The Origins of DNA Damage in Mammalian Spermatazoa

Sasha Ann King BSc Hons

Doctor of Philosophy
University of Edinburgh
2003

Declaration

The research described in this thesis, except where reference has been made, is solely the work of the author and has not been submitted for any other degree or professional qualification at the University of Edinburgh or any other educational institution.

Sasha Ann King
July 2003

Acknowledgements

I would like to express my gratitude to my supervisors, Philippa Saunders and Stewart Irvine. Thank you for giving me the opportunity to carry out this work, for your support, words of wisdom and patience throughout.

Many thanks to David Melton and colleagues for supplying the Ercc-1 and Prion mice used in this research.

I would hazard a guess that approximately 90% of the staff at "the unit" have helped me in some way during the course of this project, whether it was practical, emotional or moral support, it was always thoroughly appreciated. I dare not put down specific names for fear of missing anyone out and causing upset. You know who you are and I thank you all.

I doubt that anyone has ever got through a PhD without a huge amount of support, encouragement, love and chocolate cake from their friends and family. I am no exception. They say that you learn who your friends are in hard times – all I can say is that I certainly know mine. My thanks to you all for passing the hankies, pouring the wine, keeping me sane, making me smile, and most importantly, just being there.

Alex, you got me started, and you certainly got me finished.
Big present, big, big, present coming your way.

Publications Arising from this Thesis

Meeting Abstracts

King, S. A.,	11th European Workshop on Molecular and Cellular Endocrinology of the Testis, Saint-Malo, France, May 2000.
King, S. A.,	Society for the study of Fertility, Edinburgh, UK, July 2000.
King, S. A.,	International Society , Montreal, Canada, June 2001.

Research Papers

Hsia, K. T., Millar, M. R., King, S. A., Selfridge, J., Redhead, N. J., Melton, D. W. and Saunders, P. T. K. 2002. DNA repair gene ERCC1 is essential for normal spermatogenesis and oogenesis functional integrity of germ cell DNA in the mouse. *Development*, 2003 Jan; 130(2):369-78.

Abstract

The development of in vitro fertilisation (IVF) and intra-cytoplasmic spermatozoa injection (ICSI) techniques has revolutionised treatment for couples with so-called male-factor infertility. However concern over the integrity of the DNA in spermatozoa used for ICSI has been expressed. Poor semen quality and morphologically abnormal spermatozoa are associated with poor embryonic development following IVF. Furthermore, studies have demonstrated that the transmission of defective DNA (e.g. adducts, gene deletions) from the spermatozoa to the developing embryo can occur and that this may lead to developmental failure of the embryo or future health risks for the child. The aims of this project were; a) to develop a Comet assay for the study of DNA integrity of murine spermatozoa, b) to use this assay to assess DNA integrity in spermatozoa from mice with known sub/infertility and to examine the susceptibility of these spermatozoa to heat-induced DNA damage, c) to identify the stages of spermatogenesis in wild type mice which are susceptible to heat-induced DNA damage, and d) to determine whether this damage is present in the mature spermatozoa developed from heat-treated germ cells.

The single-cell gel-electrophoresis (Comet) assay was originally developed to study DNA damage in somatic cells and has been modified to study both endogenous and induced DNA damage in human ejaculated spermatozoa. DNA packaging and condensation in mouse spermatozoa is not identical to that in the human. The human spermatozoa Comet assay was therefore not suitable for use on murine spermatozoa. A series of modifications were made to the human spermatozoa Comet assay and a usable assay based on a commercially-available Comet Assay Kit (Trevigen) was developed for the study of murine spermatozoa.

The motility, morphology and DNA integrity of motile spermatozoa recovered from the epidiymes of four transgenic lines of mice, all of which suffered from male infertility/subfertility, were examined. Deleted in azoospermia-like autosomal (*Dazla*)-deficient mice (-/-) are infertile, and males fail to produce any mature spermatozoa; heterozygous (+/-) males are fertile but exhibit reduced numbers of

spermatozoa with high incidence of morphological abnormality. Reduced numbers of motile spermatozoa and increased incidence of morphological abnormalities in motile spermatozoa from +/- *Dazla* mice was observed. Compared to wild type (+/+) mice, the level of DNA damage in the spermatozoa of +/- mice was significantly higher. Levels of DNA damage in both +/+ and +/- were increased following *in vitro* heat treatment. Similar findings were also observed in mice with excision repair cross-complementation gene 1 (*ercc-1*) genotypes (+/- and -/-). ERCC1 is involved in the nucleotide excision repair (NER) pathway and mitotic recombination process, and is highly expressed in the testis. Deletion of the Prion protein (PrP) or the PrP-related gene Doppel (PrnD) also resulted in lower numbers of motile spermatozoa with higher levels of DNA damage. However, following *in vitro* heating, levels of DNA damage in the motile spermatozoa from these mice did not increase.

