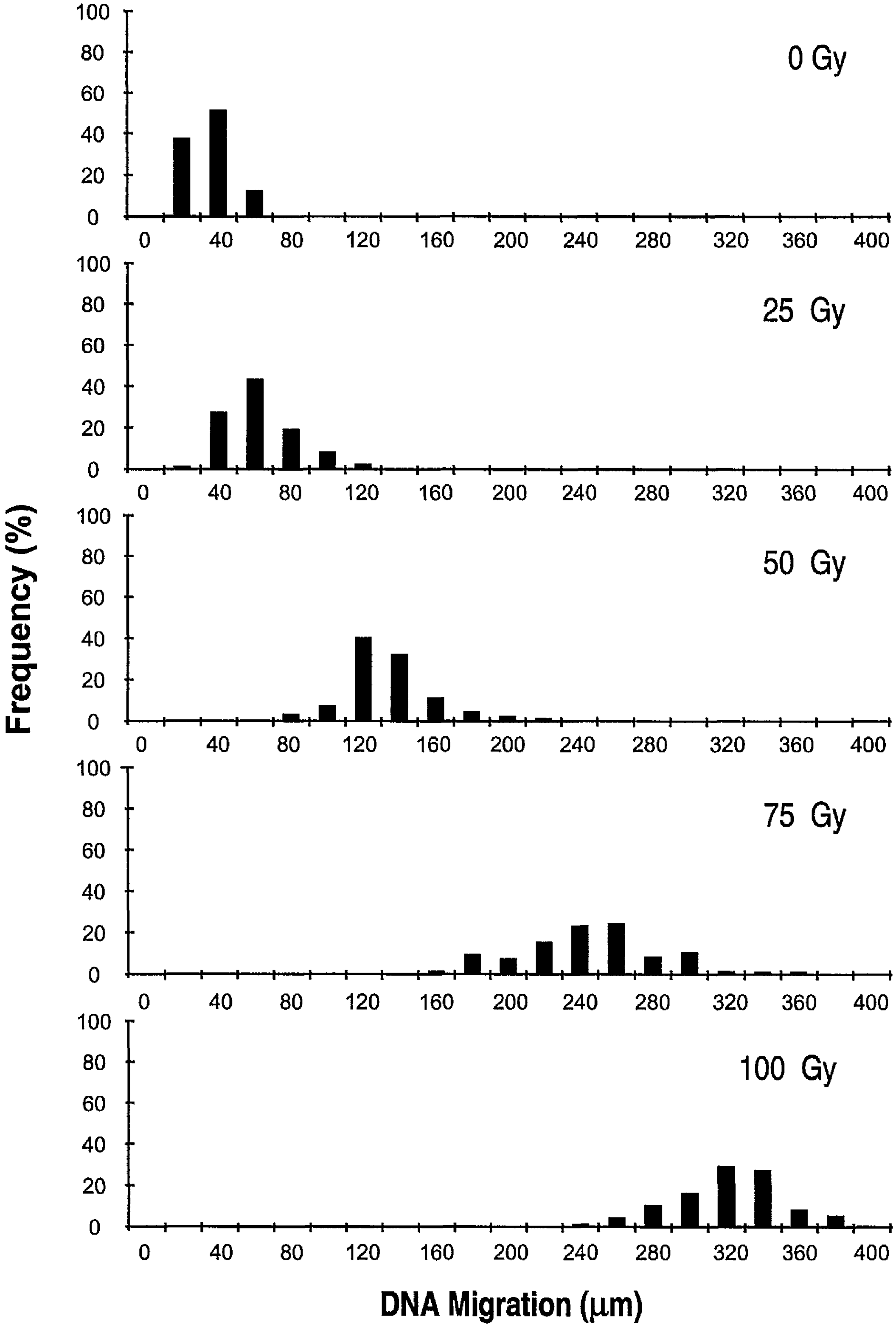

Figure 3.2.2.4 *: Distribution of comet tail lengths at each radiation dose for mouse spermatozoa analysed by neutral comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.*

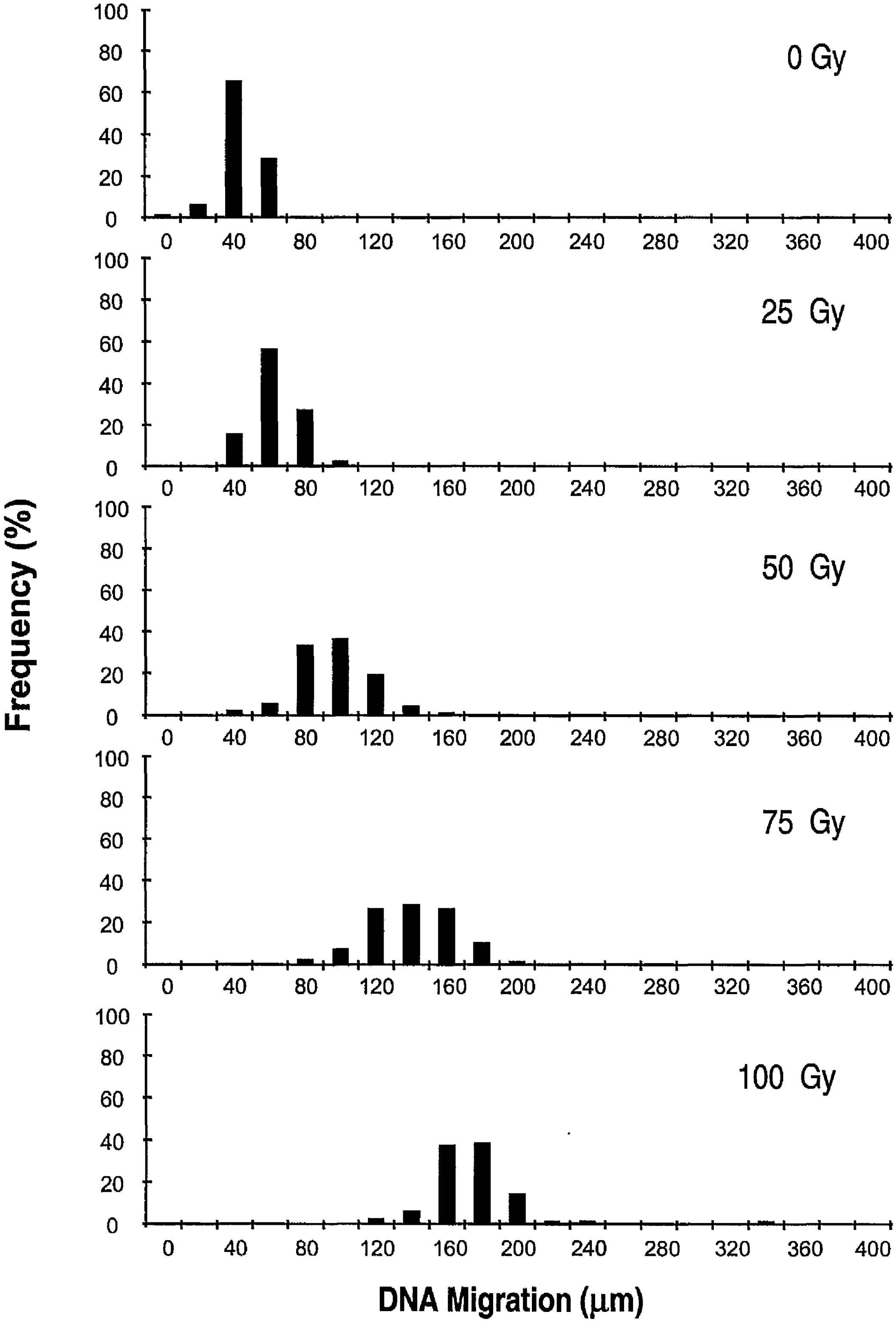

Figure 3.2.2.5 : Distribution of comet tail lengths at each radiation dose for human spermatozoa. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.

3.2.3 *In vitro* Irradiation of Human and Mouse Spermatozoa - Alkaline Comet Assay.

In the previous sections results have been described using the neutral comet assay in which the DNA remains double stranded and therefore only double stranded DNA breaks are theoretically measured. Another version of the assay commonly used is the alkaline comet assay. This version of the assay uses an alkaline unwinding step in which the samples are exposed to alkaline (pH >12.3) and allowed to unwind (convert to single-strands) for a period of time before electrophoresis in alkaline conditions. This version of the assay permits the measurement of single-stranded DNA breaks and alkali-labile sites.

Figure 3.2.3.1 shows the appearance of *in vitro* irradiated mouse and human spermatozoa after alkaline comet assay. In contrast to control samples from the neutral version of the assay where round controls cells were observed with very little DNA migration, unirradiated samples in the alkaline comet assay showed extensive migration of DNA from the nucleus forming large comet tails, indicative of cells with large numbers of DNA breaks. This was evident in both mouse and human spermatozoa but not in lymphocytes from both species subjected to alkaline unwinding and electrophoresis (see sections 3.2.4 & 3.2.5). Mean values for DNA migration were 139.6μm (Mouse) and 176.1μm (Human) which are representative of values obtained with irradiation doses above 50Gy in the neutral assay. The values for % Tail DNA (Table 3.2.3.1) show that approximately 95% of the total DNA migrated out of the nucleus in these unirradiated cells. This extensive migration of DNA from control spermatozoa apparently suggests that spermatozoa from both mice and humans possess extremely high numbers of single strand breaks within their DNA.

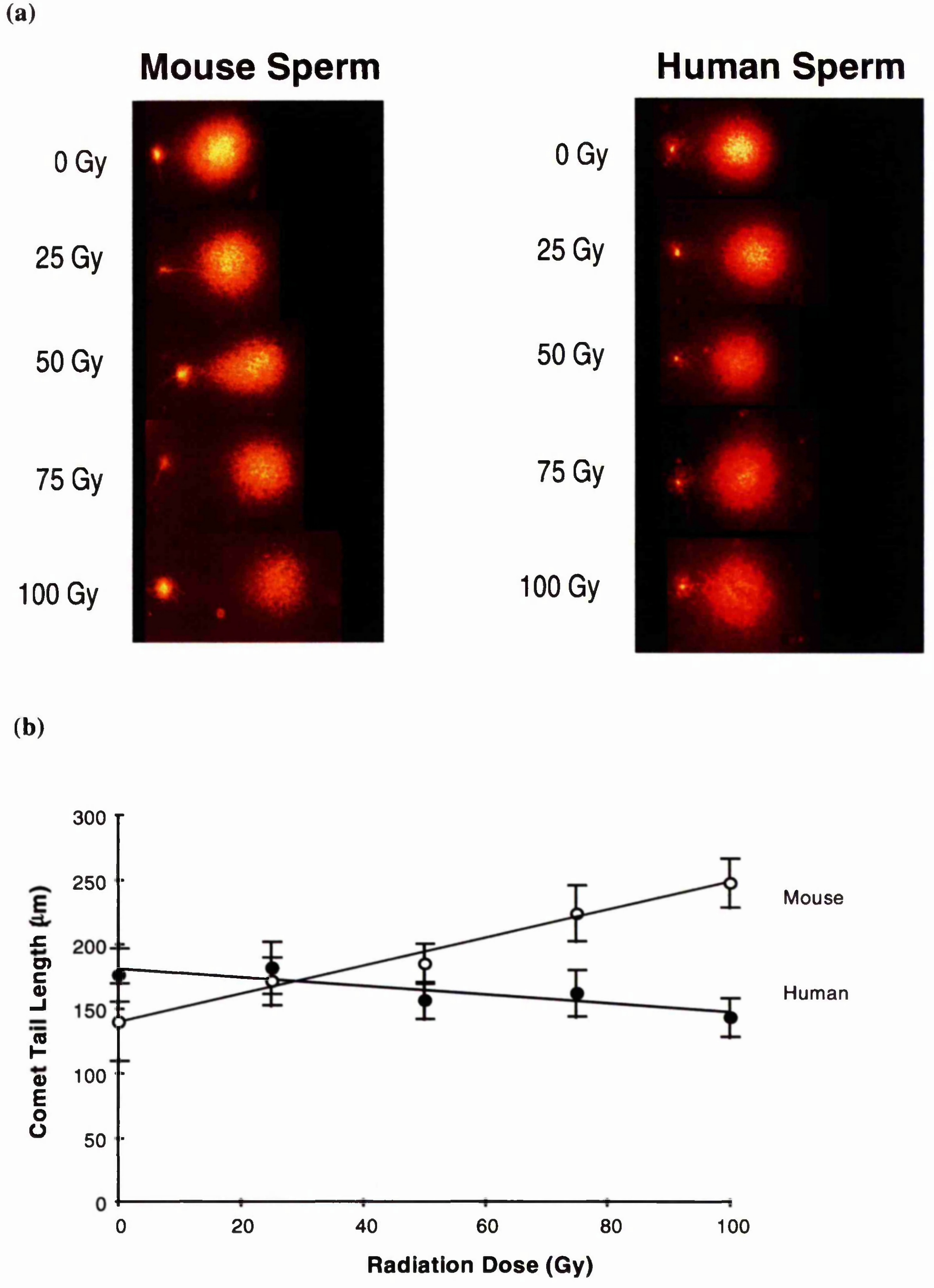

Figure 3.2.3.1 *: DNA damage in mouse and human spermatozoa after in vitro irradiation with* ^{60}Co *γ-rays as measured by alkaline sperm comet assay. (a) Appearance of comets after alkaline electrophoresis of irradiated sperm cells and (b) the relationship between radiation dose and comet tail length. Each data point represents the mean results from 3 experimental subjects, 100 cells scored per subject.*

(a)

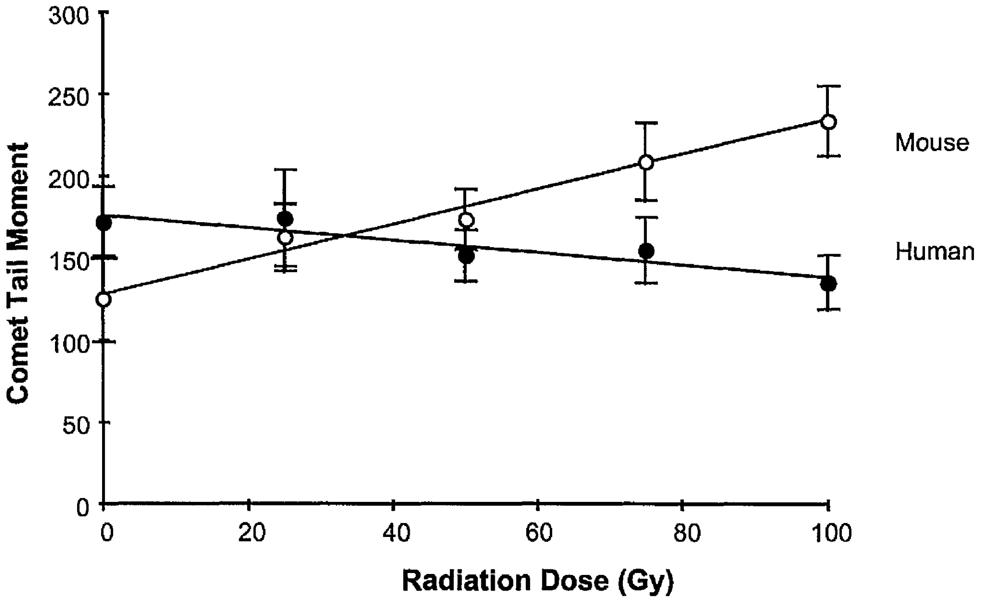

Figure 3.2.3.2 *: Effects of In vitro irradiation of mouse and human spermatozoa with ^{60}Co γ-rays upon alkaline comet tail moment. Each data point represents the mean results from 3 experimental subjects, 100 cells analysed per subject.*

Table 3.2.3.3 *: Effects of in vitro irradiation of mouse and human spermatozoa upon percentage of the total DNA found in the tail of the comet as assessed by alkaline sperm comet assay.*

	TAIL DNA (%)	
Dose (Gy)	*Mouse*	*Human*
0	90.8 ± 6.4	97.4 ± 1.3
25	94.8 ± 1.7	95.6 ± 6.5
50	94.0 ± 2.6	97.0 ± 1.1
75	93.1 ± 2.1	95.5 ± 1.9
100	94.3 ± 2.5	94.4 ± 1.7

Results for each dose represent the mean ± S.E.M from 3 experimental subjects, 100 cells analysed per subject. Data analysed using one way analysis of variance.

Despite the high background levels of damage produced when sperm are subjected to alkaline electrophoresis, irradiation still seemed to produce an effect on comet size at least in mouse spermatozoa. As can be seen in Figure 3.2.3.1 dose dependent increases in comet tail length with increasing doses of radiation were observed with mouse spermatozoa although this was not the case for human sperm. If anything a decrease in comet tail length at higher doses was observed in human spermatozoa. As with the neutral assay, there again appeared to be a linear relationship between comet tail length and radiation dose. The values for comet tail moment essentially reflect the values for tail length since the percentage of DNA in the tail is effectively constant at all doses of radiation (Table 3.2.3.1). The only difference in the amounts of tail DNA was observed in control samples, with human sperm having slightly more DNA in their comet tails than mouse sperm. This was also observed in the neutral assay and may suggest that human sperm have higher background levels of DNA damage than mouse spermatozoa.

As in the neutral comet assay the frequency histograms for comet tail length suggest that spermatozoa have a homogenous response to *in vitro* irradiation with each dose represented by a single normal distribution. For the mouse it can be seen that the median values increase with radiation dose (Figure 3.2.3.3) although this is not the case for human (Figure 3.2.3.4). Although the data for human sperm generally fit a normal distribution at some doses there is a skewing of the distribution. However there is clearly no increase in median values for comet tail length with increasing radiation dose.

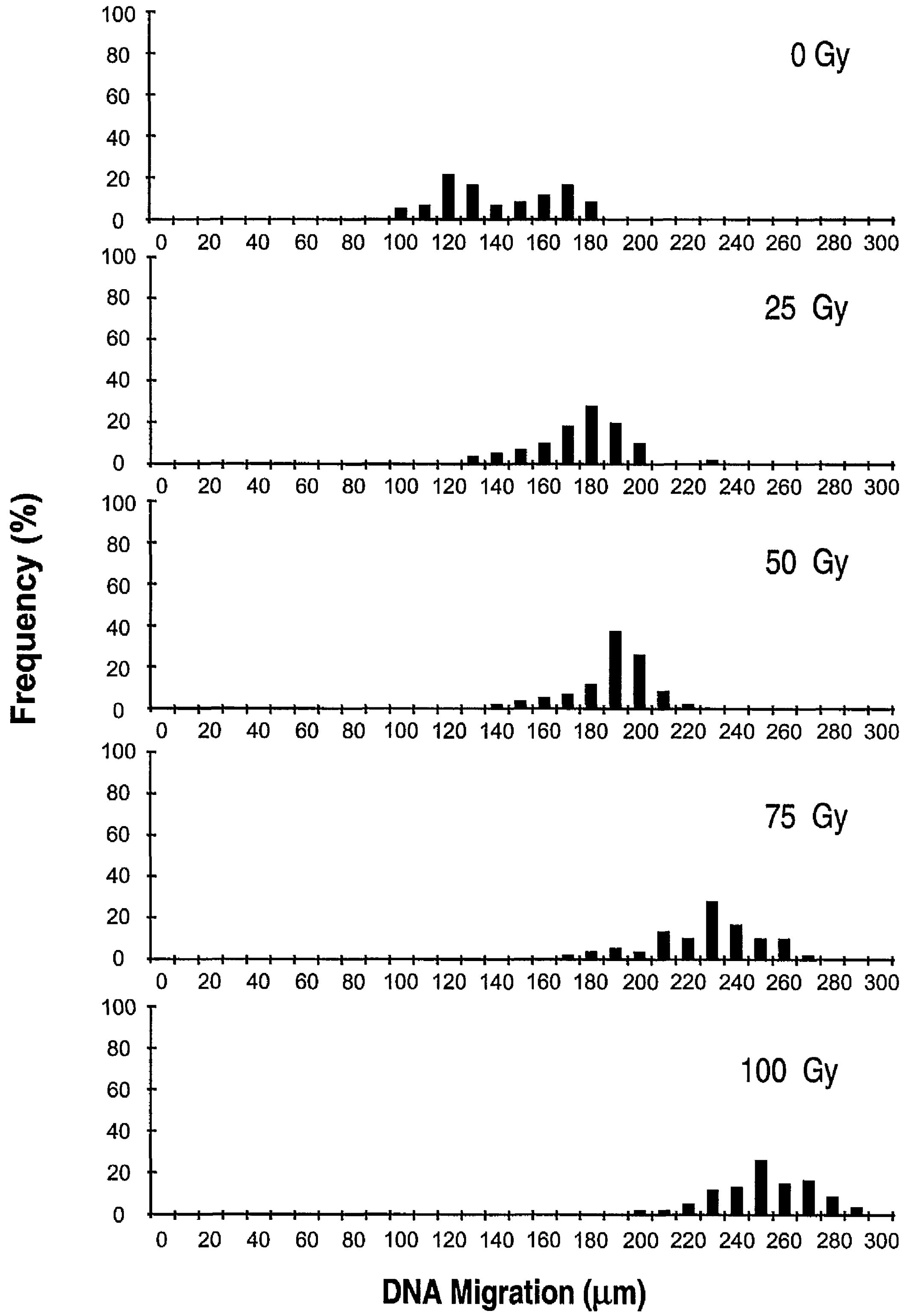

Figure 3.2.3.3 : Distribution of comet tail lengths at each radiation dose for mouse spermatozoa analysed by alkaline comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.

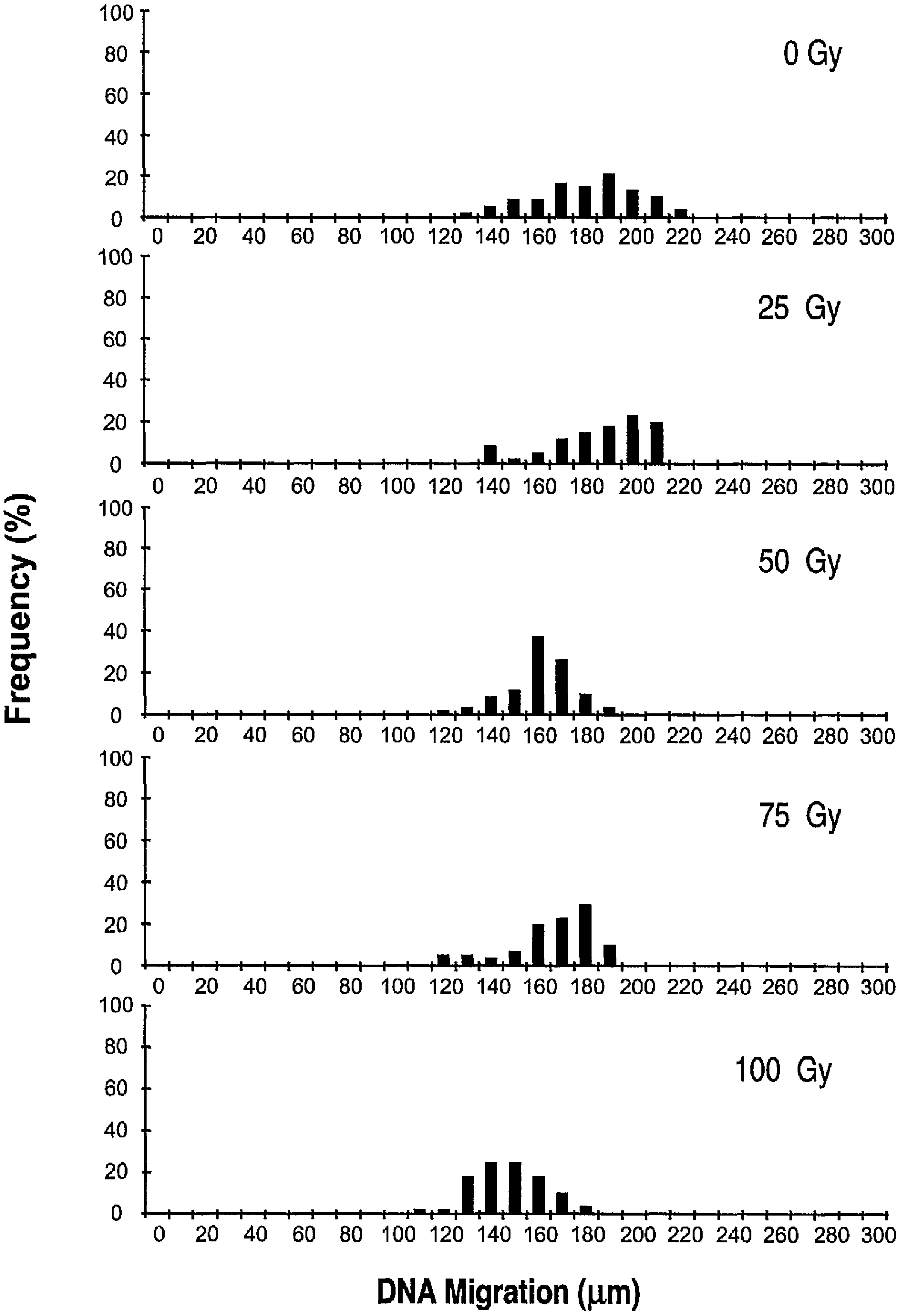

Figure 3.2.3.4 : *Distribution of comet tail lengths at each radiation dose for human spermatozoa analysed by alkaline comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.*

3.2.4 In vitro Irradiation of Human and Mouse Lymphocytes - Neutral Comet Assay.

The comet assay has been widely used to look at DNA damage and repair in a number of somatic cells types (e.g. lymphocytes, fibroblast, cultured cell lines). Therefore mouse and human lymphocytes were also exposed to similar doses of radiation as spermatozoa in order to compare their relative responses. Lymphocytes are generally regarded as radiosensitive whilst spermatozoa are thought to be radioresistant. This appears to be reflected in the high doses of radiation required to produce measurable damage in spermatozoa in the previous experiment.

As with spermatozoa, *in vitro* irradiation of lymphocytes produced an increase in comet tail length (Figures 3.2.4.1). Control cells from both species showed a typical round undamaged appearance with mean tail length in unirradiated lymphocytes being 25.5µm (Mouse) and 32.0µm (Human). These values are comparable to the data obtained with spermatozoa. Irradiation with 75Gy of ^{137}Cs γ-rays increased mean tail lengths to 130.0µm (Mouse) and 91.6µm (Human). Although it is evident that irradiation produced increases in DNA migration, the increases in tail length were smaller in lymphocytes than those recorded with spermatozoa (234.8µm - 75Gy, Mouse and 132.1µm - 75Gy, Human). Although irradiation of lymphocytes did produce dose-dependent increases in comet tail length it is evident from Figure 3.2.4.1 that this did not follow a linear relationship, at least for human lymphocytes. There appears to be a big increase in DNA damage as detected by comet tail length over the first two doses but the tail lengths remain constant with no increase observed with increasing radiation dose. Also, dramatic differences in lymphocyte comet parameters were not observed between species as was noted earlier with mouse and human spermatozoa. The results for comet tail moment generally reflect that of comet tail length with a non-linear relationship for human lymphocytes with tail moment increasing in a dose-dependent manner up to 50Gy before falling slightly at a dose of 75Gy. Mouse lymphocytes showed dose-dependent increases in comet tail moment across all doses of irradiation used. As with % tail DNA values, tail moment results for unirradiated mouse and human lymphocytes were similar (2.9 Mouse and 4.3 Human) to each other and comparable to mouse spermatozoa (Table 3.2.4.1). However they were significantly lower than background tail moment values for control human spermatozoa.

From Table 3.2.4.1 it can be seen that irradiation causes more DNA to move out of the nucleus and into the tail of the comet. Control tail DNA values are 8.7% (Mouse) and 10.1% (Human) which are similar to that of unirradiated mouse spermatozoa (10.4%). However, these are significantly lower than the control values for human spermatozoa (33.3%) which again suggests that human sperm have a higher degree of background DNA breaks than both mouse sperm and lymphocytes from both species. If a comparison of % tail DNA is made between sperm and lymphocytes it is clear that up to a dose of 25Gy, irradiation causes similar amounts of DNA to be released into the tail. However at doses above 25Gy it appears that more DNA is released from spermatozoa than lymphocytes (55.2% Mouse sperm-75Gy compared to 32.8% Mouse Lymphocytes-75Gy).

As with spermatozoa, the frequency histograms show that *in vitro* irradiation of lymphocytes results in a universal response across the whole population with each histogram being represented as a single normal distribution (Figures 3.2.4.3 & 3.2.4.4). It is clear that there is not such a pronounced increase in comet tail length in lymphocytes compared to spermatozoa and the plateauing of response at higher doses of radiation is clearly evident from the histograms.

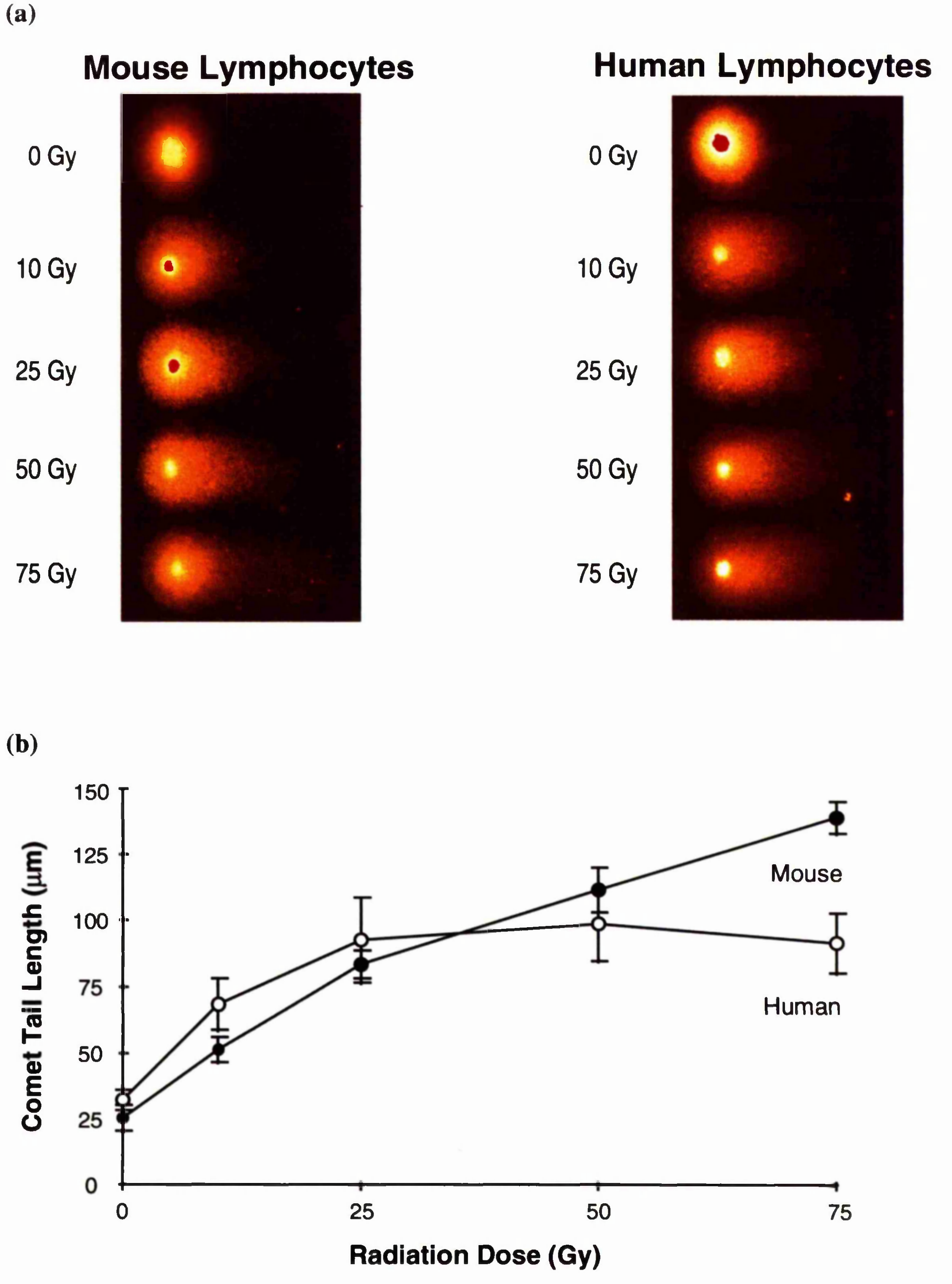

Figure 3.2.4.1 *: DNA damage in mouse and human lymphocytes after in vitro irradiation with* ^{60}*Co γ-rays as measured by neutral comet assay. (a) Appearance of comets after neutral electrophoresis of irradiated lymphocytes and (b) the relationship between radiation dose and comet tail length. Each data point represents the mean results from 3 experimental subjects, 100 cells scored per subject.*

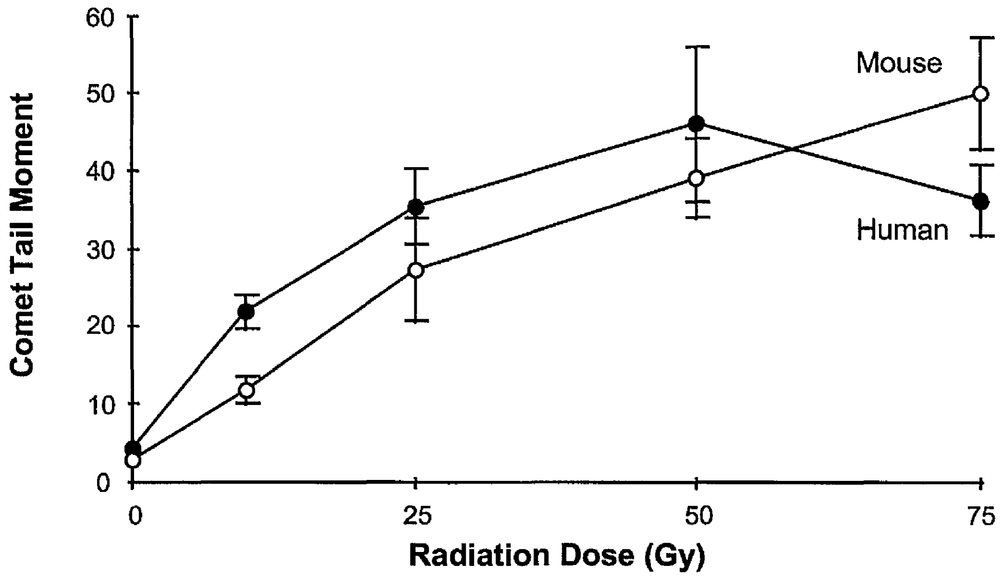

***Figure 3.2.4.2** : Effects of In vitro irradiation of mouse and human lymphocytes with ^{60}Co γ-rays upon neutral comet tail moment. Each data point represents the mean results from 3 experimental subjects, 100 cells analysed per subject.*

***Table 3.2.4.1** : Effects of in vitro irradiation of mouse and human lymphocytes upon percentage of the total DNA found in the tail of the comet as assessed by neutral comet assay.*

	TAIL DNA (%)	
Dose (Gy)	*Mouse*	*Human*
0	8.7 ± 0.8	10.1 ± 1.4
10	26.4 ± 5.7**	31.6 ± 3.2**
25	30.7 ± 9.1**	37.2 ± 3.6**
50	32.8 ± 5.4**	42.5 ± 2.5**
75	39.2 ± 3.1**	35.8 ± 1.4**

Results for each dose represent the mean ± S.E.M from 3 experimental subjects, 100 cells analysed per subject. Asterisks indicate values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's test)

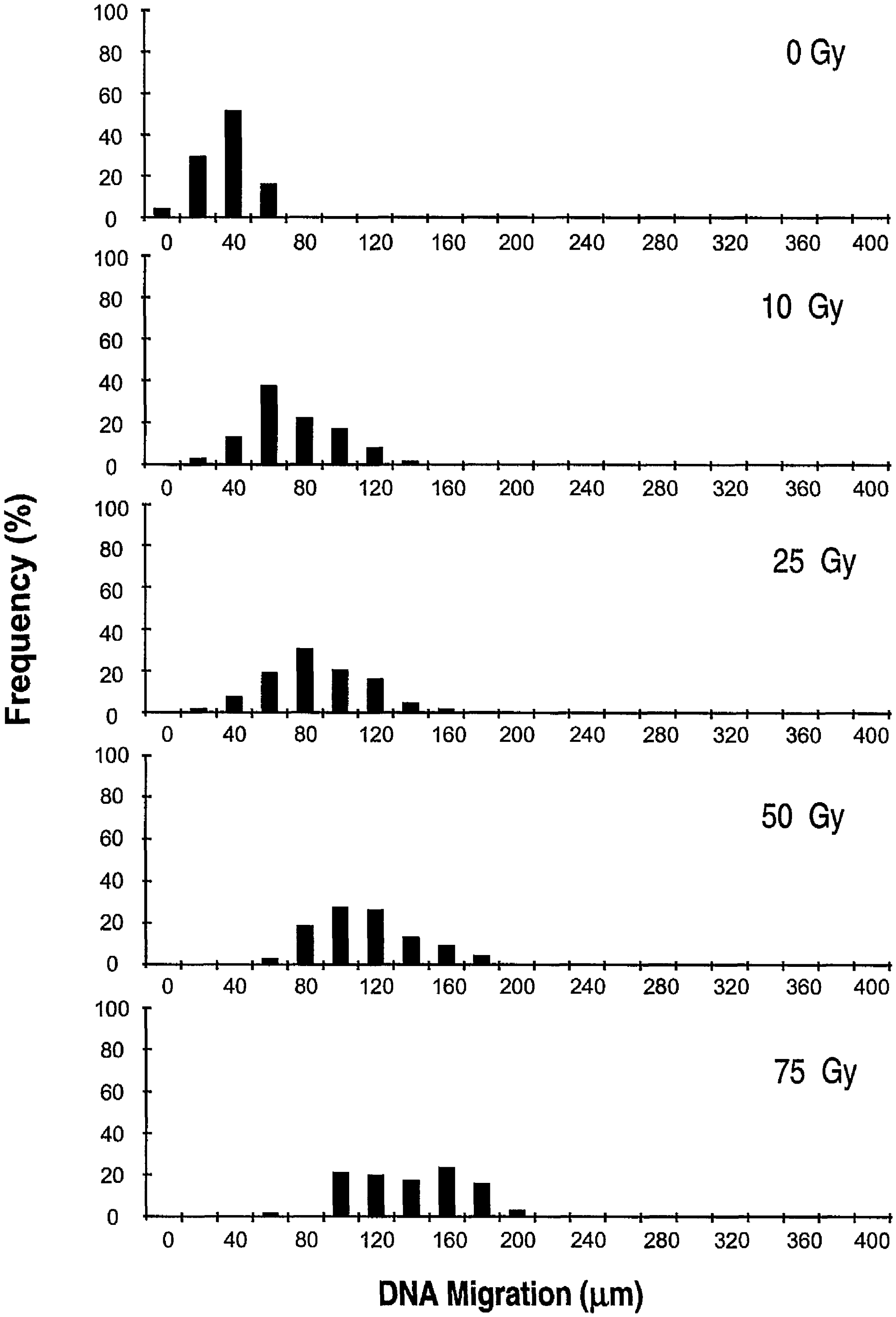

Figure 3.2.4.3 : *Distribution of comet tail lengths at each radiation dose for mouse lymphocytes analysed by neutral comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.*

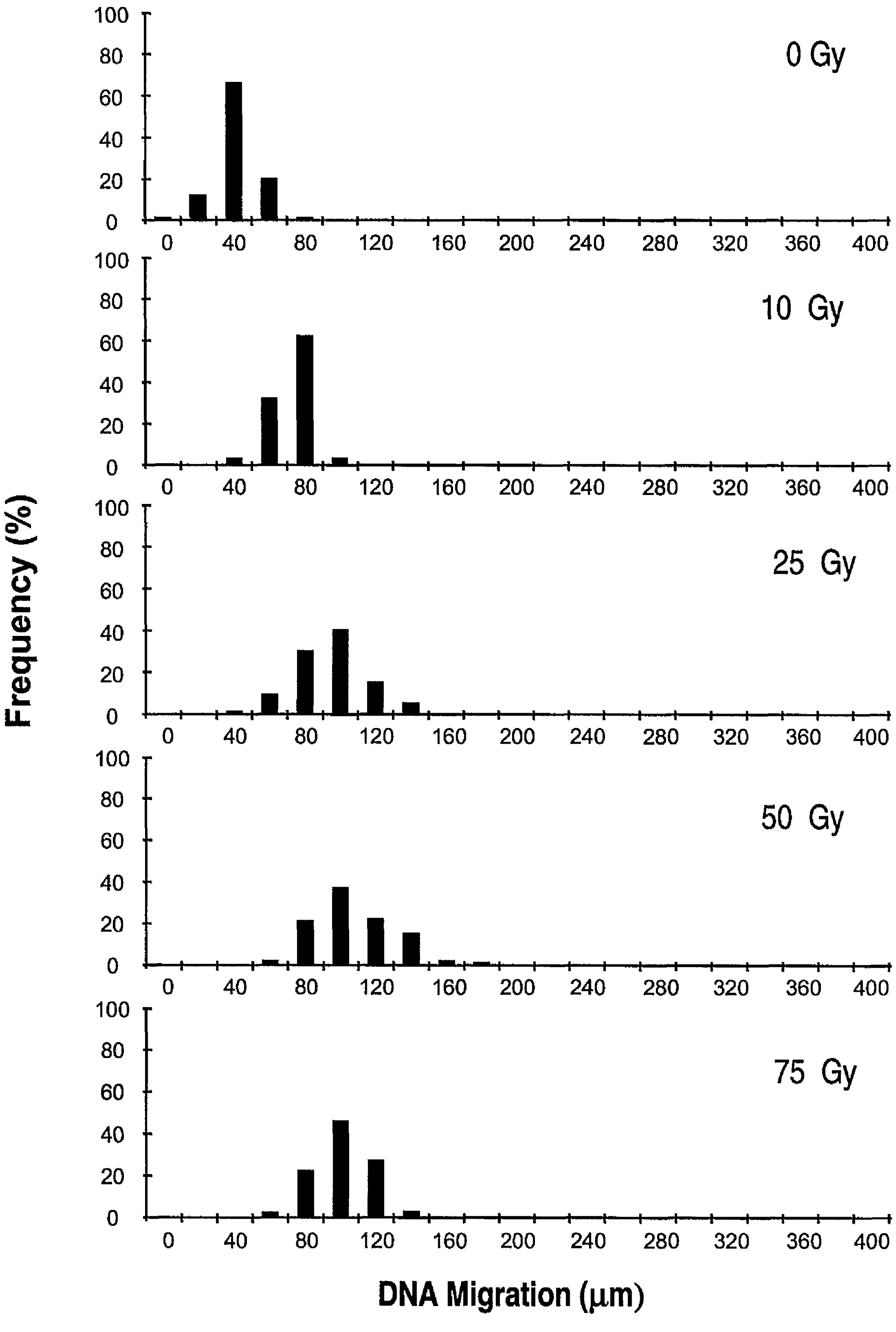

__Figure 3.2.4.4__ : Distribution of comet tail lengths at each radiation dose for human lymphocytes analysed by neutral comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.

3.2.5 *In vitro* Irradiation of Human and Mouse Lymphocytes - Alkaline Comet Assay.

Unlike spermatozoa, alkaline comet assay of lymphocytes produced totally different comets to those previously described for neutral comet assay. Alkaline unwinding and electrophoresis of unirradiated mouse and human lymphocytes produced typical round control comets characteristic of undamaged cells (Figure 3.2.5.1). This is in contrast to the comets from unirradiated spermatozoa which had large tails containing >95% of the total DNA. In contrast % tail DNA values for control lymphocytes were very low (8.3% Mouse, 5.6% Human) indicating that most of the DNA is undamaged and remains within the nucleus. This is also reflected by low values for comet tail length (10.4μm Mouse,17.1μm Human) and tail moment (1.1 Mouse, 1.0 Human).

As in the neutral assay, *in vitro* irradiation of mouse and human lymphocytes resulted in dose-dependent increases in comet tail length. From Figure 3.2.5.1 it can clearly be seen that for both mouse and human lymphocytes the relationship between comet tail length and radiation dose was non-linear with the shaper of the curve being hyperbolic. Up to a dose of 25Gy, comet tail length increased rapidly for lymphocytes from both species but at doses above 25Gy a plateau is seen with no significant increase in comet tail length with increasing radiation dose. Unlike results with mouse and human spermatozoa in both neutral and alkaline comet assays there was no significant difference in response as measured by comet tail length between mouse and human lymphocytes. As described above, control comets for both mouse and human lymphocytes showed very little migration of DNA into the tail of the comet. As expected, irradiation of lymphocytes resulted in DNA damage, which resulted in movement of DNA from the nucleus into the tail of the comet. From Table 3.5.2.1 it is evident that doses of 10 and 25Gy produce quite considerable DNA damage with up to 77% of the total DNA being able to migrate and form a comet tail. At doses above 25Gy there is not such a dramatic increase in % tail DNA as most of the DNA has already been released into the tail of the comet. As with tail length there are no significant differences in % tail DNA values for mouse and human lymphocytes at corresponding doses of radiation. The amount of DNA released into the tail of the comet in the alkaline assay is much greater than that observed with the neutral assay. This is because radiation produces more single-strand breaks compared to double-strand breaks therefore at a given radiation dose more breaks will be revealed by the alkaline form of the assay than the neutral.

Results for comet tail moment, reflect what has previously been described for both tail length and % tail DNA. From Figure 3.2.5.2 the hyperbolic nature of the curves can be seen and the plateauing of radiation response at doses >25Gy.

The frequency histograms (Figures 3.2.5.3 & 3.2.5.4) again show that the response of lymphocytes to irradiation is universal across the population with each histogram represented as a normal distribution although at higher doses the range and standard deviation of tail lengths increase. As with previous curves the plateauing of response can be seen at doses above 25Gy.

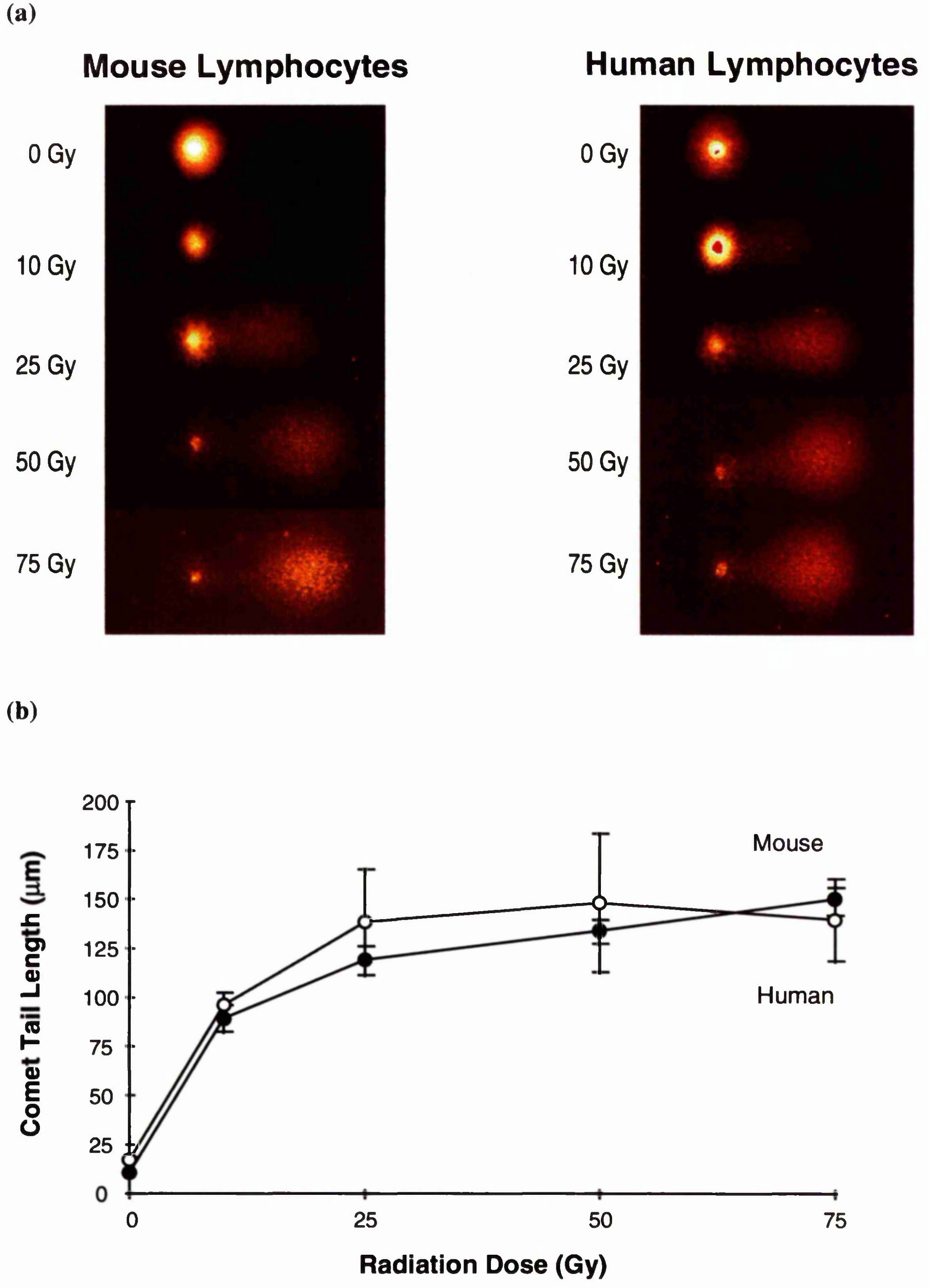

***Figure 3.2.5.1** : DNA damage in mouse and human lymphocytes after in vitro with ^{60}Co γ-rays irradiation as measured by alkaline sperm comet assay. (a) Appearance of comets after alkaline electrophoresis of irradiated sperm cells and (b) the relationship between radiation dose and comet tail length. Each data point represents the mean results from 3 experimental subjects ± S.E.M, 100 cells scored per subject.*

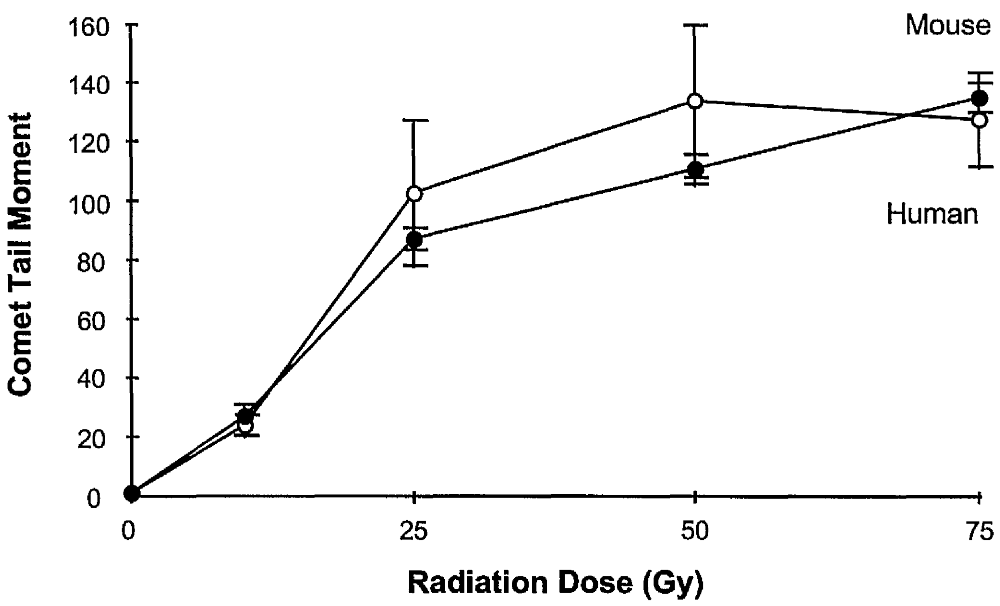

Figure 3.2.5.2 *: Effects of in vitro irradiation of mouse and human lymphocytes with ^{60}Co γ-rays upon alkaline comet tail moment. Each data point represents the mean results from 3 experimental subjects, 100 cells analysed per subject.*

Table 3.2.5.1 *: Effects of in vitro irradiation of mouse and human lymphocytes upon percentage of the total DNA found in the tail of the comet as assessed by alkaline comet assay*

	TAIL DNA (%)	
Dose (Gy)	*Mouse*	*Human*
0	8.3 ± 1.7	5.6 ± 1.9
10	32.9 ± 3.8**	24.7 ± 3.7**
25	77.4 ± 2.5**	74.3 ± 2.0**
50	87.0 ± 4.6**	90.7 ± 3.0**
75	91.6 ± 2.3**	91.4 ± 1.7**

Results for each dose represent the mean ± S.E.M from 3 experimental subjects, 100 cells analysed per subject. Asterisks indicate values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferonni's post-hoc test).

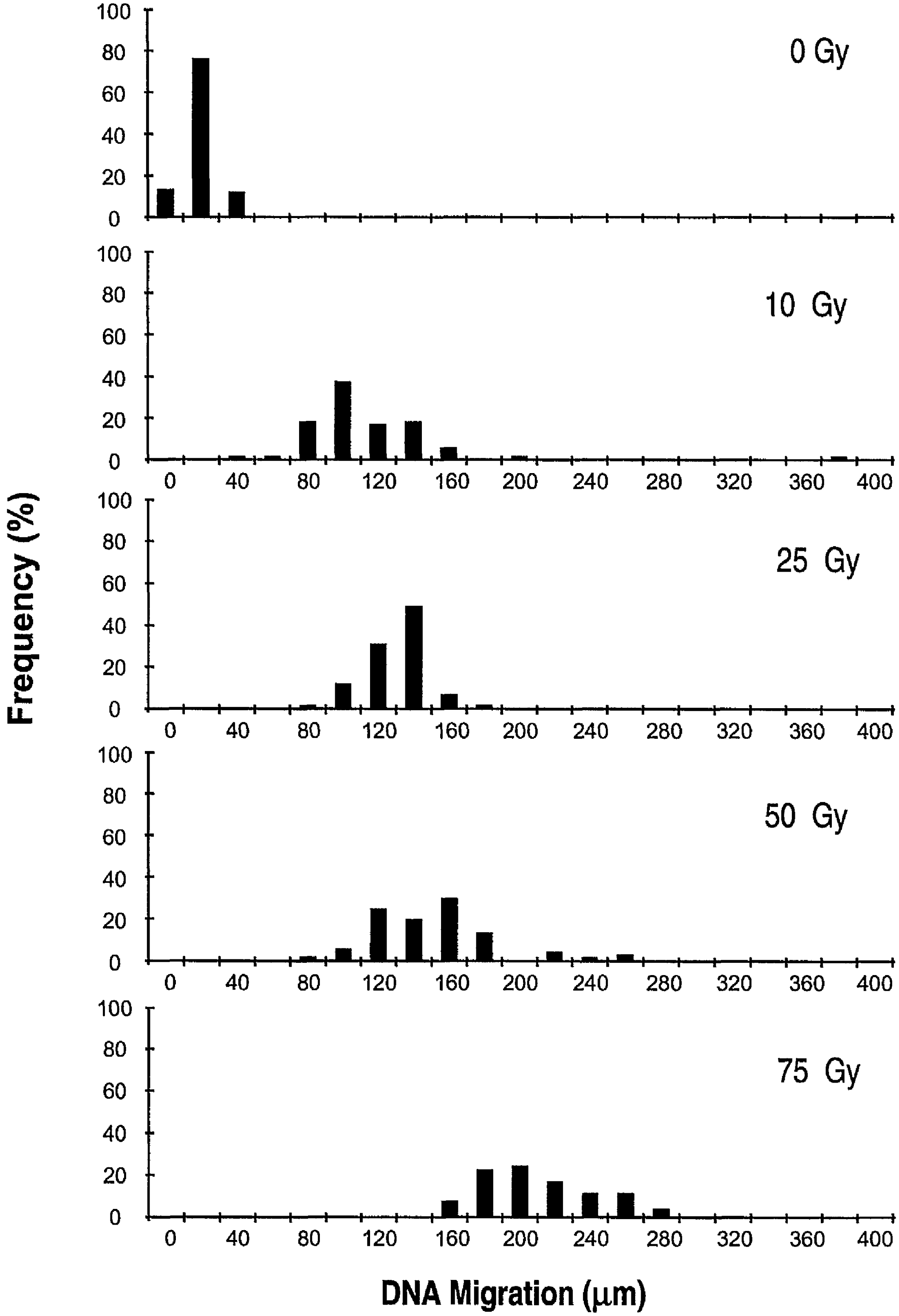

Figure 3.2.5.3 *: Distribution of comet tail lengths at each radiation dose for mouse lymphocytes analysed by alkaline comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.*

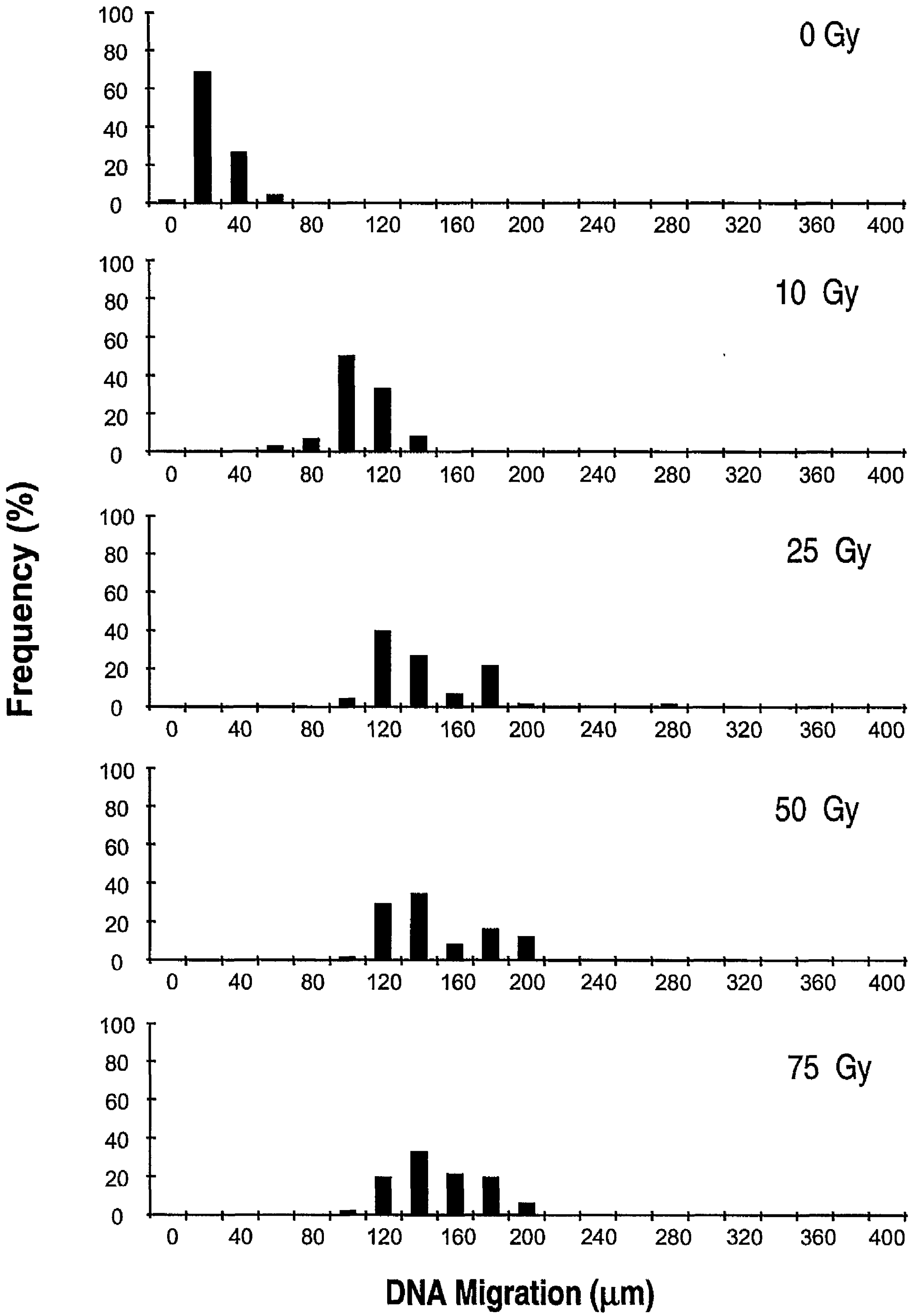

Figure 3.2.5.4 : *Distribution of comet tail lengths at each radiation dose for mouse lymphocytes analysed by alkaline comet assay. Data shown is representative of 3 experimental subjects, 100 cells analysed per subject.*

3.3 DISCUSSION.

In contrast to the majority of other germ cells within the testis, spermatozoa have no DNA repair mechanisms (Ono & Okada, 1977). This may be due to the tightly packed chromatin structure of spermatozoal DNA which restricts access of the DNA repair machinery (Wheeler & Wierowski, 1983) and/or the loss of the DNA repair machinery itself during spermiogenesis (Sega, 1976; Van Loon *et al.,* 1991). The lack of any effective DNA repair mechanisms in spermatozoa suggest there is the possibility that damage induced in nuclear sperm DNA may manifest itself in the offspring via transmission to the embryo at fertilisation. Therefore it is extremely important in the field of reproductive toxicology to have techniques that enable the screening of genotoxic compounds to examine possible effects on sperm DNA. This chapter has described the potential of the comet assay to detect DNA damage induced in mammalian sperm by *in vitro* irradiation. Effects on lymphocytes are also presented as a comparison of effects in somatic cells.

Established cell lysis techniques (Fairbairn *et al.,* 1995) are ineffective when applied to sperm cells (Sega *et al.,* 1986; Singh *et al.,* 1989; Aravindan *et al.,* 1997; Singh & Stephens, 1998). Unlike somatic cells, spermatozoal DNA is not wound round histones and packaged into solenoids but is instead bound by protamine molecules and stabilised by disulphide cross-links resulting in non-supercoiled linear side-by side arrays of chromatin (Figure 3.3.1), (Ward & Coffey, 1991). This results in the DNA being six-fold more highly condensed than in somatic cells, rendering conventional cell lysis techniques ineffective. Therefore in order to ensure effective lysis of sperm DNA, existing comet protocols were modified. Previous publications have reported the use of comet assay in measuring DNA damage in human spermatozoa from fertile and infertile patients (Hughes *et al.,* 1996) and on the protection of human spermatozoa from free radical damage by the use of antioxidant chemicals (Hughes *et al.,* 1998). The protocol employed in these studies used a high salt lysis buffer similar to that used in conventional comet assays but additionally performed an extended lysis overnight at

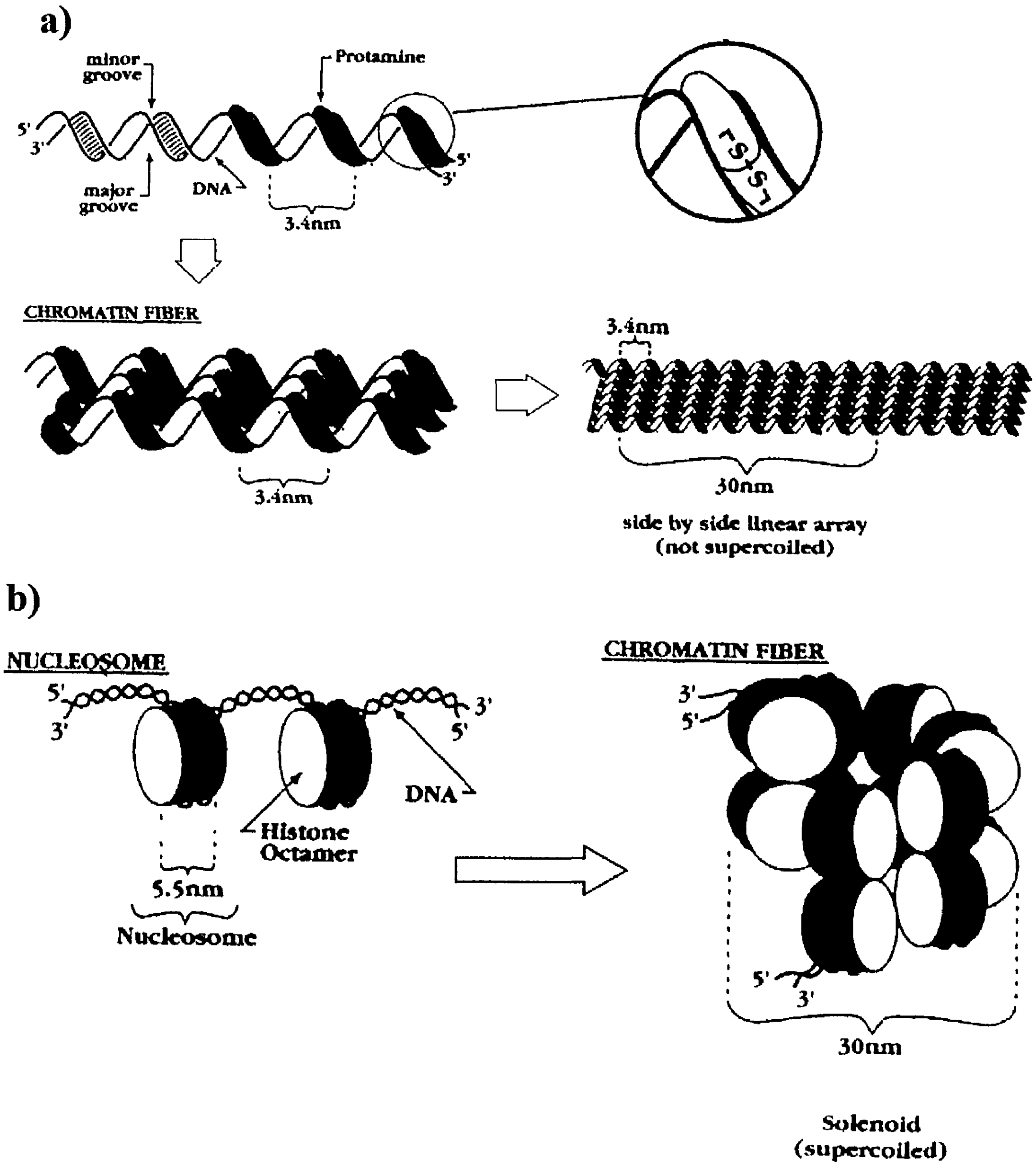
a)
minor groove
Protamine
5'
3'
5'
3'
major groove
DNA
3.4nm
CHROMATIN FIBER
3.4nm
3.4nm
30nm
side by side linear array
(not supercoiled)
b)
NUCLEOSOME
5'
3'
3'
5'
DNA
Histone Octamer
5.5nm
Nucleosome
CHROMATIN FIBER
3'
5'
5'
3'
30nm
Solenoid
(supercoiled)

Figure 3.3.1. *: Nuclear DNA structure : a) Sperm Nuclear Chromatin, b) Somatic cell Nuclear Chromatin. Reproduced from Ward & Coffey (1991).*

37°C in the presence of proteinase K to aid protamine extraction and digestion. These conditions were tried in our laboratory with mouse sperm samples but results were unsuccessful with no DNA migration observed after irradiation. With human samples, results were more encouraging with some degree of lysis achieved but when experiments were repeated a number of times the results proved inconsistent. The reason for the failure of these protocols may be explained by the structure of sperm chromatin. As previously described mouse sperm chromatin is extremely compact with disulphide bonds between protamine molecules (Ward & Coffey, 1991). Therefore even with an extended lysis in the presence of proteinase K it may not be possible to achieve adequate protein displacement and digestion without first loosening the rigid structure of the DNA by disrupting the covalent disulphide bonds. Reducing agents such as β-mercaptoethanol and dithiothreitol (DTT) may be used to achieve this (Sega *et al.,* 1986; Ward & Coffey, 1989, 1991). With human sperm samples some degree of lysis was achieved, although results were inconsistent. The difference between the human and mouse samples may possibly be explained by differences in their chromatin structure (Balhorn, 1982; Ward & Coffey, 1991). Human sperm, although packed in a linear manner like mouse sperm chromatin may not be as highly condensed. Therefore some degree of lysis may be able to take place with just high salt and proteinase K. However human spermatozoa still contain disulphide bonds and therefore complete lysis and removal of all DNA associated proteins may not be possible without the use of reducing agents.

Another publication on sperm comet assay (Singh *et al.,* 1989) used proteinase K with an extended lysis technique but also included the reducing agent β-mercaptoethanol. This method was also tried but in our laboratory resulted in extreme lysis of the DNA and together with the high electrophoresis current used in this protocol resulted in the loss of DNA from the gels. It is possible that over exposure to reducing agents may also result in DNA damage (Sega *et al.,* 1986) which may complicate the assay. Although it was felt that a reducing agent was needed, the activity of β-mercaptoethanol was too harsh and impractical to manage effectively. Therefore it was decided to try another reducing agent and dithiothreitol (DTT) was chosen instead as it is a well-established biochemical reagent. From protocols describing the extraction of sperm DNA in the literature, a concentration of 40mM DTT was chosen (Ward & Coffey, 1989). In a series of experiments it was found that 1 hour lysis at room temperature was still not sufficient even in the presence of reducing agent, whilst overnight lysis at 37°C resulted in over digestion and lysis of sperm DNA. Finally it was determined that sufficient lysis could be achieved with a 1 hour initial lysis period at room temperature followed by 2½ hours at 37°C. For consistency of methods, DTT was included in the lysis buffer of both mouse and human sperm samples.

The comet assay permits both double-stranded and single-stranded DNA breaks to be measured depending on the electrophoresis conditions used (Fairbairn *et al.,* 1995). If microgel electrophoresis is performed in neutral buffer (e.g. TBE, pH~8.5) then DNA remains double-stranded and will migrate most readily in the regions of DNA breaks permitting the visualisation of double-strand DNA breaks. However, if microgels are placed in alkaline buffer (commonly containing NaOH and pH >12.3) the high pH promotes disruption of the complementary base pairs holding the helix together and the strands can be unwound to form single-stranded DNA, allowing the detection of single-strand breaks. The experiments described in this chapter examined the effectiveness of both the neutral and alkaline forms of the assay in measuring *in vitro* induced radiation damage in human and mouse spermatozoa.

Results from the neutral comet assay (which supposedly measures double-stranded DNA breaks) showed that *in vitro* irradiation of mouse and human spermatozoa with ^{137}Cs γ-rays produced a dose-dependent linear increase in DNA damage as measured by DNA migration, comet tail moment and % tail DNA. This is in agreement with previous comet studies in somatic cells, which suggest that migration of DNA from the nucleus is proportional to the extent of DNA damage (Singh *et al.,* 1988; Fairbairn *et al.,* 1995). Irradiation produces strand breaks in DNA and these cause both relaxation of DNA loop domains and the production of DNA fragments which are free to migrate upon application of an electric current (Fairbairn *et al.,* 1995). It can clearly be seen that irradiation produced an increase in % Tail DNA. As the degree of damage increased, as well as more DNA being released into the tail the DNA was able to migrate further along the gels producing increases in both comet tail length and comet tail moment. In this study, *in vitro* irradiation of lymphocytes over a similar dose range as spermatozoa also produced dose dependent increases in comet parameters although unlike spermatozoa these did not follow a linear relationship. This may reflect differences in the relative radiosensitivities of spermatozoa and lymphocytes.

Significant differences were observed in baseline DNA damage (as measured by comet tail moment) between mouse and human sperm. Background levels of DNA damage were also higher in human sperm compared to lymphocytes from both species. It is well established that human semen is very heterogeneous in quality (World Health

Organisation, 1992) and estimates have suggested that 10-15% of spermatozoa in the human ejaculate are genetically abnormal (Brandriff *et al.,* 1985; Martin *et al.,* 1987; Mikamo *et al.,* 1990). In contrast, cytogenetic studies with sperm from laboratory animals and with human lymphocytes have suggested that much lower background incidences of chromosome aberrations are present in these cell types (Matsuda *et al.,* 1985c; Brandriff *et al.,* 1988; Mikamo *et al.,* 1991). Therefore the higher levels of baseline damage observed in this study may reflect the heterogeneity of human sperm compared to spermatozoa from other species and also other cell types.

The minimum dose of radiation examined in this study and required to produce measurable amounts of DNA damage in spermatozoa after neutral comet assay was 25Gy (preliminary data, not shown). Therefore, as a result the doses that were chosen for this study with spermatozoa ranged from 0-100Gy. These doses are a lot higher than previous studies have reported necessary to induce DNA strand breaks in somatic cells (Singh *et al.,* 1988; Vijayalaxami *et al.,* 1993) and germ cells within the testis (Van Loon *et al.,* 1991, 1993). It has previously been demonstrated however, that mature spermatozoa are extremely resistant to the effects of radiation and chemotherapy compared to other germ cells within the testis (Meistrich, 1986a, 1993; Ogilvy-Stuart & Shalet, 1993). Previous studies have reported that the LD_{50} of mouse spermatozoa to radiation is 600Gy compared to a dose of 0.5Gy for proliferating spermatogonia (Meistrich, 1986a). Another study, which also used the comet assay to detect radiation-induced DNA damage in human sperm, could only detect effects after *in vitro* irradiation with 30Gy of X-rays (Hughes *et al.,* 1996). This is comparable to the doses described in this chapter and therefore also suggests that spermatozoa are radioresistant. This high radioresistance of spermatozoa is probably a consequence of the highly condensed nature of sperm DNA and previous studies have demonstrated that chromatin structure can protect DNA from strand breaks produced by irradiation (Warters & Lyons, 1992; Elia & Bradley, 1992; Nygren *et al.,* 1995). Therefore the high doses of radiation used in this experiment may not reflect insensitivity of the technique but may instead be due to the extreme radioresistance of spermatozoa. After irradiation of lymphocytes, which are generally regarded as radiosensitive (Chuckhlovin *et al.,* 1995), DNA damage was detected at the lowest dose of 10Gy by neutral comet assay. Previous reports have demonstrated that DNA damage can be detected in human lymphocytes using comet assay after *in vitro* irradiation with 2Gy ^{137}Cs γ-rays (Plappert *et al.,* 1995).

Although this dose is much lower than the 10Gy dose used in this chapter, smaller doses were not investigated as the main aim of the experiments was to try and examine effects of comparable doses of radiation in sperm and lymphocytes. Studies have also been described in the literature reporting the detection of single-strand breaks in lymphocytes using modified alkaline comet assay techniques at dose ranging from 0.05-0.5Gy (Vijayalaxami *et al.,* 1993). These results therefore suggest that lymphocytes are more radiosensitive than spermatozoa.

A recent study compared the *in vitro* effects of estrogenic chemicals and reproductive genotoxins on human lymphocytes and spermatozoa using the comet assay (Anderson *et al.,* 1997b). The group observed positive results (increased tail moments and decreased % head DNA) in both lymphocytes and sperm comet assays over similar dose ranges with the majority of agents tested. This led them to the conclusion that lymphocytes and spermatozoa were equally sensitive to the DNA damaging effects of these agents. This is in contrast to the findings of this study and other reports in the literature as spermatozoa are generally regarded as resistant to the effects of chemotherapy and radiation (Meistrich, 1986a). However, in these studies the investigators group used high doses of the test chemicals and examined only positive effects in the comet assay (i.e. formation of a comet) to reach this conclusion rather than comparing DNA damage levels directly between the two cell types. This may explain the differences between the findings of this study and the results presented in this chapter.

Despite earlier evidence suggesting that spermatozoa are extremely radioresistant and that high doses of radiation are required *in vitro* to produce measurable DNA damage, a study has recently been published reporting the detection of DNA double-strand breaks in human spermatozoa after X-irradiation *in vitro* with doses ranging from 0.25-1Gy (Singh & Stephens, 1998). These doses are much lower than those reported in previous studies which utilised the comet assay to measure radiation-induced DNA damage in spermatozoa (Hughes *et al.,* 1996, 1997; McKelvey-Martin *et al.,* 1997). Therefore this new technique appears to permit extremely sensitive detection of DNA strand breaks in human spermatozoa. Previous studies have demonstrated that alkaline comet assays which measure single-strand breaks are able to detect DNA damage after irradiation with much lower doses than are needed to detect increases in DNA strand breaks using neutral comet assays (Fairbairn *et al.,* 1995). This reflects the fact that radiation induces preferentially single strand breaks in DNA rather than double strand breaks. Previous reports have suggested that exposure of cell to 1Gy of X-irradiation will yield approximately 1000 single-strand breaks yet only 70 double strand-breaks (Ward, 1990). Therefore these Figures suggest that this new comet method can resolve levels of DNA double-strand breaks to a detection limit of only 17 per spermatozoon. This is much more sensitive than has previously been reported with the neutral assay although the same group has published new methodologies over the years reporting improvement and development of the alkaline comet assay for maximum sensitivity (Singh *et al.,*1994, 1995). Cytogenetic studies with *in vitro* irradiated spermatozoa have reported increases in the incidence of structural chromosome aberrations after exposure to doses from 0.23 - 2Gy which are similar to the doses reported with this new comet assay. Cytogenetic techniques are regarded as highly sensitive, as chromosome aberrations can arise as a result of a single lesion in DNA. However, the methodology is extremely technical and time-consuming and therefore limits the numbers of spermatozoa that can be analysed. Therefore it appears remarkable that this new method can resolve DNA of strand breaks to levels comparable with cytogenetic studies.

Possible explanations for the difference between results reported in this chapter and this new technique which appears to offer extremely sensitive detection of DNA strand breaks (Singh & Stephens, 1998) may be attributed to differences in the methodology. Firstly, this new technique uses an extremely extended lysis period (20 hours total lysis) with exposure to RNase and extremely high concentrations of proteinase K (1mg/ml). These conditions may achieve a greater degree of nuclear protein degradation than was achieved using the methods described in this chapter although interestingly exposure to reducing agents was apparently not required during the lysis as reported by this new study even thought it is well established that disulphide bonds present in sperm chromatin allow it to maintain a highly condensed structure. Instead the authors reported that the inclusion of RNase in the lysis buffers was essential for the complete lysis of sperm DNA. This is somewhat surprising as spermatozoa lose most of their RNA when elimination of the majority of the cytoplasm occurs during spermiogenesis (Passot *et al.,* 1989). However it has been reported that sperm cells still contain small amounts of RNA and the authors of this new comet study postulate that RNA may be bound to the DNA like proteins in spermatozoa and play a role in the packaging of DNA in sperm cells. As well as an extended lysis step, this new comet protocol carried out electrophoresis for 1 hour instead of 20 minutes described in previous comet protocols and performed on samples in the experiments described in this thesis. Ammonium acetate has also been used to precipitate DNA in the gels post-electrophoresis and staining was performed with YOYO-1 (instead of SYBR Green used in this chapter) which is a new generation fluorescent DNA binding dye which allows extremely sensitive detection of DNA. These differences between the protocols may account for the differences in the sensitivities of this technique and the comet assay described in this chapter. However, due to the recent publication of this technique it has not been possible to replicate the experiments and the technique has only been described in human spermatozoa so it is not known whether it will be applicable to mouse spermatozoa. Further studies are warranted to investigate and compare this new technique with that described in this thesis. It may be possible to improve and refine the comet assay described in this chapter by performing extended electrophoresis and using alternative DNA stains to improve the sensitivity.

At comparable levels of radiation, higher levels of DNA damage were observed in mouse compared with human spermatozoa. This could suggest that mouse spermatozoa are more radiosensitive than human sperm to the effect of *in vitro* γ-irradiation. However, it is probably unwise to compare two vastly different populations of spermatozoa from two different species so closely. The quality of sperm in the human ejaculate is very heterogeneous with as little as 30% morphologically normal sperm (World Health Organisation, 1992) and other studies have suggested that approximately 10-15% of human sperm are genetically abnormal (Brandriff *et al.,* 1985; Martin *et al.,* 1987; Mikamo *et al.,* 1990). In contrast, murine sperm samples were obtained from the vas deferens at post-mortem, which provides a very homogenous sperm population with a high percentage (>95%) of normal sperm. Therefore, sample origin may contribute to the differences in results observed between the two species in this study. Secondly, the conditions for this comet assay were initially developed using the mouse as a model and then applied to human sperm. There may be differences in the DNA packaging between the two species (Balhorn, 1982) and this may contribute to the effective removal of DNA associated proteins during the lysis step which can affect DNA migration during electrophoresis. There may also be differences in the size of the nuclear DNA loop domains between the two species, which may contribute to the distance that the DNA can migrate during electrophoresis.

Although *in vitro* irradiation of both sperm and lymphocytes produced increases in comet parameters there were significant differences between spermatozoa and somatic cells. In general at equivalent doses of radiation higher comet parameters were observed in spermatozoa than lymphocytes even though spermatozoa are thought of as radioresistant (Meistrich, 1986a) and lymphocytes radiosensitive (Chuckhlovin *et al.,* 1995; Singh *et al.,* 1995). However it is difficult to directly compare results from sperm and lymphocytes because of the significant differences in DNA structure and DNA content as has previously been described (Ward & Coffey, 1991). Although there are differences in protein-DNA organisation in somatic cells and sperm, both cell types show organisation of their DNA into loop domains within the nuclear matrix. However there may be differences in the size of the loop domains between sperm and somatic cells and DNA in sperm is also organised around another structure termed the nuclear annulus which is not present in somatic cell nuclei (Ward & Coffey, 1989). Therefore these differences in nuclear structure may account for the differences observed in the neutral comet assay in this study.

In the alkaline comet assay (which detects single-strand DNA breaks) extensive migration of DNA from the nucleus was observed in unirradiated spermatozoa from both humans and mice. Normally this would be interpreted to suggest a high level of DNA damage but since in this case approximately 95% of the DNA has migrated out of the nucleus without exposure to a DNA damaging agent, it appears more likely that this high background level of DNA single-strand breaks in spermatozoa is an artefact of the assay conditions. In a previous study which examined single strand breaks in spermatozoa using an alkaline comet assay, similar results were observed to those described in this chapter with all of the DNA migrating out of the nucleus (Singh *et al.,* 1989). It is unlikely that spermatozoa carry such high levels of single-strand breaks within their DNA since this level of damage would translate to approximately 10^6-10^7 single strand breaks per cell, (Singh *et al.,* 1989). Also, high background levels of DNA damage were not observed in unirradiated sperm subjected to neutral comet assay nor lymphocytes processed by alkaline comet assay as described earlier in this chapter and in previously reported studies (Singh *et al.,* 1988; Vijayalaxami *et al.,* 1993). Therefore, these breaks may represent high numbers of alkali-labile sites present within spermatozoal DNA which are expressed as single-strand breaks during the alkaline unwinding step of the assay. Other workers have also observed high levels of background DNA damage in mature spermatozoa and elongate spermatids using alkaline elution techniques (Van Loon *et al.,* 1991, 1993). More recently, other groups have used the alkaline comet assay to examine DNA damage levels in human sperm (Hughes *et al.,* 1996, 1997, 1998; McKelvey-Martin *et al.,* 1997; Anderson *et al.,* 1997a, 1997b, 1997d). Although DNA migration from untreated samples in these studies was not seen at such a severe extent as in the experiments described in this chapter and by Singh *et al.,* 1989 study, higher background levels of DNA damage in human sperm (20-30% tail DNA) were observed compared to somatic cells which may reflect the expression of some alkali labile sites. These recent studies used a lysis protocol, which did not include a reducing agent (which was found to be a requirement for mouse sperm lysis in these experiments). Therefore the difference in lysis conditions of these studies and those described in this chapter may account for the difference in results.

Experiments also performed by Singh *et al.,* 1989 showed that extensive DNA migration observed from sperm cells after alkaline electrophoresis was absent when sperm DNA was electrophoresed under neutral conditions. In addition this group showed that when sperm DNA was denatured to single-strands using conditions (heat or formalin treatment) that did not involve alkali treatment, high levels of DNA breaks were not detected. Therefore these data strongly suggest that the high levels of background DNA damage observed in spermatozoa are an artefact of alkali treatment. Denaturation of sperm DNA using heat or formalin treatment was tried in our laboratory but it proved impossible to lyse spermatozoa on slides due to loss of the gels. The exact function of the alkali labile sites present within spermatozoal DNA is not known. However, as these sites are not abundant in somatic cells and it is well known that there are differences in DNA packaging between somatic and sperm cells (Ward & Coffey, 1991), it is possible that these sperm-specific alkali-labile sites are related to the condensed nature of spermatozoal DNA, involving the close and frequent association of DNA and protamine molecules. Other cell types that possess highly condensed DNA have also been shown to have DNA migration patterns similar to spermatozoa and therefore this data also suggests that such alkali-labile sites may be characteristic of condensed chromatin in general (Singh *et al.,* 1989; Fairbairn *et al.,* 1994).

Despite the high background levels of DNA migration seen in control cells a dose-response relationship was still evident in irradiated mouse spermatozoa subjected to alkaline comet assay. A linear increase in DNA migration and comet tail moment was observed with increasing radiation dose. However, no such relationship was seen in irradiated human spermatozoa, with if anything a small decrease in DNA migration observed with increasing radiation dose. The lack of a relationship in human spermatozoa may be due differences between the two species as described previously.

In contrast to the results observed for spermatozoa, increases in DNA damage following irradiation (as detected by tail length, tail moment and % tail DNA) were detectable in both human and mouse lymphocytes after alkali comet assay. As was observed in the neutral assay, the relationship between radiation dose and DNA damage did not follow a linear relationship. There was a dramatic increase in the amount of damage over the first 25Gy and the curve starts to flatten out. Previous studies involving lymphocytes and alkaline comet assay have shown that they are very sensitive to the effects of radiation with damage being detected at extremely low dose of radiation (Vijayalaxami *et al.,* 1993; Singh *et al.,* 1995). As irradiation produces more single strand breaks compared to double strand breaks, the damage response curves increases steeply initially and then flattens out as the DNA is saturated with single-strand breaks. Although differences in the response of sperm and lymphocytes under alkaline electrophoresis conditions make direct comparison of results between somatic and germ cells difficult. This study and the literature suggest that lymphocytes have a higher degree of radiosensitivity compared to spermatozoa.

In summary, this study has demonstrated that radiation-induced DNA damage in spermatozoa can be detected using the comet assay, although the measurement of single-stranded DNA breaks is complicated by high levels of background damage produced by alkali treatment. The high levels of radiation used in this study may reflect the high radioresistance of spermatozoa compared to somatic cells and this may be related to the method of DNA packaging within the sperm nucleus. However humans and animals are unlikely to be exposed to such high doses of radiation either accidentally or as a result of therapeutic procedures. Most radiotherapy regimes use doses lower than 5Gy (Ogilvy-Stuart & Shalet, 1993) which produce profound effects on the testis and reductions in sperm counts. Therefore this suggests that radiosensitive germ cells within the testis are affected by irradiation, rather than spermatozoa themselves. Experiments described in the next chapter were performed *in vivo* with direct testicular irradiation. This was to investigate whether irradiation of germ cells within the testis with therapeutically relevant doses of radiation results in the transmission of DNA damage through the spermatogenic process and whether such damage can be detected in the spermatozoa when subjected to comet analysis.

Chapter 4

Detection and Measurement of DNA Damage in Spermatozoa after Irradiation of Testicular Germ Cells *in vivo.*

4.1 INTRODUCTION.

In the previous chapter, it has been demonstrated how the comet assay can be used to measure DNA damage in mammalian spermatozoa induced by *in vitro* irradiation with ^{60}Co γ-rays. However, the doses of radiation required to produce measurable amounts of damage by *in vitro* irradiation were extremely high (25-100Gy). These doses are much higher than patients would receive if undergoing radiotherapy or if people are exposed to environmentally or by accidental radiation exposure. Indeed doses in the range of 25Gy and above would in fact be lethal to most whole organisms (including humans and animals).

Therefore it appears that spermatozoa themselves are extremely radioresistant to the toxic effects of radiation. However, spermatozoa are the end product of spermatogenesis and are derived from successive divisions and differentiation of individual testicular cells. Within the testis, many different cell types and cellular stages are present. It is well known that these different cell types differ in their responses to radiation because of their differing radiosensitivities (Meistrich, 1986a). Although spermatozoa themselves are extremely radioresistant, precursor cells are in fact radiosensitive and can be damaged by relatively low doses (<5Gy) of radiation. If this damage is not repaired, or repaired ineffectively, then there is the possibility that these cells may continue to develop through the spermatogenic process resulting in the formation of spermatozoa containing damaged DNA.

4.1.1 Radiosensitivity of the Testis.

Within the testis, the differentiating spermatogonia are the most sensitive cells to the killing effects of radiation (Meistrich, 1986a, 1993). This may be because these cells are rapidly dividing and radiation is very effective in killing actively mitotic and dividing cells. It is because of this property that radiotherapy and many cytotoxic drugs are the main treatment for cancer (as they will also kill rapidly dividing tumour cells). Radiation probably induces the death of these cells by damaging their DNA. As these cells are cycling very rapidly there may not be enough time for effective DNA repair to take place before the cells enter the critical phase of their cycle and therefore the cells die rather than pass on the damage to the next generation of cells.

Spermatogonial stem cells are regarded as having an intermediate radiosensitivity (Meistrich *et al.,* 1978; Meistrich, 1986a, 1993). They are more resistant to the killing effect of radiation than differentiating spermatogonia and this is probably because they are not as active mitotically (i.e. they have a lower proliferative index). The spermatogonial stem cells are responsible for repopulation of the seminiferous tubules after cytotoxic insult and therefore ultimately recovery of normal sperm counts (Meistrich *et al.,* 1978; Erickson, 1981; Meistrich, 1986b). Elimination of all the stem cells by radiation or cytotoxic therapy will result in permanent sterility. If the DNA of the stem cells is permanently damaged then this has important implications regarding future fertility as it may result in production of cells containing mutagenic lesions, which may result in abnormal reproductive outcomes.

The spermatocytes and round spermatids are also regarded as being of intermediate radiosensitivity (Meistrich, 1986a, 1993). These cells have only to pass through one round of DNA synthesis and two meiotic divisions compared to the many round of mitosis that the proliferating spermatogonia undergo. These cells may be less sensitive to killing by radiation as they can sustain damage to their DNA and chromosomes and still proceed through the process of differentiation into spermatozoa.

Finally the late spermatids and spermatozoa are regarded as the most radioresistant cells within the testis and are highly refractory to the effects of radiation (Meistrich, 1986a, 1993). This is because these cells are post-mitotic/meiotic and differentiated. Therefore as cell division is not taking place the cells do not pass through any DNA checkpoints. Also the fact that the structure of the DNA has changed by the replacement of the histones with protamine molecules resulting in a highly compacted structure (Balhorn, 1982; Ward & Coffey, 1991) which may make it more resistant to damage may also account for the low radiosensitivity of these cells.

4.1.2 Assessment of Damage after Irradiation.

The assessment of the cell killing effects of radiation has been extensively studied. Germ cell death after irradiation can be assessed by examination of sperm counts (either from the vas deferens/epididymis or testicular sperm head counts) and examination of histological sections of the testis (Oakberg, 1955; Huckins & Oakberg, 1978; Pogany, 1987). By relating the time after irradiation to decreases in sperm counts, the stage of spermatogenesis that has been killed producing a decrease in sperm counts can be elucidated. This can be confirmed by inspecting histological sections of the testis.

Whilst the cell killing effects of radiation are relatively easy to study and characterise as described above, the assessment of the level of damage in spermatogenic cells after irradiation and its effects is somewhat more complicated and has been a problem for many years. Until very recently the direct assessment of DNA damage in sperm after testicular irradiation has been impossible and instead the main test for the detection of mutagenic lesions in sperm DNA has been the dominant lethality assay (Ehling, 1971; Searle & Beechey, 1974; Russell *et al.,* 1998).

4.1.3 Effects of Radiation in Producing Dominant Lethal Mutations.

The dominant lethal assay has widely been used to assess the mutagenic effects of agents after paternal exposure (Ehling, 1971; Searle & Beechey, 1974; Goldstein *et al.,* 1978; Dobrzynska *et al.,* 1990; Dobrzynska & Gajewski, 1994; Russell *et al.,* 1998). The assay detects the failure of fertilised eggs to develop and it is inferred that paternal exposure to a toxic agent may cause an increase in dominant lethal mutations by transmission of a genetic defect, which prevents development after fertilisation.

It is widely accepted that exposure of the testis to ionising radiation leads to an increase in the frequency of dominant lethal mutations. Ehling, (1971) irradiated male mice with 2Gy, 4Gy and 8Gy of X-rays and mated irradiated animals with females at various timepoints after irradiation in order to assess the yield of dominant lethal mutations induced by irradiation of different spermatogenic stages. It was found that radiation induced dominant lethal mutations in all spermatogenic stages. After 2Gy of irradiation the yield of dominant lethals was highest in early spermatids whilst after irradiation with 4 or 8Gy the most sensitive stage for the induction of dominant lethals were the spermatocytes.

Searle and Beechey, (1974) also investigated the induction of dominant lethal mutations after paternal irradiation with X-rays (2Gy). This group argued that the high levels of dominant lethals observed in spermatocytes in Ehling's study was in fact due to increased failure of fertilisation rather than embryonic death after fertilisation. This group concluded that early spermatids were the most sensitive cell stage to the effects of radiation on dominant lethal mutations. A large retrospective study published recently (Russell *et al.,* 1998) has also concluded that the most sensitive stages for radiation induced dominant lethals are early spermatids

Thus it appears that the induction of dominant lethal mutations does not mirror the radiosensitivity of the testis in terms of cell killing. It is known that proliferating spermatogonia are the most radiosensitive cells within the testis (Meistrich, 1986a, 1993) and so these would be expected to receive the most damage after a single dose. However the highest frequencies of dominant lethals are recorded following irradiation of the relatively resistant spermatid stages (Ehling, 1971; Searle & Beechey, 1974; Russell *et al.,* 1998). Indeed it has proved quite problematic to accurately assess the effects of paternal irradiation on dominant lethals from pre-meiotic stages. One problem that confounds the measurement of dominant lethals from pre-meiotic stages is the decline in sperm counts that occurs after spermatogonial irradiation (Searle & Beechey, 1974; Pogany, 1987; Meistrich, 1993). Because of their radiosensitivity many spermatogonia are killed by irradiation resulting in temporal decreases in sperm counts which has the effect of lowering the fertilisation rates and complicating the detection of embryonic arrests. Also, it is well known that spermatogonia and spermatocytes have active DNA repair mechanisms (Bradley & Dysart, 1985; Coogan & Rosenblum, 1988; Van Loon *et al.,* 1991) which are not present in later germ cell stages which may be able to effectively repair DNA damage therefore producing the low rate of dominant lethals observed for these cell stages.

4.1.4 Other Techniques for Assessing the Effects of Sperm DNA Damage.

Measurement of dominant lethal mutations is technical, generally laborious and requires analysis of large numbers of animals and cells. Sperm based methods of assessing toxicity offer an attractive alternative since they are generally non-invasive and allow the collection of large numbers of cells (individual samples) from very few animals.

One new technique that can be applied to this problem is flow cytometry, which utilises laser scanning to analyse changes in DNA when stained with fluorescent probes. Pinkel *et al.,* (1983) used flow cytometry to investigate changes in the DNA content of mouse sperm after exposure to X-rays. Animals were testicularly irradiated with doses of X-rays (0, 1, 2, 3, 4.5 and 6Gy) and spermatozoa collected from the cauda-epididymis 35 days post irradiation. At doses of 3Gy and above sperm showed abnormal DNA content compared to controls. It was also shown that radiation also produced dose-dependent increases in sperm shape abnormalities analysed by conventional light microscopy. This has also been demonstrated previously by Bruce *et al.,* (1974) who showed that doses of testicular X-rays (0.3 - 3Gy) produced significant increases in the numbers of abnormally shaped epididymal sperm particularly during the period 3-8 weeks post-irradiation.

4.1.4.1 Sperm Chromatin Structure Assay.

Another flow cytometric method that has been applied to measure DNA abnormalities in spermatozoa is the sperm chromatin structure assay (SCSA), (Evenson, 1990a; Evenson & Jost, 1994). The SCSA utilises the properties of the dichromatic DNA stain, acridine orange (AO). Acridine orange intercalated into double stranded DNA fluoresces green (Lerman, 1963) whilst AO bound to RNA or single stranded DNA fluoresces red (Kapuscinski *et al.,* 1982). The assessment of chromatin structure using AO is based on the principle that abnormal sperm chromatin has a greater susceptibility to partial DNA denaturability induced by acid or heat treatment *in situ.* The extent of DNA denaturation following heat or acid treatment is determined by measuring the metachromatic shift from green fluorescence to red fluorescence as the DNA is denatured from double-stranded to single-stranded (Evenson, 1990a; Evenson & Jost, 1994).

The SCSA has been used to show that sperm with abnormal chromatin structure are produced when mice are treated with the toxic chemicals, triethylenemelamine (TEM) (Evenson *et al.,* 1989b) and methylmethanesulphonate (MMS), (Evenson *et al.,* 1993). More recently the effects of testicular irradiation have been assessed by SCSA and it has been shown that sperm collected from mice 40 days after irradiation with X-rays at doses of 0.25Gy and above were significantly more susceptible to acid-induced DNA denaturation than sperm from unirradiated control animals (Sailer *et al.,* 1995a).

4.1.4.2 Minisatellite Mutation Rates.

With the advent of DNA fingerprinting, a new molecular method has become available to study mutation rate after paternal exposure to radiation. Minisatellites are tandem repeat loci found in non-coding regions of DNA and are widely distributed throughout vertebrate genomes (Jeffreys *et al.,* 1985a, 1985b). They are highly polymorphic even within a species and some minisatellites show remarkably high germline mutation rates arising from spontaneous changes in repeat copy number giving rise to new length alleles (Jeffreys *et al.,* 1988; Jeffreys *et al.,* 1995). These characteristics make minisatellites extremely suited to the measurement of radiation induced mutation rates.

It was first demonstrated in 1993, that irradiation of male mice at the spermatogonial stage with ^{60}Co γ-rays (0.5 and 1.0 Gy) led to an increase in minisatellite mutation rates in the offspring (Dubrova *et al.,* 1993). Another study in 1994, confirmed the finding that radiation caused increases in minisatellite mutation although this study found that after 3Gy of ^{60}Co γ-rays the highest mutation rates occurred in offspring derived from irradiated spermatid cells (Sadamoto *et al.,* 1994). The effects of radiation dose on minisatellite mutation rates have also been investigated. Fan *et al.,* (1995) irradiated male mice with 1, 2 and 3Gy of ^{60}Co γ-rays and examined offspring derived from mating immediately, 3 weeks and 11 weeks after irradiation (to examine spermatids and spermatogonia respectively). Again this group found that spermatids were the most sensitive stage for the induction of minisatellite mutations by radiation and the frequency of mutations increased in a dose-related manner from 1 to 3Gy. Dubrova *et al.,* (1998) also have demonstrated a dose related increase in minisatellite mutations, this time after irradiation with X-rays. Increases in mutation rates were observed in offspring derived from matings at 6 and 10 weeks post irradiation. The incidence of mutation increased in a dose-related fashion with doses of 0.5

and 1.0Gy. A doubling dose of 0.33Gy was calculated for the increase in radiation induced minisatellite mutations.

Therefore the studies described above clearly demonstrate the usefulness and sensitivity of minisatellite loci in detecting and measuring radiation induced paternal mutations. However there appears to be discrepancies in the results from the two groups with the researchers from Japan suggesting that spermatids are the most sensitive stage for the induction of mutations (Sadamoto *et al.,* 1994; Fan *et al.,* 1995) whilst the British group suggest that it is the spermatogonial stages that are the most sensitive (Dubrova *et al.,* 1993, 1998). These differences remain unexplained but in the assessment of mutation rates the Japanese group have examined only one locus (Ms6hm) whilst the British researchers have examined mutation rates at up to 4 loci. Therefore these differences in methodology may partially account for the differences in results. More recently minisatellites have been used to demonstrate an elevated mutation rate in the offspring of exposed families after the Chernobyl accident (Dubrova *et al.,* 1996) and after irradiation of male mice with high LET ^{252}Cf irradiation (Niwa *et al.,* 1996).

4.1.4.3 Other Techniques.

Other techniques that have been used to investigate DNA damage in sperm after *in vivo* exposure of the testis to a toxic agent include alkaline elution which has demonstrated DNA strand breaks in sperm after exposure of spermiogenic stages to the mutagens MMS and EtO (Sega *et al.,* 1986; Sega & Generoso, 1988). The TUNEL assay which detects apoptotic DNA breaks has also been used to demonstrate that semen from smokers contain significantly higher numbers of spermatozoa possessing these breaks than ejaculates from non-smokers (Sun *et al.,* 1997).

Despite a number of recent published studies on the use of the comet assay to detect DNA damage in sperm after *in vitro* treatments there are very few studies relating to DNA damage induced in sperm after *in vivo* treatment of testicular germ cells. Aravindan *et al.,* (1997) have described the presence of spermatozoa with abnormal DNA in ejaculates from 23 human volunteers. This group demonstrated that levels of sperm exhibiting increased susceptibility to in situ DNA denaturation (SCSA) were strongly correlated with results obtained on the same samples analysed by comet assay and TUNEL labelling. Two publications have used the comet assay to investigate the effects on the testis of the genotoxic chemical 1,3 butadiene in rats and mice (Anderson *et al.,* 1997c; Brinkworth *et al.,* 1998). Whilst these studies demonstrated the ability of the comet assay to detect DNA damage post-treatment in extracted testicular germ cells they did not examine DNA damage levels in spermatozoa. Another similar study, examined the reproductive and genotoxic effects of vanadium pentoxide using the comet assay on testicular germ cells (Altamirano-Lozano *et al.,* 1996).

4.1.5 Aims and Objectives.

The aims of the experiments in this chapter were to determine whether the comet assay could be used to detect DNA damage in sperm after *in vivo* testicular irradiation. Experimental animals were irradiated with 4Gy of X-rays in the first study and sperm collected 16, 31 and 45 days post-irradiation which corresponds to the sampled cells being spermatids, spermatocytes and spermatogonia respectively at the time of irradiation. Comet assay was performed on these samples to determine whether irradiation of germ-cell stages results in the transmission of genetic damage through the spermatogenic process and whether this can be detected by comet assay. An additional experiment was also performed by irradiating animals with 4Gy X-rays as in the first experiment but sperm were collected 120 days post-irradiation in order to look at long-term effects to spermatogonial stem cells. In the second study the effects of radiation dose on the detection of DNA damage have been investigated after testicular irradiation of animals (0, 0.25, 0.5, 1, 2 and 4Gy) and assay of sperm collected 45 days post-irradiation. In both experiments routine measurements (body weight, testis weight, sperm counts) and histological examination of the testes were performed alongside comet measurement of DNA damage.

4.2 RESULTS.

4.2.1 Effect of *In vivo* Irradiation on Body Weights, Organ Weights and Sperm Counts.

Testicular irradiation with 4Gy X-rays had no effect on the body weight of experimental animals. At all timepoints (16, 31 and 45 days post-irradiation) the body weights of irradiated animals were not significantly different to that of time matched control animals (Table 4.2.1.1).

***Table 4.2.1.1** : Effects of 4Gy testicular X-irradiation on body weights.*

	Bodyweights (g) / Treatment Group	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	29.0 ± 0.3	27.6 ± 0.4
31 days	28.9 ± 0.1	32.2 ± 1.2
45 days	32.2 ± 1.9	32.0 ± 1.0

Data shown represents the mean for each experimental group ± S.E.M, (n=5).
Results from experimental groups analysed and compared using one way analysis of variance.

Irradiation with X-rays produced significant decreases in paired testis weights. At the earliest timepoint (16 days post-irradiation) testis weights of irradiated animals had decreased by approximately 55% compared to time matched controls. This decrease in testis weight was sustained through the 31 day timepoint although 45 days after irradiation testis weights showed signs of recovery but were still significantly lower than controls (Table 4.2.1.2).

***Table 4.2.1.2** : Effects of 4Gy testicular X-irradiation on paired testis weights.*

	Paired Testis Weights (mg) / Treatment Group	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	221 ± 12	99 ± 4 **
31 days	241 ± 5	97 ± 4 **
45 days	237 ± 4	161 ± 6 **

Data shown represents the mean for each experimental group ± S.E.M, (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$ (One way analysis of variance followed by Bonferroni's post-hoc test).

As well as decreases in testis weights, *in vivo* irradiation with 4Gy X-rays also produced effects on sperm counts in the vas deferens and within the testis (Tables 4.2.1.3 and 4.2.1.4). It appears that when the animals were irradiated (12 weeks of age) then spermatogenesis may still have been maturing as over the course of the experiment vas deferens sperm counts in control animals rose from approximately 10 million to around 18 million at the end of the experiment (Table 4.2.1.3). At 16 days after irradiation no decrease in sperm counts in the vas deferens was evident despite the dramatic decreases in testis weights observed at this timepoint. However, at 31 days post-irradiation, vas deferens sperm counts in irradiated animals were two thirds lower than control animals. At 45 days after testicular X-irradiation, sperm counts had decreased further to approximately 25% of control animal values and this was despite signs of recovery in testis weights. Testicular sperm head counts also decreased after irradiation (Table 4.1.2.4). At the 16 day timepoint, sperm head counts had decreased by 40% compared to control animals, however after 31 days sperm heads were present in testicular preparations at such low concentrations that counts could not be accurately determined. By the 45 day timepoint however, testicular sperm heads were present again but counts were only 45% of those in unirradiated animals.

***Table 4.2.1.3** : Effects of 4Gy testicular X-irradiation on vas deferens sperm counts.*

	Vas Deferens Sperm Counts (x 10^6) / Treatment Group	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	9.8 ± 0.4	10.6 ± 0.5
31 days	18.4 ± 0.9	6.4 ± 0.3 **
45 days	17.8 ± 1.3	4.0 ± 0.5 **

Data shown represents the mean for each experimental group ± S.E.M, (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$ (One way analysis of variance followed by Bonferroni's post-hoc test).

***Table 4.2.1.4** : Effects of 4Gy testicular X-irradiation on testicular sperm head counts.*

	Testis Sperm Head Counts (x 10^6) / Treatment Group	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	8.9 ± 1.0	5.3 ± 0.5**
31 days	8.4 ± 0.5	_[a]
45 days	9.5 ± 0.7	4.2 ± 0.3**

[a] There were too few sperm heads in these samples to count accurately.
Data shown represents the mean for each experimental group ± S.E.M, (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$ (One way analysis of variance followed by Bonferroni's post-hoc test).

4.22 Testicular Histology.

Photographs of testis sections from animals in all experimental groups are shown in Figures 4.2.2.1-4.2.2.3. Testes from untreated control animals at all timepoints showed normal spermatogenesis with a seminiferous epithelium consisting of a full complement of developing germ cells and spermatozoa in the lumen of the tubules. However 16 days post-irradiation it can be clearly observed that loss of early germ cells (spermatogonia and spermatocytes) has occurred and a decrease in tubular size is also evident (Figure 4.2.2.1). In testis sections from animals irradiated with 4Gy X-rays 31 days previously, it is evident that early germ cells are now present in the seminiferous tubules but there is now a lack of late germ cell stages (elongate spermatids and spermatozoa) towards the centre and in the lumen of the tubules (Figure 4.2.2.2). Testis sections, 45 days post-irradiation show evidence of some recovery of spermatogenesis since earlier timepoints as indicated by the presence of early germ cells (spermatogonia and spermatocytes) and evidence of elongate spermatids. However there appears to be a lack of spermatozoa in the lumen of the tubules (Figure 4.2.2.3).

4.2.3 Effects of *In vivo* Irradiation on DNA Damage in Spermatozoa.

Sperm recovered from the vas deferens 16, 31 and 45 days after irradiation were analysed using the neutral comet assay to measure DNA strand breaks (Figure 4.2.3.1). Baseline DNA damage in unirradiated control animals (as measured by comet tail length, tail moment and % tail DNA) did not alter significantly over the course of the experiment (Figures 4.2.3.1, 4.2.3.2 & Table 4.2.3.1).

16 days after irradiation with 4Gy X-rays there were no significant differences in comet tail length (38.6 ± 1.4μm - Control; 39.6 ± 3.0μm - Irradiated) between control and irradiated animals (Figure 4.2.3.2). However, despite the lack of difference in comet tail lengths, results for comet tail moment (1.3 ± 0.1 - Control; 3.6 ± 0.8 - Irradiated) and % tail DNA (5.2 ± 0.4% - Control; 10.6 ± 1.9% - Irradiated) were significantly higher in irradiated animals (Figure 4.2.3.2 & Table 4.2.3.1) compared to untreated controls.