To investigate the effects of heat stress on DNA integrity, wild type mice (*Dazla* +/+) underwent scrotal heating (42°C, 30 min) and were then sacrificed at various time points; 1, 2, 4, 6 or 24 hours (h) or 7, 14, 21, 24, 28, and 32 days (d) after heat stress. Testes and epididymides were removed and fixed for histological analysis, and motile epididymal spermatozoa were retrieved. Altered expression of Cirp (a heat-response protein), heat shock protein 105 (HSP105) and Bax (a pro-apoptotic protein), together with increased numbers of TUNEL-positive cells were detected in testes. Expression of Cirp, Bax and the macrophage marker CD68 were also altered in the epididymis. Levels of DNA damage in motile spermatozoa increased significantly within 1h of heating, reaching a peak at 4h and then recovering to control levels at 7 and 14d. At 21d after heating, DNA damage increased again reaching a second peak at 28d and failing to recover by 32d.

These results indicate that motile spermatozoa in the epididymis are susceptible to heat-induced DNA damage. Furthermore, while DNA integrity of spermatozoa derived from spermatids subjected to heat stress is normal, loss of DNA integrity in pre-meiotic germ cells caused by heat stress is not repaired as these cells mature, resulting in motile spermatozoa with impaired DNA integrity. These findings

suggest that it may be necessary for additional criteria to be taken into consideration when selecting spermatozoa for ICSI/IVF treatment.

Abbreviations

8-OhdG	8-Hydroxydeoxyguanosine
A4	androstenedione
ABC	avidin-biotin complex
AO	acridine orange
AZF	azoospermic factor
BWW	Biggers, Whitten and Whitingham solution
cAMP	cyclic adenosine monophosphate
Cirp	cold-inducible RNA-binding protein
CS-RBD	consensus sequence RNA-binding domain
DAB	3,3'-diaminobenzidine tetrachloride/H_2O_2
DAZ/daz	deleted in azoospermia
DAZL/dazl	deleted in azoospermia autosomal
DHA	dehydroepiandrosterone
DHT	5α -dihydrotestosterone
DNA	deoxyribonucleic acid
Dpl	doppel
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
Ercc-1	excision repair cross-complementing gene
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GS/MS	gas chromatography/mass spectrometry
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSP 105	heat shock protein 105
ICSI	intracytoplasmic spermatozoa injection
ISNT	*in situ* nick translation

IVF	*in vitro* fertilisation
LH	luteinising hormone
NER	nucleotide excision repair
NMA	normal melt agarose
PBS	phosphate –buffered saline
PCR	polymerase chain reaction
PK	proteinase K
PrnD	prion protein-like protein doppel
PrP	prion protein
RNA	ribonucleic acid
ROS	reactive oxygen species
SCGE	single cell gel electrophoresis
SDF	spermatozoa diluting fluid
STF	seminiferous tubule fluid
TBS	tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
TUNEL	TdT-mediated dUTP- nick end labelling assay
WT/wt	wildtype
XPF	*xeroderma pigmentosum* factor

Table of Contents

CHAPTER 1 REVIEW OF THE LITERATURE.....1

1.1 GENERAL INTRODUCTION.....1
1.2 ROLE OF THE TESTIS.....1
1.3 TESTIS.....2
1.3.1 Interstitial compartment.....2
1.3.2 Seminiferous Tubules.....3
1.3.2.1 Somatic cells.....5
1.3.2.2 Germ cells and Spermatogenesis.....6
1.3.2.2.1 Mitosis.....7
1.3.2.2.2 Meiosis.....8
1.3.2.2.3 Spermiogenesis.....9
1.3.2.3 Functional organisation of the seminiferous tubules.....10
1.4 EPIDIDYMIS.....12
1.4.1 Structure.....12
1.4.2 Function.....14
1.4.2.1 Transport.....14
1.4.2.2 Maturation.....15
1.4.2.3 Storage and protection of spermatozoa.....16
1.5 SPERMATOZOA.....17
1.5.1 Structure.....17
1.5.1.1 Head.....18
1.5.1.2 Tail.....20
1.5.2 Function.....21
1.5.3 Susceptibility to insult.....21
1.6 MAINTENANCE OF NORMAL SPERMATOGENESIS/EPIDIDYMAL FUNCTION.....22
1.6.1 Hormonal support.....22
1.6.1.1 Testis.....22
1.6.1.2 Epididymis.....23
1.7 STRESS RESPONSES IN THE TESTIS/EPIDIDYMIS.....24
1.7.1 Apoptosis.....24
1.7.1.1 Genes involved in the regulation of apoptosis.....24
1.7.2 Antioxidant enzymes.....25
1.8 IMPACT OF HEAT STRESS ON TESTICULAR AND EPIDIDYMAL FUNCTION.....26

1.8.1 *Causes of raised scrotal temperature in man* ... 26
1.8.1.1 Clothing ... 27
1.8.1.2 Posture ... 27
1.8.1.3 Occupation ... 27
1.8.1.4 Lifestyle ... 28
1.8.1.5 Medical conditions ... 29
1.8.2 *Animal models of heat stress* ... 31
1.8.3 *Effects of heat stress on testicular function* ... 31
1.8.4 *Effects of heat stress on epididymal function* ... 33
1.8.5 *Effects of heat stress on fertilising ability/embryonic development* ... 34
1.9 MALE INFERTILITY ... 35
1.9.1 *Assisted reproduction techniques (ART)* ... 36
1.9.2 *Selection of spermatozoa for ART* ... 36
1.10 AIMS OF THE PROJECT ... 38

CHAPTER 2 MATERIALS AND METHODS ... 39

2.1 ANIMALS AND ANIMAL WELFARE ... 39
2.1.1 *Dazl mice* ... 39
2.1.2 *Ercc-1* ... 39
2.1.3 *Prion mice* ... 40
2.1.4 *Animal accommodation* ... 40
2.1.5 *Animal genotyping* ... 41
2.1.5.1 Dazl mice ... 41
2.1.5.2 Ercc-1 mice ... 42
2.1.5.3 Prion mice ... 42
2.1.6 *Sacrifice of animals* ... 43
2.2 TREATMENTS ... 43
2.2.1 *Anaesthetic* ... 43
2.2.2 *Testicular heating method* ... 43
2.3 METHODS FOR TISSUE FIXATION, PROCESSING AND STAINING ... 45
2.3.1 *Tissue fixation for paraffin embedding* ... 45
2.3.2 *Tissue processing and sectioning of paraffin blocks* ... 45
2.3.3 *Haematoxylin and eosin (H&E) staining* ... 45
2.4 IMMUNOHISTOCHEMISTRY ... 46
2.4.1 *Immunohistochemistry protocol for paraffin sections* ... 46

2.4.2 *Procedure for detection using fluorescence* 47
2.4.3 *TUNEL* 48
2.5 IMAGE ANALYSIS 49
2.5.1 *Digital photomicroscopy* 49
2.5.2 *Fluorescent photomicroscopy* 49
2.6 METHODS FOR THE PREPARATION OF SPERMATOZOA 49
2.6.1 *Preparation of Biggers, Whitten and Whitingham solution* 49
2.6.2 *Preparation of human spermatozoa (centrifugation)* 49
2.6.3 *Preparation of human spermatozoa (percoll gradient)* 50
2.6.4 *Preparation of murine spermatozoa* 50
2.6.5 *Storage of murine spermatozoa* 50
2.6.6 *Study of concentration of spermatozoa* 51
2.6.7 *Study of morphology of spermatozoa (DiffQuik staining)* 51
2.7 IN VITRO HEATING OF SPERMATOZOA 52
2.8 SINGLE CELL GEL ELECTROPHORESIS (COMET) ASSAY 52
2.8.1 *Comet assay (Kit method)* 52
2.8.2 *Comet analysis* 52

CHAPTER 3 COMET ASSAY DEVELOPMENT 54

3.1 INTRODUCTION 54
3.1.1 *Measures of DNA abnormalities* 55
3.1.1.1 8-Hydroxydeoxyguanosine (8-OhdG) 55
3.1.1.1 Spermatozoa chromatin structure assay (SCSA) 56
3.1.2 *Measures of DNA integrity* 56
3.1.2.1 Enzyme-linked immunosorbent assay (ELISA) 56
3.1.2.2 In situ nick translation (ISNT) 58
3.1.2.3 TdT-mediated dUTP- nick end labelling (TUNEL) assay 58
3.1.2.4 Single cell gel electrophoresis (SCGE or 'Comet') assay. 59
3.2 MATERIALS AND METHODS 60
3.2.1 *Preparation of spermatozoa* 60
3.2.2 *Development of a Comet Assay for murine spermatozoa* 61
3.2.2.1 Modification of the Human Spermatozoa Comet Assay 61
3.2.2.2 Development of novel Comet assay for murine spermatozoa 62
3.2.2.3 Modification of method supplied with a Comet assay kit 63
3.2.3 *Comet analysis* 64

3.2.4 *Requirements of the Comet assay* 65
3.3 RESULTS 66
3.3.1 *Modification of human spermatozoa Comet assay* 66
3.3.1.1 Reproducibility of the assay 67
3.3.2 *Development of a novel Comet assay for study of murine spermatozoa* 68
3.3.2.1 Cumulative cell analysis 72
3.3.2.2 Reproducibility of the assay 75
3.3.3 *Modification of a Comet assay kit for use on murine spermatozoa* 76
3.3.3.1 Reproducibility of the assay 80
3.4 DISCUSSION 81