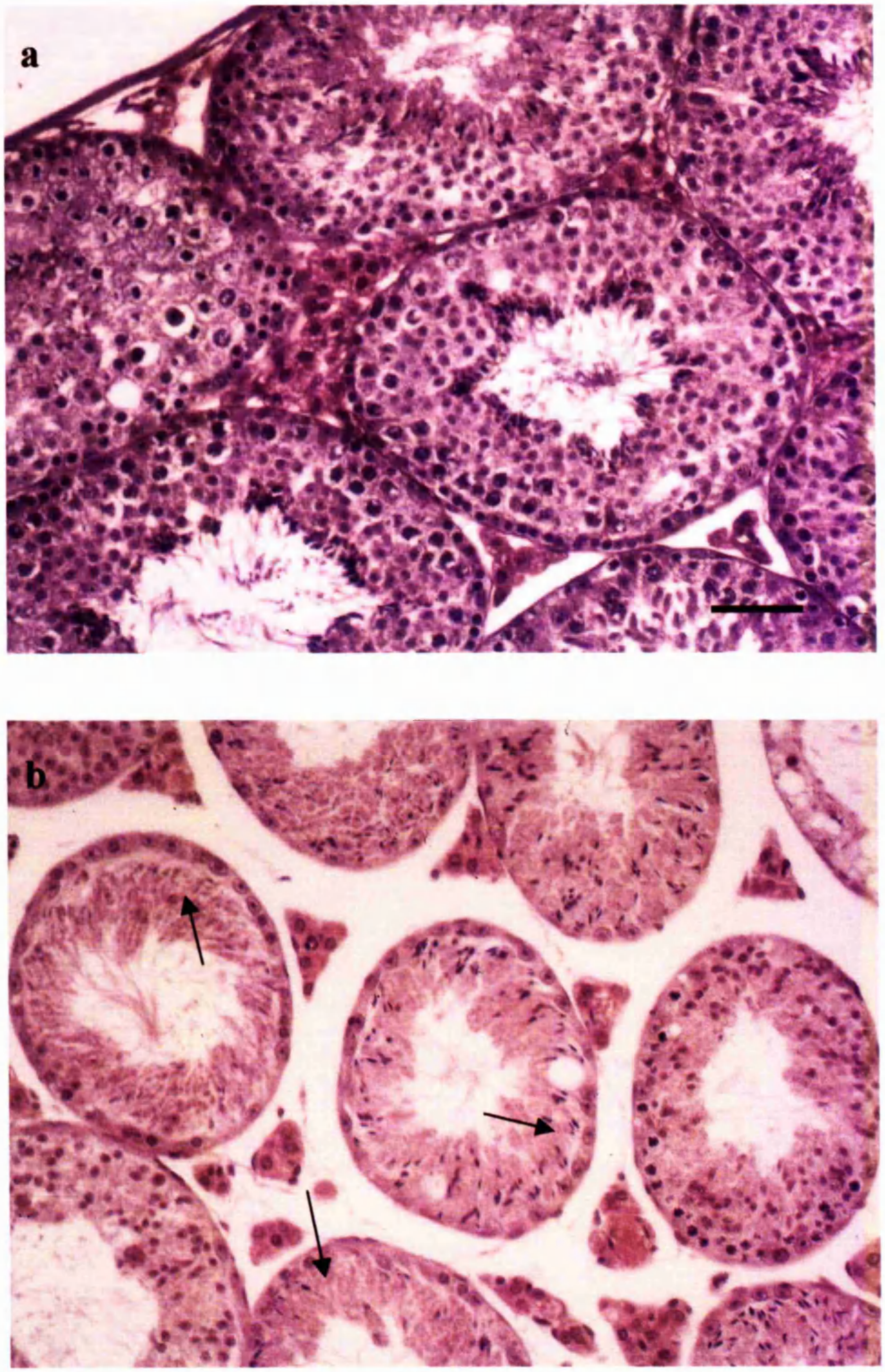

Figure 4.2.2.1 *: Effects of 4Gy X-rays on testicular histology. Panel a - control testis, 16 days post-irradiation of experimental animals; Panel b - irradiated testis, 16-days post-exposure to 4Gy X-rays. The loss of early germ cells in the regions shown by arrows can clearly be seen. Magnification x260. Scale Bar = 50µm.*

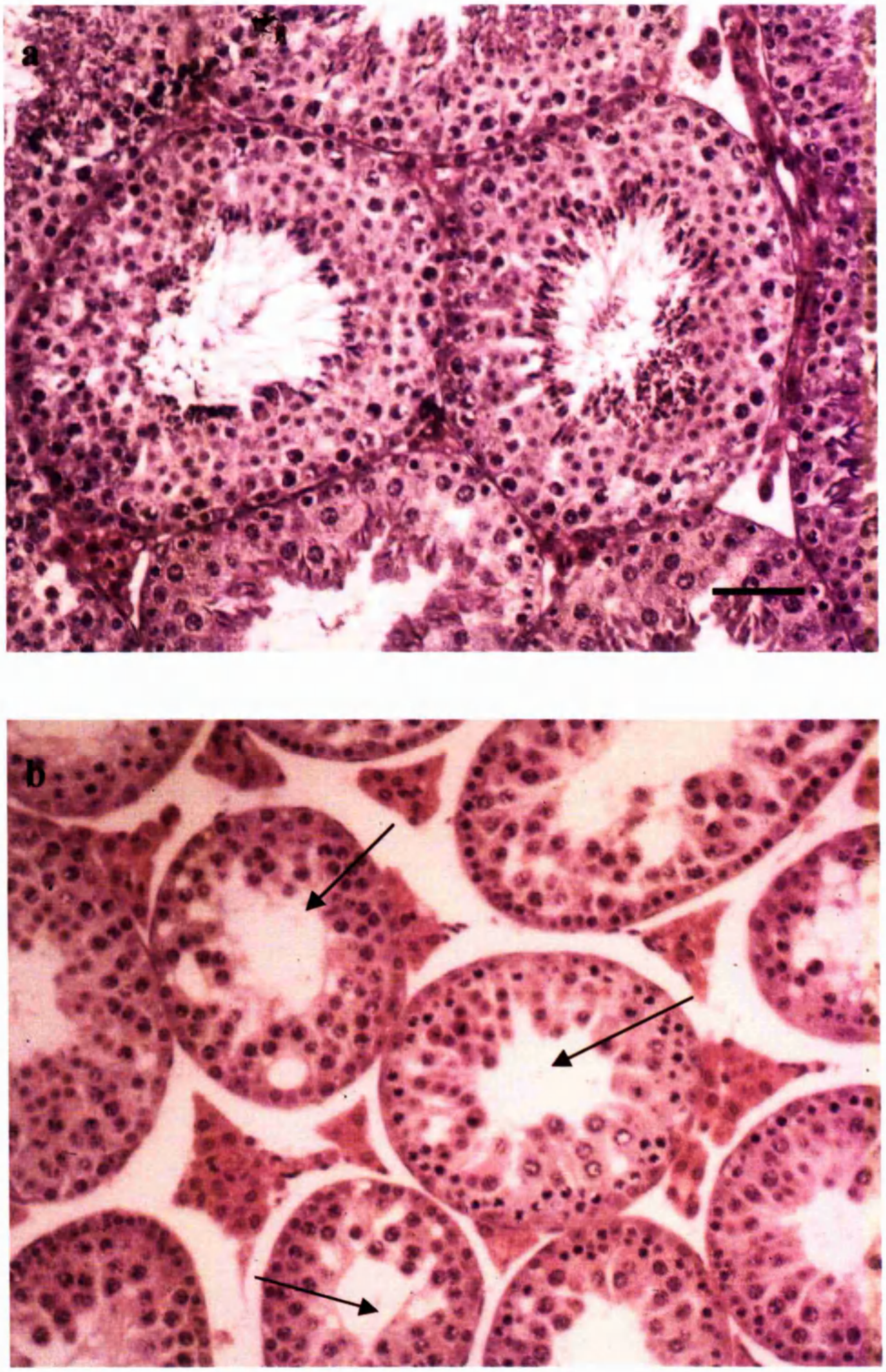

***Figure 4.2.2.2** : Effects of 4Gy X-rays on testicular histology. Panel a - control testis, 31 days post-irradiation of experimental animals; Panel b - irradiated testis, 31-days post-exposure to 4Gy X-rays. The lack of late germ cells (elongate spermatids and spermatozoa) in the middle and luminal parts of the tubules are indicated by arrows. Magnification x260. Scale Bar = 50μm.*

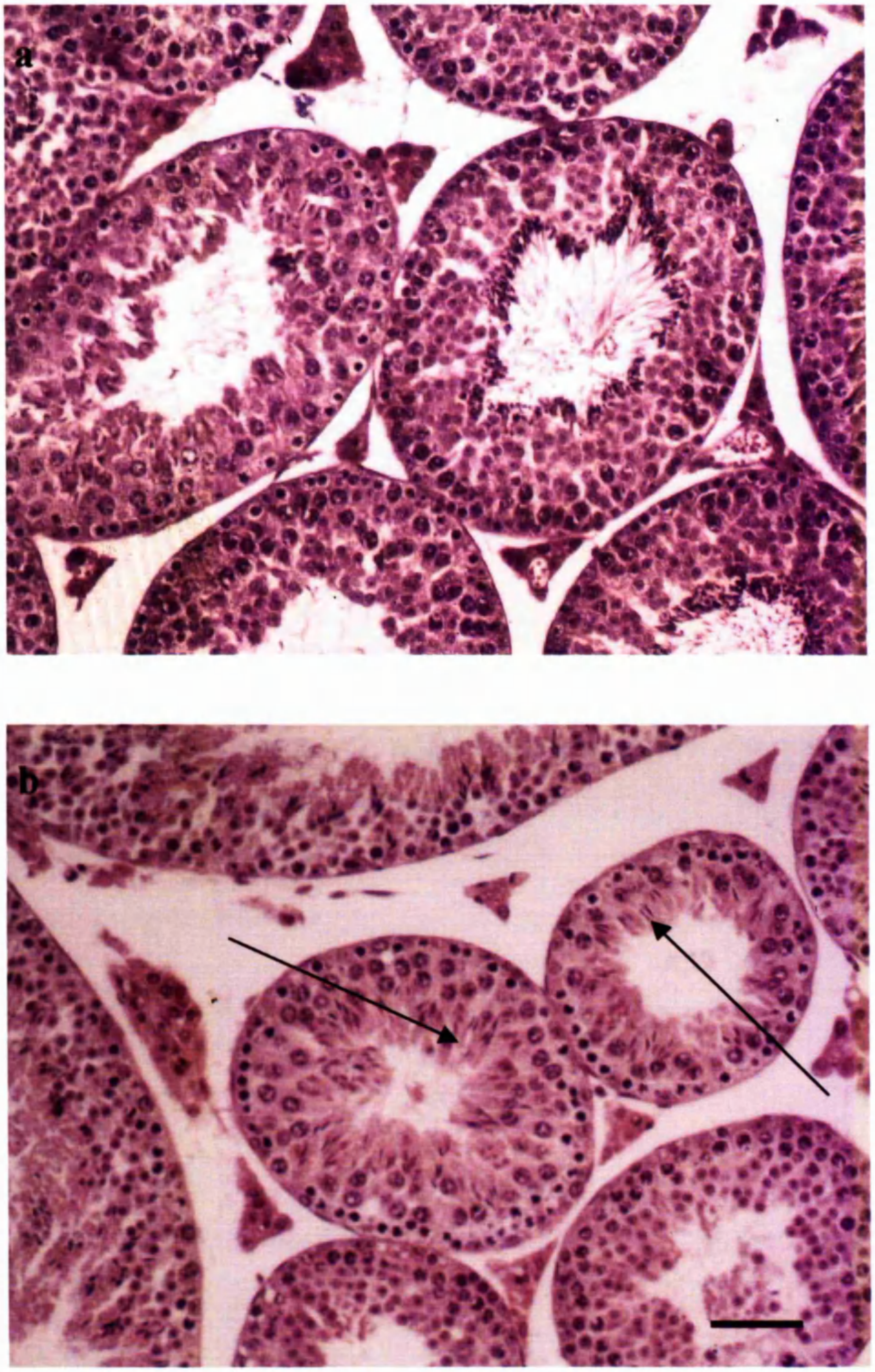

Figure 4.2.2.3 *: Effects of 4Gy X-rays on testicular histology. Panel a - control testis, 45 days post-irradiation of experimental animals; Panel b - irradiated testis, 45-days post-exposure to 4Gy X-rays. Some recovery of spermatogenesis can be observed as indicated by reorganisation of the testis and the appearance of elongate spermatids as indicated by the arrow. However, spermatozoa still appear to be lacking from the lumen of the tubules. Magnification x260. Scale Bar = 50μm.*

At 31 days post-irradiation higher levels of DNA damage were observed in sperm from irradiated animals than were measured at the earlier 16 day timepoint (Figures 4.2.3.1 & 4.2.3.2). Tail lengths of comets from irradiated animals were now significantly higher than those from unirradiated controls (39.1 ± 0.9μm - Control; 57.4 ± 2.0 - Irradiated). DNA damage levels as measured by comet tail moment (1.2 ± 0.1 Control; 13.3 ± 1.2 - Irradiated) and % tail DNA (4.9 ± 0.2% - Control; 31.0 ± 2.8% - Irradiated) had also increased and were higher than levels recorded 16 days post-irradiation (Figure 4.2.3.2 & Table 4.2.3.1).

Sperm samples recovered from the vas deferens 45 days after irradiation with 4Gy X-rays showed the highest levels of DNA damage as measured by comet assay at any of the three timepoints (Figures 4.2.3.1 & 4.2.3.2). Comet tail lengths were approximately double those observed in sperm from control animals (39.5 ± 1.2μm - Control; 88.1 ± 3.9μm - Irradiated) whilst comet tail moments (Figure 4.2.3.2) reflected a greater difference between controls (1.4 ± 0.1) and irradiated animals (30.0 ± 1.4). As with tail length and tail moment, results for % DNA were much higher in comets from irradiated animals (56.9 ± 1.9%) than controls (5.4 ± 0.2%) (Table 4.2.3.1).

Unlike the results described above for the neutral assay. No significant differences were observed between spermatozoa from control and irradiated animals using alkaline comet assay at any timepoints (Table 4.2.3.2). As described in Chapter 3 exposure of sperm DNA to alkaline treatment followed by electrophoresis results in migration of almost all the DNA out of the nucleus and this makes the measurement of DNA single strand breaks in spermatozoa very difficult. Unfortunately due to extensive migration of DNA from the sperm head, the image analysis software could not accurately determine head and tail regions of the comet. Therefore only measurement of tail length (DNA migration) was possible.

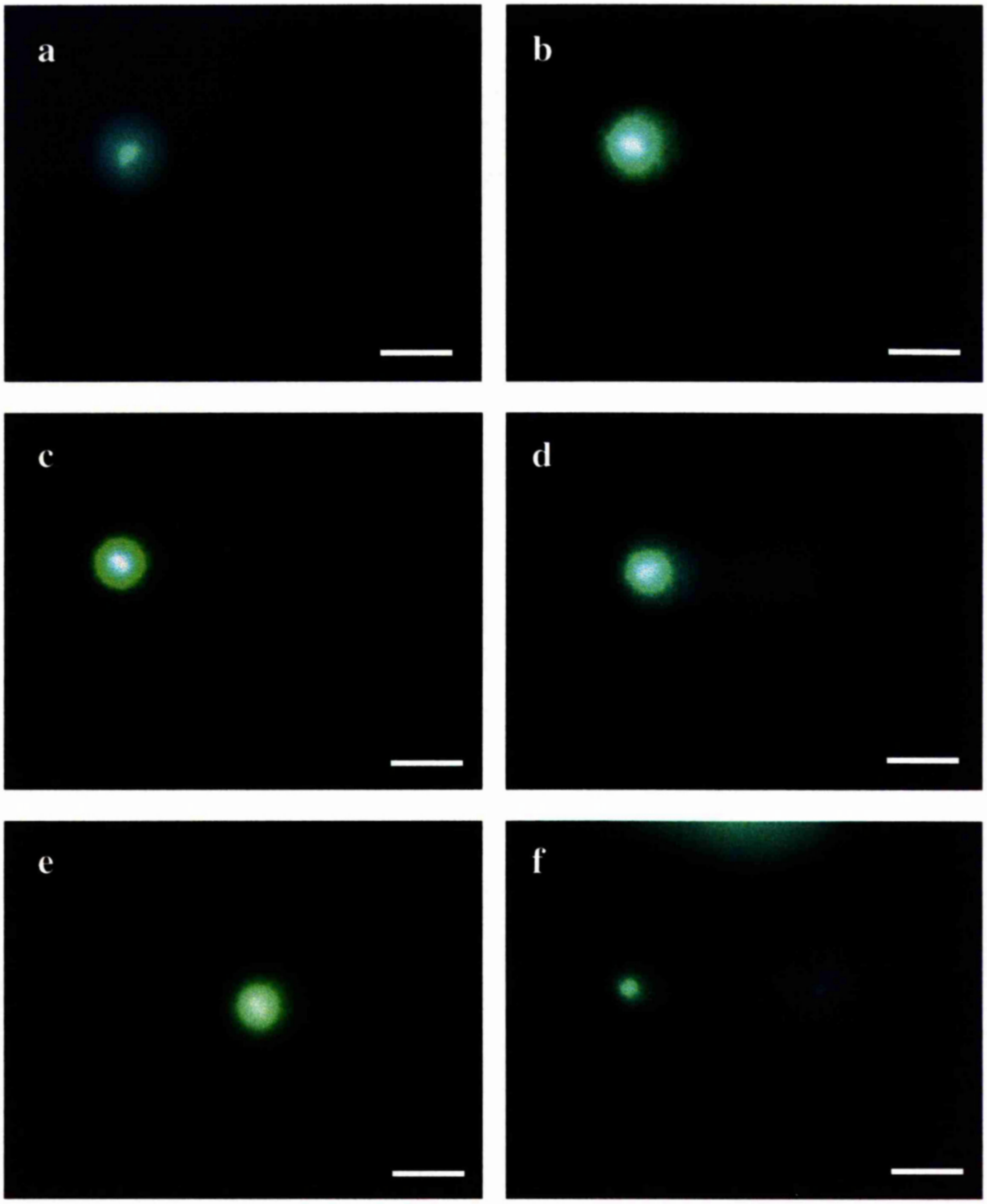

***Figure 4.2.3.1** : DNA damage in individual spermatozoa measured by comet assay after irradiation of various germ cells stages in vivo after testicular irradiation with 4Gy X-rays. Panel a - control spermatozoa, 16 days post-irradiation; Panel b - spermatozoa recovered from the vas deferens 16 days post irradiation; Panel c - control spermatozoa, 31 days post-irradiation; Panel d - spermatozoa recovered from the vas deferens 31 days post-irradiation. Panel e - control spermatozoa, 45 days post-irradiation; Panel f - spermatozoa recovered from the vas deferens 45 days post-irradiation. All images are shown at the same magnification x440. Scale Bar =25µm.*

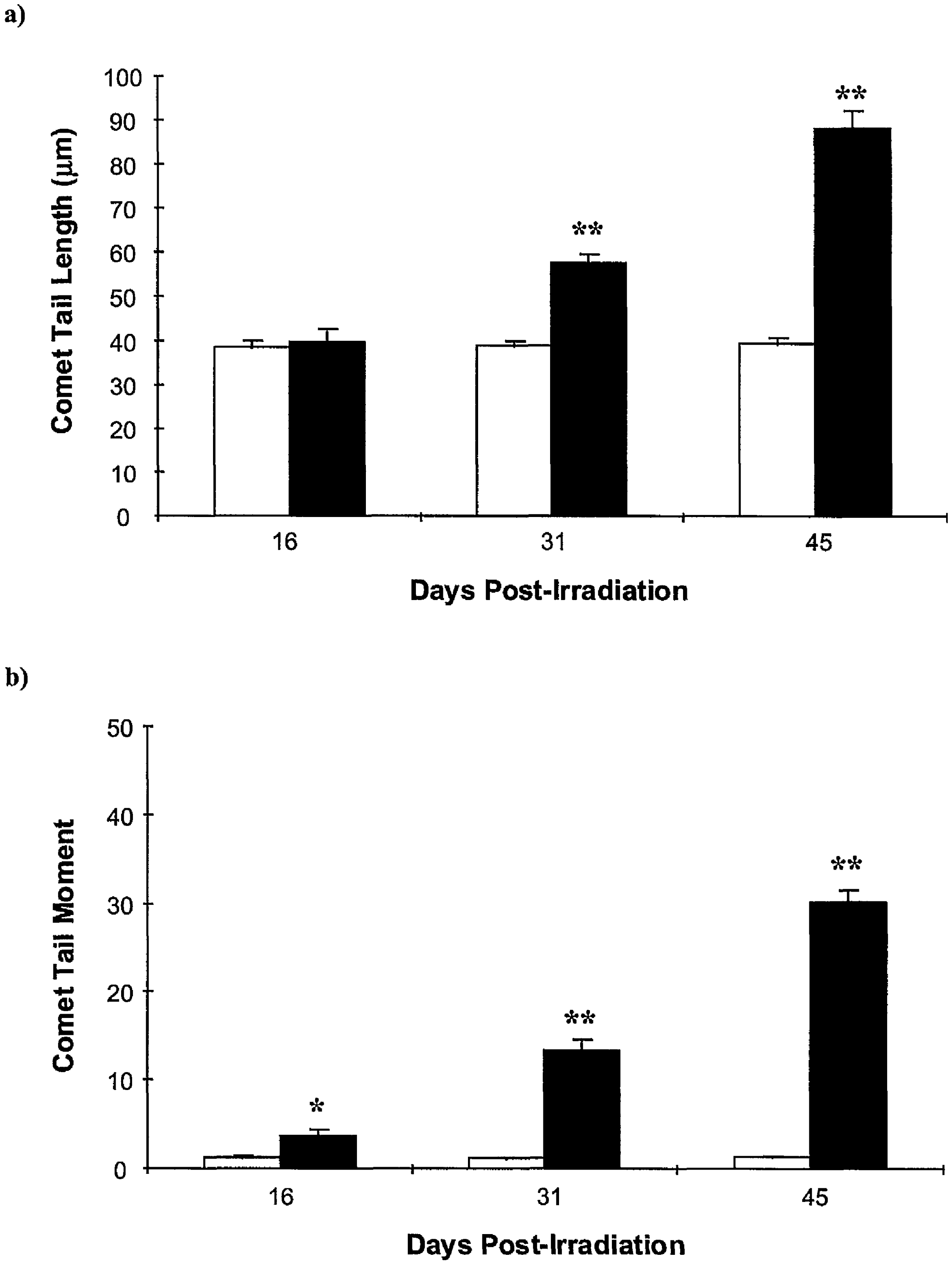

*Figure 4.2.3.2 : DNA damage assessed by neutral comet assay in vas deferens spermatozoa after testicular irradiation with 4Gy X-rays. a) Effects on comet tail length and b) effects on comet tail moment. Data shown represents the mean ± S.E.M for each experimental group (n=5). Asterisks denote values significantly different to time-matched control, * = $P<0.05$, ** = $P<0.01$ (Kruscal-Wallis one-way ANOVA and Mann Whitney U-test).*

***Table 4.2.3.1** : Effects of testicular irradiation with 4Gy X-rays on % Tail DNA in mouse spermatozoa after neutral comet assay.*

	% Tail DNA / Treatment Group	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	5.2 ± 0.4	10.6 ± 1.9 *
31 days	4.9 ± 0.2	31.0 ± 2.8 **
45 days	5.4 ± 0.2	56.9 ± 1.9 **

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$ (Kruscal-Wallis one-way ANOVA and Mann Whitney U-test)

***Table 4.2.3.2** : Effects of testicular irradiation with 4Gy X-rays on comet tail length of mouse spermatozoa after alkaline comet assay.*

	Comet Tail Length (μm)	
Time Post-Irradiation	***Control***	***Irradiated (4Gy X-rays)***
16 days	79.43 ± 1.28	75.90 ± 3.48
31 days	78.12 ± 1.57	77.63 ± 2.14
45 days	76.79 ±1.26	84.88 ±2.42

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Experimental data analysed and compared using Kruscal-Wallis one-way ANOVA.

4.2.4 Distribution of DNA Damage in Spermatozoa from Irradiated Animals.

It was described in chapter 3 how *in vitro* irradiation of spermatozoa resulted in all the sperm analysed having similar levels of DNA damage. However, following *in vivo* irradiation it was evident that despite the mean values for comet parameters increasing with each timepoint post-irradiation, not all of the spermatozoa had the same levels of DNA damage (Figures 4.2.4.1 & 4.2.4.2). By performing frequency histogram analysis of the comet tail length results, it is possible to analyse the range and frequency of comet tail lengths within the sperm samples. This allows heterogeneity of DNA damage within the samples to be examined.

16 days after irradiation mean values for comet tail lengths were not significantly different than controls. Despite this results for tail moment and % tail DNA were significantly higher in irradiated animals compared to controls. When the frequency distribution for comet tail lengths is analysed (Figure 4.2.4.2) it is clear that the majority of spermatozoa (90%) are undamaged and have tail lengths between 30 and 60μm. However there is a small but separate population of cells (10% of total spermatozoa) with higher levels of DNA damage (tail lengths 70-100μm).

At 31 days post-irradiation, all comet parameters were significantly higher in sperm from irradiated animals compared to controls. However, analysis of the frequency distribution shows two separate populations of cells showing differing levels of DNA damage (Figure 4.2.4.2). Approximately 50% of spermatozoa have tail lengths ranging from 30μm to 60μm which are representative of undamaged cells whilst 50% have tail lengths of 70μm and above. This results in a mean value for tail length of 57.4 ± 2.0μm, even though only approximately 10% of sperm had comets of this length.

At 45 days post-irradiation, the shape of the frequency distribution chart has changed once again (Figure 4.2.4.2). Now less than 10% of sperm have comets representative of undamaged cells (tail lengths 30-60μm) whilst the majority of cells show a high degree if DNA damage (tail lengths 80μm and above).

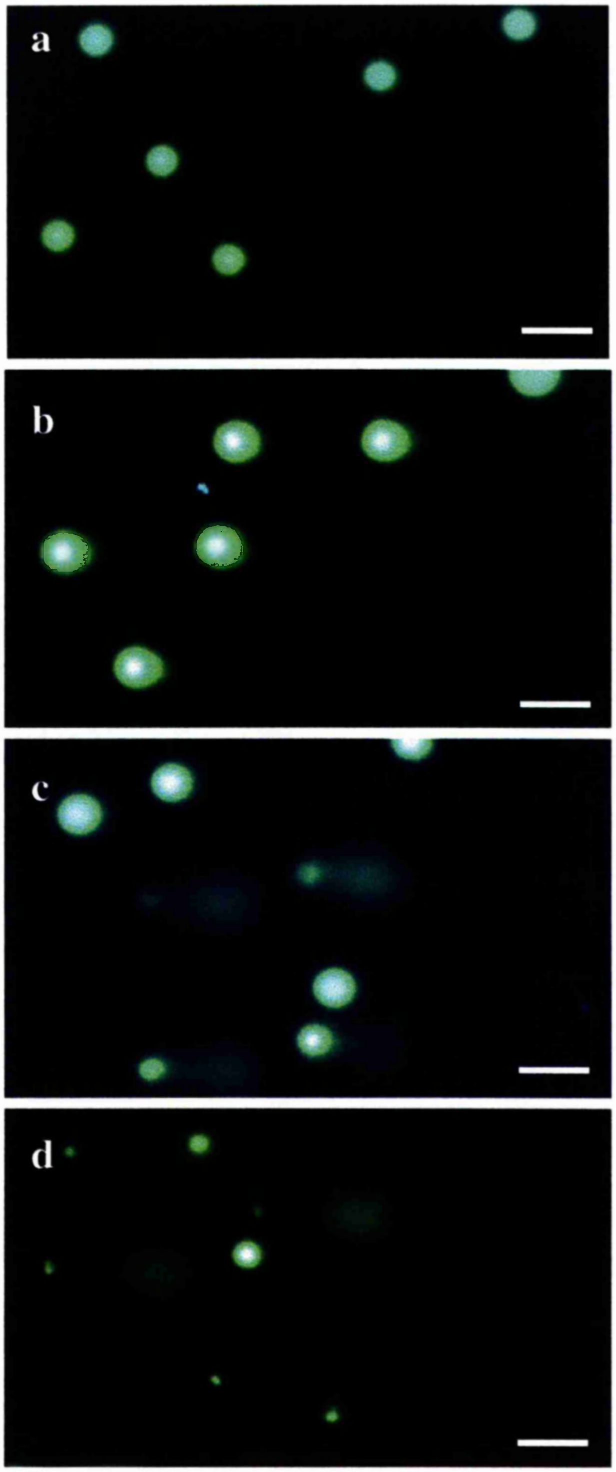

Figure 4.2.4.1: *Incidence of DNA damage in spermatozoa measured by comet assay after irradiation of various germ cells stages in vivo after testicular irradiation with 4Gy X-rays. Panel a - spermatozoa from a unirradiated control animal; Panel b - spermatozoa recovered from the vas deferens 16 days post irradiation; Panel c - spermatozoa recovered from the vas deferens 31 days post-irradiation; Panel d - spermatozoa recovered from the vas deferens 45 days post-irradiation. All images are shown at the same magnification x190. Scale Bar = 50µm.*

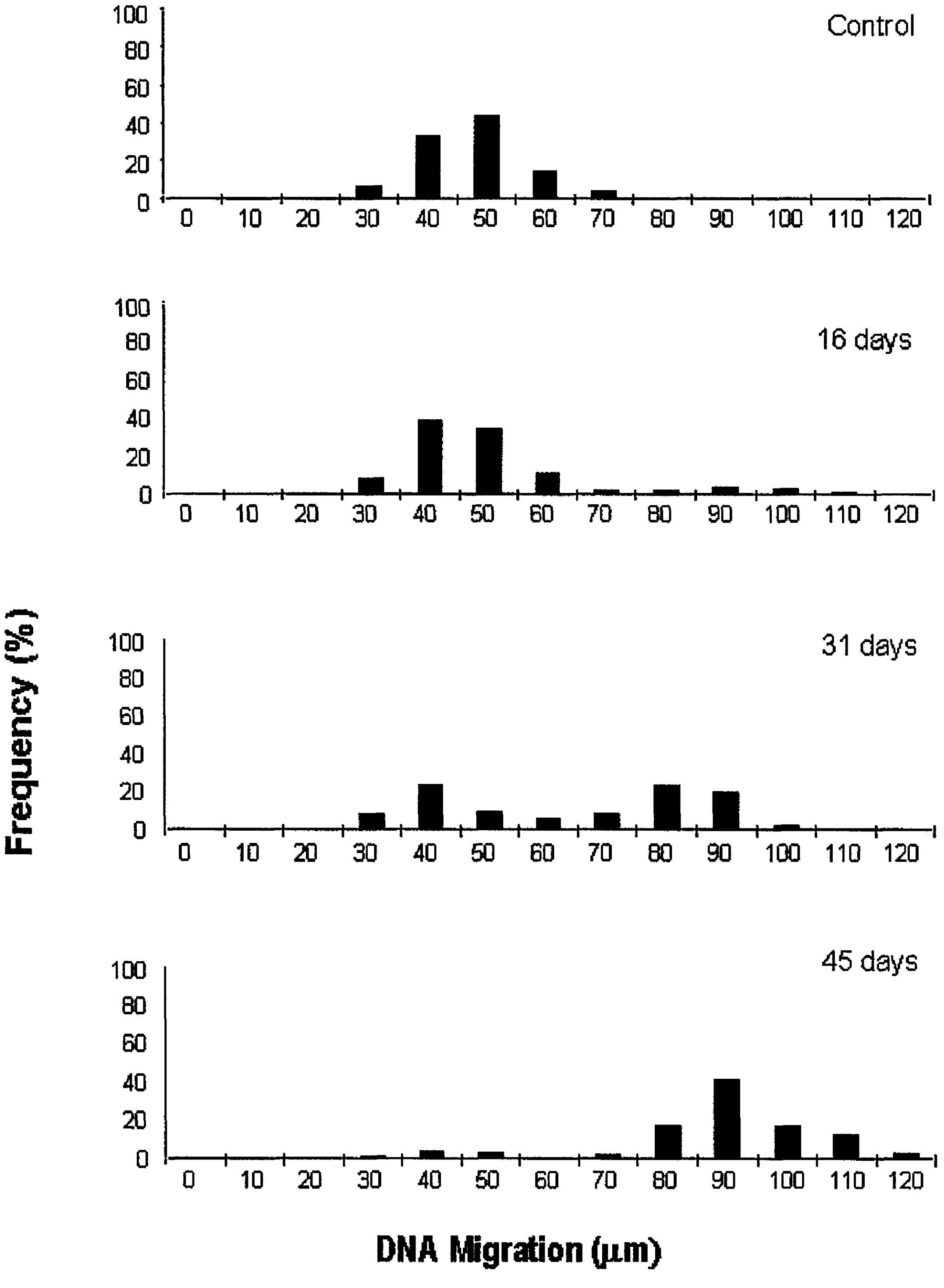

Figure 4.2.4.2 : Distribution of comet tail lengths at each experiment timepoint post-irradiation with 4Gy testicular X-rays. Data shown is representative of 5 experimental subjects, 100 cells analysed per subject.

4.2.5 Effect of Long Term Recovery (120 Days) after Testicular Irradiation with 4Gy X-rays on Body weights, Testis weights and Sperm counts.

In the previous experiment analyses were performed at three different timepoints (16, 31 and 45) days. These were all within one spermatogenic cycle in order to obtain sperm developed from spermatids, spermatocytes and spermatogonia respectively. In order to look at the long term effects of testicular irradiation a longer timepoint (120 days) was chosen. This allows the seminiferous epithelium to undergo at least two full cycles of spermatogenesis and therefore any sperm collected will be derived from spermatogonial stem cells that survived the irradiation and not irradiated spermatogonia, spermatocytes or spermatids.

In vivo irradiation had no significant long term effects on body weight as no significant differences were detectable between control and irradiated animals (Table 4.2.5.1). Paired testis weights, which were significantly decreased at earlier timepoints showed considerable recovery towards results from unirradiated control animals. However, despite testis weights from irradiated animals being only slightly lower than controls these results still achieved statistical significance indicating that testicular irradiation with 4Gy X-rays may have resulted in some permanent damage to the seminiferous epithelium within the testis (Table 4.2.5.2).

Table 4.2.5.1 *: Effect of long term recovery (120 days) after irradiation with 4Gy testicular X-rays on body weights.*

	Bodyweights (g)
Control	35.2 ± 0.7
Irradiated (4Gy X-rays)	33.1 ± 0.7

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Experimental data analysed and compared using Students t-test.

Table 4.2.5.2 : Effect of long term recovery (120 days) after irradiation with 4Gy testicular X-rays on paired testis weights.

	Paired Testis Weights (mg)
Control	254.8 ± 11.3
Irradiated (4Gy X-rays)	231.4 ± 6.8 *

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$ (Students t-test).

Table 4.2.5.3 *: Effect of long term recovery (120 days) after irradiation with 4Gy testicular X-rays on vas deferens sperm counts.*

	Vas Deferens Sperm Counts (x10^6)
Control	18.7 ± 0.8
Irradiated (4Gy X-rays)	17.1 ± 1.3

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Experimental data analysed and compared using Students t-test.

Table 4.2.5.4 *: Effect of long term recovery (120 days) after irradiation with 4Gy testicular X-rays on testicular sperm counts.*

	Testis Sperm Head Counts (x10^6)
Control	9.6 ± 0.6
Irradiated (4Gy X-rays)	8.4 ± 0.8

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Experimental data analysed and compared using Students t-test.

Despite their being a small but significant difference in testis weights no decrease in vas deferens or testicular sperm counts were observed 120 days after irradiation with 4Gy X-rays (Tables 4.2.5.3 & 4.2.5.4). Histological sections confirmed that recovery of spermatogenesis had taken place since earlier timepoints. However, there were no obvious differences in the testicular histology of control and irradiated animals (Figure 4.2.5.1).

4.2.6 DNA Damage in Spermatozoa after Long Term Recovery (120 Days).

120 days after irradiation differences were still detectable between spermatozoa from control and irradiated animals (Figures 4.2.6.1, 4.2.6.2 & Table 4.2.6.1). Comet tail lengths were slightly higher in sperm from irradiated animals (48.0 ± 4.9μm) compared to controls (37.9 ± 1.0μm) although this was not statistically significant. Significant differences however were observed with comet tail moment (1.8 ± 0.2 - Control; 4.3 ± 0.8 - Irradiated) and % tail DNA (6.8 ± 0.6 - Control; 13.5 ± 2.3 - Irradiated). These results suggest that irradiation with 4Gy X-rays may have resulted in some permanent transmissible damage to the DNA of some spermatogonial stem cells.

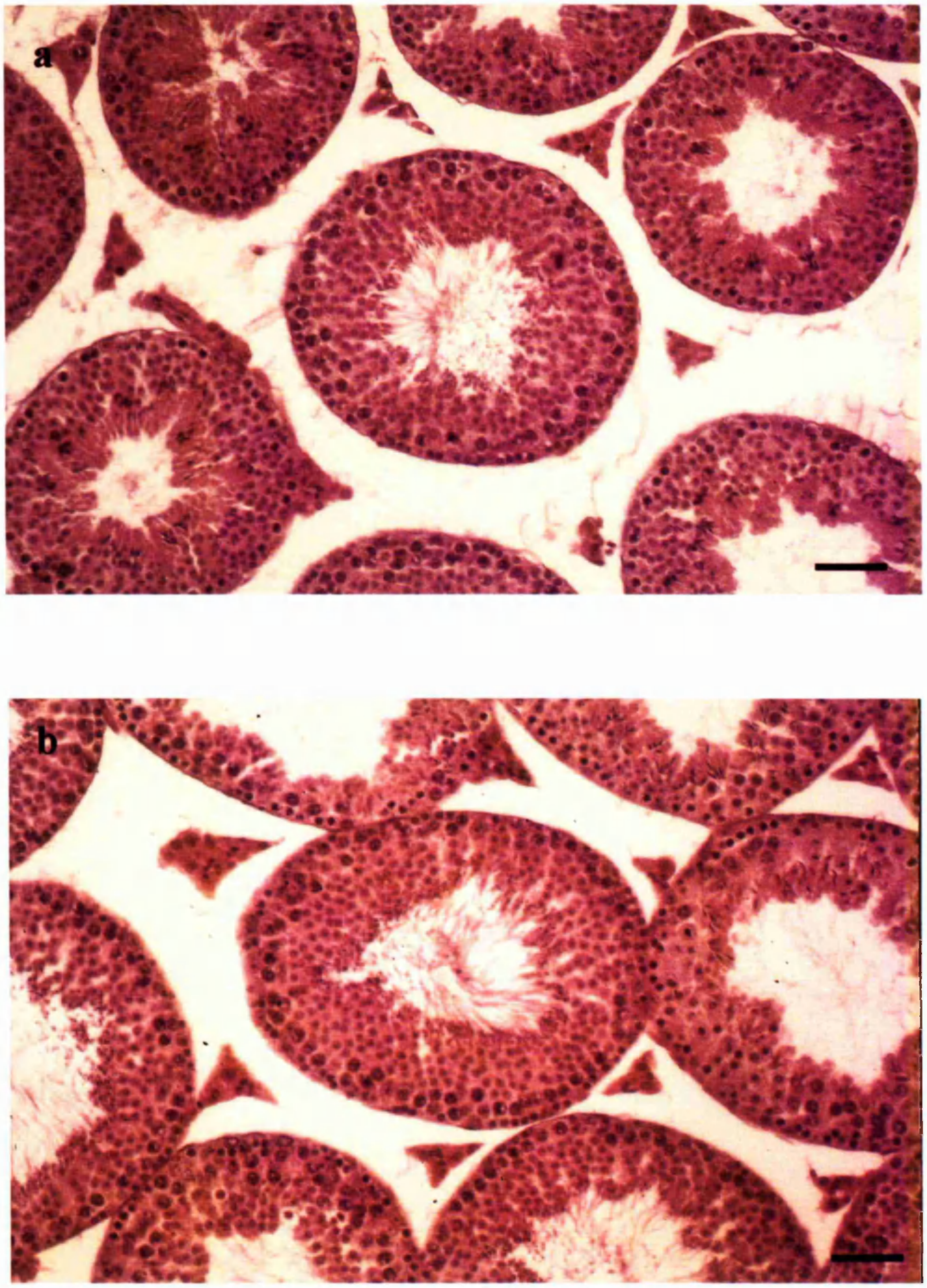

Figure 4.2.5.1 *: Effects of 4Gy X-rays on testicular histology, 120 days post-irradiation. Panel a - control testis, 120 day post-irradiation of experimental animals. Panel b - Irradiated testis, 120 days after exposure to 4Gy X-rays. Recovery and repopulation of the tubules can clearly be seen to have occurred since earlier timepoints and spermatozoa are present in the lumen of the tubules. Magnification x200. Scale Bar =50μm.*

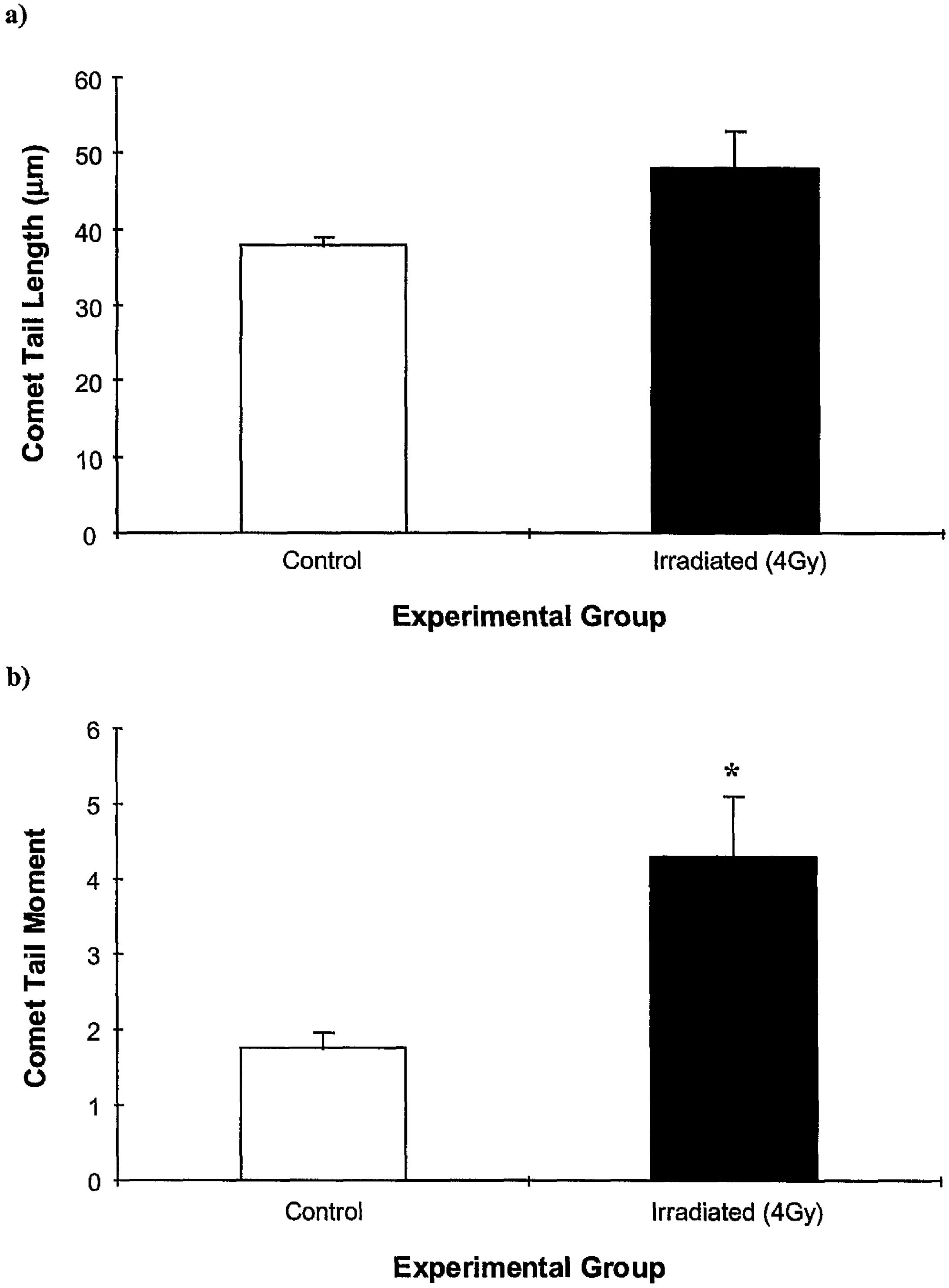

***Figure 4.2.6.1** : DNA damage assessed by neutral comet assay in vas deferens spermatozoa 120 days after testicular irradiation with 4Gy X-rays. a) Effects on comet tail length and b) effects on comet tail moment. Data shown represents the mean ± S.E.M for each experimental group (n=5). Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (Mann Whitney U-test).*

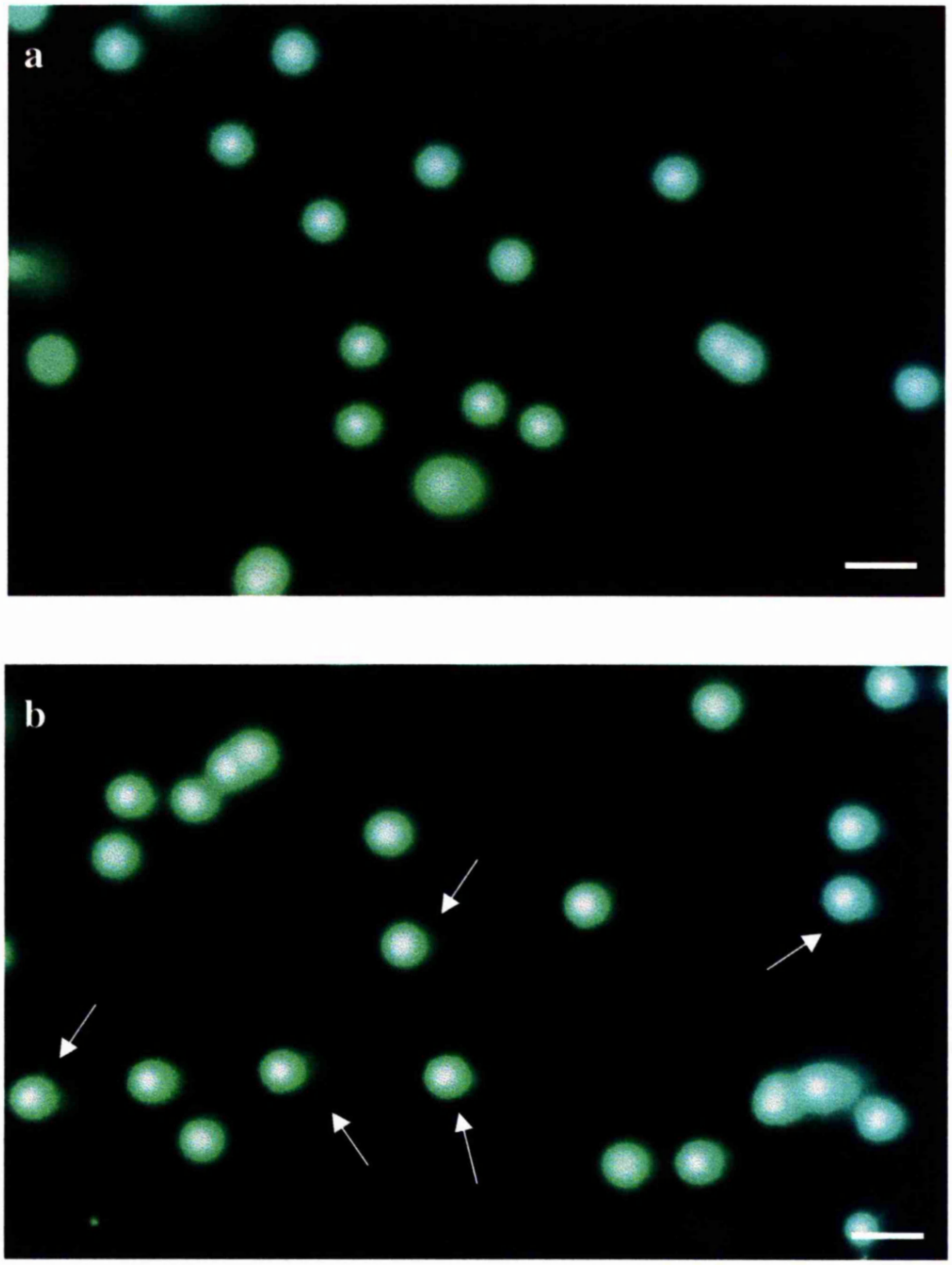

***Figure 4.2.6.2** : Chronic effects of radiation on spermatozoa produced by the testis 120 days post-irradiation with 4Gy testicular X-rays. Panel a - control spermatozoa, 120 days post-irradiation of experimental animals. Panel b - spermatozoa collected from the vas deferens 120 days post-irradiation. The arrows indicate sperm with DNA damage as indicated by the presence of comet tails. Both panels are shown at the same magnification x200. Scale Bar = 50μm.*

***Table 4.2.6.1** : Percentage Tail DNA in comets of mouse spermatozoa 120 days after 4Gy testicular X-rays.*

	% Tail DNA
Control	6.8 ± 0.6
Irradiated (4Gy X-rays)	13.5 ± 2.3 *

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (Mann Whitney U-test).

4.2.7 Effect of Dose of Radiation on Body Weights, Testis Weights and Sperm Counts.

Testicular irradiation with doses of X-rays ranging from 0 to 4Gy produced no detectable effects on the body weights of animals 45 days after irradiation (Table 4.2.7.1). Significant dose-dependent effects however were detected on the testis (Table 4.2.7.2) and sperm counts (Tables 4.2.7.3 & 4.2.7.4). Irradiation with 1.0Gy X-rays resulted in testis weights being 12% lower in irradiated animals compared to controls when measured 45 days post-irradiation. With a dose of 2.0Gy testis weights from irradiated animals were 20% less than controls whilst the highest dose of 4.0Gy resulted in 30% lower testis weights from irradiated animals.

All doses of irradiation produced significant decreases in both vas deferens (Table 4.2.7.3) and testicular sperm head counts (Table 4.2.7.4). Despite no observable decrease in testis weights, after 0.25Gy vas deferens sperm counts were reduced by approximately 10%. Further reductions in vas deferens sperm counts were observed with increasing radiation dose and at the highest dose of 4Gy, sperm counts had fallen by over 80% compared to unirradiated animals. Dose dependent decreases were also observed when testicular sperm head counts were examined, however the effects were not as dramatic as in the vas deferens. After 4Gy X-rays, testicular sperm head counts were reduced by approximately 35% compared to controls.

***Table 4.2.7.1** : Effect of testicular X-rays on body weight, 45 days post-irradiation.*

Radiation Dose (Gy)	Bodyweights (g)
0	29.0 ± 0.8
0.25	28.7 ± 0.5
0.50	27.6 ± 0.9
1.0	30.8 ± 0.8
2.0	31.6 ± 0.9
4.0	31.7 ± 1.0

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Data analysed and compared using one way analysis of variance.

***Table 4.2.7.2** : Effect of testicular X-rays on paired testis weights, 45 days post-irradiation.*

Radiation Dose (Gy)	Paired Testis Weights (mg)
0	226 ± 5
0.25	213±11
0.50	199 ± 18
1.0	200 ± 4**
2.0	180 ± 6**
4.0	160 ± 6**

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = $P<0.05$, ** = $P<0.01$.

***Table 4.2.7.3** : Effect of testicular X-rays on vas deferens sperm counts, 45 days post-irradiation.*

Radiation Dose (Gy)	Vas Deferens Sperm Counts ($x10^6$)
0	18.9 ± 0.7
0.25	16.6 ± 0.4**
0.50	14.9 ± 0.9**
1.0	10.1 ± 0.7**
2.0	6.5 ± 0.6**
4.0	3.0 ± 0.4**

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferonni's post-hoc test).

***Table 4.2.7.4** : Effect of testicular X-rays on testicular sperm counts, 45 days post-irradiation.*

Radiation Dose (Gy)	Testis Sperm Head Counts ($x10^6$)
0	10.2 ± 0.6
0.25	8.5 ± 0.6*
0.50	7.9 ± 0.5**
1.0	8.0 ± 0.5**
2.0	7.7 ± 0.7**
4.0	6.6 ± 0.4**

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferonni's post-hoc test).

4.2.8 Effect of Dose on DNA Damage in Sperm after *In vivo* Irradiation.

The effects of radiation dose on comet tail length can be seen in Figure 4.2.8.1. Irradiation of testis with X-rays between 0 and 4Gy resulted in dose-dependent increases in comet tail length of spermatozoa recovered from the vas deferens 45 days after irradiation. The smallest dose at which a significant increase in comet tail length was detected was 0.5Gy, which resulted in an increase in comet tail length of approximately 25%. At the maximum dose of 4Gy, comet tail lengths were approximately double those produced by spermatozoa from unirradiated animals (Figure 4.2.8.2).

As with previous experiments, the results for comet tail moment showed more clear-cut differences between the experimental groups than comet tail lengths (Figure 4.2.8.2). The lowest dose of radiation that produced a statistically significant increase in comet tail moment was 0.5Gy (5.4 ± 0.5; Unirradiated Control - 2.86 ± 0.81). Irradiation with 1, 2 and 4Gy resulted in 3 fold, 7 fold and 10 fold increases in comet tail moment respectively. Using the data for comet tail moment and linear regression it was possible to calculate a doubling dose of 0.35Gy (dose of radiation required to double the background level of DNA damage) for the induction of DNA damage in spermatozoa after testicular irradiation (Figure 4.2.8.3).

The results for % tail DNA reflect those previously described for both comet tail length and tail moment (Table 4.2.8.1). Again the lowest dose at which a significantly different result to the control was observed was 0.5Gy with higher doses result in dose dependent increases in % tail DNA.

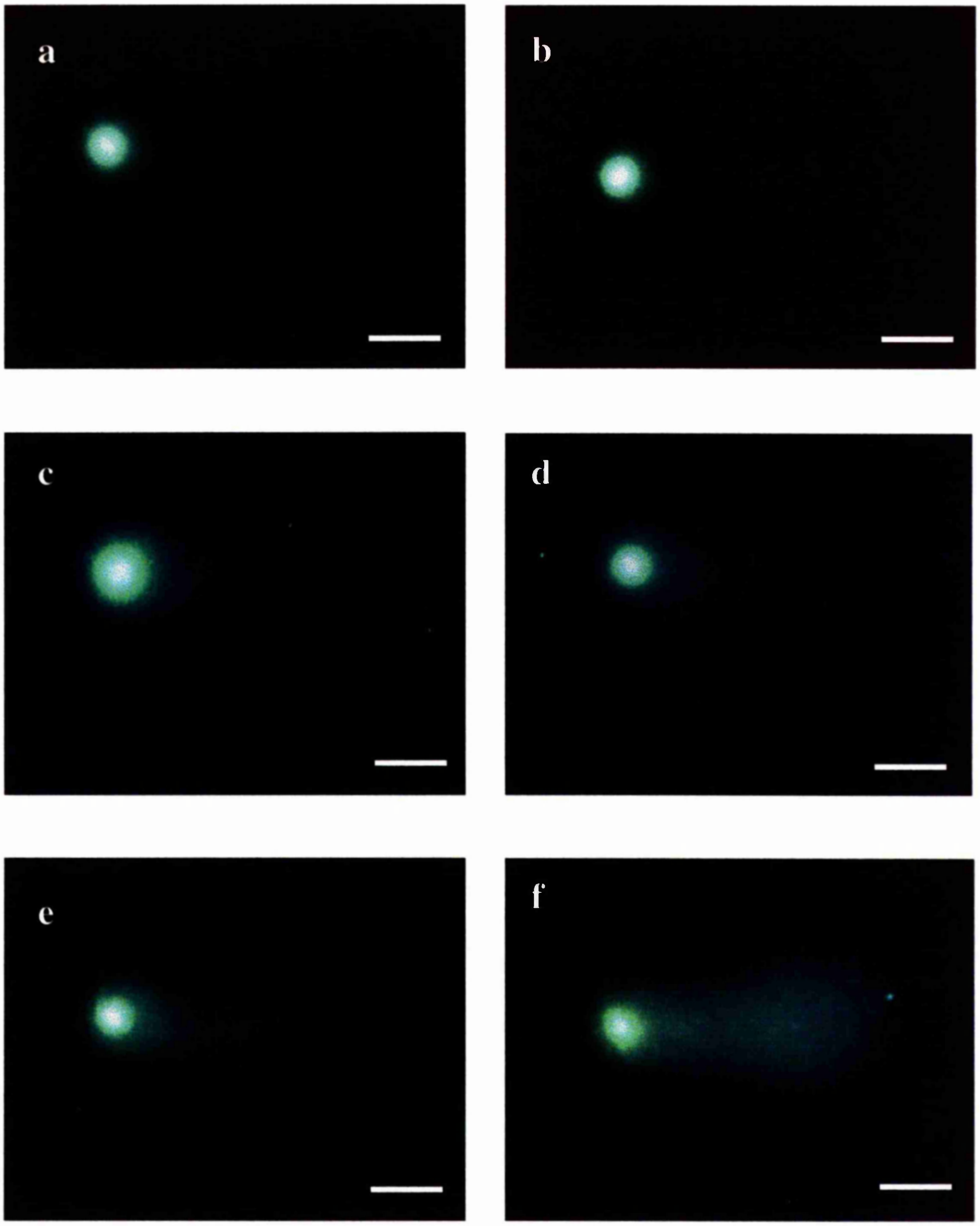

***Figure 4.2.8.1** : Dose response effects of spermatogonial irradiation in vivo assessed by neutral comet assay of spermatozoa, 45 days post-irradiation. Panel a - 0Gy X-rays; Panel b - 0.25Gy X-rays; Panel c - 0.5Gy X-rays; Panel d - 1Gy X-rays; Panel e - 2Gy X-rays; Panel f - 4Gy X-rays. All images are shown at the same magnification, x440. Scale Bar = 25μm.*

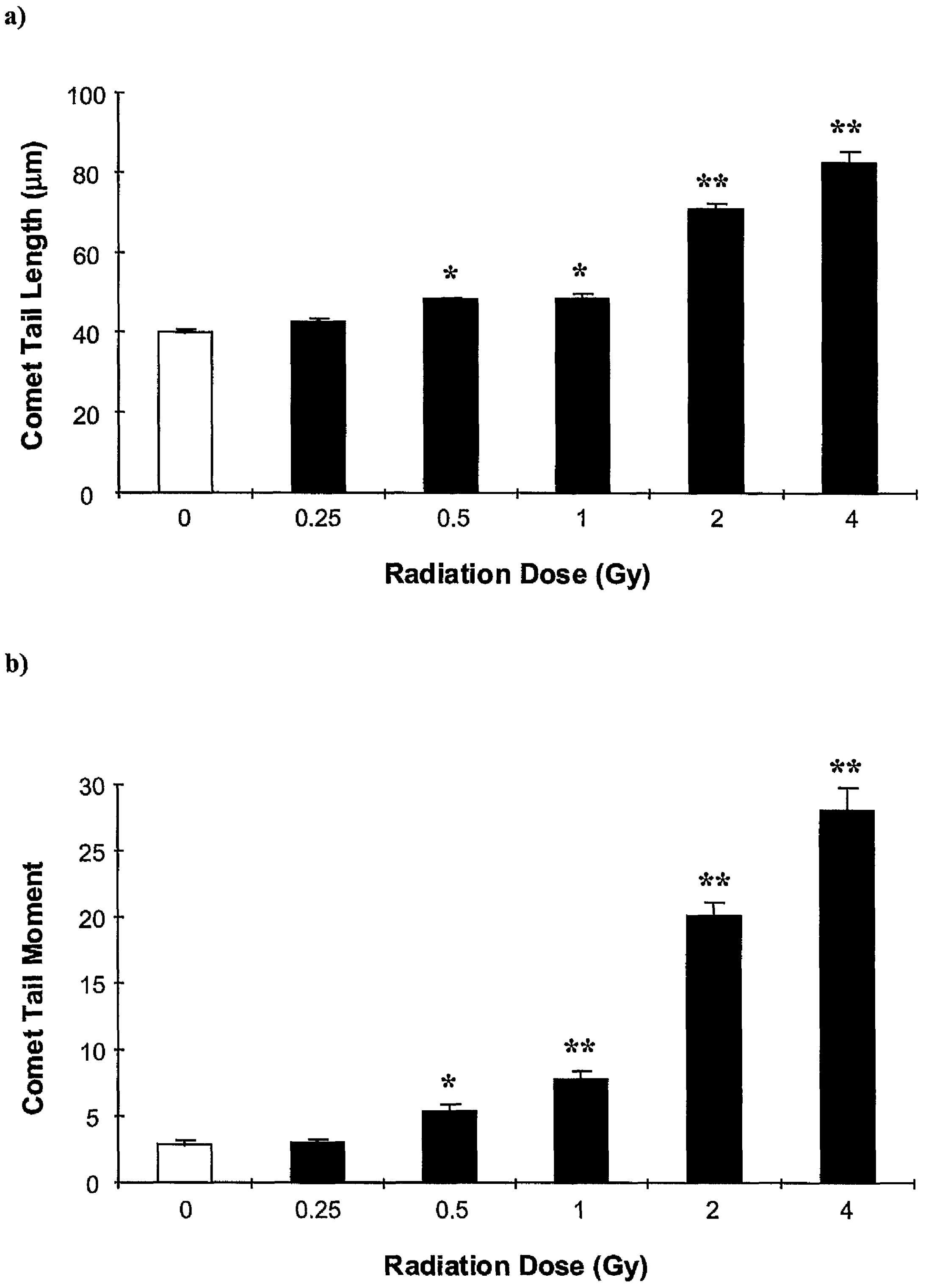

***Figure 4.2.8.2** : Dose response effects of testicular X-rays on DNA damage assessed by neutral comet assay in vas deferens spermatozoa 45 days post-irradiation. a) Effects on comet tail length and b) effects on comet tail moment. Data shown represents the mean ± S.E.M for each experimental group (n=5). Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (Kruscal-Wallis one-way ANOVA and Mann Whitney U-test).*

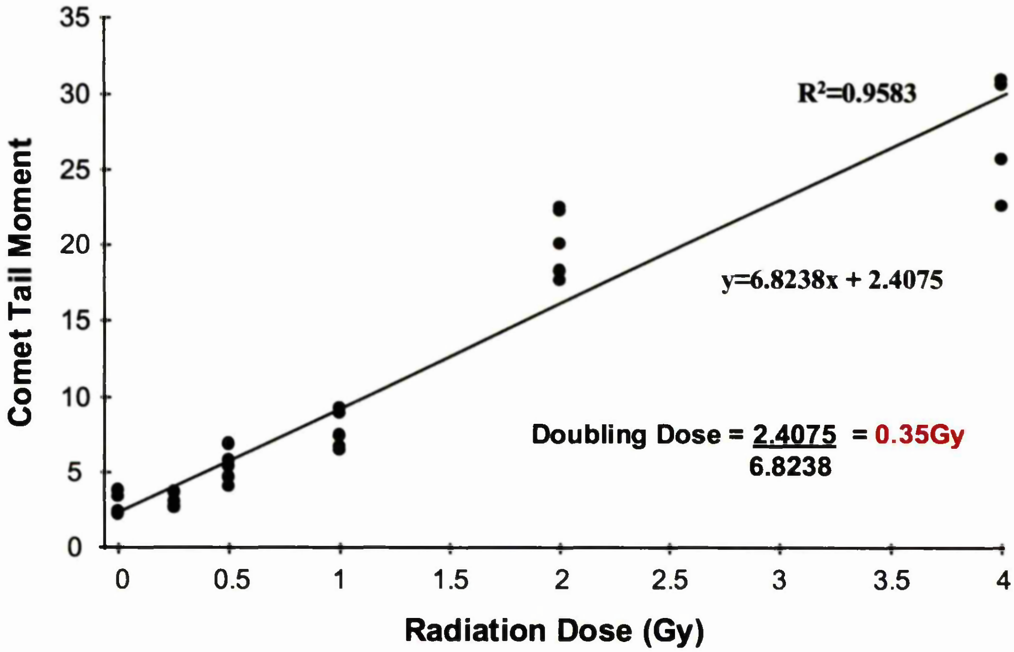

Figure 4.2.8.3. *: Fitting of linear regression line to dose response data in order to calculate the doubling dose. Each data point represents the mean comet tail moment for one experimental animal after the scoring of 100 comets.*

Table 4.2.8.1 *: Dose response effects of testicular X-rays on % Tail DNA of neutral comets from vas deferens spermatozoa, 45 days post-irradiation.*

Radiation Dose (Gy)	**% Tail DNA**
0	10.4 ± 0.9
0.25	11.1 ± 0.7
0.50	17.2 ± 1.3 **
1.0	21.6 ± 1.4 **
2.0	47.9 ± 2.2 **
4.0	59.3 ± 1.6 **

Data shown represents the mean ± S.E.M for each experimental group (n=5).
Asterisks denote values significantly different to control, * = P<0.05, ** = P<0.01 (Kruscal-Wallis one-way ANOVA and Mann Whitney U-test).

4.2.9 Distribution of DNA damage in Spermatozoa at Different Doses of *In vivo* Irradiation.

Again, unlike *in vitro* irradiation of spermatozoa, *in vivo* testicular irradiation at different doses did not result in a homogenous distribution of DNA damage in the sampled spermatozoa population (Figure 4.2.9.1). Spermatozoa obtained from unirradiated animals show a normal frequency distribution for comet tail length with a mean of approximately 40μm. Increasing the dose of *in vivo* irradiation does not simply cause a rightward shift of the normal distribution as was seen with *in vitro* irradiation of spermatozoa. At 0.5Gy which was the first dose at which statistically significant increases in comet parameters were detected, it can be seen that although a normal distribution is still evident it appears to be skewed rightwards with a long rightward tail (comet tail lengths > 70μm). This has the effect of raising the mean comet tail length whilst the median stays similar to control levels. This effect is even more exaggerated with a dose of 1.0Gy with a even larger tail on the right side. Again the mean comet tail length is significantly different to the control whilst the median is the same as the control. It appears that irradiation causes a separate population of cells to emerge, that have higher levels of DNA damage (tail lengths >60μm). However *in vivo* irradiation does not produce similar effects in all cells with some remaining undamaged whilst other display large comet tails. At higher doses (2.0 and 4.0Gy) we see the disappearance of the subpopulation of undamaged cells with almost all the cells showing large comet tails and high degrees of DNA damage.

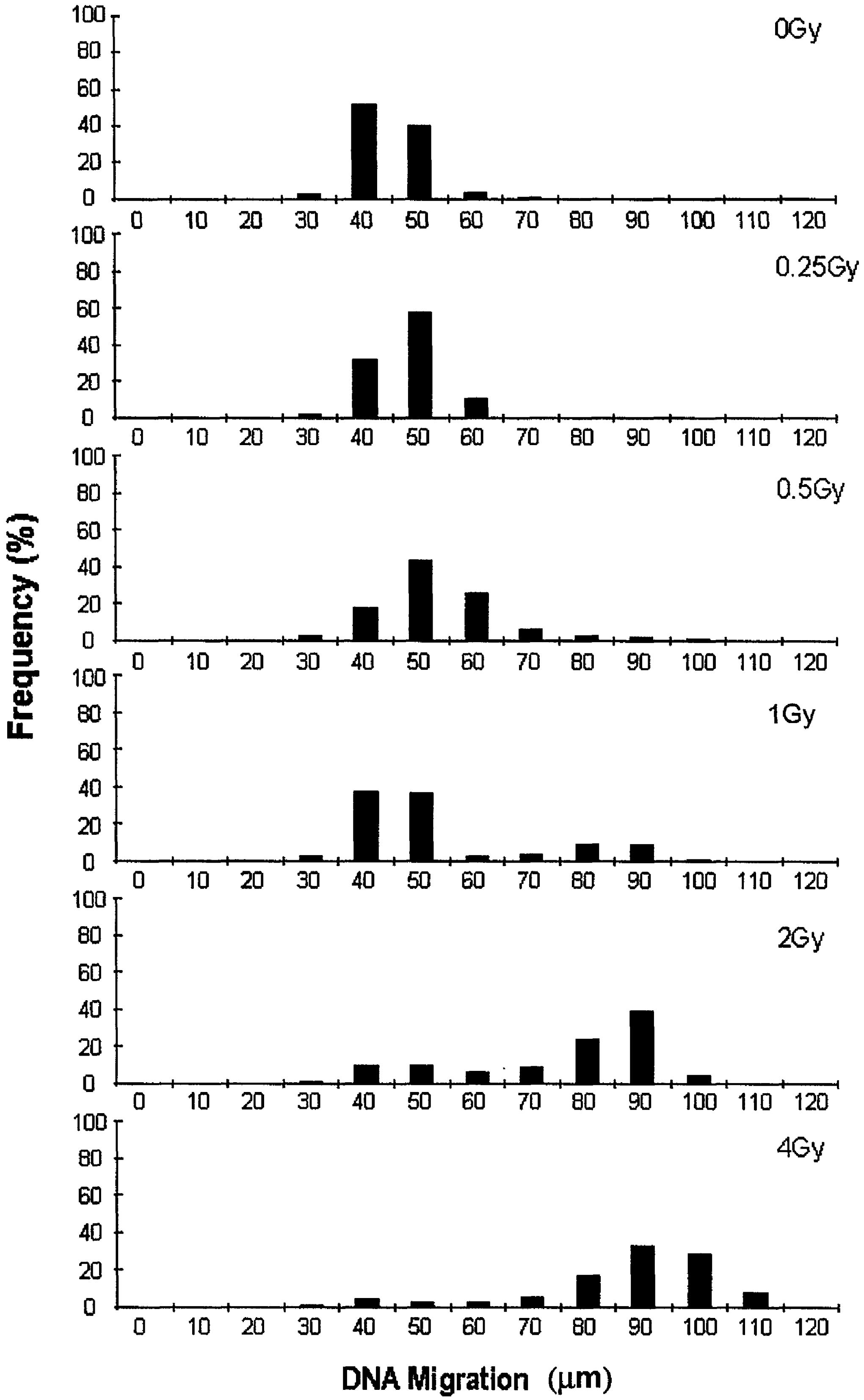

Figure 4.2.9.1 : *Distribution of comet tail lengths with increasing dose of testicular X-rays, 45 days post-irradiation. Data shown is representative of 5 experimental subjects, 100 cells analysed per subject.*

4.3 DISCUSSION.

Radiation can have adverse affects upon the male reproductive system and it has been demonstrated that exposure of germ cells within the testis to ionizing radiation may result in the production of sperm with damaged DNA which may lead to effects post fertilisation (Ehling, 1971; Nomura, 1988; Dubrova *et al.,* 1993; Sailer *et al.,* 1995a). We have developed an assay that enables the detection of DNA damage induced by ionising radiation in spermatozoa exposed *in vitro* (Chapter 3). However as spermatozoa are extremely radioresistant (Meistrich, 1986a) high doses of radiation were required *in vitro* to produce measurable levels of DNA strand breaks. The aim of the experiments in this chapter therefore, were to determine whether *in vivo* irradiation of testicular germ cells results in the transmission of DNA damage through the spermatogenic process and whether the comet assay can be used to detect this damage.

4.3.1 Effects of 4Gy Testicular X-rays Assessed 16, 31 and 45 Days Post-Irradiation.

The effects of radiation on the testis have been well documented (Meistrich, 1986a, 1993; Ogilvy-Stuart & Shalet, 1993). Irradiation with doses as low as 0.2Gy may cause a significant depression in sperm counts and temporary azoospermia (Oakberg, 1955; Ogilvy-Stuart & Shalet, 1993). Differentiating spermatogonia are regarded as the most radiosensitive cell type within the testis (Meistrich, 1986a) and at a dose of 0.5Gy their numbers and that of their daughter cells, the preleptotene spermatozoa are severely reduced. The doses of radiation required to kill spermatocytes are higher than for spermatogonia. Doses of approximately 2-5Gy are required to kill spermatocytes and produce a subsequent reduction in spermatid numbers. Post-meiotic germ cells stages are generally regarded as radioresistant to the cytotoxic effects of radiation although doses of 5-8Gy may cause some damage to round spermatids leading to a reduction in sperm counts. Due to the differing radiosensitivities of the germ cells within the testis, spermatogonia, spermatocytes and ultimately spermatids disappear from the testis after an acute dose of radiation. After doses of 3-5Gy to the testis sperm counts in laboratory animals remain above 50% of control values for the first 2-3 weeks post-irradiation but then drop dramatically due to the kinetics of sperm production (Meistrich, 1993). Studies have suggested that the kinetics of spermatogenesis are not affected by irradiation (Edwards & Sirlin, 1958).

In this experiment irradiation of the testis with 4Gy of X-rays lead to a decrease in testis weights and sperm counts which is consistent with effects described in the literature (Meistrich, 1986a, 1993; Pogany, 1987). Testes and sperm from experimental animals were examined at 3 timepoints post-irradiation. These were firstly at 16 days and then at 31 days and 45 days post-irradiation. The duration of a spermatogenic cycle in the mouse is 41 days (Clermont, 1972; Meistrich, 1993). These timepoints were chosen so that sperm collected from the vas deferens at the different timepoints would be derived from different germ cell stages within the testis. The radiosensitivity of germ cells within the testis is known to be specific to germ-cell stage. Proliferating spermatogonia are known to be more radiosensitive that post-meiotic germ cells such as spermatids and spermatozoa (Meistrich, 1986a). By collecting sperm at these timepoints it was aimed to determine whether irradiation of different germ cell types would result in spermatozoa with differing degrees of DNA damage and whether this was related to irradiation of specific germ cell stages. Taking into account the length of the spermatogenic cycle of the mouse and the time taken for the sperm to pass out of the lumen of the tubule and through the epididymis into the vas deferens sperm collected 16 days post-irradiation would be derived from spermatids, sperm collected 31 days post-irradiation derived from spermatocytes and sperm collected 45 days post-irradiation derived from proliferating spermatogonia (Clermont, 1972; Meistrich, 1986a).

A decrease in testicular weights was evident at 16 days post-irradiation and remained at this level through the 31 day timepoint as well. The decrease in testicular weight is as a result of the death of radiosensitive germ cells within the testis and this is reflected in the histological sections of the testes at these timepoints, which show decreased tubular size, irregular spermatogenic tubules and loss of testicular germ cells. Previous studies have suggested the most radiosensitive germ cells within the testis are the proliferating spermatogonia which have an LD_{50} to ionising radiation of 0.4Gy (Meistrich, 1986a). Therefore it is highly likely that after the dose of radiation used in this experiment (4Gy) the majority of these cells will have been killed. However the proliferating spermatogonia only account for a relative small proportion of testicular mass (Russell *et al.,* 1990) and therefore killing of this cell type alone will not result in the decrease in testis weights observed after 16 days. As well as the death of proliferating spermatogonia, a dose of 4Gy X-rays will also kill other germ cells within the testis such as spermatocytes, although they will not be depleted to the extent of the proliferating spermatogonia. However, after 16 days this will lead to a depletion in the numbers of elongating spermatids which together with spermatozoa account for the majority of the mass of the testis (Russell *et al.,* 1990). Therefore the loss of both proliferating spermatogonia and some spermatocytes may account for the decreases in testis weight observed after 16 days. Additionally. the LD_{50} of stem spermatogonia is 2-3Gy, so a significant proportion of these cells are likely to have been killed by X-irradiation. This is supported by the presence of some tubule devoid of germ cells in the 45 day testes sections. Presumably all early spermatogonia and stem cells in these tubules were killed by the radiation dose and therefore after all the late germ cells had passed into the lumen of the tubule no repopulation could occur. Spermatids and spermatozoa are radioresistant so it is unlikely that irradiation will have resulted in significant killing of these germ cell stages. This is reflected in the results for vas deferens sperm counts as after 16 days no decrease was observed. Decreases in testis sperm head counts observed at this timepoint probably reflect the killing of spermatocytes and round spermatids. At 31 days however, sperm counts in the vas deferens had fallen by 66% and at 45 days sperm counts were even lower. The decrease in sperm counts observed at 31 and 45 days represents killing of spermatocytes and spermatogonia respectively. Indeed it has previously been shown that after acute irradiation of the testis a gradual decrease in sperm counts is observed over the following weeks with the greatest decrease in sperm counts observed 5-7 weeks post-irradiation with a recovery of sperm numbers afterwards (Searle & Beechey, 1974; Meistrich, 1993). At 45 days post-irradiation an increase in testes weights from 16 and 31 day levels was observed. This is probably as a result of the repopulation of the tubules from surviving spermatogonial stem cells. Previous studies have demonstrated that the recovery potential of spermatogenesis is directly related to the surviving numbers of spermatogonial stem cells (Meistrich *et al.,* 1978; Meistrich, 1986b).

4.3.2 Effect of *In vivo* Irradiation on DNA Damage in Spermatozoa.

Testicular irradiation has been shown to produce and increased frequency of dominant lethal offspring in the offspring and this is due to fertilisation with sperm containing damaged DNA (Ehling, 1971; Searle & Beechey, 1974; Goldstein *et al.,* 1978; Russell *et al.,* 1998). Irradiation of the testis with 4Gy X-rays resulted in sperm with increased levels of DNA strand-breaks as detected by comet assay at all timepoints. This confirms results from the previous chapter (Chapter 3) that the comet assay is able to detect DNA damage induced in spermatozoa and also supports the evidence that the high doses of radiation required to induce DNA damage in sperm irradiated *in vitro* are due to the high radioresistance of spermatozoa compared to other testicular germ cells.

At the 16 day timepoint, significant increases in comet tail moment and % tail DNA were observed but no significant increase in comet tail length was recorded. Previous comet studies using the neutral assay in other cell types have also demonstrated increases in comet tail moment with no corresponding increase in comet tail length at low levels of DNA damage (Collins *et al.,* 1997). Unlike the alkaline assay (where very distinct head and tail regions of the comet are visible at low levels of damage) at low levels of damage in the neutral assay there is no distinct formation of a separate tail region of the comet. Instead what appears to occur is a stretching of the head region of the comet towards the anode with only a small increase in tail length but significant increases in % tail DNA and comet tail moment. This is thought to be due to the movement of attached strands of DNA as strand breaks increase the ability of the loop domains of DNA to move whereas at higher levels of damage, individual "DNA fragments" are formed and are able to migrate a form a characteristic tail (Fairbairn *et al.,* 1995). At the other timepoints (31 and 45 day's post-irradiation) damage levels were high enough to produce significant increases in all comet parameters.

Testicular X-irradiation produced DNA damage in spermatozoa at all timepoints as detected by increases in comet tail length, tail moment and % tail DNA. Sperm from irradiated animals were processed by neutral comet assay, which permits the measurement of double-stranded DNA breaks. Estimates suggest that no more than 70 double strand breaks are induced by every 1Gy exposure to X-rays (Ward, 1990).

Therefore it can be calculated that approximately 280 double strand breaks will have been induced in every cell exposed to 4Gy X-rays. However, it is unlikely that such numbers of breaks would produce the large comets and % tail DNA values (approximately 57%) seen in sperm collected at the 45 day timepoint after irradiation of spermatogonial cells. Previous studies with *in vitro* irradiation of CHO-K1 and V79-171b cell lines have suggested that approximately 50Gy of irradiation would be required to produce equivalent comet parameters to those described after 4Gy irradiation of spermatogonia (Olive *et al.,* 1990; Olive & Banath, 1993; Kent *et al.,* 1995). Therefore this neutral form of the assay may also be measuring other forms of DNA damage as well as double-strand breaks. Single strand breaks are induced at the rate of approximately 1000 per Gy by X-rays (Ward, 1990). Although ssb's are much more easily repaired than double strand breaks they may also contribute an effect in the neutral comet assay. It has previously been reported that single strand breaks can have an effect in the neutral comet assay since relaxation of DNA loop domains induced by ssb's will occur at neutral as well as alkaline pH (Ostling & Johanson, 1984; Collins *et al.,* 1997). Also extra DNA strand breaks may be induced during attempted repair of DNA lesions (e.g. the excision repair pathway) and therefore these may also explain the large comets produced in this experiment (Gedik *et al.,* 1992; Green *et al.,* 1992).

DNA damage levels measured by comet assay in spermatozoa after *in vivo* testicular irradiation reflected the relative radioresistance of the different germ cell types (Meistrich, 1986a). The highest levels of DNA damage were observed in spermatozoa derived from spermatogonia (high radiosensitivity) with sperm collected at 31 days (spermatocytes-intermediate radiosensitivity) showing an intermediate level of DNA damage and spermatozoa derived from spermatids (radioresistant) showing the least amount of DNA damage. These results however are in contrast to previous studies which examined incidences of dominant lethal mutations after testicular irradiation (Ehling, 1971; Searle & Beechey, 1974; Goldstein *et al.,* 1978; Russell *et al.,* 1998). Ehling (1971) investigated the incidence of dominant lethal mutations after testicular irradiation of mice with either 2Gy, 4Gy or 8Gy X-rays and suggested that the highest incidence of dominant lethal mutations was observed after irradiation of early spermatids and late spermatocytes. Searle and Beechey (1974) and Russell *et al.,* (1998) also examined the effects of X-irradiation (0-4Gy) on the induction of dominant lethal mutations in mice and similarly concluded that the most sensitive spermatogenic stage were early spermatids. The difference between the results described in this chapter and studies investigating dominant lethal mutations after X-rays may be explained by effects of radiation on spermatozoa and sperm counts.

The measurement of dominant lethality is based on the assessment of the numbers of live embryos obtained from treated groups relative to the number of embryos produced by control matings. This assay make the assumption all oocytes ovulated will be successfully fertilised. However, it has been demonstrated that a decrease in sperm counts to 10% of control levels may lead to an increased incidence of unfertilised oocytes which will be classified as pre-implantation losses by the dominant lethal assay (Searle & Beechey, 1974). Therefore after irradiation of spermatogonia which results in dramatic decreases in sperm counts and therefore reductions in fertilisation rates, a high incidence of dominant lethals is observed which is falsely due to failure of fertilisation rather than transmitted genetic damage. For this reason spermatogonial irradiations have been largely omitted from dominant lethal studies because of the difficulty in accurately assessing true dominant lethality. Therefore it is quite possible that sperm produced after irradiation of spermatogonia cells may contain higher levels of genetic damage than after irradiation of other germ cell stages but this may lead to increases in unfertilised eggs rather than preimplantation losses as a result of genetic damage transmitted at fertilisation.

Another study, which examined genetic damage transmitted at fertilisation after X-irradiation of different germ-cell stages by assessing minisatellite mutation rates found that the highest incidences of minisatellite mutations were found in offspring, derived from irradiated spermatogonial cells (Dubrova *et al.,* 1993, 1998). Minisatellite mutation rates after irradiation of spermatogonial cells were 4-fold higher than spontaneous mutation rates in offspring derived from control matings. Mutation rates after irradiation of spermatids although slightly elevated were not significantly different to controls. Another study which examined the genetic integrity of spermatozoa after testicular X-irradiation has also demonstrated increased chromatin damage after exposure of spermatogonia cells to X-rays (Sailer *et al.,* 1995a). These results are consistent with the data presented in this chapter that irradiation of spermatogonial cells leads to the production of spermatozoa with the high levels of DNA damage. This demonstrates that radiosensitive cells can survive irradiation and develop into spermatozoa despite the high levels of cell killing observed in these experiments (shown by the decrease in testis weights). The observation that spermatozoa with DNA damage are produced after testicular irradiation with 4Gy suggests a failure of effective DNA repair mechanisms and genetic checkpoints present within the testis.

At the present date this is the only study of which we are aware, that has described the use of the comet assay to detect DNA damage in spermatozoa after exposure of testicular germ cells *in vivo* to a reproductive toxin. A number of publications in recent years have described the use of the comet assay to measure DNA damage in spermatozoa but experiments have concentrated on the *in vitro* exposure of spermatozoa to genotoxic agents (Hughes *et al.,* 1996, 1997; McKelvey-Martin *et al.,* 1997; Anderson *et al.,* 1997a, 1997b, 1997d; Singh & Stephens, 1998). The comet assay has been used to investigate the *in vivo* effects of the chemical 1,3 butadiene on the rat testis and results suggested that after 10 weeks chronic daily exposure, increased levels of DNA damage could be detected by comet assay in testicular cells from treated animals (Brinkworth *et al.,* 1998). However, this study examined DNA damage in germ cells prepared by enzymatic digestion of the testis, rather than in spermatozoa themselves as performed in the experiments described in this chapter. Also no attempt was made to classify different germ cell stages so measurement and comparison of damage in different cell types was not performed. However, this study does demonstrate the ability of the comet assay to detect DNA damage in germ cells within the testis. Further studies could be performed on testicular germ cells irradiated both *in vitro* and *in vivo* to examine the relative radiosensitivities of the germ-cell stages and to examine repair of DNA strand breaks by testicular germ cells.

Studies with alkaline elution protocols have demonstrated increases in DNA strand breaks in spermatozoa after exposure of testicular cells *in vivo* to the cytotoxic chemicals ethylene oxide (Sega & Generoso, 1988) and MMS (Sega *et al.,* 1986). However these studies only examined exposure of spermatids and not earlier germ cells stages. As described earlier Sailer *et al.,* (1995a) examined the effects of testicular X-irradiation on sperm chromatin structure using the SCSA. It was found that after irradiation with 4Gy, sperm collected 40 days post-irradiation showed increased susceptibility to DNA denaturation compared to unirradiated sperm from control animals. Whilst this study differs in the mode of measurement to the experiments described in this chapter it also suggests that testicular exposure to X-rays can induce DNA damage in germ cells that can be transmitted through the spermatogenic process resulting in sperm with damaged DNA. Unfortunately however, this SCSA study did not examine sperm collected at different timepoints post-irradiation to examine the susceptibility of different germ cell types.

Unlike *in vitro* exposure to ionising radiation, exposure to X-rays *in vivo* did not produce a normal distribution of DNA damage across the sample population. Instead it can be seen that the shape of the frequency distributions are biphasic. It is interesting to note that two separate populations of sperm appear to emerge after irradiation *in vivo* rather than a wide distribution of cells with differing levels of DNA damage as was observed *in vitro* (Chapter 3). This may reflect differences in the capacity of the various germ cell stages to repair their DNA. Previous studies have suggested that spermatogonia, spermatocytes and round spermatids are able to repair DNA damage induced by irradiation (Ono & Okada, 1977; Bradley & Dysart, 1985; Coogan & Rosenblum, 1988). However repair is not always complete and significant levels of strand-breaks may remain after the repair process. In contrast, it has been reported that elongating spermatids and spermatozoa are incapable of repairing DNA damage due to a lack of DNA repair enzymes (Ono & Okada, 1977; Van Loon *et al.,* 1991, 1993). This may occur due to the loss of cytoplasm and DNA repair machinery during spermiogenesis. Therefore this information suggests that two separate populations of spermatozoa may emerge, as a proportion of early germ cells are able to repair their DNA effectively after irradiation, whilst other cells do not undergo DNA repair as the levels of strand breaks induced are too high to repair effectively. Additionally, there may differences in the induction of DNA damage amongst the different germ cell types reflecting their relative radiosensitivities (Meistrich, 1986a). Also there are also numerous subtypes of spermatids, spermatocytes and spermatogonia (Russell *et al.,* 1990) and they may differ in both their radiosensitivity and induction of DNA damage by ionising radiation. Therefore these factors may explain why a uniform distribution of DNA damage is not observed across the whole sperm population.

4.3.3 Effects of 4Gy Testicular X-rays 120 Days Post-Irradiation.

As marked changes were observed in both testicular and sperm parameters at all timepoints after acute irradiation, effects on the testes and spermatozoa were also examined 120 days post-irradiation. This was in order to determine whether there were any chronic effects on reproduction. As the duration of a seminiferous cycle in the mouse is 41 days (Clermont, 1972; Meistrich, 1993), at this 120 day timepoint the only cells still present in the testis from the time of the original irradiation will be the spermatogonial stem cells. These stem cells will have been used to repopulate the tubule and aid recovery of the seminiferous epithelium. It has previously been demonstrated that recovery of spermatogenesis by stem cells starts rapidly after insult and is related to surviving stem cell numbers (Meistrich *et al.,* 1978; Meistrich, 1986b).

At 120 day post-irradiation, testis weights and sperm counts had made marked recovery towards control values showing that tubular repopulation by stem cells was occurring. However, testis weights were still significantly lower in irradiated animals than controls. This difference in testis weights may be as a result of X-irradiation producing the complete ablation of spermatogenesis in some tubules. This is evident in the histological sections of testes from irradiated animals. Other investigators have suggested that the LD_{50} of stem cell spermatogonia is 2-3Gy (Meistrich, 1986a). Therefore after the dose of 4Gy X-rays used in this experiment a significant proportion of stem cells will have been killed.

Sperm collected from the vas deferens at 120 days had much lower levels of DNA damage as assessed by comet assay than sperm derived from spermatogonia collected after 45 days. This suggests that repopulation of the tubule occurs from either undamaged stem spermatogonia or stem cells that have repaired their DNA. Spermatozoa, derived from irradiated stem cells however, showed slightly but significantly increased comet tail moments and % tail DNA values. No significant increases in comet tail length were observed but this may be due to the phenomenon of "stretching" which occurs at low levels of DNA damage (Fairbairn *et al.,* 1995) and has previously been discussed. All comet parameters were considerably lower than observed in spermatozoa derived from irradiation of proliferating spermatogonia (45 days). This may reflect the higher radioresistance of stem cell spermatogonia relative to proliferating spermatogonia (Meistrich, 1986a) but also probably reflects repair of induced DNA damage in the period post-irradiation. These results suggest that acute irradiation of the testis with 4Gy X-rays may have produced some damage to spermatogonial stem cells which has not been repaired. Genetic damage present in stem cells may be transmitted to future generations of germ cells derived as a result of mitotic divisions from stem spermatogonia. This has important implications for fertilisation and the genetic integrity of future offspring. Other studies in mice have demonstrated that offspring born after paternal X-irradiation of stem spermatogonia have significantly higher minisatellite mutation rates than control offspring (Dubrova *et al.,* 1998) and experiments performed by Ehling, (1971) have also demonstrated an increase in dominant lethal mutations after paternal irradiation of stem cell spermatogonia. These reports are consistent with the results of the experiments presented in this thesis and demonstrated that damage to spermatogonial stem cells may persist and lead to chronic reproductive effects.

4.3.4 Dose-Response Effects of Testicular Irradiation.

The previous experiment used a single dose of 4Gy of X-irradiation and demonstrated that damage can be induced during spermatogenesis resulting in the production of sperm with damaged DNA. In order to investigate the sensitivity of the comet assay, it was required to examine the effects of different doses of radiation. Previous studies using other techniques (e.g. SCSA) have demonstrated that effects can be detected in sperm after irradiation of the testis with doses as low as 0.25Gy (Sailer *et al.,* 1995a). Therefore, mice were irradiated testicularly with doses ranging from 0Gy up to 4Gy. Sperm were collected from animals 45 days post-irradiation which corresponds to spermatogonial irradiation which were shown to be most sensitive germ cell stage to the DNA damaging effects of radiation in the previous study.

Significant changes in testis weights were observed with doses as low as 0.5Gy and increased in severity with dose. Reductions in sperm counts were also observed in a dose-related manner although decreases in vas deferens and testis sperm head counts were observed after the lowest dose of 0.25Gy. The effects of 4Gy X-rays on the testis and sperm counts in this experiment were similar to those described in the previous experiment. As described previously, radiation produces decreases in testis weights by killing radiosensitive germ cells. Therefore dose-response effects were expected as the cytotoxic potential of radiation is related to dose. As the dose of irradiation increases more germ cells will be killed. At doses up to 1Gy only spermatogonia will be affected because of their extremely high radiosensitivity. At higher doses cytotoxic effects on spermatocytes will also be observed. Previous studies have demonstrated dose-dependent decreases in testicular weight following X-irradiation over a similar dose range (Sailer *et al.,* 1995a) consistent with the results described in this chapter. It is also not surprising that effects were observed with doses as low as 0.5Gy. This is because proliferating spermatogonia within the testis are extremely radiosensitive with a calculated LD_{50} of 0.4Gy (Meistrich, 1986a). Therefore after a dose of 0.5Gy over half these cells would have been killed by the irradiation. In this experiment a small, but non-significant decrease in testis weights was observed after 0.25Gy X-rays at 45 days. Previous studies have demonstrated significant levels of killing in proliferating spermatogonia in histological testes sections from mice, 48 hours post-irradiation with 0.25Gy γ-rays (Oakberg, 1955). However, from the experiments with 4Gy described earlier in this chapter, it was shown that decreases in testis are observed as early as 16 days post-irradiation. However by 45 days the testis is starting to recover with repopulation of the tubule occurring from stem spermatogonia. Therefore, 45 days is probably not the best timepoint to choose if effects on germ cell killing are to be examined and a timepoint between 16 and 31 days should be chosen. Maybe at an earlier timepoint, significant decreases in testis weights after 0.25Gy may have been observed.

4.3.5 Effects of Radiation Dose on DNA Damage in Sperm.

DNA damage as assessed by neutral comet assay was evident in sperm collected from animals irradiated with doses of X-rays as low as 0.5Gy. This is also the lowest dose at which cytotoxic effects on testis mass were observed. It might have been expected that DNA damage would be detected at lower doses than would produce cytotoxic effects. However previous studies have suggested that spermatogonia are able to repair DNA damage induced by radiation (Ono & Okada, 1977; Bradley & Dysart, 1985; Coogan & Rosenblum, 1988). Therefore although low doses of radiation may induce DNA damage in spermatogonia without producing cytotoxic effects this may not be detectable in spermatozoa descended from these cells due to repair of the damage as the cell matures through the spermatogenic process. At higher doses where DNA repair is not as effective (Coogan & Rosenblum, 1988), damage may persist throughout the spermatogenic process. With increasing dose of radiation greater increases in spermatozoal comet parameters were observed. The most sensitive parameter for the measurement of DNA damage was the comet tail moment. Again at lower doses increases in tail moment and % tail DNA were observed with no significant increases in comet tail length which are probably due to the effects of "stretching" as described previously.

Therefore the above results suggest that the comet assay is able to detect DNA strand breaks in spermatozoa after *in vivo* irradiation of spermatogonia cells with a dose of X-rays of 0.5Gy. This is the first experiment, of which we are aware, that has used this technique to look at DNA damage induced in sperm after testicular irradiation and therefore unfortunately there are no similar comet experiments available for comparison. However, studies have demonstrated the ability of the alkaline comet assay to detect DNA damage after *in vivo* irradiation of liver cells of mice irradiated with dose of 0.5Gy and 1Gy γ-rays (Carrera *et al.,* 1998); peripheral blood and bone marrow cells from dogs subjected to whole body irradiation with 3.9Gy X-rays (Kerja *et al.,* 1996); peripheral blood leukocytes from thyroid cancer patients treated with ^{131}I (Gutierrez *et al.,* 1998) and lymphocytes from patients and radiologists exposed to diagnostic X-rays (Wojewodzka *et al.,* 1998). Despite the lack of comet studies after *in vivo* testicular irradiation, a number of other investigators have looked at different markers of genetic damage in sperm after exposure of testicular germ cells to radiation. Sailer *et al.,* (1995a) used the SCSA, a flow cytometric assay to examine the denaturability of sperm chromatin after X-irradiation of testicular spermatogonial cells. Significant increases in DNA denaturability were observed with doses as low as 0.25Gy. Whilst this is lower than the dose of 0.5Gy required in this study, it is still of similar magnitude and compares well with the comet assay results described in this chapter. The difference in sensitivity of the two assays may be attributed to differences in the techniques. The comet assay measures purely DNA strand-breaks whilst the SCSA measures DNA denaturability and it is thought that a variety of factors such as strand breaks, protamine binding, DNA adduct formation may influence this (Evenson, 1990b; Evenson & Jost, 1994). However, a previous study has demonstrated that comparable results were obtained when endogenous DNA damage levels in human sperm samples were measured by both comet assay and SCSA (Aravindan *et al.,* 1997). Alternatively, the experiments described in this chapter were performed with BDF1 mice, whilst the SCSA studies utilised mice of B6C7F1/J lineage. A previous study has suggested that spermatogonia from different strains of mice may differ in radiosensitivity by up to a factor of 2 (Bianchi *et al.,* 1985). Therefore strain differences may account for the difference in sensitivity between the SCSA and comet experiments described in this thesis.

The doses of radiation employed in this experiment are also comparable to those used in minisatellite mutation studies conducted by Dubrova *et al.,* (1993; 1998). This group detected elevated minisatellite mutation rates in the offspring of parents paternally irradiated with 0.5 and 1.0Gy X-rays. After exposure of spermatogonial cells to 0.5Gy X-rays, 6 weeks prior to matings, minisatellite mutation rates in offspring derived from paternally irradiated animals were increased by a factor of 2.4 compared to controls. Spermatogonial irradiation with 1.0Gy X-rays produced a 4 fold increase in mutation rates among offspring of irradiated males. Therefore the results from this study are comparable in sensitivity with the results described with the comet assay in this chapter. As Dubrova *et al.,* (1998) also examined the dose response effects of X-irradiation (although only three dose points, 0, 0.5 and 1.0Gy were investigated compared to the six doses used in the comet assay experiment described in this chapter) from their results they were able to calculate a doubling dose for radiation-induced increases in mutation rates. The doubling dose is the dose of radiation required to double the background mutation frequency. In a similar fashion using the comet assay results presented in this chapter it was possible using linear regression analysis to calculate a doubling dose for the induction of DNA damage in mouse sperm after spermatogonial irradiation. The doubling dose of 0.35Gy calculated from the results presented in this chapter is similar to the dose of 0.33Gy described by Dubrova *et al.,* (1998). This dose is also consistent with the results of other studies (total doubling dose range 0.17-0.56Gy, mean =

0.35Gy) which used other scoring systems to measure mutations after acute exposure of spermatogonia to irradiation (Luning & Searle, 1971; Russel & Kelly, 1982; Favor, 1989). Therefore these result seem to indicate that the comet assay can detect DNA damage resident in spermatozoa after irradiation of spermatogonia with a similar level of sensitivity to other techniques such as minisatellite mutation rates Dubrova *et al.,* 1993, 1998) and the SCSA (Sailer *et al.,* 1995a).

Whilst it also appears to produce similar results for doubling dose as other mutational analysis systems (e.g. Russell 7-locus test, dominant cataract and skeletal malformations), much lower doses of radiation were required by this comet study to produce detectable changes than in these other systems (Russel & Kelly, 1982; Luning & Searle, 1971; Favor, 1989). This may reflect the fact that traditional mutational analysis systems examine targeted effects at specific loci in DNA. Since events in DNA occur at random after irradiation, these occur extremely infrequently at the loci examined by these specific mutational techniques. Therefore high doses of radiation are required to produce detectable increases in mutation rates. Minisatellite techniques offer greater sensitivity for mutational analysis since minisatellite tandem repeat loci are widely distributed throughout the vertebrate genome therefore increasing the probability of irradiation producing a "hit" and leading to a mutational event at these loci. The advantage of techniques such as the comet assay and SCSA is that they are able to examine the integrity of the whole genome rather than just specific parts of it. Another advantage of the comet assay over mutational analysis techniques is that these methods rely on matings and the production of offspring before assessment and measurement can take place. Therefore this is much more consuming of both time and experimental animals as well as being of higher technical difficulty than the comet assay where analysis of damage can be performed on sperm directly. However although the comet assay may give an indication of total DNA damage in spermatozoa, if this is relatively severe then it may result in the failure of fertilisation. Minisatellite analysis on the other hand only examines genetic damage that has been transmitted by spermatozoa to the offspring at fertilisation. Further studies should therefore be performed to determine the levels of DNA damage than can be transmitted at fertilisation and result in the production of live offspring.

As the comet assay is a single cell based assay where effects are measured on individual cells rather than across the population as a whole then heterogeneity can be studied. The frequency histograms show the distribution of the comet tail lengths at each radiation dose. Firstly it can be seen with doses of 0.5Gy and above irradiation leads to a wide variety of sperm cells with differing degrees of DNA damage as reflected by the wide range in tail moments. This may reflect heterogeneity in radiation response of spermatogonia and/or heterogeneity in repair capacity of these cells. It is known that there are up to 8 different subtypes of spermatogonia (Russell *et al.,* 1990) and therefore these different subtypes may differ slightly in their radiosensitivities therefore accounting for this range of radiation response.

Although mean values for comet tail length and comet tail moment increased with radiation dose this is not reflected as a simple increasing rightward shift of normal distributions with increasing radiation dose in the frequency histograms. Instead it appears that irradiation produced a separate population of cells with higher tail lengths rather than uniformly damaging all cells. The control frequency distribution is of normal appearance with a mode of 40μm and a range between 30 and 60μm. With increasing doses of irradiation (starting at 0.5Gy) there appears to be a decrease in this undamaged population of cells with an increase in frequency of cells showing DNA damage with tail lengths >70μm. At high dose of irradiation (>2Gy) then there are very few undamaged cells (tail lengths <60μm) with the majority of sperm showing tails with lengths between 80 and 110μm. Therefore it appears unlike *in vitro* irradiation *in vivo* does not result in a uniform response of cells. At lower doses of radiation, cells may be able to repair their DNA quite effectively so that only a small proportion of cells retain a degree of DNA damage when they have developed into spermatozoa. However at higher doses of radiation then cells may not be able to repair their DNA damage effectively and/or only the most radioresistant cells survive but show a high degree of DNA damage. At intermediate doses there appears to be a mixture of the two extremes with separate populations of undamaged and damaged sperms present and this may reflect differential response to radiation of the different subtypes of spermatogonia and inherent differences in their radiosensitivities and repair capacities.

Therefore, this chapter has demonstrated how the comet assay can be used to detect DNA damage after *in vivo* irradiation with X-rays. DNA damage in sperm after irradiation seems to mirror the radiosensitivities of the germ cells to the cytotoxic effects of radiation with the greatest DNA damage detected in sperm derived from spermatogonial cells which are also regarded as the most radiosensitive germ cells within the testis. Using different doses of radiation the comet assay is able to detect DNA strand breaks in sperm produced after irradiation of spermatogonia with doses of X-rays as low as 0.5Gy. The comet assay was also able to detect low levels of DNA damage in sperm after irradiation of the testis with 4Gy X-rays followed by long term recovery and it is suggested that this damage is as a result of persisting unrepaired lesions in spermatogonial stem cells. Overall therefore the studies described in this chapter demonstrate the potential of the comet assay as a tool to investigate DNA damage in spermatozoa after insult to the testis and it therefore may have potential as a simple and reliable genotoxic screen for the future.

Chapter 5

Effects of Paternal Irradiation on Apoptosis in the Preimplantation Embryo.

5.1 INTRODUCTION.

In previous chapters it has been demonstrated how irradiation of both germ cells and spermatozoa may lead to DNA damage. This may have very important implications since it has been reported that spermatozoa themselves have no DNA repair mechanisms (Ono & Okada, 1977). Therefore if fertilisation of an oocyte occurs with a sperm containing damaged DNA, the induced damage may be transmitted to the embryo leading to abnormal effects. Concern has been expressed that paternal exposure to radiation may cause increased incidences of cancer and genetic disorders in the offspring of humans (Gardner *et al.,* 1990; Roman *et al.,* 1993) although no hard scientific evidence has been produced at present. Studies have shown that sperm with damaged DNA are capable of fertilisation (Kamiguchi & Mikamo, 1982; Mikamo *et al.,* 1990; Kamiguchi *et al.,* 1990a). When both human and mouse spermatozoa were irradiated *in vitro* (with doses up to 4Gy) and used to fertilise oocytes *in vitro,* no significant difference in the rates of fertilisation were observed between irradiated and unirradiated spermatozoa (Matsuda *et al.,* 1985a, 1985c; Mikamo *et al.,* 1990). Also when the testis is irradiated (especially post-meiotic germ cells) increases in dominant lethal mutations are observed before decreases in sperm count (Ehling, 1971; Searle & Beechey, 1974; Russell *et al.,* 1998). This indicates that spermatozoa can tolerate induced damage and are still able to achieve successful fertilisation of the oocyte.

5.1.1 Effects of DNA Damage in Spermatozoa upon the Embryo.

DNA damage in spermatozoa can lead to effects post-fertilisation. One of the most widely described effects of paternal irradiation is an increase in embryonic death more commonly assessed as the frequency of dominant lethal mutations (Ehling, 1971; Searle & Beechey, 1974; Goldstein *et al.,* 1978; Russell *et al.,* 1998). Embryonic death may occur both pre-implantation and post-implantation but is generally assessed by excising the reproductive tract during pregnancy and comparing the tubular contents (numbers of embryos and resorption sites) with the number of corpora lutea on the ovaries (no of eggs ovulated). In this way both post-implantation losses (number of resorption sites) and pre-implantation losses (number of corpora lutea minus resorption sites and embryos) are calculated (Ehling, 1971).

Paternal exposure to irradiation can also lead to other effects on the offspring. Exposure of testicular germ cells to X-rays has been demonstrated to lead to an increased incidence of leukaemia, tumours, developmental abnormalities and predisposition to cancer in the offspring of irradiated males (Nomura, 1988, 1989; Lord *et al.,* 1998). These outcomes are very severe and represent a high degree of genetic damage. Also the methods of assessment are both time consuming and complicated (require large numbers of animals and techniques). Often DNA damage in spermatozoa will lead to more subtle effects on the embryo which will not cause death of the embryo or developmental abnormalities such as skeletal malformations. However, the above techniques will not detect such subtle effects.

Assessment of minisatellite mutation rates is a method of examining effects of radiation doses that do not produce dramatic increases in embryonic death. Exposure of males to X-rays and γ-rays has been shown to lead to increased minisatellite mutation rates in derived offspring (Dubrova *et al.,* 1993, 1998; Sadamoto *et al.,* 1994; Fan *et al.,* 1995; Niwa *et al.,* 1996). However this technique only looks at surviving offspring and therefore does not reflect the total DNA damage induced by irradiation.

One common consequence of DNA damage in the majority of cells is apoptosis or programmed cell death (White, 1996). If the DNA damage in a cell is assessed as severe or unrepairable by the cells own mechanisms it may initiate a series of events that leads to its own death. This results in the deletion of the damaged DNA and avoids possible harmful consequences as a result of transmission of the damage at mitosis to future generations.

5.1.2 Apoptosis.

Apoptosis is a physiological form of cell death characterised by unique morphological and biological hallmarks (Kerr *et al.,* 1972). It is an active process and is therefore genetically driven, unlike necrosis, from which it is also morphologically distinct.

Necrosis, or accidental cell death often occurs as a result of extreme changes to the cellular environment (e.g. cellular injury, pH balance, high toxicity) and typically results in the disruption of cellular membranes and rupture of the cells as a result of osmotic swelling (Majno & Joris, 1994). Necrotic cell death often results in the induction of a secondary inflammatory reaction in the area surrounding the dead cell. In apoptosis, however the cell death seems to be driven to avoid widespread disruption to cellular membranes in order to avoid an inflammatory reaction, which is often harmful to surrounding cells, and the tissue. Apoptosis is characterised by cytoplasmic and nuclear condensation (Kerr *et al.,* 1972). Endogenous nucleases are activated which digest the DNA into oligonucleosomal fragments, which are multiples of 180bp in length. Often nuclear fragmentation is an early event in apoptosis and can be detected as the nucleosomal fragments give the appearance of a DNA ladder when electrophoresed (Arends *et al.,* 1990). As well as nuclear changes, apoptosis is also characterised by "blebbing" of the cell membrane and condensation of the cytoplasm and breakdown of organelles into membrane-bound "apoptotic bodies". These are phagocytosed by neighbouring cells and are quickly removed without triggering an inflammatory reaction. In some cases, where apoptotic fragments are not phagocytosed they undergo secondary necrosis with time.

5.1.2.1 Apoptosis in the Embryo.

Apoptosis occurs extensively in the postimplantation embryo where it removes cells during the formation and modelling of tissues and organs (e.g. removal of interdigital tissue during limb formation and neurones during brain remodelling, Bosman *et al.,* 1996; Naruse & Keino, 1995). However very little is known about the occurrence and role of apoptosis in the very early pre-implantation stage of embryo development.

It has been proposed that the blastocyst is the first stage during mammalian embryonic development where apoptosis is observed (Parchment, 1993). As early as the mid 1970's electron microscopy studies of blastocyst sections from a number of mammalian species described the morphological characteristics of certain cells within the embryo (clumped, condensed chromatin, swelling of the endoplasmic reticulum and cellular fragment) which we now recognise as the classical hallmarks of apoptosis (El-Shershaby & Hinchliffe, 1974; Mohr & Trounson, 1982). Several newer studies have confirmed that apoptosis does indeed occur within the mammalian blastocysts using the techniques of fluorescent chromatin labelling and TUNEL assay (Jurisicova *et al.,* 1995, 1996, 1998; Brison & Schultz, 1997; Hardy, 1997; Levy *et al.,* 1998; Moley *et al.,* 1998).

More recently however, evidence has began to emerge that apoptosis may occur in embryos before the blastocyst stage. In particular it has been noted that the quality of human embryos produced by *In vitro* Fertilisation (IVF) is variable with a significant number of embryos produced containing irregular sized blastomeres and multiple cellular fragments within the zona pellucida (Jurisicova *et al.,* 1995, 1996). At present the reason for these fragmented embryos is unclear however it is assumed they are of lower quality than embryos with a normal unfragmented appearance and studies have shown that fragmented embryos often show impaired development and when replaced into the female reproductive tract rarely result in pregnancy (Plachot & Mandelbaum, 1990; Erenus *et al.,* 1991).