CHAPTER 4 STUDY OF SPERM FROM MODIFIED MOUSE MODELS 86

4.1 INTRODUCTION 86
4.1.1 *Mouse models for male infertility* 86
4.1.1.1 Deleted in azoospermia (DAZ) and daz-like autosomal (DAZL) 86
4.1.1.2 Excision repair cross-complementing gene 1 (Ercc-1) 87
4.1.1.3 Prion protein (PrP) and Prion protein-like protein Doppel (PrnD) 88
4.1.2 *Aims of this chapter* 89
4.2 METHODS 90
4.2.1 *Genotyping of mice* 90
4.2.2 *Preparation of murine spermatozoa* 90
4.2.3 *Study of concentration of spermatozoa* 90
4.2.4 *Study of morphology of spermatozoa (DiffQuik staining)* 91
4.2.5 *In vitro heating of spermatozoa* 91
4.2.6 *Comet assay* 91
4.2.7 *Comet analysis* 92
4.2.8 *Statistical Analysis* 92
4.3 RESULTS 93
4.3.1 *Dazl* 93
4.3.1.1 Spermatozoa counts 93
4.3.1.2 Spermatozoa morphology 94
4.3.1.2.1 Morphologies observed in the spermatozoa of wt mice 94
4.3.1.2.2 Morphologies observed in the spermatozoa of +/- dazl mice 95
4.3.1.3 Endogenous DNA damage 95
4.3.1.4 DNA damage after heating 96

4.3.2 *Ercc-1* 97

4.3.2.1 Spermatozoa counts 97

4.3.2.2 Spermatozoa morphology 98

4.3.2.2.1 Morphologies observed in the spermatozoa of 129wt mice. 98

4.3.2.2.2 Morphologies observed in the spermatozoa of Ercc-1 -/- mice. 99

4.3.2.3 Endogenous DNA damage 100

4.3.2.4 DNA damage after heating 101

4.3.3 *PRION* 102

4.3.3.1 Spermatozoa counts 102

4.3.3.2 Spermatozoa morphology 102

4.3.3.2.1 Morphologies observed in the spermatozoa of 129wt mice. 102

4.3.3.2.2 Morphologies observed in the spermatozoa of PrnD -/- mice. 103

4.3.3.2.3 Morphologies observed in the spermatozoa of PrP -/- mice. 104

4.3.3.3 Endogenous DNA damage 104

4.3.3.4 DNA damage after heating 105

4.3.4 *Comparison between all mouse strains* 106

4.4 DISCUSSION 108

CHAPTER 5 THE EFFECTS OF HEAT ON THE TESTIS AND GERM CELLS 114

5.1 INTRODUCTION 114

5.1.1 *Heat stress and the testis* 114

5.1.1.1 Clinical consequences 115

5.1.2 *Aims of this chapter* 115

5.1.3 *Experimental outcomes to be studied* 116

5.1.3.1 Expression of stress markers 116

5.1.3.2 Detection of apoptotic cells 117

5.1.3.3 Study of spermatozoa 117

5.2 MATERIALS AND METHODS 117

5.2.1 *Animals* 117

5.2.2 *Scrotal Heating* 118

5.2.3 *Time-points* 118

5.2.4 *Immunohistochemistry* 119

5.2.4.1 Diaminobenzidine (DAB) staining 119

5.2.4.2 Fluorescent staining 120

5.2.4.3 Detection of apoptotic cells 120

5.2.5 *Preparation of murine spermatozoa* 121
5.2.6 *Study of concentration of spermatozoa* 121
5.2.7 *Comet Analysis of spermatozoa* 122
5.2.8 *Statistical Analysis* 122
5.3 RESULTS 123
5.3.1 *Immunohistochemistry* 123
5.3.1.1 Response of the testis to heat stress 123
5.3.1.2 Stress responses in the testis 126
5.3.2 *Number of Motile Spermatozoa* 130
5.3.3 *DNA integrity of Motile Spermatozoa* 130
5.4 DISCUSSION 132

CHAPTER 6 THE EFFECTS OF HEAT ON THE EPIDIDYMIS AND SPERMATOZOA 139

6.1 INTRODUCTION 139
6.1.1 *Structure and Function of the epididymis* 139
6.1.1 *Storage and protection of spermatozoa* 140
6.1.2 *Heat stress and the epididymis* 140
6.1.2.1 Effects on epididymal function 141
6.1.2.2 Effects on the spermatozoa 141
6.1.3 *Aims of this chapter* 142
6.2 METHODS 142
6.2.1 *Animals* 142
6.2.2 *Scrotal Heating* 142
6.2.3 *Time-points* 142
6.2.4 *Immunohistochemistry* 143
6.2.4.1 Haematoxylin and Eosin Staining (H&E) 143
6.2.4.2 Stress Markers 143
6.2.5 *Preparation of murine spermatozoa* 144
6.2.6 *Study of concentration of spermatozoa* 144
6.2.7 *Comet analysis of spermatozoa* 145
6.2.8 *Statistical Analysis* 146
6.3 RESULTS 146
6.3.1 *H&E Staining* 146
6.3.2 *Immunohistochemistry* 148
6.3.2.1 Cirp 148

6.3.2.2 Bax 151
6.3.2.3 CD68 153
6.3.3 Analysis of motile spermatozoa 156
6.3.3.1 Number of motile spermatozoa 156
6.3.3.2 Levels of DNA damage in motile spermatozoa 157
6.4 DISCUSSION 158