The morphological appearance 'of these embryos (multiple cellular fragments) and reports describing nuclear abnormalities (condensed chromatin) have led to suggestions that the fragmentation occurring in these embryos is due to apoptosis (Jurisicova *et al.,* 1995, 1996). Jurisicova *et al.,* (1996) studied spare human embryos (ranging from the 2-cell to uncompacted morulae) produced by IVF and examined them by electron microscopy, chromatin staining and TUNEL labelling. Those embryos of normal appearance with regular sized blastomeres showed uniform morphology (electron microscopy) and chromatin staining and showed no TUNEL labelling. In contrast to embryos of normal appearance, those with a fragmented appearance often showed bright chromatin staining with DAPI (characteristic of condensed chromatin) and stained positive with TUNEL labelling (indicative of the presence of apoptotic DNA breaks). Ultrastructural analysis also demonstrated the presence of a high proportion of fragmented embryos with dense chromatin and multiple cellular fragments containing intact organelles. These features together are indicative of apoptotic cell death and therefore suggest that in the human at least apoptosis may occur at stages prior to blastocyst.

5.1.2.2 Role of Apoptosis in the Pre-Implantation Embryo.

Several roles have been suggested for apoptosis in the pre-implantation embryo although its exact function is at the moment unknown. Apoptosis in the blastocyst may serve a number of roles. Firstly several groups have observed that apoptosis in blastocysts occurs primarily in the inner cell mass (ICM), (El-Shershaby & Hinchliffe, 1974; Handyside & Hunter, 1986; Hardy *et al.,* 1989; Pampfer *et al.,* 1990b; Brison & Schultz, 1997; Hardy, 1997). This has led to suggestions that apoptosis may serve to eliminate cells within the ICM that retain the potential to form trophectoderm (TE). Secondly it has also been suggested that apoptosis may regulate cell number within the ICM since this gives rise to the embryo proper and a critical number of cells are required for normal postimplantation development (Tam, 1988). Thirdly, apoptosis may remove damaged cells from the embryo in order to preserve genomic integrity. Cytogenetic studies with spare human embryos have shown that fragmented embryos have a higher incidence of cytogenetic abnormalities (Michaeli *et al.,* 1990; Pellestor *et al.,* 1994; Munne & Cohen, 1994; Munne *et al.,* 1995). Together with the observation of Jurisicova *et al.,* (1996) this may suggest that apoptosis is therefore performing its common duty in somatic cells, to remove those cells with damaged DNA.

5.1.3 TUNEL Assay.

One of the key events during apoptosis is cleavage of the DNA into oligonucleosomal fragments (Arends *et al.,* 1990). The TUNEL assay is a technique that measures apoptosis by labelling the strand breaks created during nuclear degradation. Cleavage of genomic DNA during apoptosis creates double-stranded, low molecular weight fragments and single-strand breaks ("nicks") in high molecular weight DNA . These breaks are created by the action of endonucleases and can be identified by the presence of -OH groups attached to the nucleotides at the end of the break (Gorczyca *et al.,* 1993b). This causes them to differ from strand breaks induced in DNA by chemical agents or radiation (Gorczyca *et al.,* 1993a). The TUNEL assay uses free 3'-OH termini as a substrate to add modified nucleotides in an enzymatic reaction. The enzyme used is terminal deoxynucleotidyl transferase (TdT) which catalyses the polymerisation of nucleotides to free 3'-OH DNA ends in a template-independent manner. Commonly TdT is used to label the 3'-OH ends with fluorescein labelled nucleotide polymers. This allows cells containing apoptotic DNA breaks to be visualised as they will fluoresce green when excited with a fluorescent light source. The assay has been shown to be specific as the TUNEL reaction preferentially labels DNA strand breaks created during apoptosis allowing the discrimination of apoptosis and necrosis as well as discriminating apoptosis from strand breaks induced by cytotoxic drugs and radiation (Gorczyca *et al.*, 1993a).

5.1.4 Aims and Objectives.

Irradiation of cells both *in vitro* and *in vivo* has been demonstrated to cause apoptosis in a number of cell types. The degree of apoptosis will depend on both the dose of radiation and the amount of DNA damage produced. Therefore, as apoptosis is a mechanism to eliminate damaged cells it may function in the pre-implantation embryo to remove genetically abnormal blastomeres. It was hypothesised that paternal irradiation may lead to increased levels of DNA damage in preimplantation embryos. This may manifest itself as an increase in the number of cells undergoing apoptosis as a protective mechanism to remove genetically abnormal cells from the embryo and maintain genetic integrity. Male mice were irradiated with 4Gy X-rays and mated with superovulated females at timepoints corresponding to irradiation of different germ cell types. Embryos were collected after matings and classified developmentally and apoptosis examined using terminal transferase-mediated end labelling (TUNEL).

5.2 RESULTS.

Embryos were flushed from the dissected uteri of positively mated females (as identified by the presence of a vaginal sperm plug) approximately 100 hours post hCG injection. At this timepoint embryos should have developed to the blastocyst stage (Pratt, 1987; Brison & Schultz, 1997).

5.2.1 Number of Embryos Collected.

The numbers of embryos recovered from the uteri of control animals at each of the three experimental timepoints were not significantly different from each other (Table 5.2.1). The number of embryos collected from females mated with irradiated males were slightly lower than those collected from time-matched control although the differences were not statistically significant. As with embryos collected from control females, the numbers of embryos collected from females mated with irradiated males at each experimental timepoint were not significantly different from each other (Table 5.2.1.1).

Table 5.2.1.1 *: Mean numbers of embryos collected from mated superovulated females.*

	No. of embryos collected per animal / Time Post-Irradiation		
Treatment Group	***16 Days***	***31 Days***	***45 Days***
Control	32 ± 5	41 ± 9	36 ± 5
Irradiated	23 ± 5	24 ± 5	27 ± 4

Each value represents the group mean ± S.E.M (n=5).
Data analysed and compared using two-way analysis of variance.

5.2.2 Classification of Embryos.

After fixation and TUNEL labelling the embryos were mounted on microscope slides. At this stage embryos were counted and classified (using both phase contrast microscopy and fluorescent propidium iodide staining) according to which developmental stage they had reached. Four embryonic categories were used to classify the embryos and these were :-

1) Blastocysts - embryos showing a clear blastocoel cavity.
2) Morulae - embryos with blastomeres showing signs of compaction and embryos with approximately 8-32 evenly sized blastomeres that may have decompacted in preparation for the next round of cell division.
3) Fragmented - embryos with irregularly sized blastomeres and many nuclear/cytosolic fragments within the zona pellucida.
4) Unfertilised - those embryos that appear to have not undergone any cellular division that resembled the appearance of an unfertilised oocyte.

Figure 5.2.2.1 shows phase contrast micrographs of embryos as typical examples of each classification category.

5.2.3 Effects of Paternal Irradiation on Embryo Development.

100 hours post-hCG injection, greater than 90% of control embryos had reached the blastocyst stage at each experimental timepoint (Table 5.2.3.1). Morulae were also observed although at a much lower frequency than blastocyst (5-9% of total embryos at each timepoint). Embryos showing a fragmented appearance and unfertilised oocytes were hardly ever observed in control matings (3 fragmented and 2 unfertilised observed out of a total of 637 embryos analysed for the control timepoints). The percentage of embryos at each developmental stage did not alter over the three control timepoints (Table 5.2.3.1).

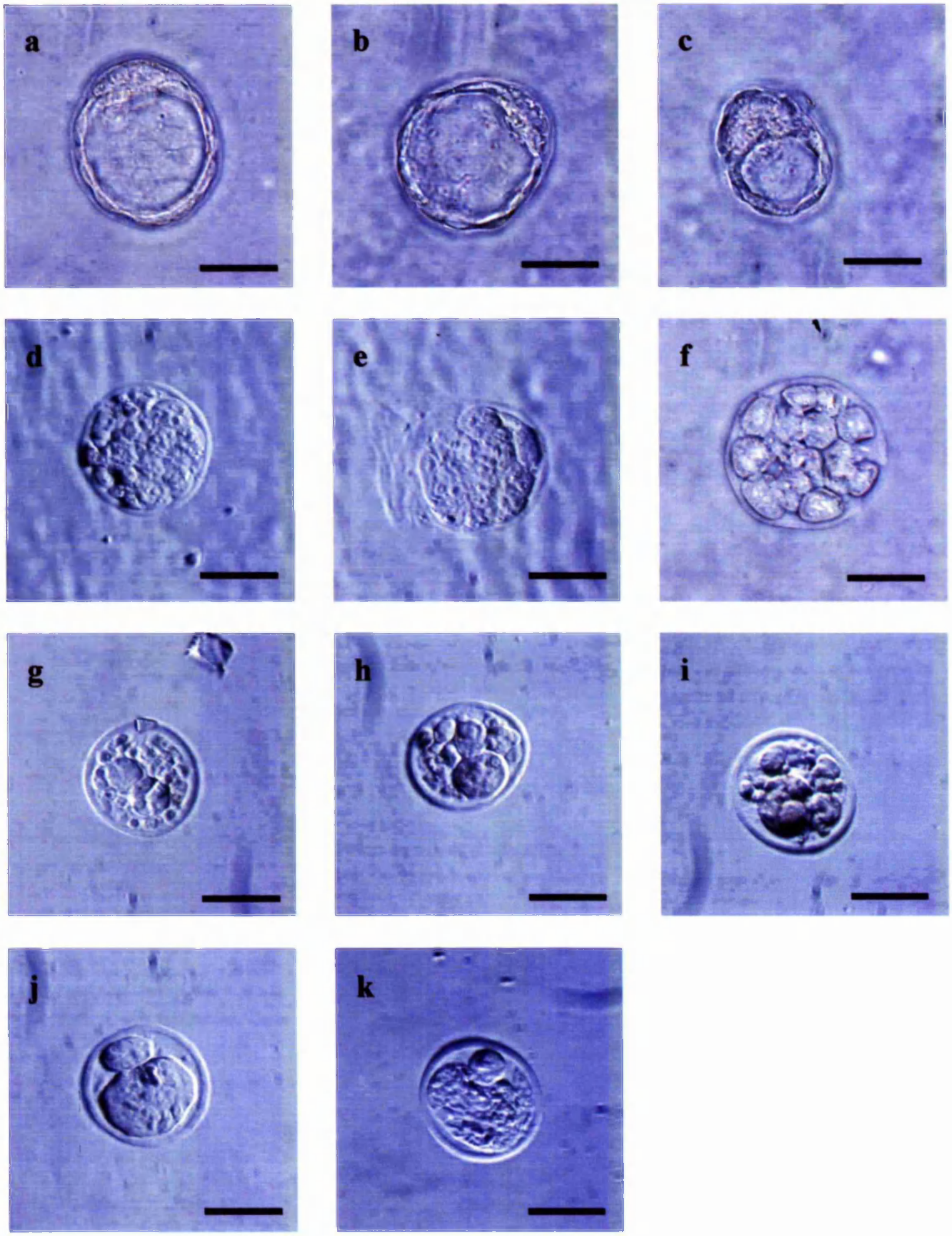

***Figure 5.2.2.1** : Phase contrast photograph showing the different classification and categorisation of embryos in this experiment. Magnification x240. Scale Bar =50μm. a-c : Blastocyst embryos; d-f : Morulae embryos (d&e show signs of compaction whilst individual blastomeres are visible in f. This may be due to decompaction as the embryo prepares to enter the next round of cell division); g-i : Fragmented oocytes/embryos; j and k: unfertilised oocytes.*

***Table 5.2.3.1** : Classification of embryos collected from matings with unirradiated control males.*

	% embryos at each developmental stage		
Embryonic Stage	***16 Days***	***31 Days***	***45 Days***
Blastocyst	95	92	90
Morula	5	7	9
Fragmented	—	1	—
Unfertilised	—	—	1

Values represent the percentage of classified embryos at each developmental stage for each experimental timepoint.

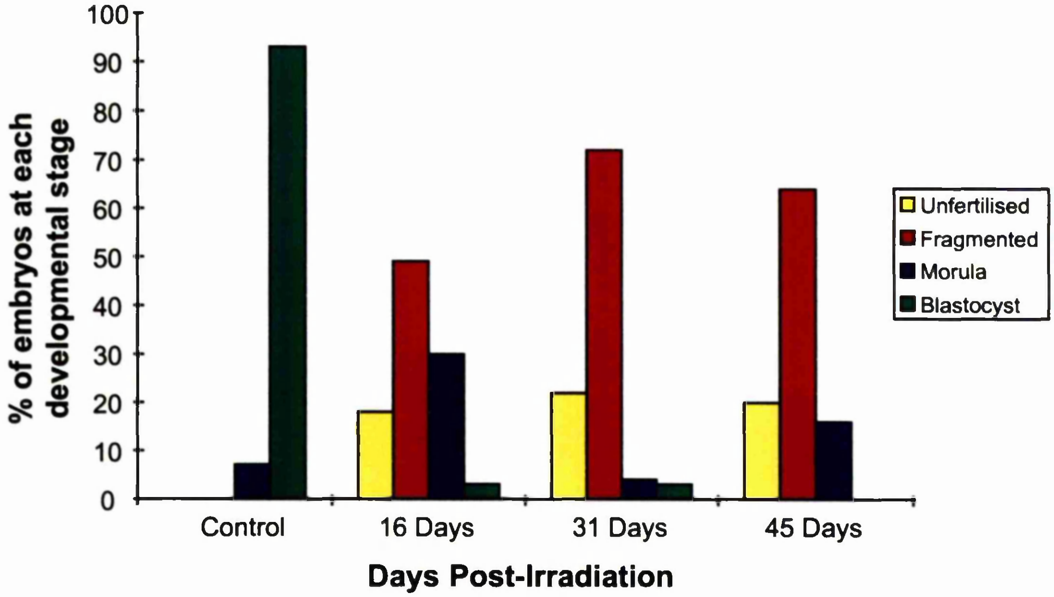

***Figure 5.2.3.1** : Effect of paternal irradiation on embryonic development. Results show the percentage of classified embryos at each developmental stage for each experimental timepoint.*

Figure 5.2.3.1 shows the stages of embryos collected after irradiation and then mating of males, 16, 31 and 45 days after 4Gy testicular X-rays. For simplification of the graph the control data has been pooled as it did not alter significantly over the three experimental timepoints (Table 5.2.3.1). In contrast to control embryos where over 90% had reached the blastocyst stage, 100 hours post hCG injection, blastocysts were rarely observed from matings derived from irradiated males. Only 3% of embryos derived from matings 16 and 31 days post-irradiation had reached the blastocyst stage whereas no blastocysts were observed from matings 45 days post-irradiation. Instead from matings 16 days post-irradiation 30% of embryos were at the morula stage (compared to around 7% in control matings) with the majority of embryos (49%) classified as fragmented and 18% of embryos classified as unfertilised. These two stages were almost never observed in embryos derived from control matings.

Matings performed 31 days post-testicular X-irradiation also yielded few blastocysts with an increased number of fragmented embryos observed over the 16 days timepoints (Figure 5.2.3.1). Only 4% of embryos were classified as morula with the majority of embryos (72%) again classified as showing a fragmented appearance. Unfertilised oocytes accounted for 22% of the total embryos collected which is similar to the levels of this stage observed in 16 day irradiated matings.

In 45 day post-irradiation matings no blastocyst embryos were observed. Morula accounted for 16% of the total embryos with again the majority of embryos (64%) classified as showing a fragmented appearance. Numbers of unfertilised oocytes (20%) were similar to those observed in 16 day (18%) and 31 day (22%) irradiation matings (Figure 5.2.3.1).

5.2.4 Apoptosis in Embryos.

As was observed with embryonic development, no significant differences were observed in the percentage of control embryos containing apoptotic (TUNEL labelled) nuclei at each of the three timepoints (16 day - 35%, 31 day - 43%, 45 day - 37%). Therefore the data has been pooled to simplify graphical presentation of the results (Figure 5.2.4.1).

Apoptosis was frequently observed in control blastocysts (Figures 5.2.4.1, 5.2.4.2a-c & 5.2.4.3) however it was rarely encountered in morulae derived from control matings with only a single morula observed containing 1 apoptotic nuclei from the control matings. No fragmented embryos or unfertilised oocytes derived from control matings contained apoptotic nuclei.

All blastocysts derived from matings 16 days post-irradiation contained at least one apoptotic nucleus (Figure 5.2.4.1 & 5.2.4.2d-f). From the 31 day matings however only 43% of collected blastocysts contained apoptotic nuclei. No blastocyst stages were observed in embryos from 45 day irradiation matings. As the total numbers of blastocysts collected from irradiated matings however was very small it is difficult to determine whether the change in the percentage of embryos with apoptotic nuclei observed at the 16 and 31 day timepoints give a true reflection of radiation-induced changes.

Compared to control matings, a higher percentage of morulae from irradiated matings contained apoptotic nuclei (2% - control; 17-84% irradiated). From matings performed 16 days post-irradiation 44% of morulae contained apoptotic nuclei. In 31 day post-irradiation matings, 17% of morulae contained apoptotic nuclei whilst at the 45 day mating timepoints the percentage of morulae containing apoptotic nuclei had increased to 84% of the total morulae analysed. Therefore it appears that over the course of the experiment the percentage of morulae with apoptotic nuclei increased from 44% at 16 days to 84% at 45 days (Figure 5.2.4.1). Examples of TUNEL labelled morulae from irradiated matings are shown in Figure 5.2.4.4.

Fragmented embryos accounted for the majority of the embryos observed from control matings (Figure 5.2.4.1). Unlike controls where apoptosis was not observed in fragmented embryos, apoptotic nuclei were frequently observed in fragmented embryos derived from matings with irradiated males (Figure 5.2.4.5). In fragmented embryos derived from 16 day matings, 16% contained apoptotic nuclei. At 31 days the number of fragmented embryos containing apoptosis had risen to 22% and at 45 days the percentage of fragmented embryos containing apoptotic nuclei had increased further to 43% (Figure 5.2.4.1).

As with control matings where no apoptosis was observed in unfertilised oocytes, no unfertilised oocytes from irradiated matings at any timepoint showed signs of TUNEL labelling (Figure 5.2.4.1).

Despite the fact that differences in the percentage of embryos containing apoptosis were observed between control and irradiated matings, the numbers of apoptotic nuclei found in TUNEL labelled embryos derived from control and irradiated matings were similar at all timepoints (Figure 5.2.4.1).

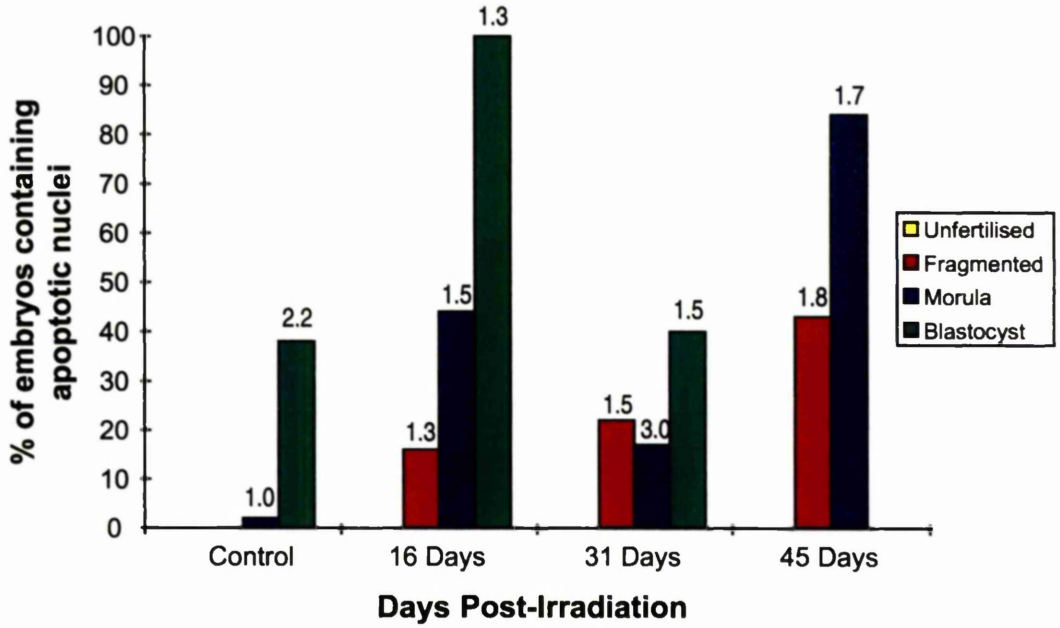

Figure 5.2.4.1 *: Effects of paternal irradiation on apoptosis in the preimplantation embryo. Results shown represent the percentage of embryos at each developmental stage containing apoptotic nuclei for each experimental timepoint. Figures above each bar indicate the mean number of apoptotic nuclei per embryo.*

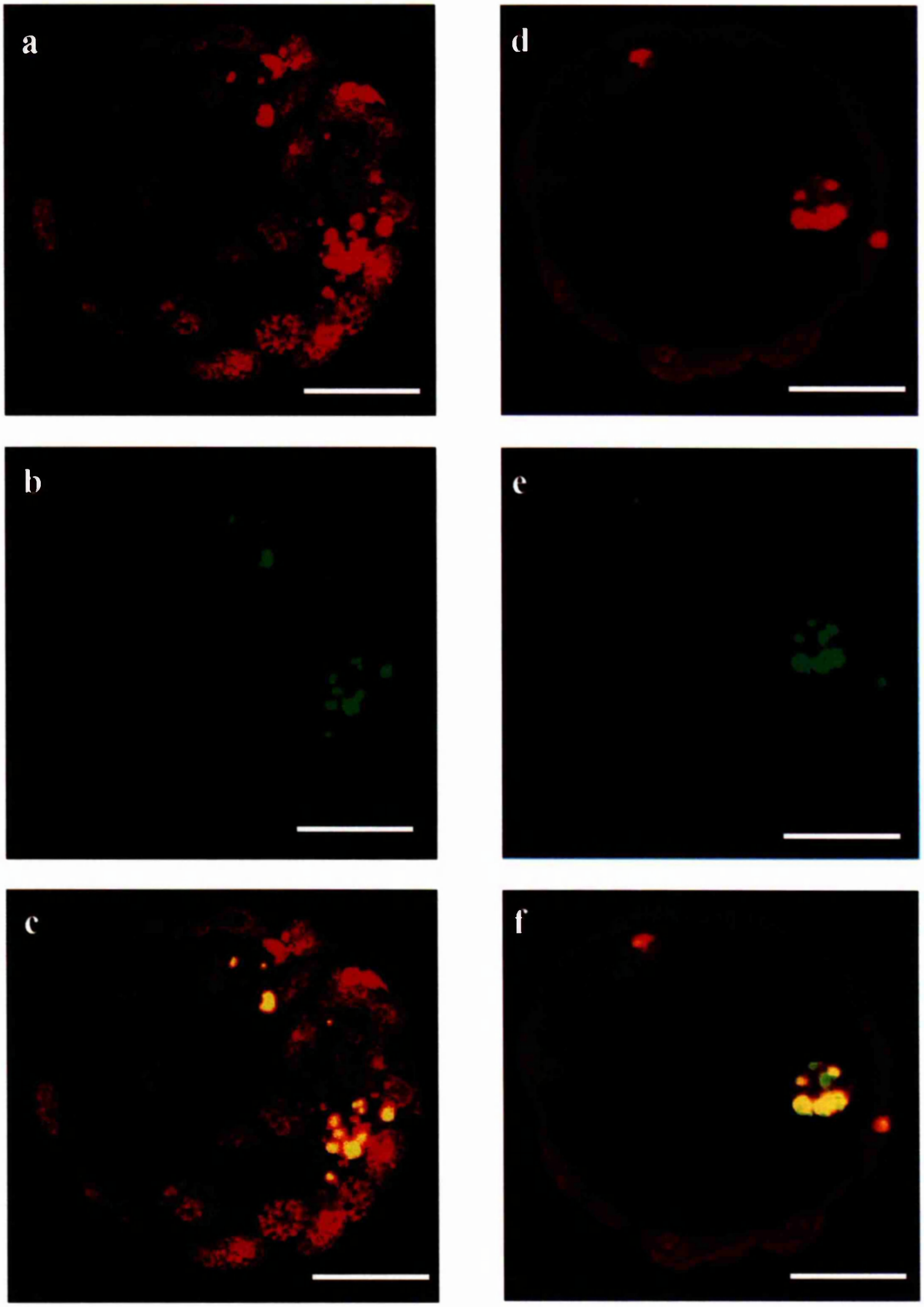

***Figure 5.2.4.2** : Apoptosis as detected by TUNEL labelling in mouse blastocysts.*
Panels a-c show a control blastocyst from a unirradiated mating with condensed/fragmented DNA labelled by TUNEL. Panels d-f show a confocal section through a blastocyst produced from an irradiated mating showing an apoptotic blastomere in the blastocoel cavity. Panels a & d - propidium iodide staining of all nuclei; Panels b & e - FITC labelled TUNEL staining of fragmented DNA; Panels c & f - Dual propidium iodide/TUNEL labelling. Magnification x720. Scale Bar = 50μm.

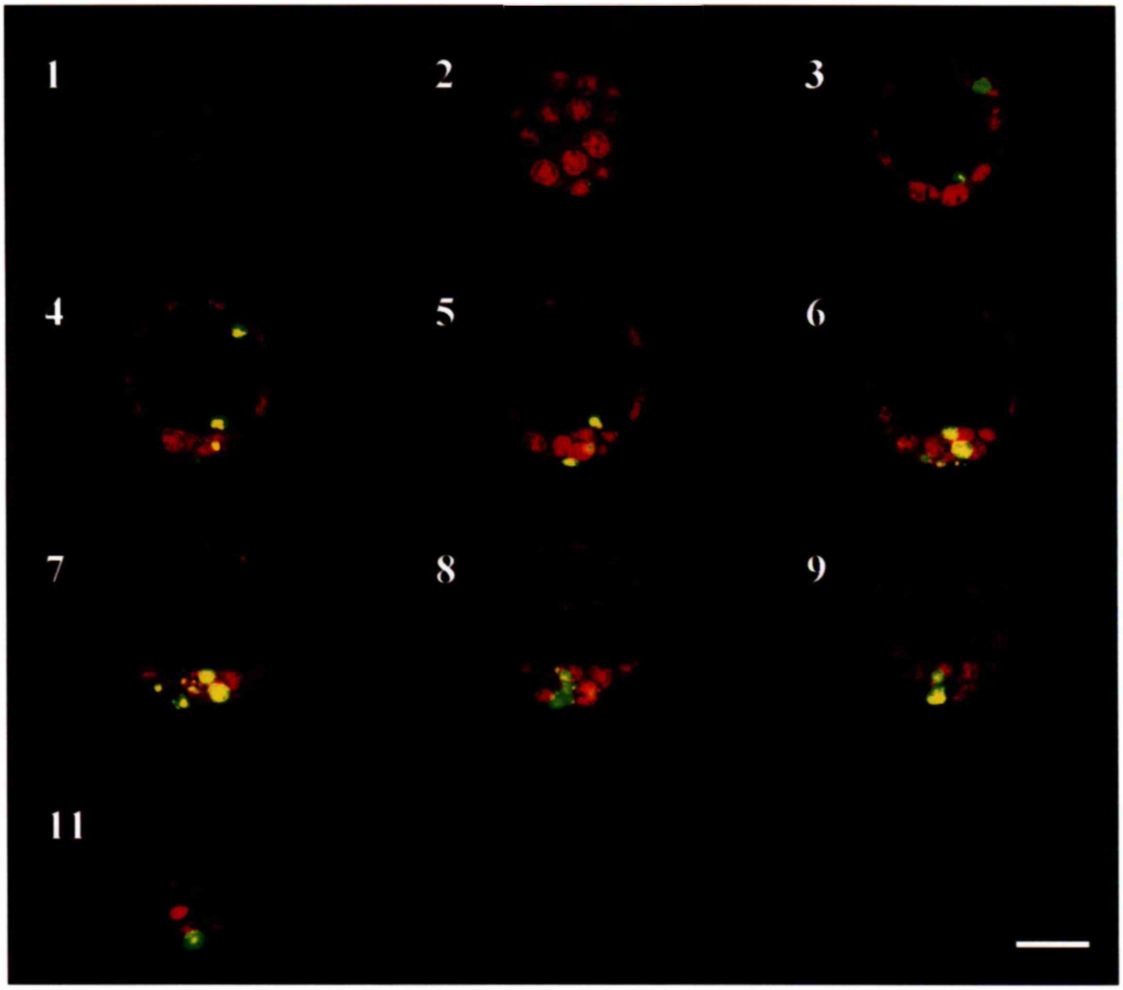

Figure 5.2.4.3. *: Serial sections (1-11) through a mouse blastocyst showing apoptotic nuclei as identified by TUNEL labelling. Section thickness = 3μm. Magnification x200. Scale Bar = 50μm.*

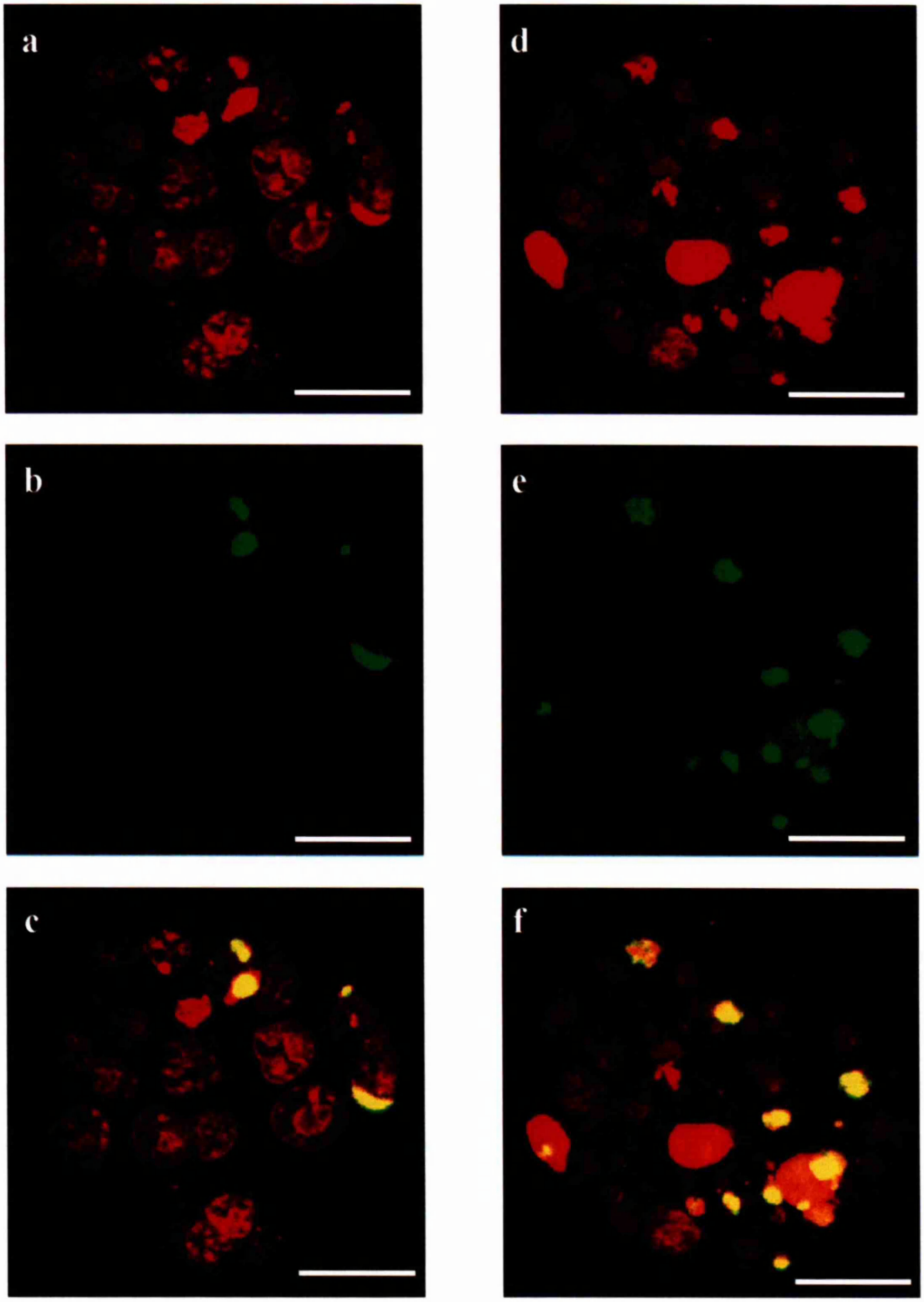

Figure 5.2.4.4 *: Apoptosis as detected by TUNEL labelling in mouse morulae produced from irradiated matings. Panels a & d - propidium iodide staining of all nuclei; Panels b & e - FITC labelled TUNEL staining of fragmented DNA; Panels c & f - Dual propidium iodide/TUNEL labelling. Magnification x720. Scale Bar = 50μm.*

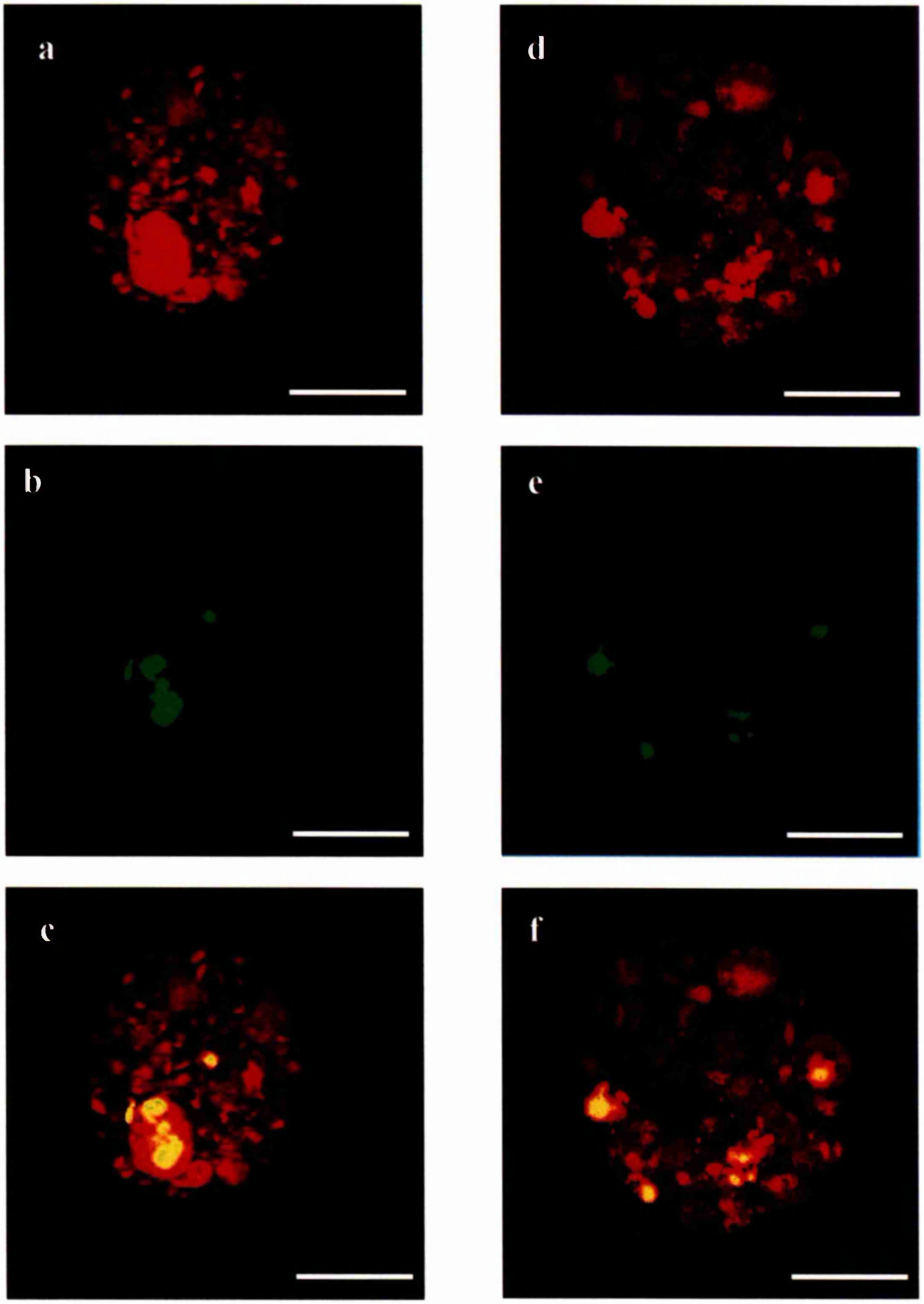

***Figure 5.2.4.5** : Apoptosis as detected by TUNEL labelling in fragmented oocytes/embryos produced from irradiated matings. Panels a & d - propidium iodide staining of all nuclei; Panels b & e - FITC labelled TUNEL staining of fragmented DNA; Panels c & f - Dual propidium iodide/TUNEL labelling. . Magnification x720. Scale Bar = 50μm*

5.2.5 120 Day Timepoint - Classification of Embryos.

Matings of control animals time-matched with irradiation at 120 days produced mainly blastocyst stage embryos (Figure 5.2.5.1) as observed in previous control matings. The numbers of blastocysts produced (84%) of total embryos recovered was slightly lower than in previous control matings but a slightly higher proportion of morulae (15%) was also observed. A small proportion of the embryos recovered (4 out of a total of 142 embryos analysed at this timepoint) displayed fragmented morphology.

Unlike previous matings with irradiated males, animals mated 120 days post testicular irradiation with 4Gy X-ray yielded predominantly blastocyst embryos (Figure 5.2.5.1). These accounted for 66% of the total embryos which is lower than values recorded for controls (84%). Morulae accounted for 30% of the embryos obtained at this timepoint which is twice that number obtained from the control mating (15%). A small proportion of fragmented embryos (5% of the total embryos at this timepoint) were also observed.

5.2.6 120 Day Timepoint - Embryo Apoptosis.

38 % of control blastocysts from the 120 day mating timepoint contained apoptotic nuclei (Figure 5.2.6.1). This is consistent with other earlier control matings where between 35 and 43% of blastocysts showed signs of apoptosis. 45% of blastocysts derived from matings with irradiated males contained apoptotic nuclei. Therefore this does not appear to be different to the numbers of apoptotic blastocysts observed in control matings.

19% of morulae from the 120 day control mating contained apoptotic nuclei (Figure 5.2.6.1). This is higher than previous control matings where only 1 morula was observed containing an apoptotic nuclei. Morula derived from 120 day irradiated males showed an increased incidence of apoptosis with 34% of morulae containing apoptotic nuclei.

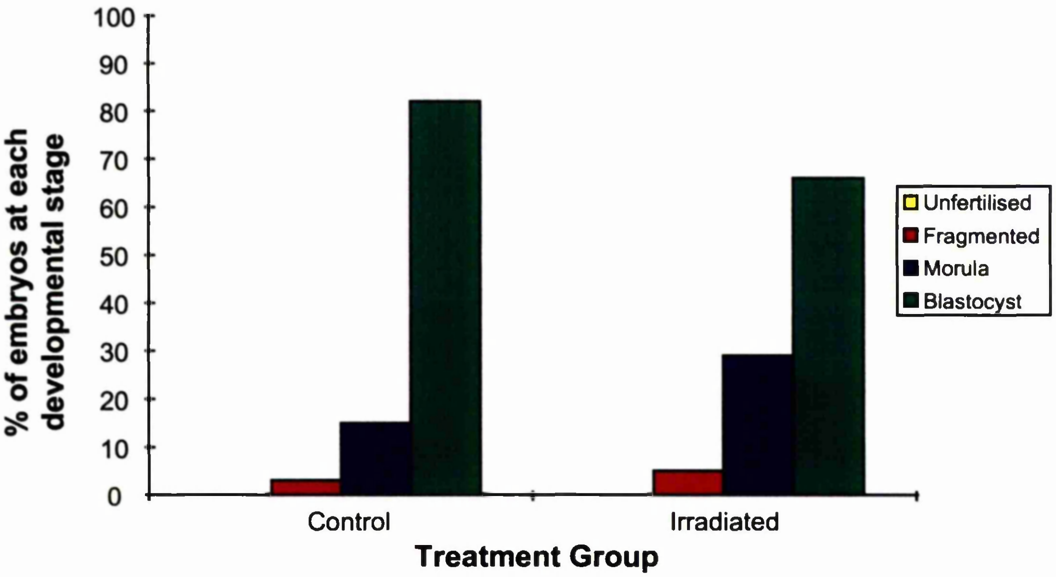

***Figure 5.2.5.1** : Effect of paternal X-rays (4Gy) on development of preimplantation embryos derived from matings 120 days post-irradiation. Results show the percentage of classified embryos at each developmental stage for control and irradiated matings.*

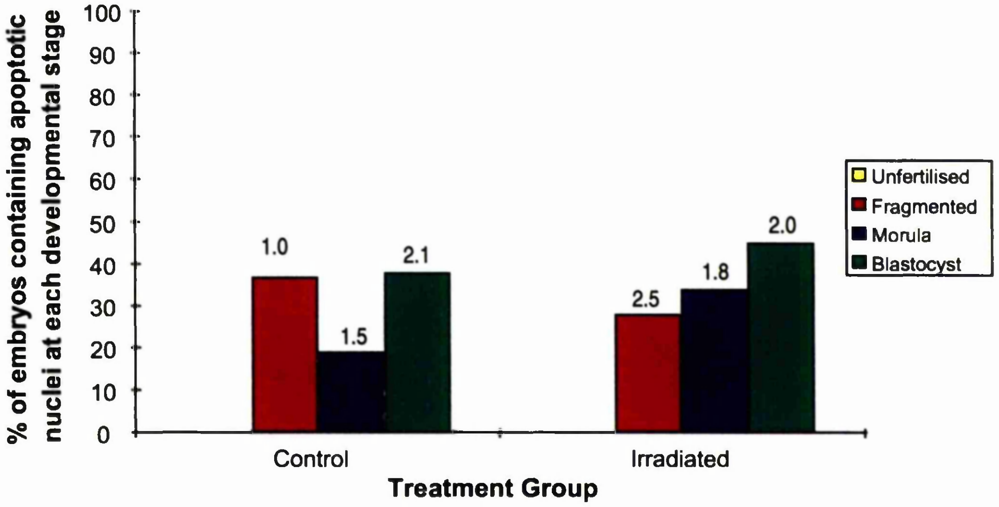

***Figure 5.2.6.1** : Effects of paternal X-rays (4Gy) on apoptosis in the preimplantation embryos derived from matings 120 days post-irradiation. Results shown represent the percentage of embryos at each developmental stage containing apoptotic nuclei for both control and irradiated matings. Figures above each bar indicate the mean number of apoptotic nuclei per embryo*

25% of fragmented embryos derived from the 120 day control mating contained apoptotic nuclei (Figure 5.2.6.1). However this is derived from only 4 fragmented embryos with one containing a single apoptotic nucleus at this control timepoint. Fragmented embryos were rarely observed at earlier control timepoints and did not contain apoptotic nuclei. 28 % of fragmented embryos derived from the 120 day irradiated matings contained apoptotic nuclei (Figure 5.2.6.1). This is lower than previously observed at earlier timepoints with irradiated males where 49-72% of fragmented embryos contained apoptotic nuclei.

As with earlier timepoints there were no differences in the numbers of apoptotic nuclei found in morulae and blastocysts derived from control and irradiated matings (Figure 5.2.6.1). However the mean numbers of apoptotic nuclei found in fragmented oocytes/embryos derived from irradiated matings (2.5) was higher than the mean number of apoptotic nuclei found in fragmented oocytes/embryos derived from control matings (1.0). However since only 4 fragmented oocytes/embryos were obtained from the 120 day control matings and that only 1 contained a single apoptotic nuclei, it is unwise to draw a meaningful conclusion from such a small pool of embryos/oocytes.

5.3 DISCUSSION.

Paternal exposure to mutagens can lead to genetic damage which results in embryonic death (Ehling, 1971; Searle & Beechey, 1974; Goldstein *et al.,* 1978; Dobrzynska *et al.,* 1990). Early foetal deaths are a direct quantitative index of dominant lethal mutations. However the assessment of dominant lethal mutations requires large numbers of animals and is time consuming. More subtle levels of genetic damage (e.g. chromosomal translocations, point mutations, base damage) may not manifest themselves as embryonic death but may affect embryonic development and produce fetal abnormalities. In this chapter the effects of 4Gy paternal irradiation was examined on embryonic development and apoptosis in the preimplantation embryo. Four timepoints were used corresponding to irradiation of different germ cell types and the production of spermatozoa with different levels of DNA damage as described in previous chapters.

Paternal exposure to radiation resulted in decreased fertility as observed by an increased incidence of unfertilised and fragmented oocytes. This was most severe in matings performed 45 days post-irradiation which was the timepoint when levels of spermatozoal DNA damage are at their highest as demonstrated by comet analysis of spermatozoa (Chapter 4). Previous experiments with paternal exposure to external radiation sources with doses of either 3 or 4Gy have demonstrated that the most dramatic effects of radiation in producing the smallest litter sizes (Russell *et al.,* 1998) and lowest number of live embryos (Ehling, 1971) are observed after matings performed during the seventh week post irradiation. This is consistent with the results of this study where the highest levels of DNA damage in spermatozoa and greatest effects upon embryo morphology, development and apoptosis were observed 45 days post-irradiation. This corresponds to the irradiation of spermatogonial cells within the testis (Meistrich, 1986a).

Paternal exposure to radiation has previously been shown to affect fertility and often the primary cause of this is a reduction of sperm counts (Searle & Beechey, 1974; Ogilvy-Stuart & Shalet, 1993). In this experiment sperm counts were reduced at the 31 and 45 day timepoints and this is due to the killing of spermatocytes and spermatogonia respectively (Meistrich, 1986a) which may contribute to the increased incidence of fragmented and unfertilised oocytes/embryos. However at 16 days sperm counts were not reduced and therefore cannot account for the decrease in fertility. Therefore it is probable that genetic damage induced in spermatozoa by irradiation could account for the decrease in fertility. Previous reports have demonstrated that paternal irradiation of spermatids with 4Gy X-rays can lead to increases in dominant lethal mutations without affecting sperm counts and fertilisation rate (Ehling, 1971; Searle & Beechey, 1974; Russell *et al.,* 1998). Exposure of germ cells to radiation has also been shown to cause developmental abnormalities in spermatozoa (Bruce *et al.,* 1974) and produce genetic damage to sperm chromatin (Bruce *et al.,* 1974; Sailer *et al.,* 1995a) which may affect their ability to fertilise and subsequent development of the embryo. In the previous chapter, comet analysis of spermatozoa demonstrated that sperm in the vas deferens at 16 days post-irradiation have higher levels of DNA damage compared to unirradiated controls. Therefore this genetic damage in spermatozoa may account for the decreased fertility observed at this early timepoint.

As sperm counts were reduced at 31 and 45 days post-irradiation it is probable that fertilisation rate also decreased. It has previously been described that reduced sperm counts can affect fertilisation rates of oocytes (Searle & Beechey, 1974). A decrease in fertility was observed in experiments described in this chapter by an increased number of unfertilised and fragmented oocytes at these time points. In this study however it was impossible to determine whether fragmentation of oocytes/embryos was occurring as a result of failure of fertilisation or due to genetic damage transmitted by spermatozoa post-fertilisation. Therefore it is likely that the oocyte/embryos classified as fragmented will be a mixture of both unfertilised oocytes and fertilised embryos. Previous studies have demonstrated that fragmentation can occur spontaneously in both unfertilised oocytes (Perez & Tilly, 1997; Perez *et al.,* 1997; Van Blerkon & Davis, 1998) and in preimplantation embryos (Jurisicova *et al.,* 1995, 1996).

Embryos produced from matings with irradiated males showed delayed development compared to time matched controls. In this study, embryos were collected from the uteri of mated females around 100 hours post-hCG injection. By this time fertilised eggs have

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normally developed to the blastocyst stage (Pratt, 1987; Brison & Schultz, 1997) and indeed in control matings >90% of embryos had reached the blastocyst stage. In contrast very few blastocysts were observed in irradiated matings and instead morulae and oocytes/embryos with a fragmented appearance were observed more frequently than in control matings. Paternal exposure of testicular germ cells to the chemical mutagen triethylenephosphoramide (TEPA) has previously been shown to lead to a retardation of preimplantation embryo cleavage and increased incidence of embryos with abnormal morphology and multinucleate blastomeres (Epstein *et al.,* 1970). Exposure of spermatid stages led to normal fertilisation rates followed by retarded and abnormal embryo development whilst spermatogonial exposure led to a marked reduction in fertilisation, probably as a result of aspermia, although a genetic component was also involved as exposure of spermatogonia to TEPA was also associated with pre- and post-implantation embryo deaths. Experiments have also shown that direct exposure of embryos to low levels of radiation leads to a delay in cleavage and a failure to undergo blastulation (Yamada *et al.,* 1982). These observations are similar to the results described in this study, where both decreases in sperm counts and effects upon embryo morphology and development are described. At 16 days when spermatozoa would have been derived from spermatids, sperm counts were normal and therefore effects on the embryo are probably a direct result of damage to spermatozoa. At later timepoints (31 and 45 days) a decrease in sperm counts was observed so there will be some contribution to the incidence of fragmented oocytes embryo due to failure of fertilisation, as well as effects post-fertilisation produced by the actions of radiation upon spermatozoa.

The majority of embryos collected from irradiated matings had a fragmented appearance. Fragmented embryos have been described before after paternal exposure to mutagens (Epstein *et al.,* 1970), direct irradiation of fertilised eggs (Yamada *et al.,* 1982) and in human embryos fertilised *in vitro* (Jurisicova *et al.,* 1995, 1996). Cellular fragments can be both anucleate or/and nucleate (Dozortsev *et al.,* 1998) and fragmentation of preimplantation embryos has been attributed to both spermatozoa and the oocyte (Janny & Menezo, 1994). The presence of nucleate fragments may be an indication that the embryo is chromosomally abnormal and will eventually become arrested (Jurisicova *et al.,* 1996). However, anucleate fragments may be benign and have no effect upon the development of the embryo (Dozortsev *et al.,* 1998). In this study, the effects on embryo development were more severe depending on the timepoint at which the matings occurred. Initially, after 16 days a small number of blastocysts were observed with the majority of embryos being at either the morula stage (30%) or showing a fragmented appearance (49%). By the 45 day mating, no blastocysts were observed and the numbers of morulae had decreased to 16% of the total embryos collected. Concomitantly, the numbers of fragmented embryos had increased to 64% of the total embryos collected. These effects on embryo development appear to reflect the situation with DNA damage levels in spermatozoa (Chapter 4) and previous studies have suggested that DNA damage in spermatozoa may be contributory to embryo fragmentation (Munne & Estop, 1991, 1993) and that embryo morphology and developmental rates are correlated with chromosome abnormalities (Munne *et al.,* 1995). It was shown in Chapter 4 that DNA damage levels in spermatozoa increased with time post-irradiation, with the highest DNA damage levels observed 45 days post-irradiation corresponding to irradiation of highly radiosensitive spermatogonial cells. Therefore, it is possible that differences in the levels of DNA damage in spermatozoa may also manifest themselves as effects of differing severity on the embryo with subsequent decreases in embryonic development and increases in the frequency of embryos with a fragmented appearance.

From this experiment, where embryos were collected at a fixed time (~100 hours) post-hCG injection, it is very difficult to determine whether the appearance of other embryonic stages apart from blastocyst reflect a slowing down of the developmental rate of the embryo or whether these cells arrested at a specific stage but continued their movements down the female reproductive tract. To address this situation experiments should be performed where embryos are removed from the reproductive tract post-fertilisation and then cultured *in vitro*. This would allow carefully monitoring of development to take place. Another drawback of this experiment is the difficulty in accurately assessing fertilisation rate. Blastocyst and morula stage embryos have clearly been fertilised whereas the definition of single-cell unfertilised oocytes is also equally clear-cut. However, with fragmented embryos it is difficult to say whether the fragmentation has occurred post-fertilisation and is a direct result of damaged paternal DNA or whether an unfertilised oocyte has spontaneously degraded in the reproductive tract. Both fragmented embryos and degradation of unfertilised oocytes have been described previously in *in vitro* culture (Perez & Tilly, 1997; Perez *et al.,* 1997; Van Blerkon & Davis, 1998). Again to obtain a true assessment experiments should be performed where the embryos are cultured *in vitro* after fertilisation.

As well as effects on development, embryos were also TUNEL labelled to assess levels of apoptosis in embryos from control and irradiated matings. Apoptosis has previously been described in preimplantation embryos from mouse, rat and human (Pampfer *et al.,* 1990a, 1990b; Jurisicova *et al.,* 1995, 1996; Brison & Schultz, 1997; Hardy, 1997). It is normally first seen at the blastocyst stage where it occurs predominantly within the inner cell mass (ICM) although its exact function in the embryo is at the moment unclear. Consistent with the findings of previous studies (Brison & Schultz, 1997; Pampfer *et al.,* 1997) TUNEL positive nuclei were frequently observed in control blastocyst embryos. Approximately 40% of blastocysts contained TUNEL positive nuclei with approximately 2 apoptotic nuclei in each blastocyst that stained positive for TUNEL labelling . This is in agreement with a previous study which examined *in vivo* developed mouse blastocysts and suggested that TUNEL labelled blastocysts on average contained 1 or 2 positively stained nuclei (Brison & Schultz, 1997). Also from this study, results with TUNEL labelled morulae and 8-cell embryos suggested that apoptosis did not occur in these stages. This is also consistent with the results reported in this chapter where TUNEL positive morulae derived from control matings were not observed. Apoptotic nuclei in blastocysts were most frequently observed in the inner cell mass (ICM) which also agrees with previously reported findings (Handyside & Hunter, 1986; Hardy *et al.,* 1989; Brison & Schultz, 1997). However, occasionally TUNEL positive nuclei were seen in trophectoderm cells.

In contrast to controls, TUNEL positive nuclei were often observed in morula and fragmented embryos as well as blastocysts. It has previously been reported that the blastocyst is the first stage of development at which apoptosis is observed (Handyside & Hunter, 1986; Parchment, 1993; Hardy, 1997). A study which examined mouse 8-cell embryos and morulae developed both *in vitro* and *in vivo* by TUNEL assay also reported that apoptosis was not seen in these earlier developmental stages (Brison & Schultz, 1997). Therefore the observations reported in this chapter are in contrast to previous findings. One obvious reason for the discrepancy amongst these finding is that most previous studies have examined morulae produced from matings with parents who have not been exposed to toxic chemicals or other agents. Indeed as described above, morulae produced from control matings in this study did not show signs of TUNEL positive nuclei. Therefore it is an obvious assumption that this abnormal expression of apoptosis may be as a result of matings with paternally irradiated males and that the apoptosis is a consequence of genetic damage in the spermatozoa. However, although previous studies have not demonstrated apoptosis in pre-blastocyst stages in the mouse it has been observed in early human embryos (Jurisicova *et al.,* 1995, 1996) suggesting that apoptosis may occur at earlier stages of development. Also it has been implied that expression of apoptosis in mouse embryos may differ according to strain with some strain of mice exhibiting apoptosis as early as the 4-cell and 8-cell stages (Jurisicova, personal communication).

As with effects upon embryo morphology and development, the incidence of apoptosis in mouse embryos also differed depending on the mating timepoint after paternal irradiation. 16-days post-irradiation when DNA damage levels in spermatozoa were much lower than at later timepoints 44% of morulae and 16% of fragmented embryos contained apoptotic nuclei. At the 45 day timepoint when DNA damage levels were at there highest in spermatozoa, apoptosis was now observed in 84% of morulae and 43% of fragmented embryos. Therefore it appears that as DNA damage in spermatozoa increased there was also an increase in the percentage of embryos containing apoptotic nuclei. Previous studies in somatic cells have demonstrated that DNA damage may induce apoptotic cell death (Evan & Littlewood, 1998) and studies with human embryos have suggested that fragmentation and the presence of apoptotic blastomeres may be linked to genetic abnormalities (Jurisicova *et al.,* 1995, 1996). Therefore from these observations and the results in this chapter, it is possible to suggest that apoptosis may be occurring in embryos as a result of DNA damage transmitted by spermatozoa at fertilisation.

Despite an increase in the frequency of embryos containing apoptosis, paternal irradiation did not increase the numbers of apoptotic nuclei per embryo which were approximately 1.7 apoptotic nuclei per embryo from irradiated matings compared to values from controls of around 2 apoptotic nuclei per embryo. Also the number of apoptotic nuclei did not alter over the three timepoints of the study (16, 31 and 45 days). If apoptosis in the preimplantation embryo can occur in response to genetic damage then we might have expected to see increased numbers of apoptotic nuclei in embryos from irradiated matings. However, this does not appear to be the case although we do see expression of apoptosis at early stages of embryo development when it does not usually occur. This may suggest that the embryo is detecting the genetic damage at an early stage and activating the cell death pathway as a protective measure. Alternatively, other studies have examined the apoptotic index of embryos, which is a measure of the percentage of cells in the embryo displaying apoptosis (Hardy *et al.,* 1989; Hardy, 1997; Brison & Schultz, 1997). Therefore since total cell counts were not performed on embryos from experiments described in this thesis it is impossible to calculate an apoptotic index for each embryonic stage. However, since apoptosis was observed in earlier stages in embryos from irradiated matings compared to controls, these embryos have fewer cells and therefore it is safe to assume that the apoptotic index will therefore be higher. When comparing blastocysts from control and irradiated matings, for an accurate comparison of the levels of apoptosis, total cell counts and a calculation of apoptotic index should be performed. Previous studies have suggested that poor quality embryos have reduced cell numbers (Hardy *et al.,* 1989). Therefore it is possible that whilst the actual numbers of apoptotic nuclei in blastocysts from control and irradiated matings are similar the apoptotic indexes of blastocysts from irradiated matings may be higher reflecting differences in total cell numbers.

One complication of the present study and the assessment of the effects of apoptosis in the pre-implantation embryo was that as irradiation of spermatozoa produced effects upon embryonic development, it was impossible to directly compare apoptosis in alike embryonic stages. In unirradiated control embryos apoptosis was commonly encountered in blastocysts. Irradiated blastocysts also showed apoptosis but since very few embryos of this stage were produced from irradiated matings it is impossible to directly compare the effects of irradiation on apoptosis in blastocysts accurately. In future it may be possible to conduct studies where lower doses of radiation are employed which do not have such a severe effect on embryonic development. This may allow the direct comparison of apoptosis in the same embryonic stages produced from unirradiated and irradiated matings.

In order to look at the longer term effects of paternal irradiation, animals were mated 120 days post-irradiation in order to look at residual effects from irradiated stem cell spermatogonia (Meistrich, 1993). Results described in Chapter 4 of this thesis have demonstrated how DNA damage levels after 4Gy of X-irradiation are lower than observed at earlier timepoints post irradiation (16,31 and 45 days) but still significantly higher than spermatozoa from unirradiated animals. In matings performed 120 days post-irradiation, blastocyst-stage embryos were observed much more frequently than at other irradiated timepoints and accounted for 66% of the total embryos collected. This however was lower than the amount of blastocysts (82%) obtained from 120 day time-matched control matings. There was also a greater frequency of morula-stage embryos from the irradiated timepoint (29%) compared to the control (15%). The occurrence of many more blastocysts in 120 day post-irradiation matings may reflect a reduction in the levels of DNA damage in spermatozoa at this timepoint compared to earlier matings (Chapter 4). It has previously been demonstrated that DNA damage in spermatozoa can produce effects upon embryo development and morphology (Munne & Estop, 1991, 1993; Munne *et al.,* 1995) and the implications of this have been discussed earlier. However whilst these results suggest that the effects 120 days post-irradiation are less severe than at earlier timepoints. Observations that slightly lower numbers of blastocysts and an increased frequency of morulae from the irradiated mating 120 days post-irradiation compared to controls, demonstrate that there may be some longer-term persistent effects on embryo development as a result of permanent spermatogonial stem cell damage. This is consistent with studies by Dubrova *et al.,* (1998) who detected increased minisatellite mutation rates in offspring derived from male mice after stem cell irradiation with 1Gy X-rays.

The percentage of blastocysts containing apoptotic nuclei (38%) from the control 120 day mating was not different from the levels observed at other control timepoints. 120 day irradiated matings produced 45% of blastocysts with apoptotic nuclei, which is comparable to the time-matched control and levels from earlier control matings. Interestingly 19% of control morulae at 120 days contained apoptotic nuclei whereas at previous control timepoints, control morulae with apoptotic nuclei were not observed. The exact reason for this observation is unknown and previous studies have suggested that apoptosis in normal embryos (unexposed to physical or chemical agents) does not occur before the blastocyst stage. Studies with human embryos however, have suggested that apoptosis may occur at pre-blastocyst stages(Jurisicova *et al.,* 1995, 1996) although if this is the case the reason why apoptotic cells were not observed in control morula from earlier matings is unexplained. Despite this observation, a higher proportion of morula from irradiated matings (34%) contained apoptotic nuclei than control matings (19%) at 120 days. These levels of apoptosis although higher than controls are lower than observed at previous irradiated timepoints and offer further evidence of a reduction in sperm DNA damage levels and that it may be DNA damage in sperm that contributes to embryo apoptosis. As with previous timepoints, no difference was observed in the mean number of apoptotic nuclei per embryo between controls (1.5) and irradiated (2.0).

In summary, the experiments described in this chapter have reported the effect of paternal irradiation with 4Gy of X-rays on pre-implantation embryo development and apoptosis in the pre-implantation mouse embryo. Paternal irradiation decreased fertility and resulted in a retardation of embryo development and higher incidence of abnormal embryos with a fragmented appearance. Expression of apoptosis was also altered by paternal irradiation and was frequently observed in morulae and fragmented embryos unlike control embryos. Effects on embryo development and apoptosis were greatest in embryos from mating performed 45 days post-irradiation which also corresponds to the highest levels of DNA damage in spermatozoa. A long term effect on embryo development (although more subtle than previous timepoints) was also noted with matings at 120 days post-irradiation also showing some retardation of development and higher incidence of apoptosis in morula-stage embryos. This suggests a permanent effect of paternal irradiation on stem spermatogonial cells. The exact function of apoptosis in the preimplantation embryo is at the moment unknown although it commonly acts to remove damage or unwanted cells in other tissue. The data presented in this chapter are consistent with a role for apoptosis in eliminating damaged cells from the preimplantation embryo.

Chapter 6

Effects on the Testis of Internally Incorporated Radionuclide, Indium-114m.

6.1 INTRODUCTION.

The previous experiments in this thesis have investigated the effects of external radiation sources (γ-rays and X-rays) on spermatozoal DNA. This reflects the easy access to such sources and their ease of use and dose delivery. The majority of the research in the literature has also mainly focused on the adverse effects of external radiation upon male reproduction. Presumably, this reflects concerns that external radiation sources pose the greatest threat of producing harmful effects to humans as their exposure is not easily controlled. However, there are also many radioisotopes in widespread use (within the nuclear industry and in diagnostic medicine) but it is generally thought that because these isotopes emit radiation products with short ranges they do not pose as great a threat as external sources because their radiation will not penetrate the body and contamination by ingestion can be easily prevented. However, recently in the event of the Chernobyl nuclear incident, widespread global contamination of both the population and the environment has occurred with a wide range of these isotopes (INSAG, 1986; Mascanzoni, 1987; Kenigsberg & Minenko, 1994). Therefore the risk assessment for these radionuclides may have to be revised as it is possible that people may have already been exposed and may be subjected to continued exposure as a result of isotopes with long half-lives contaminating the environment as a result of such incidents. The population may potentially be exposed to these isotopes if they contaminate the body either by pollution of the food chain or through inhalation of radioactive particles. A primary concern is that these agents may produce specific tissue effects if they become localised within the body.

One of the isotopes released into the environment after the Chernobyl accident was Iodine-131 (^{131}I) Thousands of people living in the Chernobyl area in Russia were exposed to relatively high levels of radio-iodine and as a result, large increases in the rates of childhood thyroid cancer were recorded (Bleuer *et al.,* 1997). This is because after ingestion, iodine becomes localised to the thyroid gland, therefore in children exposed to airborne pollution with ^{131}I a high concentration of radioactive iodine was achieved in the thyroid and the organ subjected to a high dose of radioactivity. Iodine-131 has a relatively short half-life compared to other isotopes (e.g. Caesium-137 and

Plutonium-238) released at Chernobyl. There are concerns that radioisotopes released into the environment may have effects upon germ cells within the testes giving rise to mutations, which may cause cancer and other abnormalities amongst the offspring. These concerns have recently been reinforced by a study suggesting that the offspring of parents exposed as a result of the Chernobyl incident have higher minisatellite mutation rates compared to the unexposed population (Dubrova *et al.,* 1996). Therefore long acting radioactive isotopes contaminating the environment may continue to pose a threat to health into the future. Furthermore through internal ingestion, these radioisotopes may be able to contaminate the testis and pose an increased risk to the germ cells than external radiation sources.

6.1.1 Effects of Internal Exposure of the Testis to Radioisotopes.

Germ cells within the seminiferous epithelium have previously been considered protected from toxicants and radioisotopes by the blood-testis barrier. This barrier is formed by tight junctions between adjacent somatic Sertoli cells at their basolateral surface (Dym & Fawcett, 1970; Setchell & Waites, 1975). This forms a wall around the base of the seminiferous tubule that excludes toxins and large proteins delivered to the testis via the bloodstream. However the blood-testis barrier does not provide a completely impenetrable layer of protection, as molecules essential for the development of the germ cells must be able to pass across and gain access to the seminiferous epithelium. Therefore, there are present a number of mechanisms which act to deliver essential factors to the developing germ cells. However, recent studies have suggested that some radionuclides may be able to utilise these physiological transport mechanisms and gain entry to the seminiferous tubule (Jackson *et al.,* 1991; Hoyes *et al.,* 1996b).

One of these transport mechanisms that has been studied by our research group is the transferrin pathway which delivers iron to the testis (Morales *et al.,* 1987). Iron required by the body is obtained through the diet. It is bound to the protein transferrin in the bloodstream and delivered to the testis. The iron-transferrin complex itself is unable to cross the blood-testis barrier (Petrie Jr & Morales, 1992). However, the Sertoli cells express transferrin receptors on the surface of their basolateral membrane which bind the transferrin-iron complexes and internalise them via receptor-mediated endocytosis. Within the tubule, the delivered iron is released from its complex and binds to Sertoli cell transferrin (Hoyes *et al.,* 1996b). It is then delivered to the germ cells developing within the cytoplasm of the Sertoli cell. Recent research by our group however, suggests that a variety of radionuclides may hijack the iron-transferrin mechanism to gain access across the blood-testis barrier and become associated with the developing germ cells (Jackson *et al.,* 1991; Hoyes *et al.,* 1996a, 1996b).

Hoyes et al, (1995, 1996a, 1996b, 1998) have extensively studied the radionuclides of indium which are widely used in medical diagnostics. It has been shown that after systemic administration, indium can utilise the iron-transferrin pathway to gain access to the seminiferous tubules where it becomes associated with the developing spermatids and spermatozoa (Hoyes *et al.,* 1994, 1995). Additionally, it appears that indium, taken up by the testis may be retained for long periods of time and therefore there is a potential hazard posed by continual low dose irradiation of germ cells within the testes by ingested radioisotope (Hoyes *et al.,* 1995, 1996b). Similarly it has also been shown that the radioisotopes of the actinides, iron-59, plutonium-238 and caesium-137 which are all found in the environment because of the activities of nuclear installations can also cross the blood-testis barrier (Hoyes *et al.,* 1996a, 1996b). Iron and plutonium appear to utilise the iron-transferrin transporter, like indium. Caesium however is actively transported across the barrier and concentrated intraluminally at a rate of transport that far exceeds that for both indium and iron (Hoyes *et al.,* 1996b). Therefore, it is thought that caesium gains access to the tubules not by the iron-transferrin system but by acting as a potassium analogue and utilising transport mechanisms by which Sertoli cells maintain high intraluminal potassium concentrations required for optimal germ cell development (Kaul *et al.,* 1966). The ability of the testis to concentrate caesium is a potential cause for concern as ^{137}Cs was a major contaminant released into the environment at Chernobyl (INSAG, 1986; Mascanzoni, 1987; Kenigsberg & Minenko, 1994; Dubrova *et al.,* 1996).

6.1.2 Radiation Quality.

Radioisotopes emit a variety of radiations including α- and β-particles as well as γ-rays. It is known that α-particles are more damaging than β- or γ- radiation as they are more densely ionising and are regarded as having radiation of high LET quality (Hall & Cox, 1994). However, they only have a very short range and are not very penetrating so therefore it has previously been thought that the risk of harmful exposure is very low. With recent data that some radionuclides may become localised in the testis (Jackson *et al.,* 1991; Hoyes *et al.,* 1996a, 1996b), the effects of exposure of germ cells to high LET radiation needs to be considered.

The biological hazards of testicular localisation of α-particle emitting radionuclides are obviously very high. However, there are other less well known radioactive emissions that may be equally as damaging but have not been considered. Many environmentally relevant radioisotopes decay by processes of electron capture and internal conversion (Howell, 1992). These modes of decay are characterised by the emission of low energy Auger and Coster-Kronig electrons with subcellular ranges of up to 10μm. Therefore the energy from the decay is deposited within a very short distance with a biological efficiency similar to that of α-emitting radionuclides. Rao *et al.,* (1985, 1988) have shown that after intratesticular injection, Auger emitting radionuclides are much more effective at reducing testicular sperm head counts that β-emitting radioisotopes. Although the range of these electrons are very short, if localisation with such a radionuclide is associated with a nucleus (such as a sperm head) then the potential hazard is far greater than that presented from external radiation sources. Relative biological effectiveness (RBE) values of around 7 (equivalent to that for α-particles) have been reported for DNA bound Auger emitters (Howell *et al.,* 1993), but the risk is still very high if radionuclides are localised within the nucleus but not associated with the DNA (RBE value ~ 4). This is of particular concern as results from recent studies have demonstrated the ability of human spermatozoa to take up and bind indium *in vivo* (Hoyes *et al.,* 1998).

6.1.3 Effects of Indium-114m on the Testis.

As described above indium and other radioisotopes (e.g. plutonium and iron) may become localised by the testis and retained (Rao *et al.,* 1988; Jackson *et al.,* 1991; Hoyes *et al.,* 1996a, 1996b). Indium decays emitting both β-particles and Auger electrons (Rao *et al.,* 1988) which are potentially more harmful than radiation produced by external sources.

Rao *et al.,* (1988) examined the cytotoxicity of the indium radiopharmaceuticals (^{111}In oxine, ^{111}In citrate and ^{114m}In citrate) on mouse testes. All compounds were cytotoxic to germ cells within the testes as shown by reductions in testis sperm head counts 29 days post treatment. Comparable studies with external X-rays suggested that indium radiopharmaceuticals were more effective at reducing sperm head counts than X-rays. This is similar to the results of an earlier study which suggested that the auger emitting radionuclide, Iron-55 was more cytotoxic to the testis than its β-emitting counterpart, Iron-59 (Rao *et al.,* 1985).

Hoyes et al, (1994) examined the spermatogenic and mutagenic effects of systemic injection of Indium-114m on male rats. After intraperitoneal injection of indium, 0.25% of the total systemic dose was localised within the testis within 24 hours where it was retained with an effective half life of 49.5days. Administration of 14.8MBq/kg ^{114m}In resulted in significant decreases in testis weights which were first observed 45 days post-injection. Maximal effect on testis weights were observed 87 days post-injection when testis weights were 70% that of control animals. Similar effects upon epididymal weight were also observed. Decreases in epididymal sperm counts were observed after 66 days and were at their lowest 108 days post-treatment where they were 33% of those recorded from control animals. After this timepoint sperm counts showed some evidence of recovery. Therefore it is quite clear that indium-114m has toxic effects upon the testis. The effects of radiation upon the testis are well documented and the decreases in testis weight and sperm counts observed after ^{114m}In treatment are a result of radiation-induced killing of germ cells.

The mutagenic effects of ^{114m}In were also examined in breeding studies with exposed animals. As well as the higher potential hazards induced by the emission of Auger electrons the germ cells will also be continually exposed to low dose irradiation by β-particles throughout their development as it has been shown that testicularly localised indium is retained by the testis. After injection of 14.8MBq/kg ^{114m}In it was calculated that the radiation dose to the testis was effectively 1.6Gy, 2.1Gy and 2.23Gy after 45, 87 and 129 days respectively

Treatment with ^{114m}In resulted in a decrease in litter size. This was first detected if offspring born to females mated with treated males 66-84 days post-injection. The greatest decrease in litter size was recorded to parents mated 87-126 days post-treatment where litter size declined to around 75% of controls. Indium-114m also caused an increase in the frequencies of pre-and post-implantation losses and dominant lethal mutations. An increase in the frequency of dominant lethals was first observed in males mated 24 days post-treatment with a 21% increase in the frequency of dominant lethal mutations observed in animals mated 66-84 days post treatment. Transgenerational effects were also observed with offspring born to males mated 24-42 days post-injection displaying decreased testis weights and sperm counts as well as higher frequencies of dominant lethal mutations amongst the F_2 offspring.

6.1.4 Aims and Objectives.

Results from previous chapter have demonstrated the ability of the comet assay to detect DNA damage induced in spermatozoa by external radiation sources both *in vivo* and *in vivo*. It was the aim of the experiments described in the following chapter to investigate whether similar effects are observed after internal irradiation of the testis with the radionuclide, indium-114m. Male mice were injected intraperitoneally with 50μCi ^{114m}In (equivalent to a dose of 70MBq/kg) and reproductive effects examined 45 days after dosing. This timepoint is representative of the highest levels of DNA damage observed in spermatozoa after *in vivo* testicular irradiation with X-rays. A group of animals were also irradiated with 4Gy X-rays as a positive control and example of an external radiation source.

6.2 RESULTS.

6.2.1 Body Weights, Organ Weights and Sperm Counts.

No significant differences in body weight were observed between control animals and those irradiated with 4Gy X-rays. Animals injected with ^{114m}In however had significantly lower body weights compared to animals in the other treatment groups (Table 6.2.1.1).

***Table 6.2.1.1** : Effects of ^{114m}In and X-rays on body weights of treated animals.*

Treatment Group	Bodyweight (g)
Control	30.3 ± 1.1
Indium-114m (5µCi)	25.6 ± 0.9*
X-Rays (4Gy Testicular)	29.3 ± 1.0

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = $P<0.05$, ** = $P<0.01$ (One way analysis of variance followed by Bonferroni's post-hoc test).

Testis and epididymis weights were reduced in all treated animals (Tables 6.2.1.2 & 6.2.1.3) but testicular damage was more severe in animals dosed with ^{114m}In where testis weight was reduced by over 75% compared to control values whilst animals irradiated with X-rays showed a 24% decline in testis weights at the end of the experimental period compared to controls. Exposure to either ^{114m}In or testicular X-rays had no effects on the weight of the livers of exposed animals although a small increased in the weight of the spleen was detected in ^{114m}In treated animals compared to control (Table 6.2.1.4)

***Table 6.2.1.2** : Effects of ^{114m}In and X-rays on paired testis weights of treated animals.*

Treatment Group	**Paired Testes Weight (mg)**
Control	216.7 ± 12.3
Indium-114m (5μCi)	51.2 ± 3.4**
X-Rays (4Gy Testicular)	167.1 ± 7.6*

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

***Table 6.2.1.3** : Effects of ^{114m}In and X-rays on paired epididymis weights of treated animals.*

Treatment Group	**Paired Epididymis Weights (mg)**
Control	50.2 ± 2.2
Indium-114m (5μCi)	24.7 ± 0.7**
X-Rays (4Gy Testicular)	41.2 ± 1.4**

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

***Table 6.2.1.4** : Effects of ^{114m}In and X-rays on liver and spleen weights of treated animals.*

	Organ Weights (mg)	
Treatment Group	***Liver***	***Spleen***
Control	1317 ± 57	74 ± 3
Indium-114m (5μCi)	1306 ± 44	83 ± 3*
X-Rays (4Gy Testicular)	1287 ± 40	79 ±5

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

In order to account for the significantly lower body weight of the indium-treated animals, testis and epididymis weights were also calculated per gram of body weight to compensate for differences between the treatment groups (Tables 6.2.1.5 and 6.2.1.6). This transformation of the data did not affect the outcome of the results as it also shows that testis and epididymal weights were reduced in both treated group with more severe decreases in testis and epididymis weights observed in animals treated with ^{114m}In.

***Table 6.2.1.5** : Effects of ^{114m}In and X-rays on paired testis weights per gram of body weight of treated animals.*

Treatment Group	**Paired Testes Weights (mg) per g Bodyweight**
Control	7.2 ± 0.6
Indium-114m (5μCi)	2.0 ± 0.1**
X-Rays (4Gy Testicular)	5.7 ± 0.3**

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

***Table 6.2.1.6** : Effects of ^{114m}In and X-rays on paired epididymis weights per gram of bodyweight of treated animals.*

Treatment Group	**Paired Epididymis Weights (mg) per g Bodyweight**
Control	1.7 ± 0.1
Indium-114m (5μCi)	1.0 ± 0.0**
X-Rays (4Gy Testicular)	1.4 ± 0.1*

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

Sperm counts were also reduced in animals irradiated with X-rays and ^{114m}In. As with the testis and epididymis the greatest effects were observed in animals dosed with ^{114m}In where the vas deferens sperm content was reduced by over 90% compared to control values (Table 6.2.1.7). Vas deferens sperm reserves in animals irradiated with X-rays had declined to 85% of control levels. Effects on testis sperm head counts also demonstrated that ^{114m}In produced a greater effect than X-rays on the testis (Table 6.2.1.8). Testicular sperm head counts from indium treated animals could not be accurately determined as they were present at such a low frequency. Counts from X-irradiated mice were approximately 50% of values from control animals.

Table 6.2.1.7 *: Effects of ^{114m}In and X-rays on vas deferens sperm counts of treated animals.*

Treatment Group	**No. of Sperm recovered from the Vas Deferens (x10^6)**
Control	19.4 ± 0.8
Indium-114m (5μCi)	1.1 ± 0.2**
X-Rays (4Gy Testicular)	2.6 ± 0.7**

Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

Table 6.2.1.8 *: Effects of ^{114m}In and X-rays on testicular sperm head counts of treated animals.*

Treatment Group	**Testicular Sperm Counts (x10^6)**
Control	12.3 ± 1.4
Indium-114m (5μCi)	<0.06**$^{(a)}$
X-Rays (4Gy Testicular)	6.1 ± 0.7**

$^{(a)}$ There were too few sperm head in these samples to counts so result shown is the minimum detection limit of the counting chamber.
Data shown represent the means ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different from the controls, * = P<0.05, ** = P<0.01 (One way analysis of variance followed by Bonferroni's post-hoc test).

6.2.2 Testicular Histology.

Photographs of testis section from animals in all experimental groups are shown in Figure 6.2.2.1. Testes from untreated controls showed normal spermatogenesis with a seminiferous epithelium consisting of a full complement of developing germ cells and spermatozoa in the lumen of the tubules. However testis sections from indium treated animals showed complete ablation of spermatogenic activity in all tubules with only Sertoli cells present and no germ cells. Leydig cells were still observed in the interstitial spaces between tubules. The tubules however were decreased in size compared to control sections and the epithelium vaculous in appearance. Compared to indium treated animals, testes from X-irradiated animals showed evidence of spermatogenesis. However there were some tubules which seemed to be devoid of germ cells. The tubules also appeared to be reduced in size compared to control animals and there was evidence of abnormal spermatogenesis as indicated by a lack of late germ cell stages in some tubules.

6.2.3 Distribution of Radioactivity.

Indium was injected intraperitoneally at the beginning of the experiment from where it would have been distributed throughout the body. At the end of the experiment tissues were collected and their radioactivity's measured. Tables 6.2.3.1 and 6.2.3.2 show the radioactivity counts for the individual organs and also the calculated biodistribution of the indium as a percentage of the total injected activity for each organ.

All organs from indium treated animals showed increased levels of associated radioactivity compared to control and X-irradiated animals had radioactivity levels comparable to background radioactivity measurements. The majority of indium was associated with the liver (~12% of injected dose) with the testes containing ~0.5% of the injected radioactivity. Increased levels of radioactivity were also detected in the epididymis and sperm collected from the vas deferens. Muscle, which is a non-transferrin binding tissue displayed the lowest levels of associated radioactivity (Table 6.2.3.1).

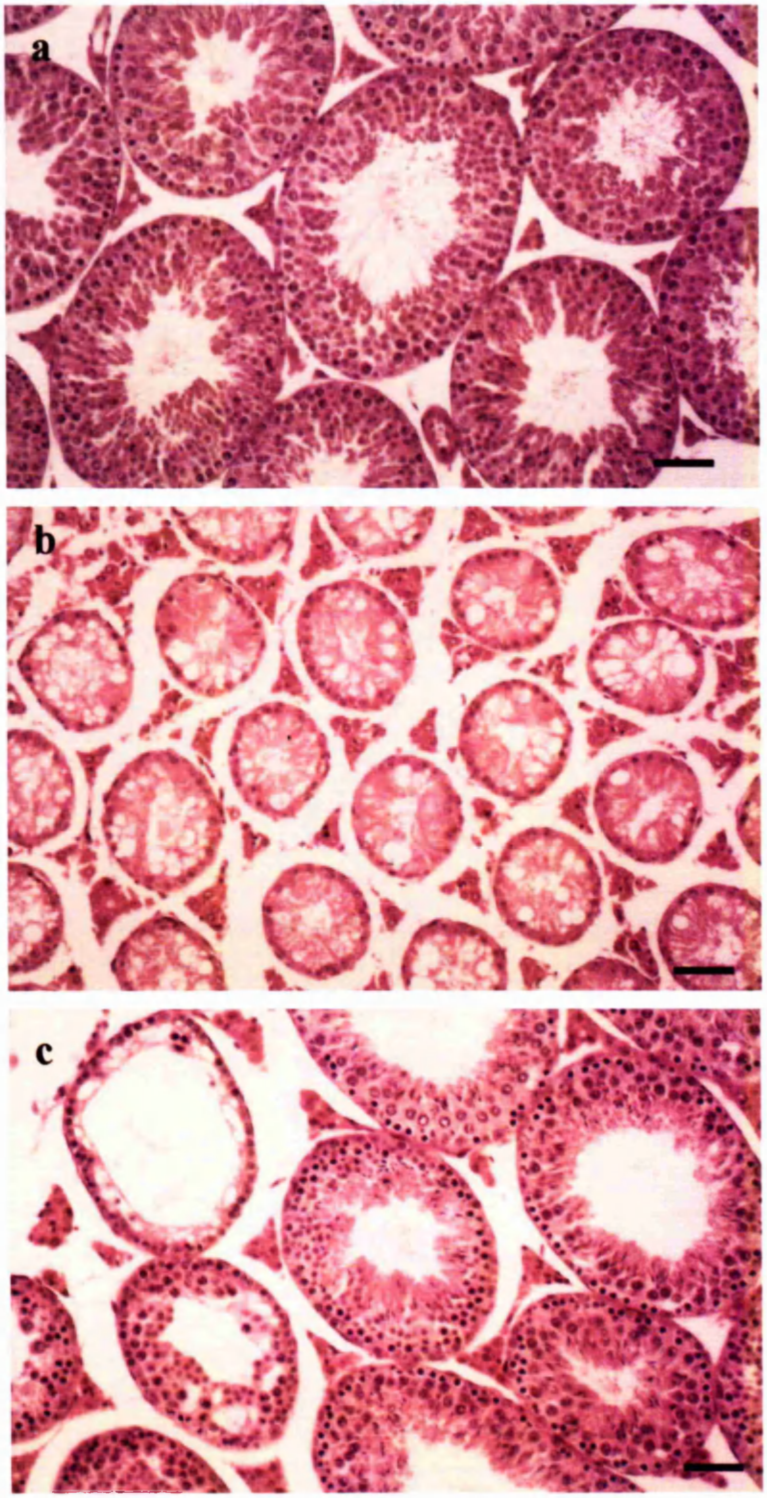


Figure 6.2.2.1 *: Effects of irradiation with either internally incorporated ^{114m}In or external X-rays on testicular histology. Panel a - Control testis, 45 days post-irradiation of experimental animals; Panel b - Testis section, 45 days post-injection of animal with ^{114m}In; Panel c - X-irradiated testis, 45 days post-treatment. All photographs are displayed at the same magnification x160. Scale Bar = 50μm.*

Table 6.2.3.1 *: Radioactivity counts of dissected organs at the end of the experiment period.*

	Radioactivity Count (γ-counter) **cpm per g tissue** **(absolute cpm per organ)**		
Organ	***Control***	***Indium-114m***	***X-ray (4Gy)***
Testes	–	509835 ± 40828 *(25751 ± 1785)*	1.82 ± 7.8 *(0.4 ± 1.2)*
Epididymis	–	360874 ± 76781 *(8668 ± 1617)*	-5.2 ± 14.8 *(-0.2 ± 0.6)*
Liver	–	439486 ± 16206 *(570768 ± 10927)*	0.16 ± 0.50 *(0.2 ± 0.6)*
Spleen	–	479736 ± 24796 *(39898 ± 2023)*	17.6 ± 5.0 *(1.4 ± 0.4)*
Muscle	–	57400 ± 8034 *(7660 ± 1365)*	5.6 ± 1.8 *(1.0 ± 0.3)*
Sperm	–	2306000 ± 566027[a] (2306 ± 566)	–

[a] = weight of spermatozoa pellet was taken as equivalent of minimum sensitivity of the balance (1mg)

Data shown is the mean count per minute per gram tissue ± S.E.M (n=6). Values in parentheses refer to the absolute radioactivity counts for each organ.

Table 6.2.3.2 *: Tissue distribution of injected ^{114m}In in mice 45 days post injection of 50μCi per animal*

Organ	**Percent of injected activity**
Testes	0.52 ± 0.04
Epididymis	0.18 ± 0.03
Liver	11.61 ± 0.22
Spleen	0.81 ± 0.04
Muscle	0.16 ± 0.02
Sperm	0.05 ± 0.01

Data shown is the mean ± S.E.M for each tissue (n=6).

In order to compare radioactivity levels in the individual tissues the data was transformed to counts per g of tissue in order to take into account differences in the mass of the tissues. Although the liver retained considerably more of the injected activity than the testis, relative levels of radioactivity per gram of tissue were actually higher in the testis. In fact the epididymis, spermatozoa and spleen all showed higher levels of relative radioactivity than the liver. Sperm from the vas deferens showed extremely high levels of radioactivity per g of tissue almost 5 fold higher than levels observed in other tissues.

6.2.4 DNA Damage in Spermatozoa.

To investigate whether internal irradiation of the testis with localised ^{114m}In would produce detectable DNA damage in spermatozoa, sperm from the vas deferens of treated animals was analysed by comet assay at the end of the experimental period. X-irradiated animals (4Gy) were also included in the study as positive controls.

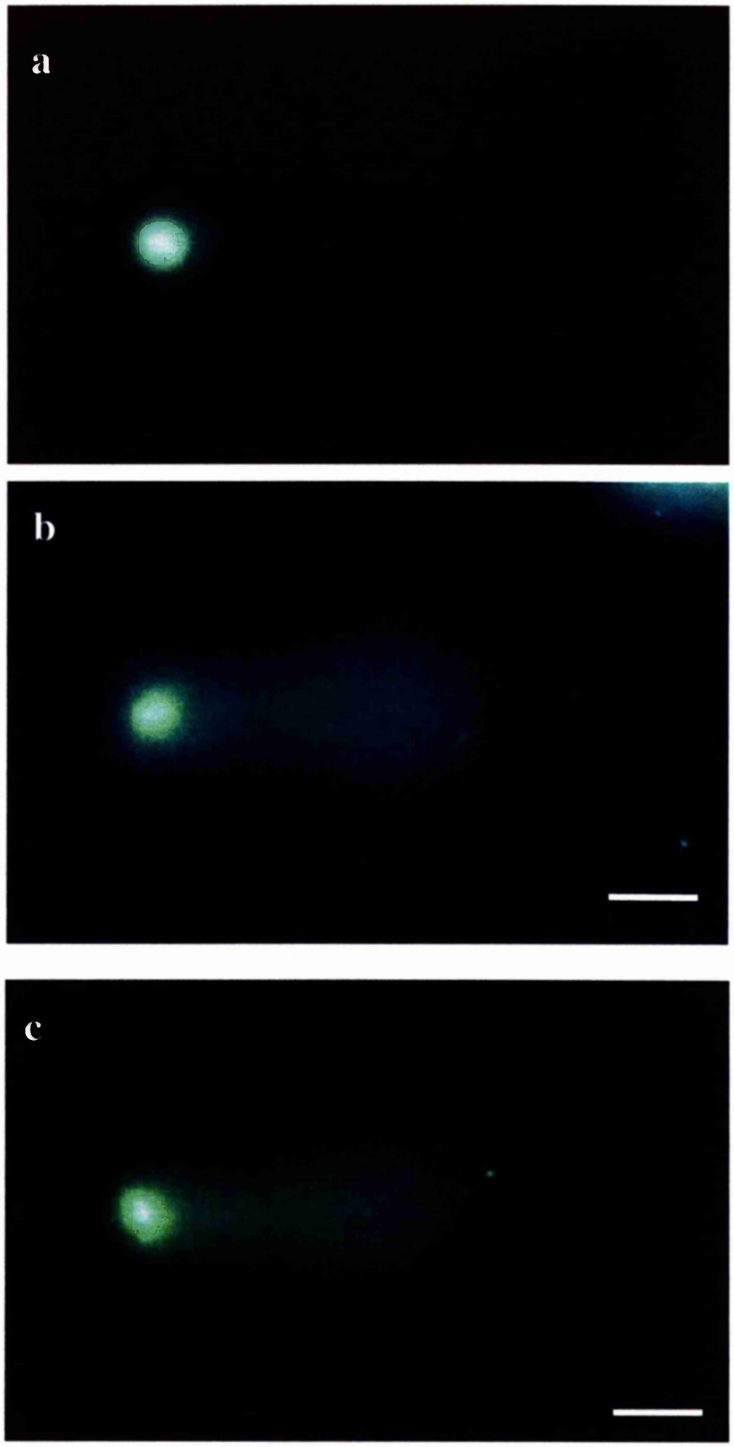
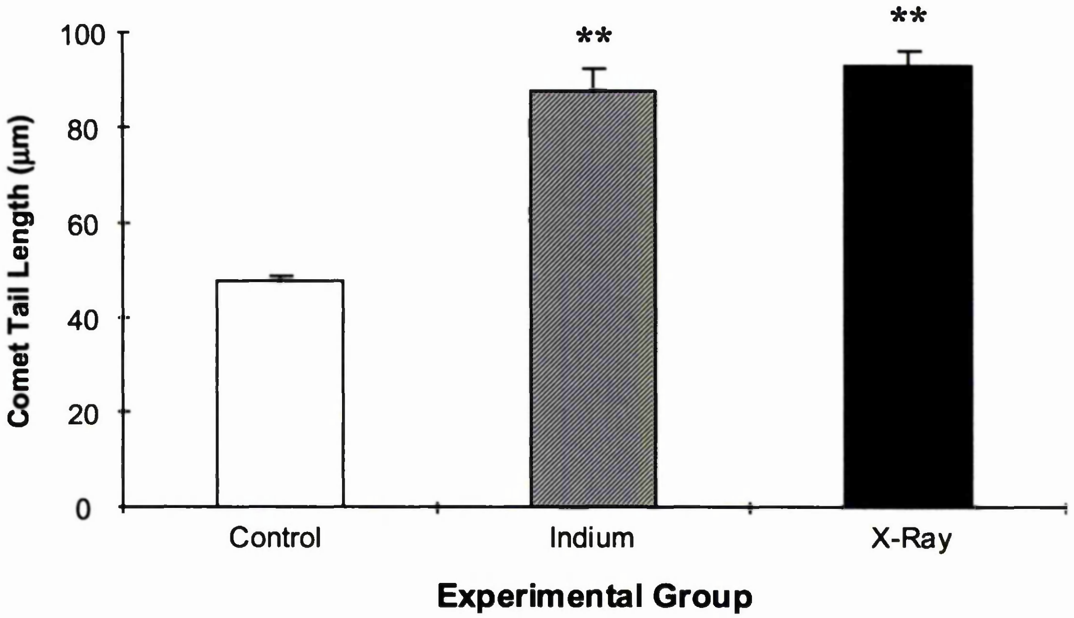


Figure 6.2.4.1 *: DNA damage in spermatozoa as measured by neutral comet assay after testicular irradiation with internally incorporated ^{114m}In or external X-rays. Panel a - spermatozoa from an unirradiated control animal; Panel b - spermatozoa from an ^{114m}In treated animal, 45 days post injection; Panel c - spermatozoa from an animal testicularly irradiated with 4Gy X-rays, 45 days previously. All images are shown at the same magnification x480. Scale Bar = 25μm.*

a)



b)

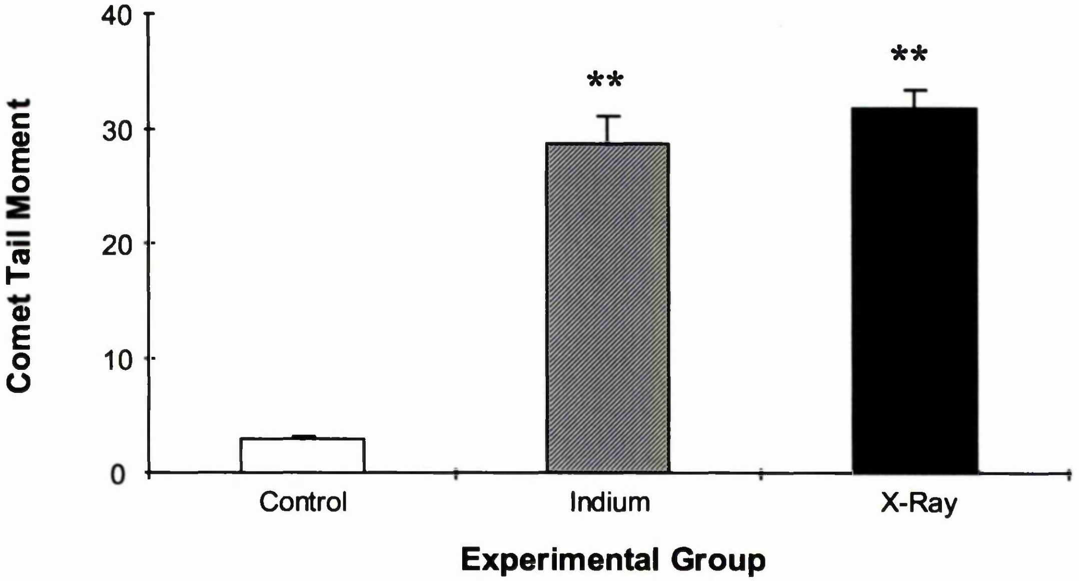


Figure 6.2.4.2 *: Effects of ^{114m}In and testicular X-rays on DNA damage in spermatozoa assessed by neutral sperm comet assay. a) Effects on comet tail length, b) Effects on comet tail moment. Data shown is the mean ± S.E.M for each experimental group (n=6). Asterisks denote values significantly different to controls. * = P<0.05, ** = P<0.01 (Kruscal-Wallis one-way ANOVA and Mann Whitney U-test).*

***Table 6.2.4.1** : Effects of ^{114m}In and testicular X-rays on % Tail DNA in comets from spermatozoa assessed by neutral sperm comet assay*

Treatment Group	% Tail DNA
Control	10.0 ± 0.7
Indium-114m (5μCi)	55.1 ± 2.0**
X-Rays (4Gy Testicular)	57.2 ± 1.9**

Data shown is the mean ± S.E.M for each experimental group (n=6).
Asterisks denote values significantly different to controls. * = $P<0.05$, ** = $P<0.01$ (Kruscal-Wallis one-way analysis of variance and Mann Whitney U-test).

Control animals showed low levels of DNA damage as assessed by comet assay parameters (comet tail length, comet tail moment, % tail DNA) which were comparable to normal levels observed in previous studies. Levels of DNA damage in spermatozoa were elevated in animals treated with ^{114m}In or X-rays (Figures 6.2.4.1-6.2.4.3).

All comet parameters showed that sperm from animals exposed to either ^{114m}In or X-rays had similar levels of DNA strand breaks. Comet tail moments showed a 9 fold increase compared to control values in the ^{114m}In dosed group and a 10 fold increase for animals exposed to X-rays. The frequency distribution of comet tail lengths show clearly how spermatozoa from both experimental groups showed increased DNA migration compared to control sperm. The distribution of control sperm shows a normal distribution with a mode of 50μm. Irradiation of germ cells with either ^{114m}In or X-rays resulted in a rightward shift of the frequency distribution with the majority of cells showing comet tail lengths of >70μm and a modal value of 110μm in both ^{114m}In and X-ray treated groups. In both cases very few undamaged spermatozoa (less than 5% of total sperm with tail lengths <60μm) were observed in samples from these treatment groups.

6.2.5 Presence of Non-Sperm Cells in the Vas Deferens after Irradiation.

Scoring of the comets revealed significant numbers of large, brightly staining cells on the comet slides (Figure 6.2.5.1). Unlike mouse spermatozoa, these cells were round in shape, unflagellated, many times bigger and more intensely staining. High numbers were observed in sperm samples from both ^{114m}In and X-ray treated groups but these round cells were not observed frequently in control sperm samples (Figure 6.2.5.2). The comets of these cells were excluded from the analysis.

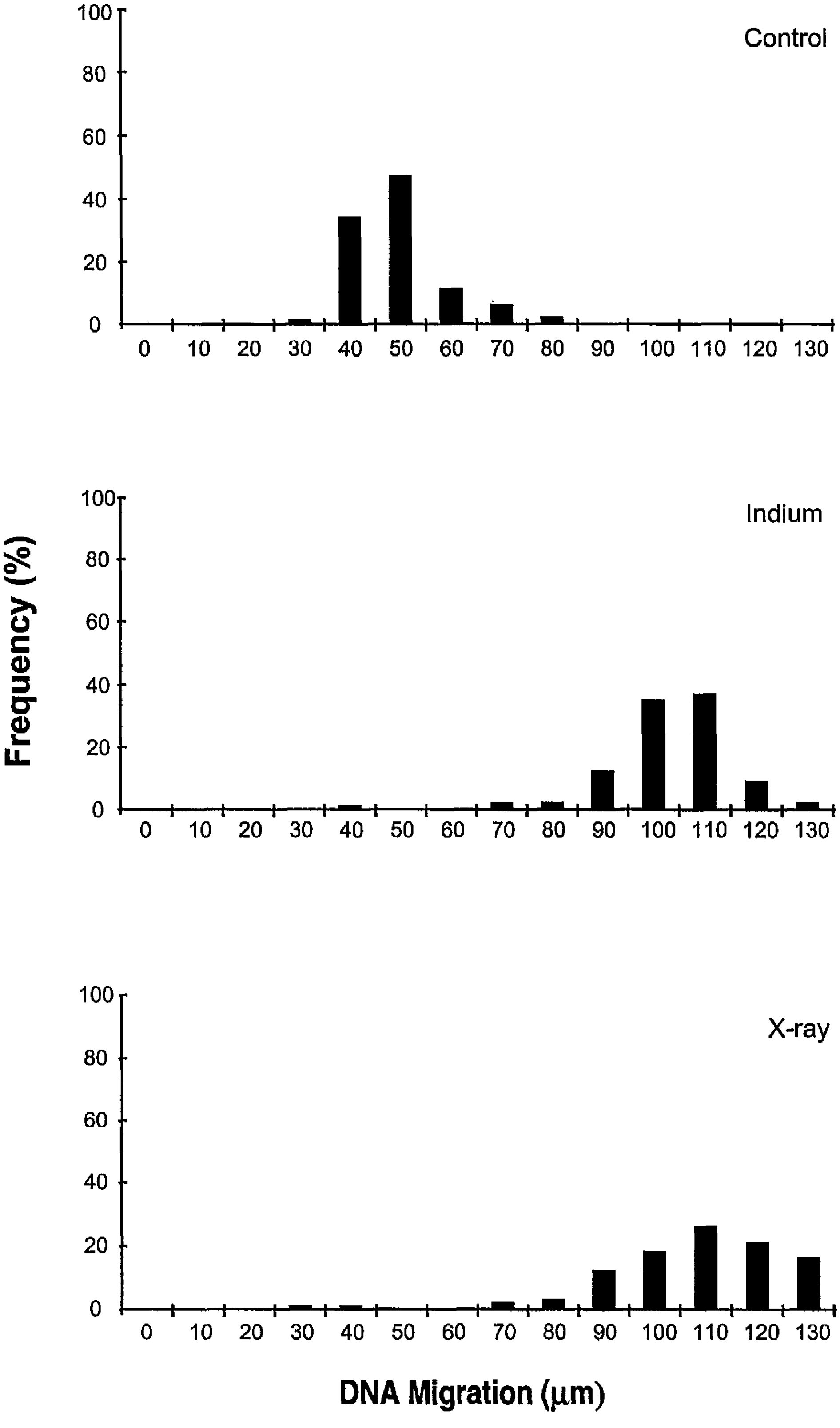


***Figure 6.2.4.3** : Frequency distributions of comet tail lengths after treatment with ^{114m}In and testicular X-rays.*

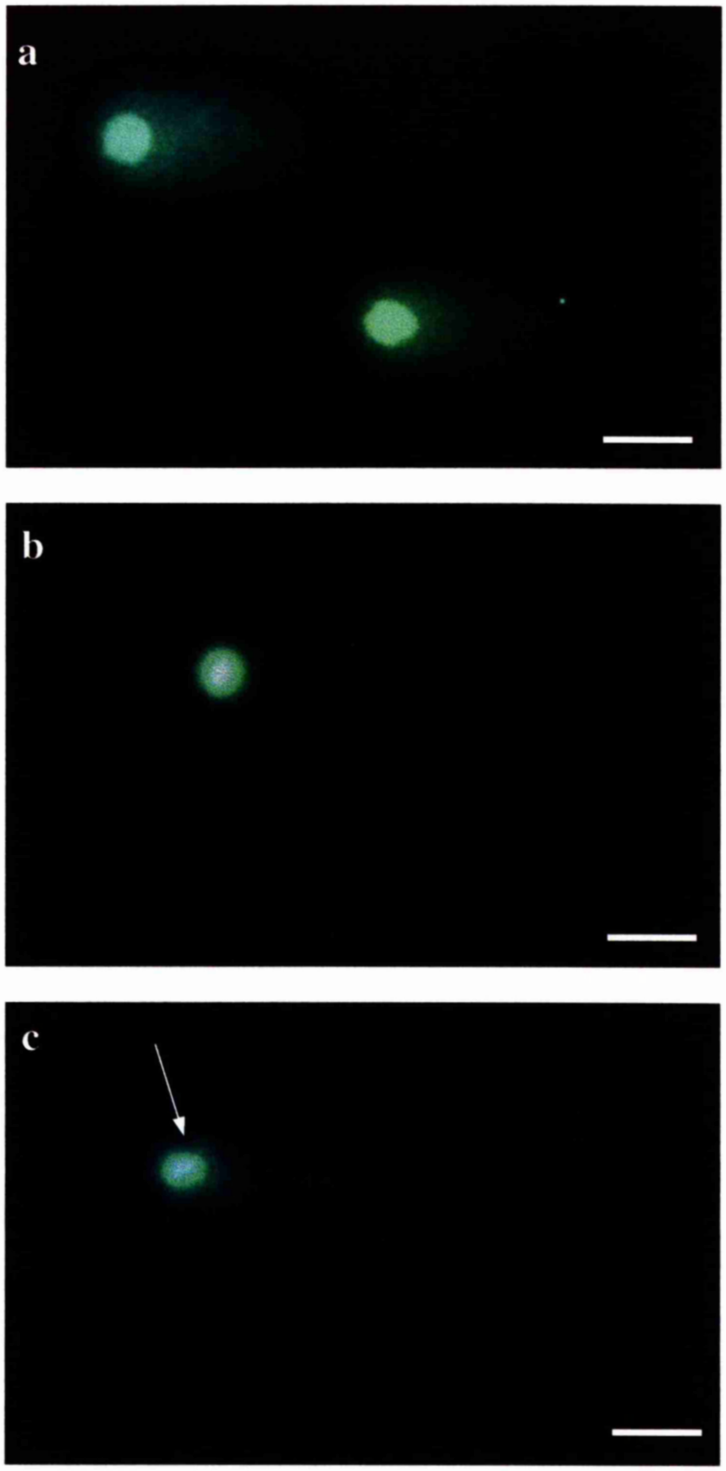


Figure 6.2.5.1 *: Presence of non-sperm cells in samples collected from the vas deferens of* ^{114m}*In treated and X-irradiated animals. Panel a - Two non-sperm cells as identified by the presence of a large round nucleus. Panel b - undamaged sperm cell as identified by much smaller nucleus and non round shape. Panel c - spermatozoa showing DNA damage as identified by small irregular nucleus and presence of a tail which can just be detected as indicated by the arrow. Magnification x480. Scale Bar = 25μm.*

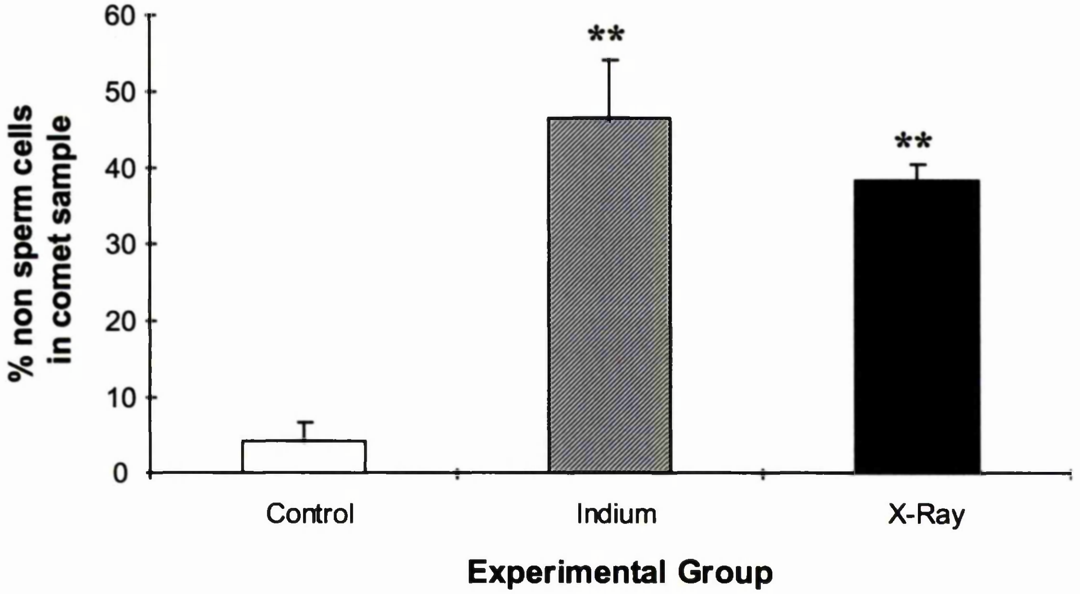


***Figure 6.2.5.2** : Percentage of non-sperm cells in vas deferens sperm samples processed by neutral comet assay. Data shown is the mean ± S.E.M for each experimental group (n=6). Asterisks denote values significantly different to controls. * = P<0.05, ** = P<0.01 (One way analysis of variance and X^2 test).*

6.3 DISCUSSION.

In this study, irradiation with either 4Gy of external X-rays or 50μCi of internal ^{114m}In caused marked reductions in testicular weight, epididymal weight and vas deferens and testicular sperm reserves. Effects of testicular irradiation with external radiation sources such as γ- and X-rays are well documented in a wide variety of species (Meistrich, 1986a, 1993; Van Alphen & De Rooij, 1986; Pogany, 1987) and it has been shown that irradiation leads to the death of early germ cells. Proliferating spermatogonia are the most sensitive germ cell stage and are killed with doses as low as 0.1Gy. With doses in the range of 4-5Gy as used in this study there may also be a cytotoxic effect on later germ cell stages such as the spermatocytes and round spermatids (Meistrich, 1986a). The killing of these early germ cell stages results in a maturation-depletion effect on late germ cells and spermatozoa with a resulting decrease in testis and epididymal weight. As well as external irradiation, internally incorporated radionuclides have also been demonstrated to be cytotoxic to the testis. The radioisotopes indium-111 and 114m (Rao *et al.,* 1988), Iron-55 and 59 (Rao *et al.,* 1985), plutonium-238 (Green *et al.,* 1975; Pomerantseva *et al.,* 1989), iodine-123 and 125 (Narra *et al.,* 1992) and astatine-211 (Cobb *et al.,* 1990) have all been demonstrated to cause germ cell death and a reduction in testicular sperm head counts in the mouse testis. Therefore the results with indium-114m presented in this chapter are consistent with these data and demonstrate the cytotoxicity of ^{114m}In in the mouse testis.