CHAPTER 7 GENERAL DISCUSSION 164

7.1 AIM OF THE WORK 165
7.2 DNA DAMAGE IN THE SPERM OF GENETICALLY MODIFIED MOUSE MODELS 165
7.3 THE EFFECT OF MILD HEAT STRESS ON THE TESTIS AND EPIDIDYMIS 168
7.4 GENERAL CONCLUSIONS 171

BIBLIOGRAPHY 172

Figures And Tables

FIGURE 1.1 COMPARTMENTALISATION OF THE TESTIS 2
FIGURE 1.2 THE ORGANISATION OF THE SEMINIFEROUS EPITHELIUM 4
FIGURE 1.3 SPERMATOGENESIS IN THE MOUSE 7
FIGURE 1.4 THE PROCESS OF SPERMIOGENESIS 9
FIGURE 1.5 SPERMATOGENIC CYCLE OF THE MOUSE 11
FIGURE 1.6 THE MAMMALIAN TESTIS AND EPIDIDYMIS 13
FIGURE 1.7 THE EPIDIDYMAL TRANSIT TIME OF CERTAIN MAMMALS 15
FIGURE 1.8 STRUCTURE OF HUMAN SPERMATOZOA SHOWING MAIN REGIONS 18
FIGURE 1.9 PACKAGING OF DNA IN SOMATIC VERSUS SPERMATOZOAN NUCLEI 19
FIGURE 1.10 DIAGRAM OF THE HEAD OF MURINE SPERMATOZOA 20
FIGURE 1.11 RAISED SCROTAL TEMPERATURES IN MAN 30

FIGURE 2.1 DIAGRAM OF AGAROSE GEL SHOWING LOCATION OF BANDS FOLLOWING GENOTYPING. 42
TABLE 2.1 VOLUME OF ANAESTHETIC GIVEN TO ANIMALS BEFORE TESTICULAR HEATING. 43
FIGURE 2.2 ANAESTHETISED MOUSE SHOWING EXPOSED SCROTUM 44
FIGURE 2.3 POSITION OF MOUSE ON POLYSTYRENE RAFT 44
FIGURE 2.4 POSITION OF MICE ON POLYSTYRENE RAFT IN WATER BATH 44

TABLE 3.1 METHODS USED FOR THE DETECTION OF DNA DAMAGE IN SPERMATOZOA 57
FIGURE 3.1 DIAGRAM OF A COMET INDICATING THE HEAD AND TAIL REGIONS 65
FIGURE 3.2 EXAMPLES OF COMETS INDICATING APPROXIMATE LEVELS OF DNA DAMAGE 65
TABLE 3.2 MODIFICATION OF HUMAN SPERMATOZOA COMET ASSAY - SUMMARY OF RESULTS 67
FIGURES 3.3-3.22. EXAMPLES OF COMETS OBTAINED USING RANGE OF ASSAY CONDITIONS. 69
TABLE 3.3 DEVELOPMENT OF NOVEL COMET ASSAY - SUMMARY OF RESULTS 70
FIGURES 3.23-3.28. EXAMPLES OF COMETS OBTAINED USING A RANGE OF ASSAY CONDITIONS 71
TABLE 3.4. SUMMARY OF RESULTS OBTAINED USING A RANGE OF DTT CONCENTRATIONS 71
TABLE 3.5. EXAMPLE OF CUMULATIVE AVERAGE CALCULATION 72
FIGURE 3.29 EXAMPLE OF A CUMULATIVE CELL ANALYSIS GRAPH 73
FIGURES 3.30-3.34 CUMULATIVE ANALYSIS OF 100 COMETS FROM VARIOUS ASSAY TRIALS 73-74
FIGURES 3.35-3.36 CUMULATIVE ANALYSIS OF 200 COMETS FROM VARIOUS ASSAY TRIALS 73-74
FIGURES 3.37-3.38 EXAMPLES OF COMETS OBTAINED USING OPTIMUM ASSAY CONDITIONS 75
FIGURE 3.39 CUMULATIVE ANALYSIS OF 100 COMETS FOLLOWING 30 MIN LYSIS 77
TABLE 3.6 SUMMARY OF RESULTS OBTAINED USING A RANGE OF LYSIS INCUBATION PERIODS 77

TABLE 3.7 MODIFICATION OF A COMET ASSAY KIT - SUMMARY OF RESULTS 78
FIGURE 3.40 CUMULATIVE ANALYSIS OF 100 COMETS FOLLOWING 60 MIN LYSIS........ 79
FIGURE 3.41 CUMULATIVE ANALYSIS OF 100 COMETS FOLLOWING 120 MIN LYSIS 79
FIGURE 3.42 CUMULATIVE ANALYSIS OF 100 COMETS FOLLOWING 180 MIN LYSIS 79
FIGURE 3.43 REPEATED CUMULATIVE ANALYSIS OF 200 COMETS FOLLOWING 180 MIN LYSIS........ 80
FIGURE 3.44 REPEATED CUMULATIVE ANALYSIS OF 200 COMETS FOLLOWING 180 MIN LYSIS........ 80
TABLE 3.8 SUMMARY OF RESULTS FOR OPTIMIM ASSAY REPRODUCIBILITY........ 80