Indium-114m was more cytotoxic than 4Gy X-rays in this study as it produced a greater reduction in testis weights and sperm counts and the histological sections showed total ablation of the seminiferous tubules. Unlike X-rays, which are pure low LET radiation, ^{114m}In emits high LET auger electrons as well as low LET β-particles during its decay process. Previous studies with high LET auger emitting radionuclides have suggested that thallium-201(^{201}Tl) and iron-55(^{55}Fe) were almost 3 times as effective at reducing testicular sperm head counts than equivalent doses of their β-emitting counterparts ^{204}Tl and ^{59}Fe (Rao *et al.,* 1985). Similarly indium radiopharmaceuticals (^{111}In oxine, ^{111}In citrate and ^{114m}In citrate) have also been demonstrated to have a higher relative biological effectiveness in the mouse testes than X-rays (Rao *et al.,* 1988). These data therefore clearly suggest that radionuclides that decay with a high energy LET component are more cytotoxic than external low LET sources and that conventional risk assessment techniques based on external radiation sources are inadequate for safety evaluation of these radionuclides.

However based on the results presented in this chapter it is unwise to draw the conclusion that indium-114m is more cytotoxic to the testes than X-irradiation. This is because firstly, the doses of radiation emitted by ^{114m}In and X-rays were not equivalent and secondly, the testicular X-irradiation was delivered as an acute dose to the testis whereas irradiation produced by intratesticularly incorporated ^{114m}In would have been delivered as continual chronic low dose irradiation over the course of the experiment. Previous studies using acute and fractionated radiation with the same total delivered dose have demonstrated that fractionated irradiation is more cytotoxic to the testis than acute irradiation (Ogilvy-Stuart & Shalet, 1993). This is because the intrinsic radiosensitivities of the testicular germ cells differ depending on their position in the cell cycle. An acute dose of 0.5Gy X-rays will kill actively dividing spermatogonia whereas resting spermatogonia will survive (Meistrich, 1986a). Also some highly damaged cells will be able to repair their DNA before they pass into the next round of mitosis. Conversely continual low dose irradiation makes the repair of DNA difficult as damage is being continually accrued. Chronic irradiation will also kill actively dividing spermatogonia (Meistrich, 1986a) but those resting spermatogonia that are resistant to acute irradiation will be killed when they pass through the next round of cell division in the cell cycle. Therefore these aspects make it difficult to accurately compare the testicular effects of indium and X-rays in this study. The dose of 4Gy X-rays used in this experiment was chosen as previous studies have demonstrated this dose causes germ cell death and dominant lethal mutations (Ehling, 1971; Searle & Beechey, 1974; Meistrich, 1986a; Pogany, 1987). Experiments described in Chapter 4 of this thesis have confirmed this and also suggest that this dose results in the production of DNA-damaged spermatozoa. Previous studies with ^{114m}In in rats have also demonstrated that it is cytotoxic to the testes and can produce an increase in the frequency of dominant lethal mutations (Hoyes *et al.,* 1994, 1995). A dose of 50μCi ^{114m}In was chosen for use in this study, which is higher than doses used previously. However, the main aim of this study was to determine whether internal irradiation (like external irradiation sources) could also produce DNA damage to germ cells which was transmitted through the spermatogenic process resulting in spermatozoa with damaged DNA. Therefore a higher dose was employed to ensure positive cytotoxic and damaging effect upon the testis. In future experiments should be performed with lower doses of ^{114m}In, which do not have such dramatic cytotoxic effect upon the testis. The dose should be matched with that of X-rays to allow direct comparison of the relative biological effectiveness of high and low LET radiation in the testis. However there may still be difficulties in comparing the two sets of data because of the difference in the nature of the two irradiations.

As well as reducing testis and epididymis weights, indium also had an effect on body weight. It may be possible to explain the reduced body weights of animals in the indium treated group due to the general systemic toxicity of ^{114m}In as it would have been distributed throughout the body. Previous studies *in vitro* have demonstrated the cytotoxicity of indium radiochemicals on bone marrow cells (Birch *et al.,* 1986), lung fibroblasts (Kassis & Adelstein, 1985) and human lymphocytes (Ten Berge *et al.,* 1983). Therefore it is possible that injected ^{114m}In may have produced effects on the haemopoeitic system and other organs which were not investigated in this study. No effects were observed in the liver, which received most of the injected dose. However a small but significant increase in spleen weight was observed which at the moment is unexplained. Previous studies have demonstrated that indium can produce effects in the spleen as studies with ^{114m}In labelled lymphocytes injected into rats found that they accumulated in the spleen producing a resulting lymphocytopenia (Birch *et al.,* 1986).

The majority of the injected indium was found in the liver. This is not surprising giving the large size of this organ and it has previously been shown to express large amounts of transferrin receptors (Gatter *et al.,* 1983; Heubers & Finch, 1987). Transferrin receptors have also been found to be expressed in the spleen and both this organ also showed a significant level of radioactivity. These results are consistent with previous studies and support the hypothesis that ^{114m}In may utilise the iron-transferrin system. Much lower levels of radioactivity were found in muscle which does not accumulate transferrin-binding radionuclides due to the low levels of transferrin receptors in this tissue (Gatter *et al.,* 1983; Heubers & Finch, 1987). Radioactivity was also found associated with the spermatozoa and the epididymis. Presumably ^{114m}In bound to spermatozoa in the epididymis accounts for a large proportion of the radioactivity measured in this tissue. This would appear to indicate that spermatozoa express transferrin receptors. Experiments with antitransferrin-receptor antibodies have suggested the presence of transferrin receptors on spermatocytes and round spermatids but were unable to find evidence of receptor expression on elongate spermatids and spermatozoa (Brown, 1985; Vanneli *et al.,* 1986). However, previous autoradiographic studies have demonstrated the presence of radiolabelled epididymal sperm after ^{114m}In administration (Jackson *et al.,* 1991) and studies with human sperm *in vitro* have shown that they are able to take up indium and other transferrin-binding radionuclides (Hoyes *et al.,* 1998). Furthermore, the uptake of indium by human spermatozoa was inhibited by the addition of transferrin to the incubation medium. Therefore, this evidence suggests that spermatozoa do express transferrin receptors and that indium is able to become associated with spermatozoa. All cells have a requirement for iron as part of biological redox systems and often the highest levels of transferrin receptor expression are found in rapidly proliferating cells (i.e. in tissues such as spleen, testis and bone marrow) (Heubers & Finch, 1987). Mature cells such as spermatozoa may also express transferrin receptors albeit at low levels in order to obtain sufficient iron for the maintenance of essential intracellular systems (Hoyes *et al.,* 1998). *In vitro* uptake studies are now being performed with isolated mouse spermatozoa to try to add further evidence for the expression of transferrin receptors on spermatozoa and their ability to bind ^{114m}In.

Although most of the injected ^{114m}In was found associated with the liver the highest levels of radioactivity (cpm per g/tissue) were found in the sperm, testes and spleen respectively. Previous experiments have demonstrated that the testis is able to accumulate and retain indium whilst the spleen may be able to retain ^{114m}In due to the phagocytosis of red blood cells by splenic macrophages (Birch *et al.,* 1986; Jackson *et al.,* 1991; Hoyes *et al.,* 1994, 1995, 1996b). The extremely high levels of radioactivity associated with spermatozoa are due to relatively small mass of these cells compared to other tissues. This indicates a considerable amount of association of indium with spermatozoa. In the testis, indium is localised within the seminiferous tubules where it is concentrated in the cytoplasm of Sertoli cells (Jackson *et al.,* 1991; Hoyes *et al.,* 1996b). Developing germ cells within the cytoplasm of Sertoli cells may bind this indium and retain it through the development process into spermatozoa or alternatively spermatozoa may be able to bind indium directly from the Sertoli cell cytoplasm themselves.

The close association of indium particles with germ cells and spermatozoa has potentially serious implications. The results reported in this study and by other investigators using other radioisotopes have demonstrated the considerable ability of internally incorporated radionuclides to cause cell death within the testis (Green *et al.,* 1975; Rao *et al.,* 1985, 1988; Cobb *et al.,* 1990; Narra *et al.,* 1992; Hoyes *et al.,* 1994, 1995). Whereas the dose of ^{114m}In used in this chapter produced dramatic cytotoxic effects, lower doses of ^{114m}In when administered may result in a smaller degree of germ cell death but may also produce mutagenic effects through damage to DNA. Treatment of male rats with ^{114m}In followed by mating trials has demonstrated that indium can produce increases in the frequency of dominant lethal mutations suggesting that it can damage spermatozoal DNA (Hoyes *et al.,* 1994). As previously described, indium emits high LET Auger electrons during its decay. These have a very short range and are not usually damaging because of their limited penetration potential. However, when internally incorporated into the body and in close association to the nucleus these particles have considerable potential to cause damage. Because these particles are considerably more damaging than X-rays, even small amounts of these agents that internally contaminate the body may pose a serious hazard if they become localised within the body.

If indium is able to bind directly to spermatozoa or access the nuclear DNA then it may pose a threat to the genetic integrity of the spermatozoa itself. Spermatozoa themselves are regarded as extremely radioresistant (Meistrich, 1986a) and this maybe due to the fact that they are post-differentiated and also contain lots of protein in close association with their DNA which can act as free radical scavengers (Nygren *et al.,* 1995). Whilst the compact nature of spermatozoal DNA may confer radioresistance, if high energy emitters such as indium can bind to sperm they may pose a much greater threat than external radiation sources. Indium during its decay process emits high energy auger electrons, which only have a short range of penetration. However in association with spermatozoa either through binding to cell surface receptors or by becoming internalised, these electrons may be able to damage DNA. Low LET radiations (e.g. γ- and X-rays) produce particle tracks with well separated ionization events. On the contrary high LET radiations (e.g. α-particles and Auger electrons) give rise to dense clusters of ionizations (Nikjoo *et al.,* 1998). As the chromatin fibres in the nuclei of spermatozoa are much closer to one another than in the nuclei of somatic cells (Ward & Coffey, 1991), then these high energy particles may be able to cause considerably more damage than in a conventional nuclear structure due to the condensed nature of spermatozoal DNA. Therefore the damage may be transmitted at fertilisation leading to effects upon the embryo. Additionally, there may also be a further threat to the embryo if sperm with bound indium can fertilise, as this may lead to irradiation of maternal oocyte chromatin and also embryonic DNA.

Despite the emission of high LET radiation by indium and the observations of more severe effects on testis weights and histology, DNA damage levels in spermatozoa from indium and X-ray irradiated animals were similar. This is despite other studies that suggest that auger emitters are much more damaging than low LET radiations (Rao *et al.,* 1988, 1989; Pampfer *et al.,* 1989; Narra *et al.,* 1992). It might have been expected that because of the more severe effects of ^{114m}In that were observed on the testis that DNA damage levels in spermatozoa would have been much higher in ^{114m}In treated animals compared to X-irradiated mice. Previous studies have suggested that high LET radiations produce greater damage to DNA than low LET radiations and that the lesions produced by high LET radiation are less repairable than those induce by low LET radiations (Goodhead *et al.,* 1993; Prise *et al.,* 1994; Nikjoo *et al.,* 1998). However, despite these observations no significant differences were observed in the levels of DNA damage in spermatozoa measured by comet assay after testicular irradiation with either ^{114m}In or X-rays. This may possibly reflect differences in the dose of radiation delivered to the testis by X-rays and indium. As stated previously the doses of ^{114m}In and X-rays were not comparable. However, it may also reflect differences in the response of the germ cells to these different radiations. Firstly, since indium would have produced continual low dose irradiation of germ cells, the germ cells may have been able to repair the majority of the DNA damage before it entered the next round of mitotic divisions. However previous studies have suggested that high LET radiations largely produce complex lesions in DNA which are largely unrepairable (Goodhead *et* *al.,* 1993; Prise *et al.,* 1994; Nikjoo *et al.,* 1998). Therefore alternatively, what may have occurred is that the majority of germ cells received or accumulated high levels of DNA damage, which prevented the cells progressing through mitosis and meiosis resulting in cell death. From the testicular histology it is evident that the large majority of the germ cells in the testis were killed by treatment with ^{114m}In and previous studies have demonstrated the greater RBE of indium compounds for cell killing than low LET radiations (Rao *et al.,* 1988). Therefore the surviving cells which developed into spermatozoa may have been a radioresistant subpopulation of germ cells. However, whilst it is possible to speculate as to why DNA damage levels were similar in X-irradiated and ^{114m}In treated animals, the only way to really test whether ^{114m}In induces higher levels of DNA damage in spermatozoa is for further experiments to be performed with matched doses of X-rays and ^{114m}In. The experiments described in this chapter, do demonstrate, as was shown with testicular X-irradiation in Chapter 4, that internal irradiation of germ cells within the testis by ^{114m}In can result in the transmission of DNA damage throughout the spermatogenic process. This is consistent with reports of increased dominant lethal mutations and reduced litter size after paternal treatment with ^{114m}In (Hoyes *et al.,* 1994).

From the previous chapter we have seen that *in vivo* irradiation of the testis produces a spectrum of DNA damage levels. However the frequency distributions for DNA migration show exposure to either indium or 4Gy X-rays causes a rightward shift in the control distribution with almost all the cells showing high levels of DNA damage. Previous results from earlier chapters have demonstrated that irradiation with lower doses of X-rays leads to a mixture of cells that are damaged and undamaged. However 4Gy, of X-rays is quite a high dose of radiation and as shown almost all the cells show a high degree of DNA damage with very few undamaged cells. A similar distribution is seen with indium and may reflect the severity of the dose. Alternatively as the doses of X-rays and indium are quite severe, many cells that were irradiated may have received high degrees of DNA damage leading to the death of the cell. This may be reflected by the dramatic decreases observed in sperm counts. Therefore the sperm that survived may represent a radiosensitive subpopulation of cells.

One interesting effect of testicular radiation exposure that was observed in both indium treated and X-irradiated animals was the appearance of large numbers of non-sperm cells in the vas deferens of irradiated animals. Other cells types such as white blood cells are often found in semen (World Health Organisation, 1992). However in sperm preparation from control animals much lower frequencies of non-sperm cells were observed. The non-sperm cells had roundly stained nuclei and were larger than spermatozoa. Previous studies have noted that after irradiation of the testis, sloughing of germ cells from the germinal epithelium into the lumen of the tubule may occur (Pogany, 1987). Studies performed in mice after acute testicular irradiation with 6Gy X-rays demonstrated that high levels of non-sperm cells were observed in the epididymis for up to 9 weeks post-irradiation. These non-sperm cells were identified as stage 1 and stage 2 round spermatids. Therefore it is possible that the increases in round non sperm cells found in sperm preparations from indium-treated and X-irradiated animals may represent germ cells "sloughed" from the germinal epithelium as a result of radiation exposure. A previous study has also suggested that the presence of immature germ cells in human ejaculates is strongly correlated with a low fertilization index of such semen samples and may be an indicator of poor quality ejaculates (Tomlinson *et al.,* 1992). In this study, high levels of round cells were only found in sperm samples obtained from animals exposed to either ^{114m}In or X-rays and not control samples. In order to ensure that only sperm cells were analysed, these round cells were excluded from comet analysis.

In summary, this chapter has presented evidence that indium, a radionuclide that becomes localised to the testis after internal contamination can have toxic effects on the testis including the production of sperm with damaged DNA which may have serious consequences for fertility and the offspring. Previous studies have expressed concern about the effects of internal contamination of humans with radionuclides. The controversial Gardner report published in 1990, which suggested a link between paternal exposure of workers in the nuclear industry and leukemias in their children suggested that exposure to internally incorporated radionuclides may be to blame. Other studies have demonstrated effects and germline mutations after radionuclide contamination after the Chernobyl nuclear incident (Dubrova *et al.,* 1996) although there is no evidence for increases in germline mutations amongst survivors of the

Hiroshima and Nagasaki atomic blasts (Kodaira *et al.,* 1995). Further experiments are required to compare the relative effectiveness of indium in producing DNA damage compared to external radiation sources and also the consequences of indium binding directly to spermatozoa.

Chapter 7

DNA Damage and Spermatogenesis in the Testes of Gene Knockout Mice.

7.1 INTRODUCTION.

The maintenance of the genetic integrity of spermatozoa is of uppermost importance for the success of fertilisation and development of the embryo. The testis has a variety of mechanisms (active DNA repair in early germ cells, cell cycle checkpoints and antioxidant systems) which operate during spermatogenesis to protect against DNA damage (Coogan & Rosenblum, 1988; Van Loon *et al.,* 1991; Almon *et al.,* 1993; Bauche *et al.,* 1994; Sjoblom & Lahdetie, 1996;). Apoptosis is an active form of cell death in which unwanted or superfluous cells are removed by an organism. Apoptosis has been shown to occur as part of the normal physiology of the testis where it may function to delete damaged germ cells (Bartke, 1995; Woolveridge & Morris, 1999).

7.1.1 Apoptosis in the Testis.

During spermatogenesis, germ cell numbers in the testis are rapidly multiplied as a result of numerous rounds of mitosis and meiosis. However several groups have noted that the actual number of spermatozoa produced by the testis is far less than that that would be expected from synchronous development of spermatogonial germ cells (Russell *et al.,* 1990). Therefore it appears that are large number of germ cells that are lost or deleted during spermatogenesis and it has been suggested that apoptosis plays an important role in removing these cells.

Apoptosis occurs spontaneously in the testis albeit at a low frequency as shown by tissues obtained from rodents. In the rat testis, apoptosis has been described in A2, A3 and A4 spermatogonia (Allan *et al.,* 1992) as well as primary and secondary spermatocytes and occasionally spermatids (Kerr, 1992; Brinkworth *et al.,* 1995). Apoptosis in the testis may be under some form of hormonal control since in rodents removal of LH and FSH by hypophysectomy leads to an increase in apoptosis in pachytene spermatocytes and spermatids (Russell & Clermont, 1977; Tapanainen *et al.,* 1993). This can be reversed by treatment with exogenous LH and FSH and administration of testosterone also prevents hypophysectomy-induced germ cell apoptosis (Russell & Clermont, 1977; Tapanainen *et al.,* 1993). This suggests a role for hormones in the maintenance of germ cell viability. In the human testis, apoptosis has been described in tissues obtained after orchidectomy for prostate cancer (Brinkworth *et al.,* 1997; Woolveridge *et al.,* 1998a) where it has been observed in a wide variety of germ cell types but not Sertoli or Leydig cells. Interestingly it has been reported that testicular biopsies from infertile men with azoospermia or severe oligospermia showed increased frequencies of apoptotic cells compared to normal fertile men (Lin *et al.,* 1997a, 1997b) suggesting that apoptosis may play a role in male infertility.

As well as occurring as part of the normal physiology of the testis, many physical agents have been shown to cause apoptosis of germ cells within the testis. Irradiation of the testis with 4Gy of X-rays has been shown to produce apoptosis of spermatogonia in mice within 9-18 hours post-irradiation (Beumer *et al.,* 1997). Similar to the effects of radiation, many cytotoxic drugs that are also used to treat cancer have been shown to cause apoptosis of germ cells in the testis. These include mitomycin C (Nakagawa *et al.,* 1997), cyclophosphamide (Cai *et al.,* 1997), adriamycin and etopocide (Sjoblom *et al.,* 1998).

7.1.2 Genetic Control of Apoptosis.

Regulation of cell death is essential for normal development and is also an important defence against cancer and genetically-induced damage. Too much cell death can lead to impaired development and degenerative diseases, whereas too little can lead to hyperplasia and cancer (White, 1996). An ever expanding family of genes controls the process of apoptosis with new regulators being identified all the time. Whilst this means that there may be many mechanism controlling apoptosis there are a number of gene products that are commonly (but not always) involved in the process of active cell death. Figure 7.1.2.1 shows a common pathway proposed for the regulation of apoptosis.

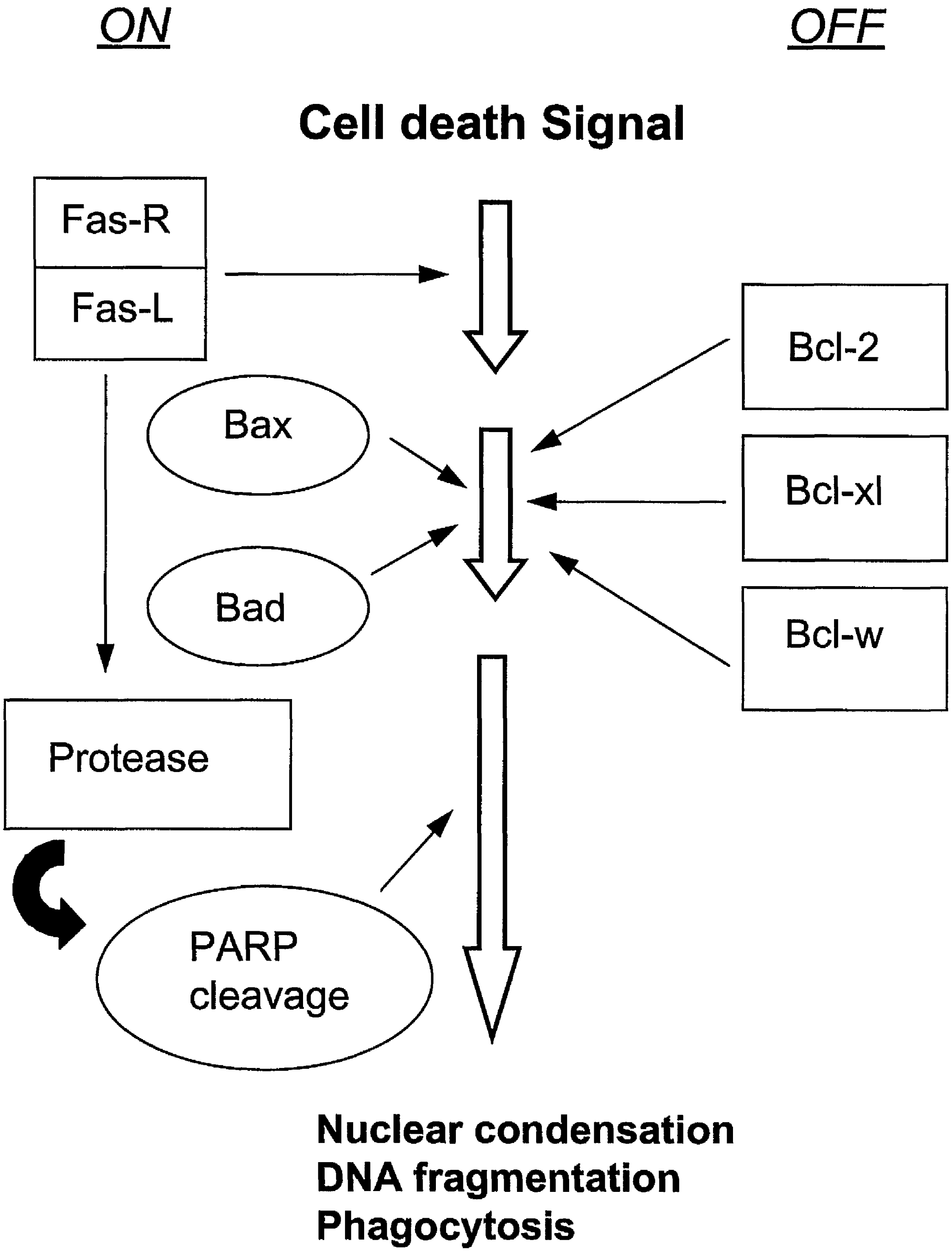

ON
OFF
Cell death Signal
Fas-R
Fas-L
Bax
Bad
Bcl-2
Bcl-xl
Bcl-w
Protease
PARP cleavage
Nuclear condensation
DNA fragmentation
Phagocytosis

***Figure** 7.1.2.1 : Genetic regulation of the apoptotic cell death pathway.*

Bcl-2 was the first member of a family of related gene products found to be involved in the regulation of apoptosis (Vaux *et al.,* 1988; Hockenbery *et al.,* 1990). Expression of

Bcl-2 has been shown to enhance cell survival by suppression of apoptosis, suggesting that Bcl-2 is an inhibitor of apoptosis. Since the discovery of Bcl-2 other related proteins have been discovered that also function to regulate apoptosis. The proteins Bcl-xl and Bcl-w which are both structural analogues of Bcl-2 are also able to inhibit apoptosis and promote cell survival (Boise *et al.,* 1993; Gibson *et al.,* 1996). These proteins are commonly referred to inhibitors of cell death. As well as members of the Bcl-2 family that inhibit apoptosis there are other related proteins that act as promoters of apoptosis. Bax, which shows sequence homology to Bcl-2, appears to suppress the ability of Bcl-2 to block apoptosis (Oltvai *et al.,* 1993). Two other proteins Bak and Bad which also show homology to Bcl-2 also appear to promote apoptosis in most cases (Chittenden *et al.,* 1995; Farrow *et al.,* 1995; Kiefer *et al.,* 1995; Yang *et al.,* 1995). The exact mechanism by which these proteins regulate apoptosis is unclear however it appears that both protectors and promoters of cell death are able to interact with each other and the eventual outcome may depend on the ratio of death promoters to death supressors. A model has been suggested where members of the Bcl-2 family regulate apoptosis by forming homodimers and heterodimers with each other (Oltvai *et al.,* 1993).

It has been shown that agents that induce DNA damage can induce apoptosis. The p53 tumour suppressor gene has been termed "The Guardian of the Genome" and appears to function as a sensor of genetic damage (Lane, 1992). When DNA damage is detected, p53 can either arrest the cell cycle to allow repair of the damage by DNA repair mechanisms (Kuerbitz *et al.,* 1992; Chernova *et al.,* 1995; Zolzer *et al.,* 1995) or alternatively p53 can also activate a biochemical cascade leading to apoptosis (El-Deiry *et al.,* 1993; Miyashita & Reed, 1995; Guillouf *et al.,* 1995).

Much evidence suggests that p53 functions as a transcription factor by upregulating the expression of death promoters such as Bax or repressing transcription of survival promoters such as Bcl-2 (Miyashita & Reed, 1995). Although p53 is often involved in apoptosis as a result of genetic damage p53-independent pathways also exist (Odorisio *et al.,* 1998).

7.1.3 Expression and Function of Apoptotic Regulators in the Testis.

7.1.3.1 Bcl-2

Western blot studies have shown that the Bcl-2 protein is found in testicular extracts from immature and adult rats (Woolveridge *et al.,* 1998c). Evidence for the expression of Bcl-2 in human and mouse testes is contradictory with some groups unable to display immunoreactivity of testis extract (Hockenbery *et al.,* 1991; Knudson *et al.,* 1995; Furuchi *et al.,* 1996; Rodriguez *et al.,* 1997) whilst other group suggest that Bcl-2 protein is present in the testis (Krajewski *et al.,* 1995; Woolveridge *et al.,* 1998a). An immunohistochemistry study on mouse testis sections suggested that Bcl-2 protein was localised to the spermatids and mature spermatozoa within the seminiferous tubules (Krajewski *et al.,* 1995). Bcl-2 is thought to function as a suppresser of apoptosis and it has been suggested that Bcl-2 may regulate the developmental wave of apoptosis in spermatogonia and spermatocytes which occurs during the first round of spermatogenesis and is essential for the development of normal spermatogenesis (Rodriguez *et al.,* 1997). Transgenic mice which overexpress Bcl-2 in all testicular germ cells or specifically in spermatogonia show highly abnormal spermatogenesis which is accompanied by infertility (Furuchi *et al.,* 1996; Rodriguez *et al.,* 1997). This has been shown to be due to an accumulation of spermatogonia and giant multinucleated cells with an absence or severe depletion of spermatocytes, spermatids and spermatozoa suggesting that Bcl-2 may function as a regulator of spermatogonial apoptosis. However, Bcl-2 knockout mice have been generated which display normal spermatogenesis suggesting that lack of Bcl-2 does not result in excess cell death in the testis. These mice however do have other physiological defects however including growth retardation, lymphoid apoptosis and polycystic kidneys (Nakayama *et al.,* 1993; Kamada *et al.,* 1995) highlighting the importance of Bcl-2 as a cell death regulator.

7.1.3.2 Bax

Bax has been shown to be highly expressed in reproductive tissues (Tilly *et al.,* 1995; Woolveridge *et al.,* 1998c). Testicular extracts from human, mouse and rat have been shown to display immunoreactivity for bax protein (Knudson *et al.,* 1995; Woolveridge *et al.,* 1998a, 1998c). However, one study has suggested that bax is barely detectable in the mouse testis (Rodriguez *et al.,* 1997). Immunohistochemistry studies of testes sections demonstrated that bax staining was only present in germinal cells located near the basement membrane of the seminiferous tubules (Krajewski *et al.,* 1994a). Bax knockout mice are infertile as a result of the accumulation of atypical premeiotic germ cells and a lack of haploid spermatids and spermatozoa (Knudson *et al.,* 1995). Also TUNEL labelling of testis sections demonstrated an increased frequency of apoptotic cells in the testes of bax knockout mice. Bax is thought to function as a promoter of cell death, however results from the knockout mice seem to suggest that bax may be required to block apoptosis in spermatogenesis. This may therefore indicate that bax can either promote or inhibit cell death depending on the cell type. Alternatively bax may be required to eliminate a specific germ cell type in development that if retained in bax knockout mice leads to abnormal cell death during spermatogenesis. This would maintain the role of bax as purely a promoter of cell death.

7.1.3.3 Other Bcl-2 Family Members.

Bcl-xl has been shown to be associated with spermatocytes and spermatids of human and mouse testes (Krajewski *et al.,* 1994b; Woolveridge *et al.,* 1998a). As with Bcl-2, overexpression of Bcl-xl by testicular germ cells lead to abnormal spermatogenesis and sterility as a result of accumulation of early germ cell stages (Rodriguez *et al.,* 1997). This suggests that Bcl-xl, like Bcl-2 may play a role in regulating apoptosis during spermatogenesis. Bcl-w, another promoter of cell survival has been shown to be expressed by elongating spermatids and Sertoli cells in the testis (Ross *et al.,* 1998). Bcl-w knockout mice are infertile and this appears to be a result of the loss of spermatocytes and spermatids by apoptosis resulting in a Sertoli cell only seminiferous epithelium. Eventually Sertoli cells are also lost from the testis by apoptosis (Ross *et al.,* 1998). Bak and Bad have been shown to be expressed in the rat testis (Woolveridge *et al.,* 1998b) although studies with mice could only find Bad present (Rodriguez *et al.,* 1997). In the human testis Bak has been found to be associated with the Sertoli and Leydig cells (Krajewski *et al.,* 1996).

7.1.3.4 p53

p53 protein is found associated with the pachytene spermatocytes in normal rodents (Almon *et al.,* 1993; Schwartz *et al.,* 1993; Sjoblom & Lahdetie, 1996) although there is also some expression by spermatogonia and spermatids (Stephan *et al.,* 1996). It has been suggested that p53 in the testis may play a role in regulating meiosis during the spermatogenic process since it is found associated with pachytene primary spermatocytes (Almon *et al.*, 1993; Schwartz *et al.*, 1993; Sjoblom & Lahdetie, 1996). It has been suggested that during normal spermatogenesis, p53 may function to halt the cell cycle to allow time for DNA recombination and repair events. Some strains of p53 knockout mice exhibit testicular giant cell degenerative syndrome which is thought to occur as a result of the inability of tetraploid pachytene spermatocytes to complete their meiotic division (Rotter *et al.*, 1993). This further suggests a role for p53 in the regulation of meiosis during spermatogenesis. However, p53 knockouts derived from other strains exhibit normal patterns of spermatogenesis (Beumer *et al.*, 1998; Hasegawa *et al.*, 1998) suggesting that their may be alternative pathways that can also regulate meiosis during spermatogenesis.

p53 has been implicated in the sensing of DNA damage and the control of apoptosis (Lane, 1992). Indeed, apoptosis of primary spermatocytes in the rat testis induced by X-irradiation is associated with increased expression of p53 in the testis 3 hours post-irradiation (Sjoblom & Lahdetie, 1996; West & Lahdetie, 1997). p53 levels have also been shown to increase in mouse spermatogonia after 4Gy (Beumer *et al.*, 1998) and using knockout mice radiation-induced apoptosis of spermatogonia has been shown to be p53-dependent (Beumer *et al.*, 1998; Hasegawa *et al.*, 1998; Odorisio *et al.*, 1998). However in contrast to results from the rat testis (Sjoblom & Lahdetie, 1996), apoptosis of spermatocytes has been demonstrated to occur independently of p53 in the mouse testis (Odorisio *et al.*, 1998).

Irradiation of knockout mice has demonstrated that stem spermatogonia of p53 null mice produce fewer progeny after irradiation than wild type mice (Hasegawa *et al.*, 1998). It has also been shown that the survival of clonogenic spermatogonia after irradiation is reduced in p53 null mice compared to heterozygote and wild-type mice although the radiosensitivity of the cells is similar in all three genotypes (Hendry *et al.*, 1996). It is thought that the reduced survival of p53 null spermatogonia post-irradiation is a result of fewer functional radioresistant clonogenic spermatogonia in the testis of the unirradiated null mouse compared to the wild type.

7.1.3.5 SCID Mice.

Severe combined immune deficiency (SCID) is a rare congenital disorder of the immune system that was first recognised in human infants in the mid-1950's (Bosma & Carrol, 1991). In 1983, the mutation that results in SCID was first described in mice and homozygous scid mice (scid/scid) are severely deficient in both T- and B-lymphocytes (Bosma *et al.,* 1983). This has lead to the use of the scid mouse as a model for the study of autoimmune diseases (Bosma & Carrol, 1991; Taylor, 1992; Vladutiu, 1993). Another property of the scid mouse is an increased sensitivity to ionising radiation (Biedermann *et al.,* 1991; Disney *et al.,* 1992) and to DNA double strand break-inducing agents (e.g. bleomycin), (Biedermann *et al.,* 1991; Chang *et al.,* 1993; Tanaka *et al.,* 1993). Other studies have shown that that scid cells have a deficiency in the repair of DNA dsb's (Fulop & Phillips, 1990; Disney *et al.,* 1992). Since testicular cells are extremely radiosensitive and the repair of DNA dsb's is important during meiosis, one research group has examined the effects of irradiation on the testes of normal and scid mouse (Van Buul *et al.,* 1995). This group found that scid mice were hypersensitive for X-ray induced cell killing of both bonemarrow cells and spermatogonial stem cells.

7.1.4 Aims and Objectives.

Apoptosis plays an important part in both the regulation of spermatogenesis and cell death in the testis after cytotoxic insult (Woolveridge & Morris, 1999). Over the past years the molecular mechanisms regulating apoptosis have began to become elucidated and a number of genes and gene products have been identified (White, 1996). Many of these gene products have been found to be expressed in the testis and knockout mice have been created which often have altered testicular physiology (Rotter *et al.,* 1993; Knudson *et al.,* 1995; Rodriguez *et al.,* 1997; Ross *et al.,* 1998). Since apoptosis appears to play a crucial role in spermatogenesis and may function as a mechanism to protect the genomic integrity of spermatozoa we hypothesised that knockout mice deficient in gene products involved in DNA repair and apoptosis may have elevated levels of DNA damage in their spermatozoa. In order to test this hypothesis, spermatozoa and testes were collected from knockout mice (p53, Bcl-2 and Bax knockouts which are absent in genes involved in DNA repair and apoptosis and SCID mice which have an immune disorder but have also been shown to be highly radiosensitive and inefficient at repairing double strand breaks (Fulop & Phillips, 1990; Bosma & Carrol, 1991; Biedermann *et al.,* 1991). These strains of mice and corresponding wild-type strains have been examined a) by comet assay (to examine DNA damage levels in spermatozoa) b) histologically and c) using the TUNEL labelling of testis sections to look at spermatogenesis and apoptosis in these animals.

7.2 RESULTS

7.2.1 Comparison of Body Weights, Testes Weights and Sperm Counts between Knockout Animals and their Wild-Type Counterparts.

Bcl-2 and Bax knockout mice had significantly lower bodyweights than corresponding wild-type animals of the same age (Table 7.2.1.1). All other knockouts ($p53^{-/-}$ and SCID) were of similar bodyweight to wild-type mice of the same age. Not surprisingly given the difference in body weights, testes from Bcl-2 knockouts were significantly smaller than those of wild types controls. However testes from Bax knockout animals were much smaller (~60%) than those from corresponding wild type animals (Table 7.2.1.2). No difference in paired testes weights were detected between p53 and SCID knockouts and corresponding wild-type animals.. Vas deferens from Bax knockout animals were completely devoid of spermatozoa. Bcl-2 with their smaller testes unsurprisingly had lower vas deferens sperm counts than their larger wild-type counterparts (Table 7.2.1.3). As with other physical parameters no significant differences were observed in sperm counts between p53 knockouts, SCID mice and their wild-type counterparts.

7.2.2 Testicular Histology of Knockout and Wild-Type Mice.

Testicular histology appeared normal for all wild type animals (Figures 7.2.2.1-7.2.2.4). Evidence of normal spermatogenesis could clearly be seen with developing germ cells present within the seminiferous tubules and spermatozoa present in the lumen of the tubules. Within the testes spermatogenic tubules at different stages could be identified. Testicular histology from p53 (Figure 7.2.2.1) and SCID mice (Figure 7.2.2.4) also appeared normal and similar to that of wild type animals.

***Table 7.2.1.1** : Comparison of transgenic mice body weights.*

Transgenic Mouse Strain	**Mean Body Weight (g)**
$p53^{+/+}$	29.9 ± 0.3
$p53^{-/-}$	27.7 ± 1.2
$Bcl\text{-}2^{+/+}$	26.5 ± 0.9
$Bcl\text{-}2^{-/-}$	17.6 ± 0.4**
$Bax^{+/+}$	29.2 ± 0.6
$Bax^{-/-}$	25.4 ± 0.5*
SCID Control	29.2 ± 0.3
SCID	29.0 ± 1.1

Data shown represents the group mean ± S.E.M (n=4).
Asterisks denote values significantly different than wild-type controls. * = $P < 0.05$, ** = $P < 0.01$ (One way analysis of variance followed by Bonferonni's post-hoc test).

***Table 7.2.1.2** : Comparison of transgenic mice paired testis weights.*

Transgenic Mouse Strain	**Mean Paired Testis Weights (mg)**
$p53^{+/+}$	250 ± 12
$p53^{-/-}$	256 ± 7
$Bcl\text{-}2^{+/+}$	252 ± 6
$Bcl\text{-}2^{-/-}$	110 ± 4**
$Bax^{+/+}$	256 ± 10
$Bax^{-/-}$	96 ± 4**
SCID Control	230 ± 6
SCID	224 ± 8

Data shown represents the group mean ± S.E.M (n=4).
Asterisks denote values significantly different than wild-type controls. * = $P < 0.05$, ** = $P < 0.01$ (One way analysis of variance followed by Bonferonni's post-hoc test).

***Table 7.2.1.3** : Comparison of transgenic mice vas deferens sperm counts.*

Transgenic Mouse Strain	**Vas Deferens Sperm Counts (x10^6)**
p53$^{+/+}$	14.0 ± 0.9
p53$^{-/-}$	13.4 ± 0.9
Bcl-2$^{+/+}$	13.6 ±1.0
Bcl-2$^{-/-}$	6.7 ± 0.8**
Bax$^{+/+}$	13.0 ± 0.5
Bax$^{-/-}$	0.0 ± 0.0**[a]
SCID Control	13.8 ± 0.6
SCID	13.6 ± 1.0

[a] Vas deferens from Bax knockout mice were completely lacking in spermatozoa.
Data shown represents the group mean ± S.E.M (n=4).
Asterisks denote values significantly different than wild-type controls. * = $P < 0.05$, ** = $P < 0.01$ (One way analysis of variance followed by Bonferonni's post-hoc test).

No evidence of giant cells could be found in the testis sections of p53 knockout mice as has been reported for some strains of p53 knockouts. Despite differences in body weights, testis weights and sperm counts between Bcl-2 knockouts and wild-types spermatogenesis appeared normal in testis sections from Bcl-2 knockouts (Figure 7.2.2.2). Bax knockout mice showed grossly disturbed testicular histology compared to their wild-type counterparts with abnormal spermatogenesis. Seminiferous tubules were reduced in size compared to sections from wild type counterparts and there was an absence of developing spermatids and spermatozoa from the tubules. Often the tubules displayed a vacuolated appearance often containing abnormal giant cells. Tubules absent of developing germ cells were also frequently observed (Figure 7.2.2.3).

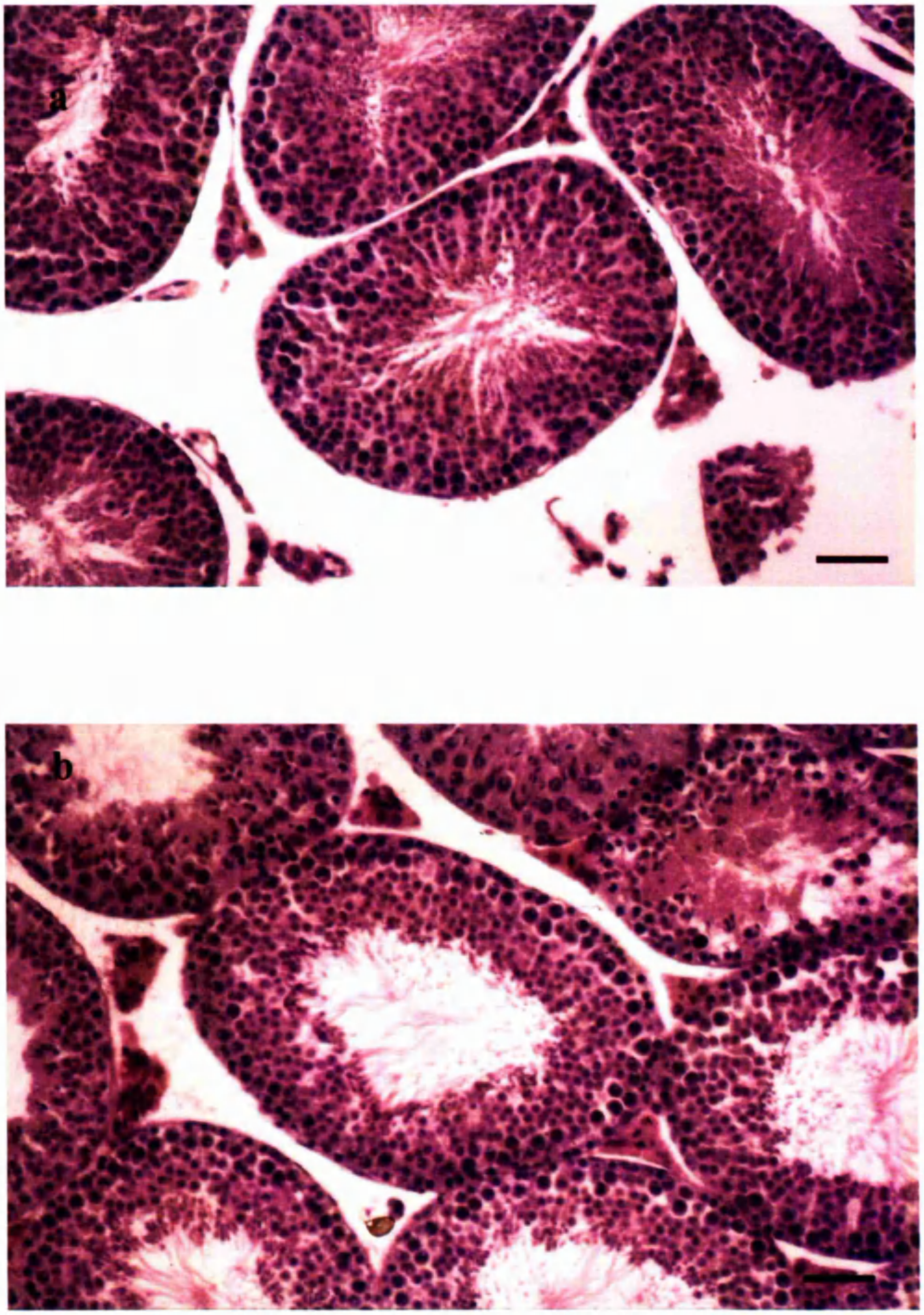
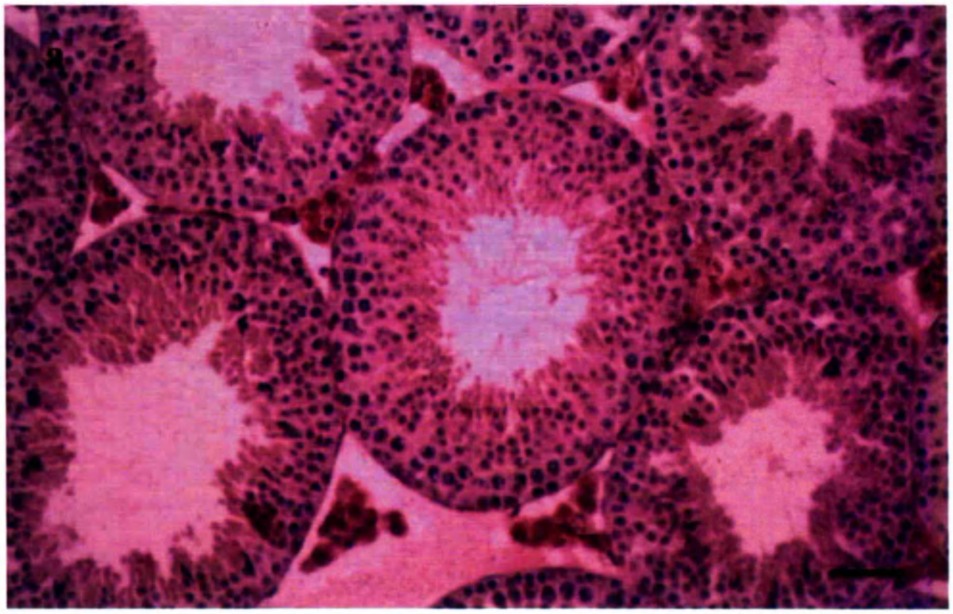


***Figure** 7.2.2.1 : Comparison of testicular histology from p53 knockout and wild-type mice. Panel a - testis from a wild-type control animal. Panel b - testis from a p53 knockout animal. Magnification x 200. Scale Bar = 50μm.*



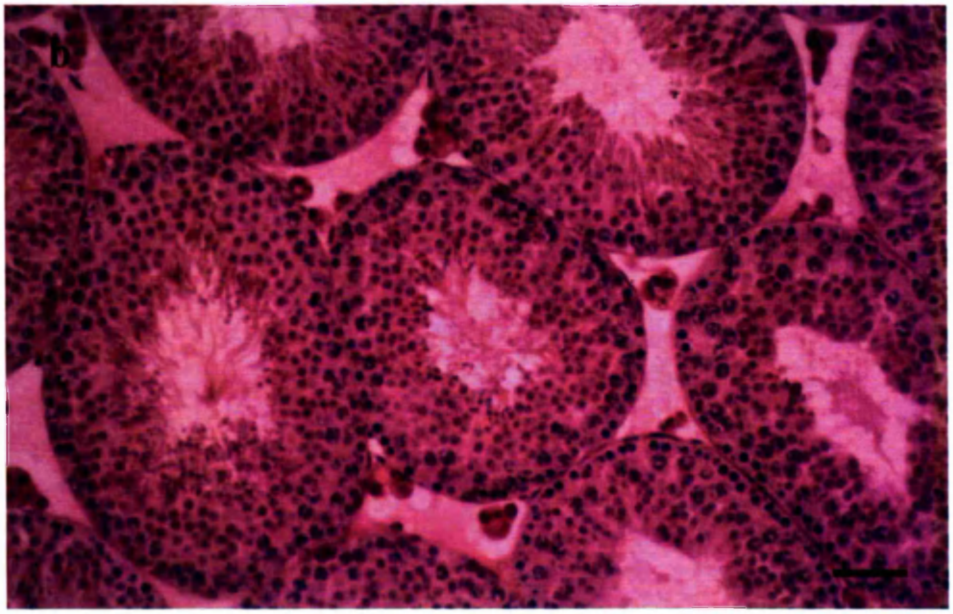


***Figure** 7.2.2.2 : Comparison of testicular histology from Bcl-2 knockout and wild-type mice. Panel a - testis from a wild-type control animal. Panel b - testis from a Bcl-2 knockout animal.*

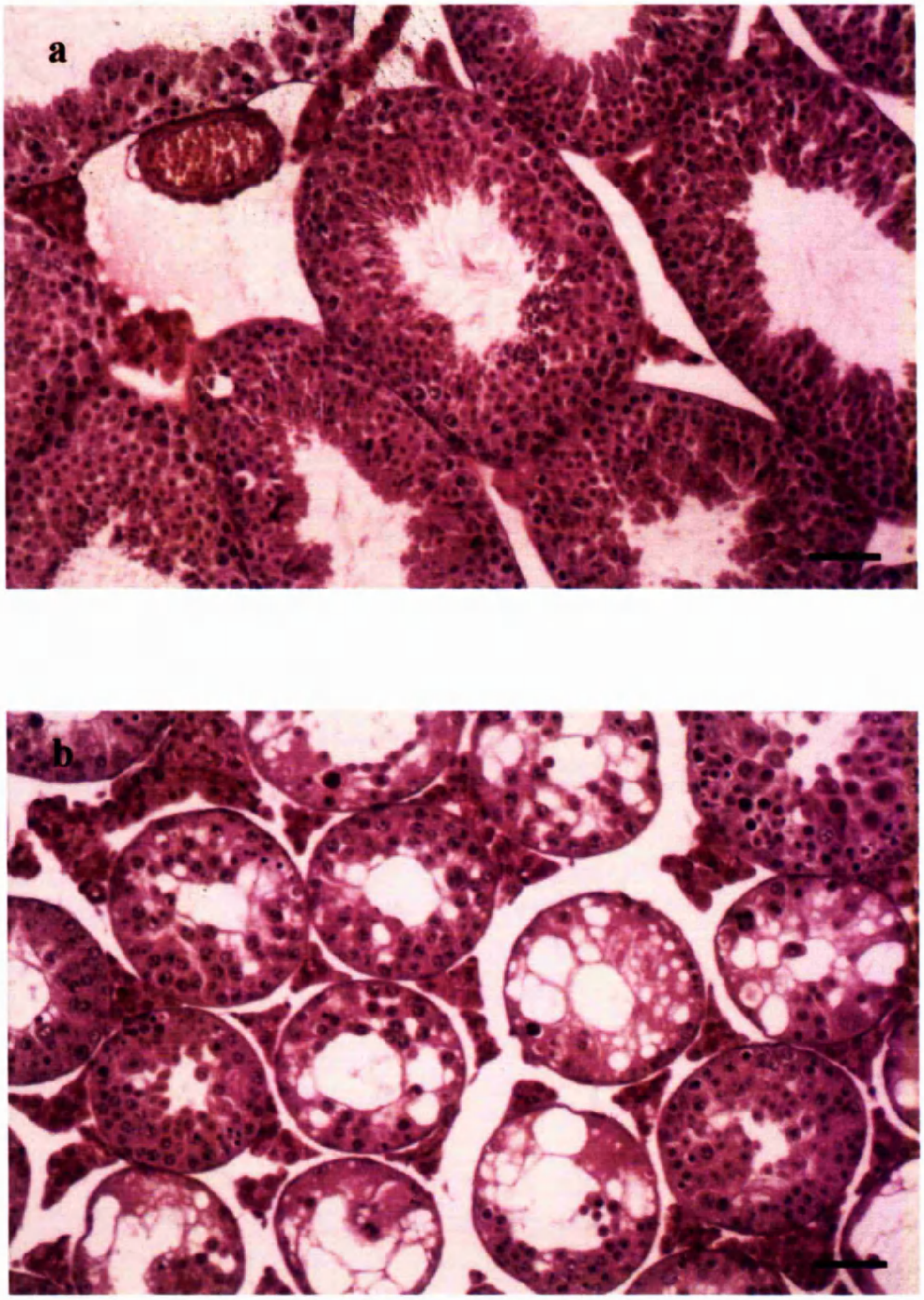


Figure 7.2.2.3 *: Comparison of testicular histology from Bax knockout and wild-type mice. Panel a - testis from a wild-type control animal. Panel b - testis from a Bax knockout animal. Magnification x200. Scale Bar = 50μm.*

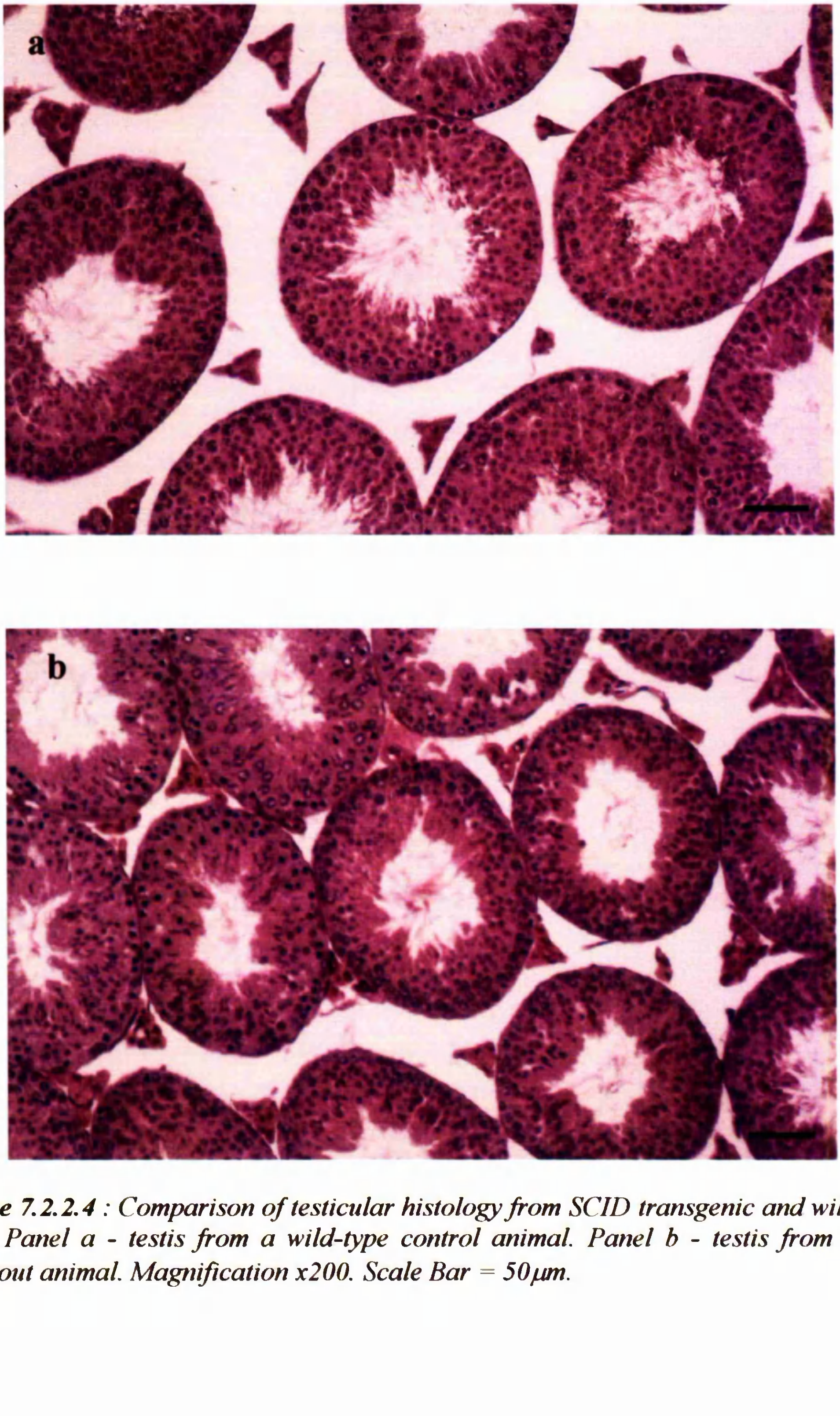


***Figure 7.2.2.4** : Comparison of testicular histology from SCID transgenic and wild-type mice. Panel a - testis from a wild-type control animal. Panel b - testis from a p53 knockout animal. Magnification x200. Scale Bar = 50µm.*

7.2.3 Neutral Comet Assay of Sperm from Knockout and Wild-Type Animals.

Due to the absence of spermatozoa in Bax knockout mice, Bax$^{-/-}$ and Bax$^{+/+}$ wild-types were not analysed by sperm comet assay. Spermatozoa collected from the vas deferens of other knockout strains and their corresponding wild-types were processed by neutral comet assay and then comet tail length, tail moments and % tail DNA compared between knockouts and wild-types to determine whether any differences in background levels of DNA damage in spermatozoa could be detected by comet assay.

No significant differences were found for all comet parameters (tail length, tail moment and % tail DNA) between knockout mice and their corresponding wild types after neutral comet assay of spermatozoa (Table 7.2.3.1).

***Table 7.2.3.1** : Comparison of transgenic mice spermatozoal DNA damage by neutral comet assay.*

	Comet Parameters		
Transgenic Mouse Strain	***Tail Length***	***Tail Moment***	***% Tail DNA***
p53$^{+/+}$	38.7 ± 0.6	2.3 ± 0.1	8.1 ± 0.3
p53$^{-/-}$	41.4 ± 1.0	2.3 ± 0.1	8.0 ± 0.4
Bcl-2$^{+/+}$	44.1 ± 0.6	2.7 ± 0.3	8.9 ± 0.8
Bcl-2$^{-/-}$	48.7 ± 1.8	2.7 ± 0.3	9.6 ± 0.8
Bax$^{+/+}$	—[a]	—[a]	—[a]
Bax$^{-/-}$	—[a]	—[a]	—[a]
SCID Control	44.9 ± 0.7	2.8 ± 0.3	10.1 ± 0.7
SCID	46.3 ± 1.2	2.9 ± 0.2	9.6 ± 0.5

[a] Due to the infertile nature of Bax$^{-/-}$ mice and lack of vas deferens spermatozoa, Bax$^{+/+}$ and Bax$^{-/-}$ were not analysed by comet assay.

Data shown represents the group mean ± S.E.M (n=4).

Data analysed and compared using Kruscal-Wallis one way analysis of variance and Mann Whitney U test.

7.2.4 Alkaline Comet Assay of Sperm from Knockout and Wild-Type Animals.

As with previous studies on alkaline electrophoresis of spermatozoa (Chapters 3 and 4 of this thesis) electrophoresis of sperm from knockouts and wild types under alkaline conditions resulted in the migration of almost all the DNA from the nucleus. The image analysis system had difficulty in identifying separate head and tail regions of the comets due to the very small origin of migration and diffuse nature of the comet fluorescence. Therefore comet tail length was the only parameter measured. Since most of the DNA has migrated from the nucleus and this has previously been shown to remain constant in spermatozoa processed by alkaline assay (Chapter 3) it can be assumed that tail moment values will simply reflect tail length effects since % tail DNA will remain constant.

Interestingly despite the lack of effects observed after neutral comet assay of sperm from knockouts and their wild-types there appeared to be a difference after alkaline comet assay at least between sperm from p53 knockouts and their wild-types (Figures 7.2.4.1 and 7.2.4.2). Alkaline comets from p53 knockouts had a considerably shorter tail length than comets produced by spermatozoa from p53 wild-type counterparts. Since DNA migration is proportional to the extent of DNA damage this would appear to indicate that p53 knockout animals have lower levels of endogenous DNA damage in their spermatozoa than wild-type animals. No differences were recorded in comet tail lengths between Bcl-2 knockouts and their wild-types (Figure 7.2.4.3) and also SCID mice and their wild-type counterparts (Figure 7.2.4.4).

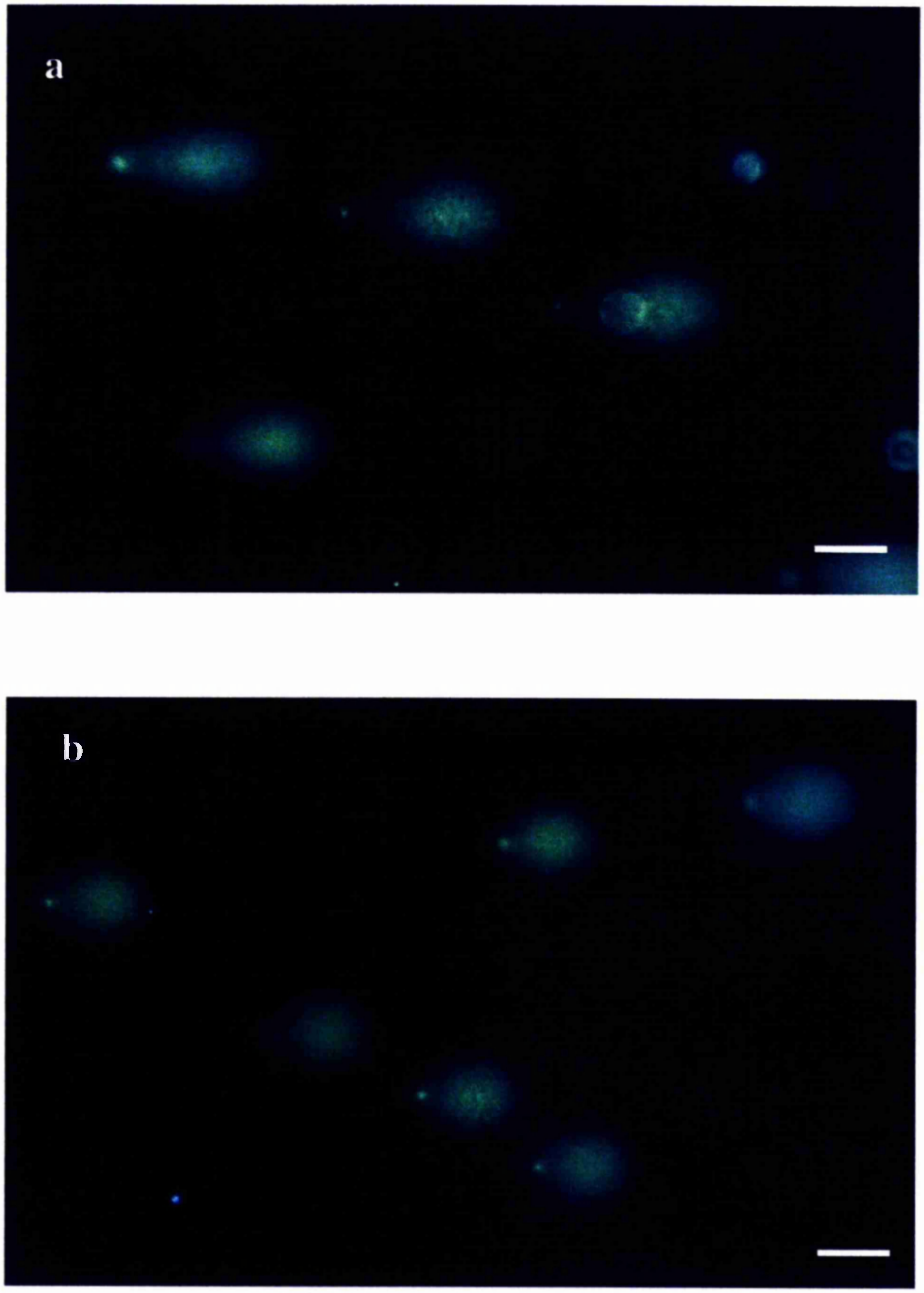


Figure 7.2.4.1 *: Comparison of comets from p53 knockout and wild-type mice after alkaline comet assay of spermatozoa. Magnification x240. Scale Bar = 50μm*

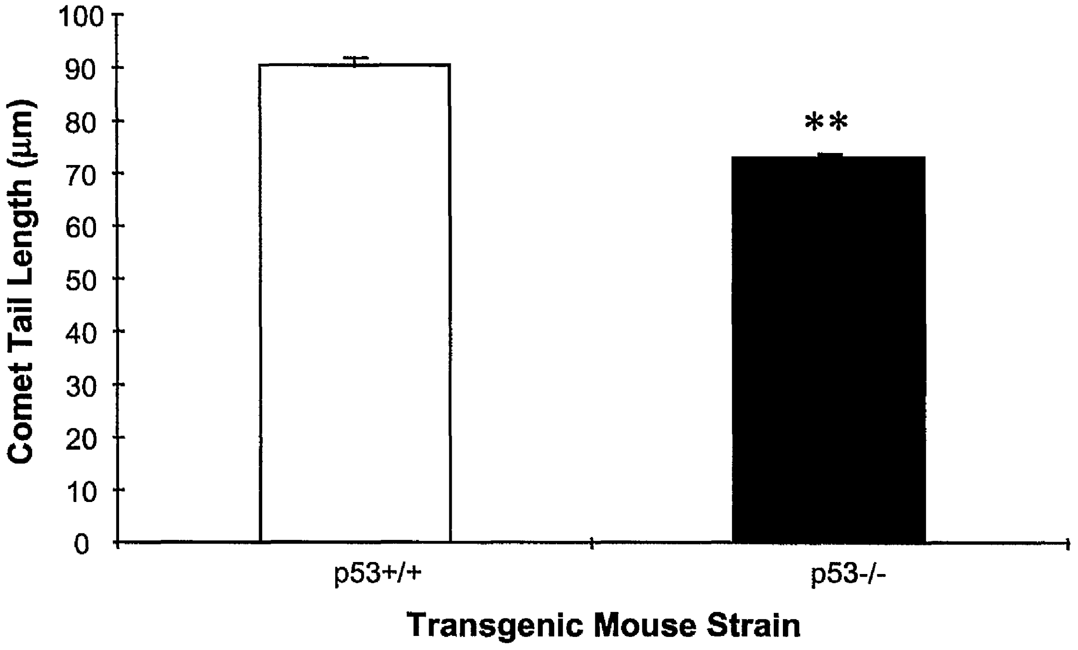


***Figure** 7.2.4.2 : Comparison of spermatozoa from p53 knockout and wild-type mice by alkaline comet assay. Data shown represents the group mean ± S.E.M (n=4). Asterisks denote values significantly different than wild-type controls. * = P <0.5, ** = P <0.01 (Mann Whitney U test).*

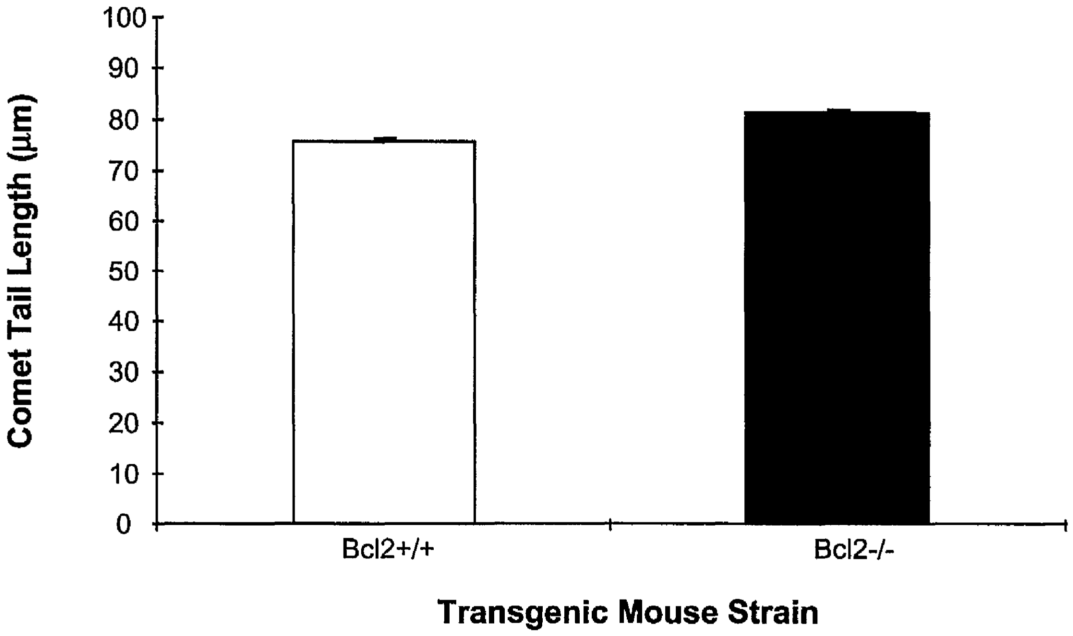


***Figure** 7.2.4.3 : Comparison of spermatozoa from Bcl-2 knockout and wild-type mice by alkaline comet assay. Data shown represents the group mean ± S.E.M (n=4). Data analysed and compared using Mann Whitney U test.*

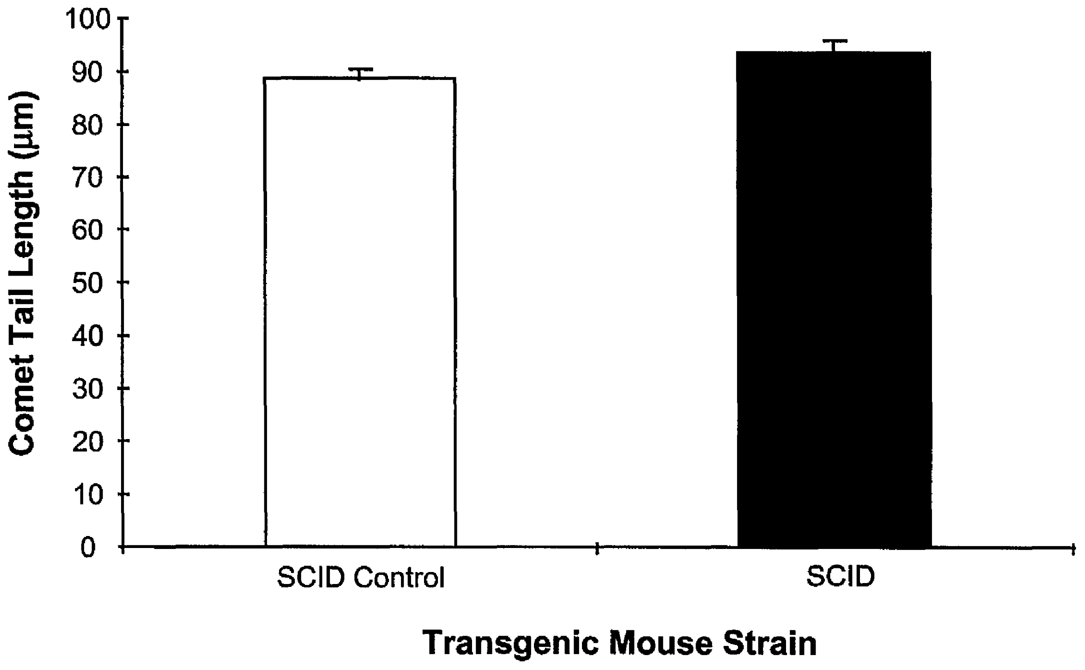


***Figure** 7.2.4.4 : Comparison of spermatozoa from SCID transgenic and wild-type mice by alkaline comet assay. Data shown represents the group mean ± S.E.M (n=4). Data analysed and compared using Mann Whitney U test.*

7.2.5 Testicular Apoptosis Identified by TUNEL labelling of Testis Sections.

Apoptosis in the testis sections of transgenic mice was quantified using the technique of TUNEL labelling. Apoptotic cells were identified by the presence of brown staining.

Apoptotic cells could be identified in testis sections from all animals, both knockout and wild-type. In wild-type animals, approximately 20% of seminiferous tubules contained TUNEL labelled cells indicating that apoptosis occurs normally during spermatogenesis (Table 7.2.5.1). Most TUNEL labelled cells were found towards the base of the tubules, typical of spermatogonia. No significant differences were observed in the percentage of tubules containing apoptotic cells between p53, Bcl-2, SCID knockouts and their corresponding wild-type counterparts (Table 7.2.5.1). There were also no significant differences in the number of apoptotic cells in positively stained tubules between knockout and wild-types from the above strains with between 2 and 3 apoptotic cells

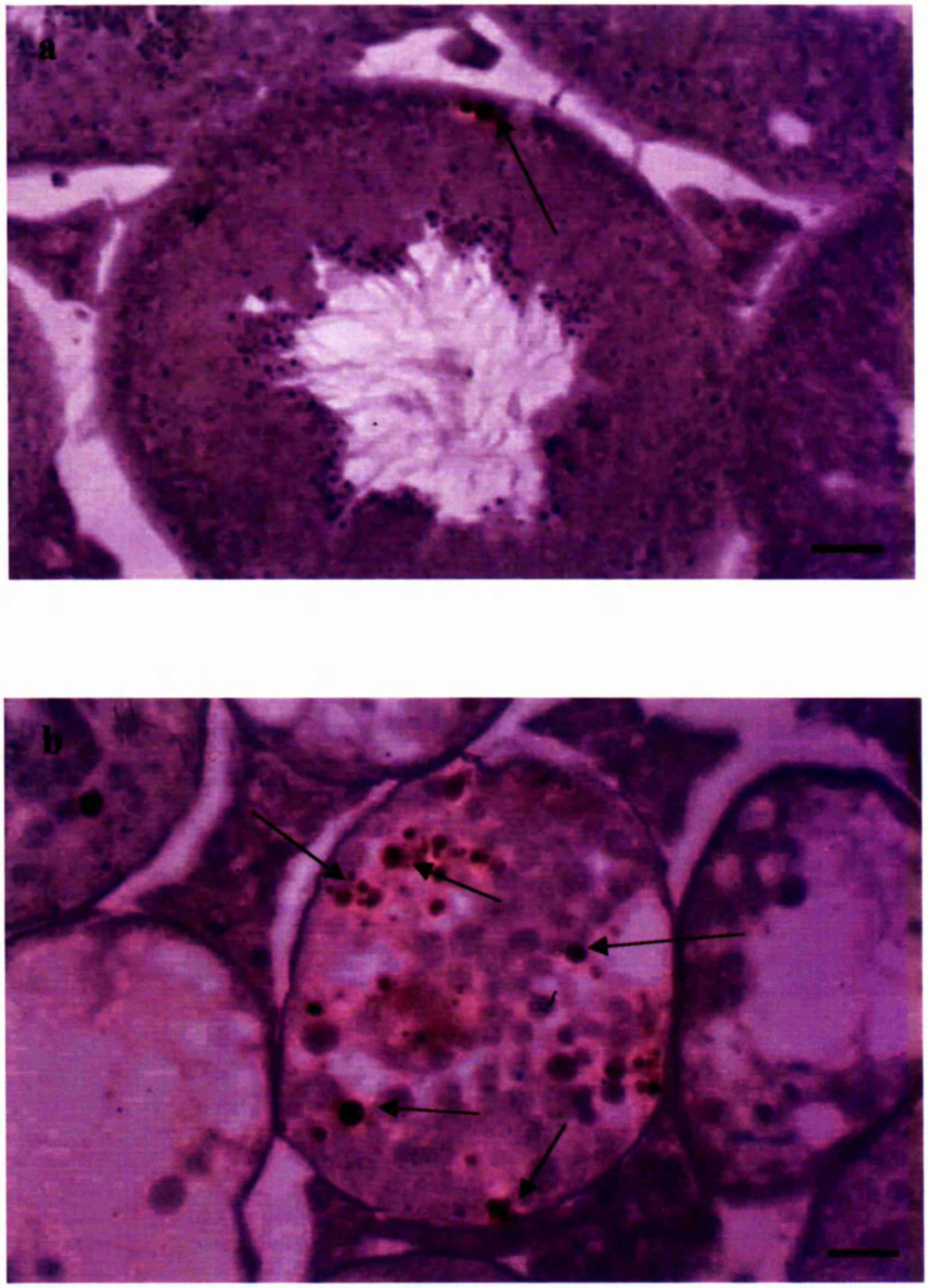


***Figure** 7.2.5.1 : Comparison of testicular TUNEL labelling from Bax knockout and wild-type mice. Panel a - testis section from wild-type control animal. Panel b- testis section from Bax knockout mouse. Arrows indicate the presence of apoptotic cells as identified by ISEL. Magnification x400. Scale Bar = 25μm.*

Table 7.2.5.1 *: Comparison of testicular apoptosis in transgenic mice assessed by TUNEL assay.*

Transgenic Mouse Strain	**Percentage of Tubules containing Apoptotic cells**	**Mean number of cells in apoptotic tubules**
$p53^{+/+}$	18.8 ± 1.8	2.4 ± 0.5
$p53^{-/-}$	19.7 ± 1.4	3.0 ± 0.6
$Bcl\text{-}2^{+/+}$	20.2 ± 2.3	2.8 ± 0.5
$Bcl\text{-}2^{-/-}$	18.4 ± 1.6	2.2 ± 0.5
$Bax^{+/+}$	17.8 ± 1.9	3.1 ± 0.4
$Bax^{-/-}$	65.2 ± 1.6 **	5.2 ± 0.8**
SCID Control	18.7 ± 1.4	2.1 ± 0.4
SCID	19.2 ± 2.7	2.6 ± 0.3

Data shown represents the group mean ± S.E.M (n=4).
Asterisks denote values significantly different than wild-type controls. * = $P < 0.05$, ** = $P < 0.01$ (Kruscal-Wallis one way ANOVA and Mann Whitney U-test).

found per tubule. In contrast, testis sections from Bax knockout animals showed considerably higher levels of apoptosis than Bax wild-type animals (Table 7.2.5.1 & Figure 7.2.5.1). In control, Bax wild-type testis sections 17.8% ± 1.9 of seminiferous tubules contained apoptotic cells. However in testis sections from knockout animals 65.2% ± 1.6 contained TUNEL labelled cells. Seminiferous tubules from bax knockout animals also contained significantly higher numbers of apoptotic cells (5.2 ± 0.8 per tubule) than wild-type counterparts (3.1 ± 0.4 per tubule.)

7.3 DISCUSSION.

Apoptosis occurs as a normal physiological process within the testes (Bartke, 1995; Woolveridge & Morris, 1999). It is possible that since spermatozoa themselves do not possess DNA repair mechanisms (Ono & Okada, 1977), germ cells containing genetic damage may be eliminated by apoptosis before they differentiate into spermatozoa. Recently some of the molecular mechanisms controlling the regulation of apoptosis have been identified (White, 1996). A number of these gene products have been found to be expressed within the testis (reviewed by Woolveridge & Morris, 1999) and more recently knockout mice models have been created which lack specific genes involved in apoptosis regulation (Donehower *et al.,* 1992; Nakayama *et al.,* 1993; Knudson *et al.,* 1995; Ross *et al.,* 1998). Some of these knockout mice have been shown to have altered testicular histology (Rotter *et al.,* 1993; Knudson *et al.,* 1995; Ross *et al.,* 1998). Therefore the aim of the experiments in this chapter was to compare spermatogenesis and background levels of DNA damage in spermatozoa between knockout mice and their wild-type counterparts using the comet assay. It was hypothesised that knockout mice lacking in vital gene products controlling apoptosis may possess higher levels of DNA damage in their spermatozoa since elimination of DNA damaged germ cells may not take place.

7.3.1 Bcl-2 Knockouts.

In this study Bcl-2 knockout were significantly smaller and displayed lower body weights than age-matched wild-type controls. This is similar to observations from previous studies which indicate that homozygous Bcl-2 knockout mice appear to develop normally until birth, but after this retardation of growth can be detected by 10 weeks of age (Nakayama *et al.,* 1993; Kamada *et al.,* 1995). Bcl-2 knockout mice have also been shown to have spleens and thymuses of drastically reduced size compared to wild-types and this may partially contribute to the lower body weights of knockout animals. The exact reason for the growth retardation of Bcl-2 knockout mice is not ascertained. However, it has been shown that certain cell types (e.g. thymocytes, lymphocytes and melanocytes) have a reduced life span (Kamada *et al.,* 1995).

Therefore if cell types within the mouse have a reduced lifespan this may impair development and delay normal growth of the mouse. Testes from Bcl-2 knockout mice were significantly smaller than those of wild-type counterparts. This is unsurprising given the smaller size of the knockout animals. Histological examination of testis sections from Bcl-$2^{-/-}$ mice suggested although tubular size was reduced, spermatogenesis was unimpaired by lack of Bcl-2. There was no evidence of increased apoptosis in testis sections from Bcl-2 knockout animals. Previous studies have shown increased apoptosis of lymphoid cells from Bcl-2 knockouts (Nakayama *et al.,* 1993) but there was no evidence of increased levels of apoptotic cells in both H&E stained testes section and TUNEL labelled testes sections from Bcl-2 knockout animals. Comet analysis of spermatozoa by both alkaline and neutral methods suggested that there were no differences in the levels of endogenous DNA strand breaks (both single- and double-) between Bcl-2 knockouts and wild-type mice. Bcl-2 is generally thought of as a cell survival factor because of its ability to inhibit apoptosis (White, 1996). Indeed Bcl-2 overexpression in many cell types leads to an increased resistance to apoptosis induced by many agents (Korsmeyer, 1992; Reed, 1994).

In the testis, overexpression of Bcl-2 produces abnormal spermatogenesis and sterility (Rodriguez *et al.,* 1997). This is due to the accumulation of spermatogonial cells, which fail to undergo apoptosis during the first round of spermatogenesis where a wave of apoptosis that is essential for subsequent rounds of spermatogenesis takes place. Therefore in this case Bcl-2 fulfils its role as a cell survival factor by preventing apoptosis of spermatogonial cells. The physiological role of Bcl-2 in the testis however is unclear. Some studies suggest that Bcl-2 is not expressed in the testis (Hockenbery *et al.,* 1991; Knudson *et al.,* 1995; Furuchi *et al.,* 1996; Rodriguez *et al.,* 1997) whilst other studies have only found it present at low levels (Krajewski *et al.,* 1995; Woolveridge *et al.,* 1998a). Therefore given the lack of evidence for Bcl-2 in the normal testis then it may be unsurprising that Bcl-2 knockout mice have normal testes and spermatogenesis. Alternatively, it is known that Bcl-2 is a member of a large family of gene products (White, 1996) and other related proteins that are pro-survival such as Bcl-x and Bcl-w may regulate apoptosis in spermatogenesis instead of Bcl-2 or may be able to assume its role in its absence.

7.3.2 Bax Knockouts.

Bax knockout mice showed decreased testis weights compared to wild-type controls and abnormal testicular histology. Again this is consistent with a previously reported study (Knudson *et al.,* 1995). Histological sections from Bax knockout testes demonstrated a lack of spermatids and spermatozoa in the tubules and also vas deferens from bax knockout mice did not contain spermatozoa which is consistent with a failure of spermatogenesis and lack of spermatozoa production. Spermatozoa account for the majority of the weight of the testes (Russell *et al.,* 1990) so it is not surprising therefore that testes from bax knockout mice weighed considerably less than wild type counterparts. It is also evident from testicular sections that tubular size was reduced in bax knockout animals. Some tubules in bax knockout animals were devoid of germ cells indicative of a complete failure of spermatogenesis and elimination of germ cells. Other tubules showed signs of germ cell accumulation and the presence of giant cells. This appears to indicate that bax knockout mice are unable to undergo meiosis and spermiogenesis.

Previous studies which have analysed the distribution of testicular cells from bax knockout testes have suggested that there is an increase in the number of 2N cells which may be due to the apparent accumulation of premeiotic cells (Knudson *et al.,* 1995). A small amount of 4N and 1N cells were present suggesting that a small proportion of cells may be able to undergo meiosis and form round spermatids although their appears to be no progression from the round spermatid stage. However this study could not find the 1N peak corresponding to the elongating spermatids and spermatozoa which is consistent with the histology which suggests a lack of these cells types. As well as the abnormal testicular histology, when bax knockout testis sections were labelled by TUNEL assay it was apparent that there was a higher frequency of apoptotic cells in the tubules of knockout animals compared to wild-types. Bax is regarded as a promoter of apoptosis (White, 1996). It is able to heterodimerize with Bcl-2 or Bcl-x (Oltvai *et al.,* 1993; Boise *et al.,* 1993) and it is thought that the ratio between hetero- and homodimers may regulate the death pathway. Overexpression of bax in cultured cell lines promotes death by apoptosis (Oltvai *et al.,* 1993). Therefore since the action of bax is pro-apoptotic it is surprising that testes of bax knockout mice display high levels of apoptosis. This would suggest that bax is required to block apoptosis in the testis suggesting an anti-apoptotic role for bax in contrast to its role in other tissues. The histological appearance of the testes from bax knockout animals are very similar to testes of animals in which bcl-2 or bcl-x has been overexpressed in germ cells (Furuchi *et al.,* 1996; Rodriguez *et al.,* 1997). Overexpression of bcl-2 or bcl-x prevents a wave of apoptosis during the first round of spermatogenesis required for future development. However studies have also suggested that in normal animals this physiological wave of apoptosis is coincident in timing and localisation with temporary high expression of Bax protein (Rodriguez *et al.,* 1997). Therefore bax may play a critical role in the testis by promoting apoptosis of cells during the first round of spermatogenesis which is required for further development of the testis. Since either removal of bax or overexpression of Bcl-2 or Bcl-x will produce the same result (i.e. prevention of the wave of apoptosis) then it is not surprising that the testes of these different transgenic animals show a similar phenotype. Also their are other pathways by which apoptosis can be regulated (White, 1996) and these may account for the increased levels of apoptosis in the bax knockout testes. Alternatively, removal of bax may upset the ratio of Bcl-2 family homo- and heterodimers resulting in increased cell death (Knudson *et al.,* 1995). This would retain the role of bax as exclusively a promoter of death, consistent with its effects in other tissues.

7.3.3 p53 Knockouts.

No differences in body weights, testis weights and sperm counts were observed between p53 knockouts and their corresponding wild-types. Testicular histology of knockout and wild-type animals also appeared similar with no evidence of abnormal spermatogenesis in p53 knockouts. Previous studies have suggested that some p53 knockout mice and p53-CAT Transgenic mice which have reduced levels of testicular p53 show abnormal spermatogenesis with the presence of giant cells within the seminiferous tubules (Rotter *et al.,* 1993). In this study no evidence of altered spermatogenesis or giant cells was found in testicular sections from transgenic mice. The reason for the discrepancy between these findings and earlier reported studies may be explained by differences in the genetic background of the knockout mice strains. p53 homozygous knockout mice derived from a 129 genetic background have been reported to display testicular giant cell degenerative syndrome (Rotter *et al.,* 1993). However, the mice used in this study were derived from a C57BL/6 background. In the previous study mice of a mixed genetic background C57BL/6 x 129 exhibited normal structure of the seminiferous tubules. More recently another study has been published suggesting that p53$^{-/-}$ knockouts derived from a pure C57BL/6 background also display normal spermatogenesis (Beumer *et al.,* 1998) in agreement with the results from this study. Therefore it appears that abnormal effects of p53 loss on spermatogenesis may only be restricted to certain strains of knockout mice. Although spermatogenesis functions normally in C57BL/6 knockout mice it has been reported that p53 knockout mice contain 40-50% more type A spermatogonia in stage VIII seminiferous tubules than wild-type counterparts (Beumer *et al.,* 1998). Detailed analyses and cell counts were not performed on testicular sections from knockout animals, and since it has been suggested that despite the increase in type A spermatogonia there is no resulting increase in preleptotene spermatocytes and that these observations can only be assessed in stage VIII tubules it is quite possible that the subtle abnormalities in spermatogenesis may have been overlooked in the present study. However if numbers of type A spermatogonia are increased in p53 knockouts but do not result in increased numbers of preleptotene spermatocytes then these cells must be lost during the developmental process. Since apoptosis has been shown to occur in developing spermatogonia and TUNEL positive cells were observed in knockout animals in this study and others (Beumer *et al.,* 1998; Hasegawa *et al.,* 1998) it seems probable that these excess cells would be eliminated by apoptosis during spermatogenesis. However, increased levels of apoptosis were not observed in p53 knockout animals compared to wild-type counterparts (Beumer *et al.,* 1998).

Again since type A spermatogonia are present in stage VIII tubules it is possible that increased apoptosis in these stage tubules may have been overlooked by this study which examined random seminiferous tubules within the testes. If apoptosis in stage VIII tubules of knockout and wild-type mice were compared then they may well show increased levels of spermatogonial apoptosis of p53 knockout mice. Some studies have reported that p53 is present only in pachytene spermatocytes in the testis (Schwartz *et al.,* 1993; Sjoblom & Lahdetie, 1996). However recent studies have shown that p53 is expressed in spermatogonia following irradiation and that apoptosis of spermatogonia is p53-dependent (Beumer *et al.,* 1998; Hasegawa *et al.,* 1998). If apoptosis of spermatogonia is p53-dependent then this would explain observations of increased numbers of type A spermatogonia in some studies with p53 knockout mice. However since apoptosis has been observed in the testes of knockout mice in this study and following irradiation it is clear that p53-independent apoptotic pathways do exist in the testis.

Analysis of sperm DNA damage by neutral comet assay found that there was no difference in comet tail length, tail moment or %tail DNA between knockout and wild-type animals. This suggests that p53 deficiency does not result in the production of spermatozoa with elevated levels of DNA damage. In contrast to the result produced by neutral comet assay of spermatozoa, when sperm from knockouts and wild types were electrophoresed under alkaline conditions, differences were detected between sperm from knockout and wild-type animals. Our initial hypothesis had been that since p53 is involved in the sensing of DNA damage and apoptosis, deficiency might cause knockout animals to produce spermatozoa with elevated levels of DNA damage. However, in contrast to this hypothesis, alkaline comets produced from knockout mice were actually smaller than comet produced by sperm from wild-type animals. This actually suggests that the knockout animals have lower levels of DNA strand breaks than their wild-type counterparts. As with previous experiment in this thesis exposure and electrophoresis of sperm under alkaline conditions results in the migration of >90% of the DNA from the nucleus. This may be because spermatozoa may have high numbers of alkali labile sites within their DNA because of the nature of their nuclear DNA structure (Singh *et al.,* 1989). Therefore it is possible that the difference observed between p53 knockout and wild-type sperm may be due to differences in the numbers of alkali labile sites and this may reflect differences in DNA packaging and structure.

P53 has been suggested to play and important role in meiosis where it may halt the cell cycle in order to allow chromosome rearranging and rejoining to take place (Schwartz *et al.,* 1993; Almon *et al.,* 1993; Sjoblom & Lahdetie, 1996). In knockout animals, this process may not be able to take place properly resulting in lack of rearrangement of the DNA. Subsequently during spermiogenesis, replacement of histones by protamines and DNA packaging may not be able to take place as effectively as in wild-type animals and therefore this may account for the difference observed in the alkaline comet assay between knockouts and wild-types. Further work is required to determine the exact reason for this difference. Other techniques such as SCSA and minisatellites would confirm whether there is a difference in endogenous DNA damage levels between p53 knockouts and wild-types.

7.3.4 SCID Mice.

No differences in any physical parameters (body weight, testis weights, sperm counts and testicular histology) were observed between SCID knockout mice and their wild type counterparts. This is in agreement with previous reports in the literature that suggest their are no obvious differences between SCID and wild-type mice apart from the reduced size of the lymph nodes and thymus which is as a result of the lack of development of T and B lymphocytes in SCID animals (Bosma & Carrol, 1991; Vladutiu, 1993). Occasionally some SCID mice show an enlarged spleen (Vladutiu, 1993). A previous study has also demonstrated that spermatogenesis is normal in SCID mice (Van Buul *et al.,* 1995) in agreement with the observations reported in this chapter. Despite apparent normal spermatogenesis, testicular cells from SCID mice are reported to be more radiosensitive than those of wild-type counterparts (Van Buul *et al.,* 1995). An increased radiosensitivity of SCID cells has also been reported for bone marrow cells (Disney *et al.,* 1992), kidney fibroblasts (Fulop & Phillips, 1990) and SCID derived tumour cells (Biedermann *et al.,* 1991). It is thought that the increased radiosensitivity of SCID mice may be due to a deficiency in DNA double strand breaks repair (Fulop & Phillips, 1990; Disney *et al.,* 1992). SCID cells have been shown to be hypersensitive to DNA dsb-inducing agents such as bleomycin and restriction enzymes (Biedermann *et al.,* 1991; Chang *et al.,* 1993; Tanaka *et al.,* 1993). However scid cells show comparable sensitivity to wild type cells for agents causing other types of DNA lesions such as U.V irradiation and MMS (Biedermann *et al.,* 1991; Tanaka *et al.,* 1993). Since spermatogenesis involves many complex rearrangements and rejoining of chromosomes and DNA it might have been expected that these steps in spermatogenesis may have been affected leading abnormal spermatogenesis. An inability to repair double-strand breaks may result in higher number of germ cells with genetic damage, which may be eliminated, from the testis by apoptosis to protect spermatozoal integrity.

However, comparable levels of apoptosis were observed in testis sections from SCID and wild-type mice. There was also no evidence of increased DNA damage in the spermatozoa of SCID mice analysed by comet assay compared to wild-type controls. The absence of DNA dsb repair and lack of effects of the SCID mutation on spermatogenesis may suggest i) that the repair pathway affected by scid mutation does not play a role in DNA rejoining during spermatogenesis or ii) that other DNA repair mechanisms are able to assume the same role in its absence. Differences between SCID and wild-type testes may however be detectable if the same study was performed after irradiation of SCID and wild-type animals.

Overall, the experiments described in this chapter have examined spermatogenesis and spermatozoal DNA integrity in transgenic animals lacking genes involved in the regulation of apoptosis, DNA damage and repair. Although differences have been observed in spermatogenesis (notably bax knockout mice) no differences were observed in DNA damage levels in spermatozoa after comet assay. Whilst this may suggest that there are no differences in DNA damage levels in sperm, it may reflect that the comet technique is not sensitive enough to detect small differences in endogenous DNA damage levels between knockouts and wild-types. Perhaps other techniques, which are able to examine DNA integrity (e.g. SCSA, TUNEL and minisatellites), may be more sensitive and able to detect subtle differences. Alternatively, in previous chapter it has been demonstrated how the comet assay is able to detect radiation-induced DNA damage in spermatozoa. Irradiation of the testis produces genetic damage in germ cells and this may lead to cell death. As apoptosis and DNA repair occur actively after irradiation of the testis then knockout and wild-type animals may differ in their response to irradiation and perhaps the comet assay could be used to examine differences post-irradiation of the testis and directly in spermatozoa.

Concluding Remarks.

8. CONCLUDING REMARKS.

Reproduction is a process vital to the existence of any species. There has been much speculation and concern about reports that sperm counts in human males have declined over the past 50 years and this may lead to a potential reproductive crisis in the future (Carlsen *et al.,* 1992; Sharpe, 1993). Compared to spermatozoa from other species, the germ cells in the ejaculates from humans are regarded as abnormal. It is widely accepted that "normal" ejaculates may possess up to 50% immotile and 30% morphologically abnormal spermatozoa (World Health Organisation, 1992). Whilst routine semen analysis may examine parameters such as concentration, motility and morphology this gives no indication of the genetic integrity of spermatozoa within the ejaculate.

The direct analysis of spermatozoal DNA integrity has proved difficult. The chromosomes of spermatozoa are not visible until pronuclear formation post-fertilisation in the oocyte (Kamiguchi & Mikamo, 1986; Zirkin *et al.,* 1989) and difficulties in obtaining sufficient human oocytes has placed a severe restraint on human chromosome studies. In 1976 however, it was demonstrated that interspecific *in vitro* fertilisation between human spermatozoa and zona-free hamster oocytes was possible (Yanagimachi *et al.,* 1976) and it was soon demonstrated that this technique could be used to examine human sperm chromosomes (Rudak *et al.,* 1978). Several studies performed to date have analysed the integrity of human sperm chromosomes from men of normal fertility. These studies have suggested that a significant proportion of human spermatozoa (10.4%) are chromosomally abnormal (Martin, 1983; Martin *et al.,* 1987; Brandriff *et al.,* 1985; Kamiguchi & Mikamo, 1986). Structural chromosome aberrations (7.6%) rather than aneuploidy (2.8%) account for the majority of chromosome anomalies found in human spermatozoa. Interestingly the incidence of chromosome abnormalities in human spermatozoa is far greater than that of laboratory animals (mouse 1.5%, chinese hamster 2.1%), (Mikamo *et al.,* 1990). However these cytogenetic techniques are extremely difficult and time-consuming to perform and are therefore unsuitable for use in routine fertility screening. Advances have been made with the advent of FISH for the assessment of aneuploidy directly in human sperm

(Spriggs *et al.,* 1996; Baumgartner *et al.,* 1999) but as described above this only accounts for a small proportion of genetic abnormalities in human spermatozoa. However, more recently there has been an increase in interest and concern over the importance of genetic damage in spermatozoa and its possible biological implications. This may in part be due to the increasing use of ICSI as a technique in assisted conception clinics and worries about sperm selection techniques (Kim *et al.,* 1998). There are also reports that genetic damage may also be a contributory factor in male infertility. Several new techniques have emerged that are currently under development and evaluation, which appear to offer relatively easy and quick determination of the genetic integrity of spermatozoa. These include the TUNEL assay (Sun *et al.,* 1997), sperm chromatin structure assay (SCSA) (Evenson, 1990b) and comet assay (Hughes *et al.,* 1996; Singh & Stephens, 1998). Together studies involving these techniques have suggested that semen samples of poor quality and ejaculates from infertile men may contain increased number of genetically abnormal sperm (Ballachey *et al.,* 1987; Evenson *et al.,* 1991; Hughes *et al.,* 1996; Aravindan *et al.,* 1997; Sun *et al.,* 1997; Lopes *et al.,* 1998). Also it has been suggested that levels of sperm with DNA damage may be correlated with reduced fertility and the production of poorer quality embryos (Ballachey *et al.,* 1987; Evenson *et al.,* 1991; Sun *et al.,* 1997; Lopes *et al.,* 1998). In respect of the concerns over ICSI, a study has suggested that ICSI sperm samples selected under normal criteria for motility and morphology are more likely to contain fragmented DNA (Lopes *et al.,* 1998). These new techniques are relatively quick and much less technically demanding that cytogenetic analysis of sperm chromosomes. Therefore with future development and testing they may offer a practical method for estimating the incidence of DNA damage in individual human ejaculates and may therefore find themselves in routine use in sub-fertility and assisted conception clinics.

The experiments described in this thesis have examined the potential use of the comet assay to detect DNA damage in spermatozoa. It has been demonstrated how the comet assay was able to measure DNA strand breaks in spermatozoa irradiated *in vitro* and after testicular irradiation *in vivo*. Other investigators using the comet assay have also described the measurement of DNA damage induced in spermatozoa by radiation (Hughes *et al.,* 1996, 1997; McKelvey-Martin *et al.,* 1997; Singh & Stephens, 1998) and genotoxic chemicals (Anderson *et al.,* 1997b, 1997d) confirming the potential of the technique for the direct assessment of sperm genetic integrity. Whilst the studies described in this thesis have examined and demonstrated the ability of the comet assay to measure genotoxin-induced damage in spermatozoa, the potential use of the comet assay in the clinic would be in evaluating endogenous levels of DNA damage in human ejaculates. Therefore as the experiments in this thesis were mainly concerned with mouse spermatozoa and the assay conditions were optimised for this species, future experiments should be conducted with human spermatozoa as it may be necessary to optimise the assay conditions because of possible differences in nuclear chromatin packaging (Balhorn, 1982; Perrault, 1997).

Experiments could be performed with ejaculates from men of normal fertility as well as various classes of infertile men and ICSI samples. This would determine the suitability of the comet assay as a clinical technique for the assessment of the genetic integrity of human spermatozoa. As well as the comet assay, it would be useful if other techniques (TUNEL and SCSA) are also examined alongside each other on the same samples. Whilst each of these three techniques (TUNEL, SCSA and Comet) have been suggested as able to evaluate the genetic integrity of spermatozoa, they all actually measure different parameters. The TUNEL assay measures specific endogenous apoptotic DNA breaks in spermatozoa (Gorczyca *et al.,* 1993c; Sun *et al.,* 1997; Sakkas *et al.,* 1999) whilst the comet assay measures actual strand breaks (single- and double-) and alkali labile sites (Singh *et al.,* 1988; Fairbairn *et al.,* 1995). The SCSA does not actually measure DNA damage itself but examines the nuclear chromatin structure of sperm DNA although there is evidence that DNA damage may alter this structure (Evenson *et al.,* 1993; Evenson & Jost, 1994; Sailer *et al.,* 1995a). Therefore although it is possible that each of these techniques may produce the same outcomes it is also possible that because they measure types of damage they may give differing results and lead to different reproductive outcomes. Therefore further studies should be performed to examine and compare all three techniques on the same sets of samples. One such study has been published which examined spermatozoa from 23 subjects examined by SCSA. When then same samples were also analysed by both TUNEL and comet assay, correlation was found between the results of all three assays (Aravindan *et al.,* 1997). Therefore with further development of the comet assay and other techniques they may have possible clinical application as new tests for the routine analysis of semen.

The quality of embryos produced by IVF treatment is variable. Reports have suggested that less than 50% of embryo produced cleave regularly to produce regularly sized blastomeres (Jurisicova *et al.,* 1995, 1996). Fragmented embryos containing anucleate and/or nucleate fragments are often encountered and it has been reported that these embryos often fail to develop properly *in vitro* and that they commonly arrest during cleavage leading to degeneration of the embryo (Puissant *et al.,* 1987; Jurisicova *et al.,* 1995, 1996; Hardy, 1997). The exact reason for the production of these "poor" quality embryos is unknown although both maternal and paternal factors have been cited (Janny & Menezo, 1994). More recently it has been demonstrated that ejaculates from men with high numbers of sperm with fragmented DNA often leads to the production of poor quality embryos (Sun *et al.,* 1997; Lopes *et al.,* 1998). The results presented in Chapter 5 of this thesis demonstrated that DNA damage induced in spermatozoa by irradiation was correlated to the production of fragmented embryos. Furthermore these embryos often contained apoptotic nuclei which is consistent with previous reports in human embryos (Jurisicova *et al.,* 1995, 1996; Hardy, 1997). At the moment very little is known about the role of apoptosis in the preimplantation embryo although the results from this thesis and from other investigators suggest that it is a common event. One possible role for apoptosis in the early embryo is the removal of damaged blastomeres which is supported by the results reported in Chapter 5. Recently advances have been made in the understanding of the regulation of apoptosis in mammalian cells and identification of several key genes and gene products has taken place (White, 1996). However, despite these advances very little is known about the expression of these genes in the preimplantation embryo and therefore further studies are warranted to examine the expression of apoptotic gene products and the regulation of apoptosis in the preimplantation embryo. This type of research may be able to produce strategies to prevent or reduce embryo fragmentation and death and therefore may lead to the production of higher quality embryos in IVF clinics and subsequent improvements in pregnancy rates.

As well as the embryo, apoptosis is frequently observed in the testis where it may function to regulate germ cell numbers and remove unwanted/damaged cells. Apoptotic gene products have been shown to be expressed in the testis (Woolveridge & Morris, 1999) although the exact regulation of cell death in this tissue is at the moment unclear. However it has recently been suggested that apoptosis may play a role in male infertility with reports that testicular biopsies from men with azoospermia or severe oligospermia contained increased numbers of apoptotic germ cells in comparison to men with normal spermatogenesis (Lin *et al.,* 1997a, 1997b). Several strains of knockout mice lacking important genes involved in the regulation of apoptosis have also been reported to be infertile and show abnormal spermatogenesis (Rotter *et al.,* 1993; Knudson *et al.,* 1995; Furuchi *et al.,* 1996; Rodriguez *et al.,* 1997; Ross *et al.,* 1998). Therefore these reports suggest that further research should be performed to determine the regulation of apoptosis in the testis and to ascertain whether it may be involved in infertility. One function of apoptosis may be to eliminate DNA damaged germ cells from the testis and prevent the formation of spermatozoa from these cells. The studies described in Chapter 7 examined whether knockout mice lacking apoptotic genes contained higher levels of DNA damage levels in their spermatozoa. Although using the comet assay increased levels of DNA strand breaks in knockout animals could not be detected. Further studies should be performed with knockout animals to investigate the role these genes have in protecting the testis from toxic insult and maintaining the genetic integrity of spermatozoa.

As well as a possible role in fertility clinics for the routine analysis of semen, studies in this thesis have demonstrated the ability of the comet assay to detect DNA damage in spermatozoa after exposure to genotoxic agents. Therefore the comet assay may also have another potential application in reproductive genotoxicity testing. Conventional methods in reproductive toxicity testing for the assessment of risk of genotoxicity and teratogenicity are time-consuming and require large numbers of animals. The comet assay offers an additional/alternative method for detecting possible genotoxic risks from exogenous agents. The experiments described in Chapters 3, 4 and 6 of this thesis have demonstrated the ability of the comet assay to detect DNA damage induced by both *in vitro* and *in vivo* irradiation and other studies have used the comet assay to detect damage induced in human spermatozoa after exposure to genotoxic agents *in vitro* (Hughes *et al.,* 1996, 1997; McKelvey-Martin *et al.,* 1997; Anderson *et al.,* 1997a, 1997b, 1997d; Singh & Stephens, 1998). It has also been demonstrated in this thesis how damage to germ cells within the testis can be transmitted throughout the spermatogenic process resulting in the production of sperm with damaged DNA. Further evaluation of the comet assay could be performed through additional animal studies with a variety of know genotoxic agents (e.g. MMS, Bleomycin, Cyclophosphamide etc). These compounds have previously been shown to lead to the death of germ cells within the testis and increases in dominant lethal mutations (Ehling *et al.,* 1968; Ehling, 1980). Therefore, it is possible that the comet assay may have a future role as a genotoxic screen to evaluate new chemicals produced by the pharmaceutical industry.

As well as toxicity screening of chemicals, there is concern that radiotherapy and chemotherapy may have long term effects on reproduction. One common effect of cancer treatment is azoospermia, which may last from a period of months up to years (Ogilvy-Stuart & Shalet, 1993; Meistrich, 1993). In extreme cases recovery of sperm counts is never achieved. Although it has been demonstrated that fertility may recover after cytotoxic therapy and patients can produce offspring, very little is known about the genetic integrity of spermatozoa after such treatments and whether there are any long term genetic effects upon spermatozoa. Cytogenetic studies have demonstrated increased incidences of structural chromosome aberrations in spermatozoa from patients undergoing radiotherapy for Hodgkin's lymphoma (Rousseaux *et al.,* 1993); 24 to 60 months post-radiotherapy in 13 patients with testicular seminoma (Martin *et al.,* 1989) and in spermatozoa from four men who had undergone radio-, chemotherapy or both for various types of cancer 5-18 years earlier (Genesca *et al.,* 1990). These studies therefore appear to indicate that chemotherapy/radiotherapy may produce DNA damage in spermatozoa during treatment. Secondly, the increased incidences of chromosome aberrations in spermatozoa observed months and even years after the cessation of treatment suggest that there may be long term damaging effects upon stem-cell spermatogonia. Experiments with the comet assay described in Chapter 4 have also described chronic effects upon spermatozoa produced by irradiation of sperm cells. Experiments could be performed with the comet assay to assess its potential to monitor DNA damage levels in spermatozoa of patients during and post-therapy. This may have important implications for fertility counselling as it may be practical to advise patients against intercourse during treatment and possibly for a period afterwards to limit the risk of genetic effects to possible conceptuses. Preliminary experiments conducted by our laboratory have demonstrated increases levels of DNA damage in spermatozoa from a patient undergoing fludrabazine treatment for a haematological malignancy (Data not shown).

As well as the risk from radiotherapy/chemotherapy, concern have also been expressed about possible transmittable genetic defects in workers in the nuclear industry and populations exposed as a result of nuclear incidents (e.g. Hiroshima/Nagasaki and Chernobyl). Studies analysing minisatellite mutation rates have demonstrated increased incidences of minisatellite mutations in offspring of parents exposed to radiation at Chernobyl (Dubrova *et al.,* 1996). Similar studies however have failed to demonstrate increased incidence of germline mutations in the offspring of survivors from the World War II Hiroshima/Nagasaki atomic bombings (Kodaira *et al.,* 1995). Studies with the comet assay have demonstrated increased levels of DNA damage in lymphocytes obtained from nuclear workers exposed to chronic low-dose irradiation (Wojewodzka *et al.,* 1998); peripheral blood obtained from Chernobyl liquidators (Plappert *et al.,* 1995) and decreased repair capacity of peripheral lymphocytes from individuals exposed to radiation (Plappert *et al.,* 1997). Therefore these studies suggest that the comet assay may be useful in a biomonitoring capacity to monitor the exposure of individuals to radiation. Additionally, it may be possible to monitor for genetic effects in spermatozoa from nuclear workers. Further experiments are required to investigate this.

In summary, this thesis has examined genetic damage in spermatozoa and its biological consequences. Using a new technique "the sperm comet assay", it has been shown how exposure to radiation can cause DNA damage in spermatozoa and that genetic abnormalities in early germ cells within the testis can be transmitted throughout the spermatogenic process. The exact relationship between the presence of genetic abnormalities in spermatozoa and effects upon fertility and reproductive outcome are at the moment unclear. However, results from this thesis have demonstrated a relationship between the levels of DNA damage in spermatozoa and effects upon morphology and programmed cell death (apoptosis) in the preimplantation mouse embryo. Therefore this suggests that genetic damage in spermatozoa can persist after fertilisation and the developmental potential of the embryo may be related to spermatozoal nuclear integrity. Further research is warranted with reports that DNA damage spermatozoa are relatively frequent in the normal human ejaculate.

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