FIGURE 4.1 SPERMATOZOA CONCENTRATIONS OF *DAZL* GENOTYPES........ 93
FIGURES 4.2 – 4.7 EXAMPLES OF SPERMATOZOA MORPHOLOGIES FROM *DAZL* GENOTYPES 94
FIGURE 4.8 LEVELS OF ENDOGENOUS DNA DAMAGE IN SPERMATOZOA FROM *DAZL* GENOTYPES........ 96
FIGURE 4.9 DNA DAMAGE BEFORE AND AFTER HEATING IN FROM *DAZL* GENOTYPES 97
FIGURE 4.10 SPERMATOZOA CONCENTRATIONS OF *ERCC-1* GENOTYPES........ 98
FIGURES 4.11 – 4.16 EXAMPLES OF SPERMATOZOA MORPHOLOGIES FROM *ERCC-1* GENOTYPES 99
FIGURE 4.17 LEVELS OF ENDOGENOUS DNA DAMAGE IN SPERMATOZOA FROM *ERCC-1* GENOTYPES. 100
FIGURE 4.18 DNA DAMAGE BEFORE AND AFTER HEATING IN SPERMATOZOA *ERCC-1* GENOTYPES 101
FIGURE 4.19 SPERMATOZOA CONCENTRATIONS OF PRION GENOTYPES........ 102
FIGURES 4.20 – 4.28 EXAMPLES OF SPERMATOZOA MORPHOLOGIES FROM PRION GENOTYPES. 103
FIGURE 4.29 LEVELS OF ENDOGENOUS DNA DAMAGE IN SPERMATOZOA FROM PRION GENOTYPES 105
FIGURE 4.30 DNA DAMAGE BEFORE AND AFTER HEATING IN SPERMATOZOA FROM PRION MICE 106
FIGURE 4.31 CHANGES IN DNA DAMAGE FOLLOWING *IN VITRO* HEATING IN ALL GENOTYPES 107

TABLE 5.1 CELL TYPES REPRESENTED BY EPIDIDYMAL SPERMATOZOA AT EACH TIME-POINT AFTER SCROTAL HEATING. 119
FIGURES 5.1-5.8 CIRP EXPRESSION IN THE TESTIS FOLLOWING IN VIVO HEAT TREATMENT. 124
FIGURES 5.9-5.16 HSP105 EXPRESSION IN THE TESTIS FOLLOWING IN VIVO HEAT TREATMENT. 125
TABLE 5.2 EXPRESSION OF CIRP AND HSP105 IN THE TESTIS FOLLOWING SCROTAL HEATING. 126
FIGURE 5.17 AVERAGE NUMBER OF TUNEL +VE CELLS IN THE TESTIS FOLLOWING HEATING 127
FIGURES 5.18-5.25 BAX EXPRESSION IN THE TESTIS FOLLOWING *IN VIVO* HEAT TREATMENT. 128
FIGURES 5.26-5.34 TUNEL +VE CELLS IN THE TESTIS FOLLOWING *IN VIVO* HEAT TREATMENT. 129
FIGURE 5.35 NUMBER OF MOTILE SPERMATOZOA IN THE EPIDIDYMIS AFTER SCROTAL HEATING 130
FIGURE 5.36 LEVELS OF DNA DAMAGE (% TAIL DNA) IN SPERMATOZOA FOLLOWING HEATING........ 131
FIGURE 5.37 DNA DAMAGE (COMET MOMENT) IN MOTILE SPERMATOZOA FOLLOWING HEATING 131
FIGURE 5.38 SUMMARY GRAPH - MOTILE SPERMATOZOA AND DNA DAMAGE FOLLOWING HEATING OF DIFFERENT CELL TYPES PRESENT IN THE TESTIS AT THE TIME OF HEATING. 135

FIGURES.6.1 – 6.12 H&E STAINING OF CONTROL AND HEATED EPIDIDYMIS.....147
FIGURE 6.13 CIRP EXPRESSION IN THE CONTROL EPIDIDYMIS.....149
FIGURE 6.14 CIRP EXPRESSION IN THE HEATED EPIDIDYMIS (+ 6 H).....150
FIGURES 6.15-6.20 BAX EXPRESSION IN THE EPIDIDYMIS FOLLOWING *IN VIVO* HEAT TREATMENT.....152
FIGURE 6.21 CD68 EXPRESSION IN THE CONTROL EPIDIDYMIS.....154
FIGURE 6.22 CD68 EXPRESSION IN THE HEATED EPIDIDYMIS (+6H).....155
FIGURE 6.23 NUMBER OF MOTILE SPERMATOZOA FOLLOWING HEATING OF EPIDIDYMIS.....156
FIGURE 6.24 LEVELS OF DNA DAMAGE (% TAIL DNA) IN MOTILE SPERMATOZOA FOLLOWING HEATING OF THE EPIDIDYMIS.....157
FIGURE 6.25 LEVELS OF DNA DAMAGE (COMET MOMENT) IN MOTILE SPERMATOZOA FOLLOWING HEATING OF THE EPIDIDYMIS.....158

FIGURE 7.1 NUMBERS OF MOTILE SPERMATOZOA AND LEVELS OF DNA DAMAGE IN THESE SPERMATOZOA AT EACH OF THE TIME-POINTS STUDIED.....169

Chapter 1 Review of the Literature

1.1 General introduction

The male reproductive system consists of the testis, epididymis, the excretory ducts (vas deferens, ampulla and ejaculatory ducts), the internal accessory organs (prostate, seminal vesicles, Cowper's glands) and the external genitalia (penis).

The main focus of this literature review will be on the structure, function and regulation of the testis and epididymis, the impact of stress (in particular heat stress) on these tissues and the clinical implications of heat stress in the testis/epididymis of man.

1.2 Role of the testis

The two main functions of the testis are the synthesis and secretion of hormones, and the production of spermatozoa (spermatogenesis).

The hormones produced in the testis are required for the production of spermatozoa and the development of secondary sexual characteristics (internal accessory organs and external genitalia) required for the delivery of the spermatozoa to the oocyte. In addition, these hormones are responsible for masculinisation and the development of sexual behaviour (Setchell, 1978). The endocrine role of the testis was first demonstrated in 1849. In an experiment using roosters, it was shown that castration resulted in the regression of secondary sexual characteristics. These effects could then be reversed by re-implantation of all or part of the testis (Berthold, 1849).

Spermatozoa were first observed over 300 years ago by van Loewnheck (1678), however, it was another 150 years before Prevost and Dumas demonstrated their role in fertility (Prevost, *et al.*, 1824). Spermatozoa are responsible for the transmission of the male genetic information to the oocyte during reproduction.

1.3 Testis

The two main compartments of the testis are the interstitial compartment and the tubular compartment (seminiferous tubules) (See Figures 1.1 and 1.2).

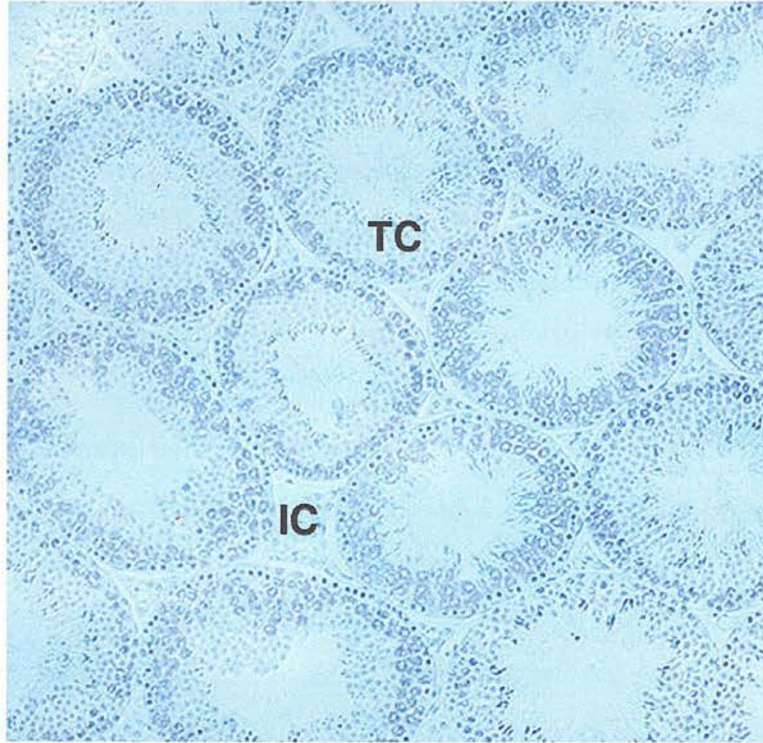


Figure 1.1 Compartmentalisation of the testis

Figure shows a cross section of a normal mouse testis indicating the seminiferous tubules (Tubular Compartment = TC) and the interstitial compartment (IC)

1.3.1 Interstitial compartment

The interstitial compartment of the testis is the location of steroidogenesis and consists of steroidogenic cells (Leydig cells), immune cells, nerves, fibroblasts, blood and lymph vessels, and loose connective tissue. The interstitial compartment comprises between 2-6% of the total testicular volume in rodents (Weinbauer, *et al.*, 2001).

Of all the components of the interstitial compartment, Leydig cells are considered the most important as they are the source of testicular testosterone. The

steroidogenic properties of Leydig cells were first observed in 1903 (Bouin, *et al.*, 1903), although it was another 30 years before the hormone produced was identified as testosterone (David, *et al.*, 1935). During puberty, precursor mesenchymal cells divide and become Leydig cells. The maturation and DNA synthesis of Leydig cells is stimulated by follicle stimulating hormone (FSH, released from the anterior pituitary), and other growth factors (produced locally) (Payne, *et al.*, 1995).

The synthesis of testosterone by Leydig cells is stimulated by luteinising hormone (LH, released from the anterior pituitary) and high-affinity LH receptors are expressed on the surface of Leydig cells. Binding of LH to these receptors triggers the production of cAMP, which in turn facilitates the transport of cholesterol into the mitochondria of the Leydig cell where testosterone synthesis begins (Johnson, *et al.*, 1995).

Within the Leydig cells, testosterone is synthesised via 2 pathways (A5; pregnenolone to dehydroepiandrosterone (DHA), and A4; progesterone to androstenedione). Intermediates of these pathways (androstenedione and DHA) are released into the blood and lymph in addition to testosterone (Johnson, *et al.*, 1995). Furthermore, the Leydig and/or Sertoli cells may then convert testosterone to oestrogen via aromatase cytochrome p450 (Papadopoulos, *et al.*, 1993;Genissel, *et al.*, 2001).

1.3.2 Seminiferous Tubules

The seminiferous tubules are the location of the spermatogenic process and consist of germ cells and somatic cells (Sertoli and peritubular cells; see figure 1.2).

The testis consists of approximately 250-300 lobules, divided by connective tissue, with each lobule containing a number of highly convoluted seminiferous tubules (1-3 in man; 5 in most species). Each tubule is between 30-80cm in length. In man, the total length of seminiferous epithelium in the testis is calculated to be around 720m (Weinbauer, *et al.*, 2001).

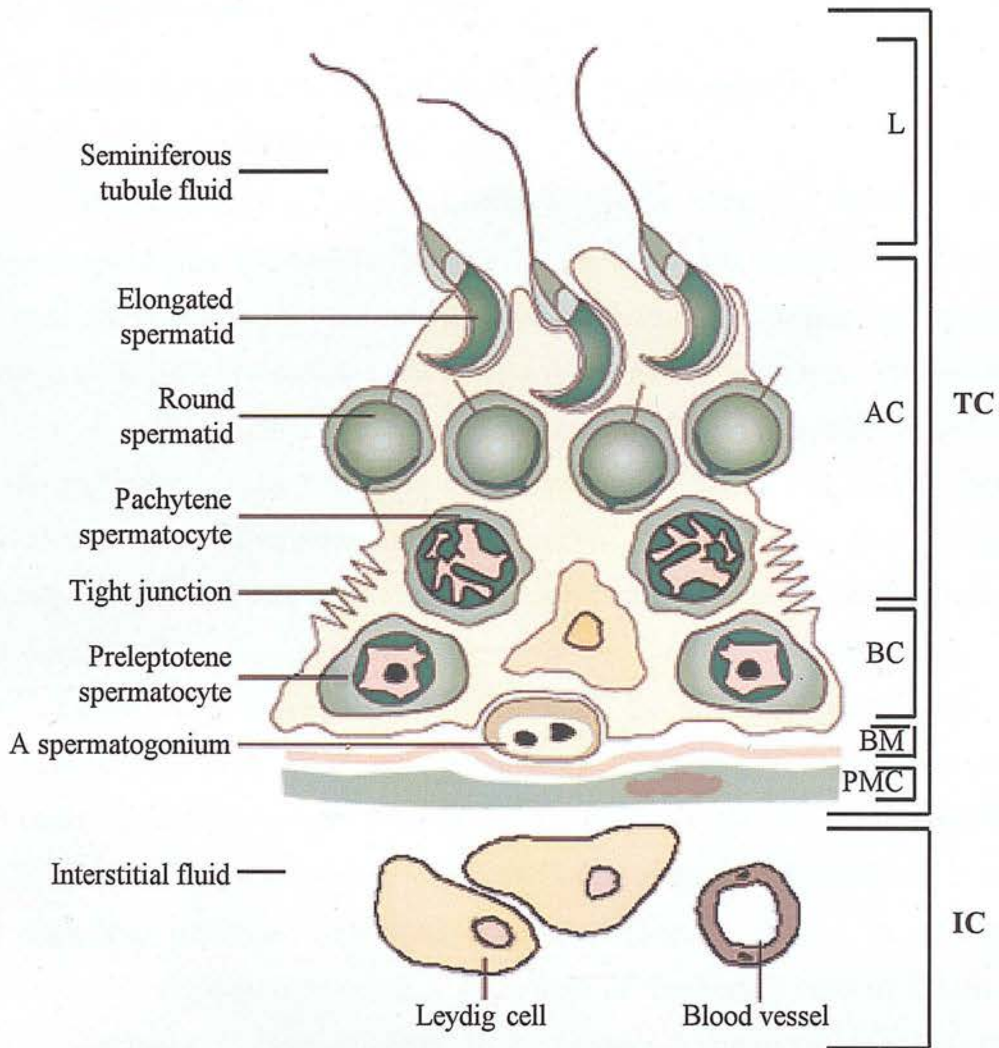


Figure 1.2 The organisation of the seminiferous epithelium

Figure shows a Sertoli cell in association with developing germ cells in the seminiferous epithelium, and the cells of the interstitial compartment with which it has contact.
(TC = tubular compartment, IC = interstitial compartment, L = lumen, AC = adluminal compartment, BC = basal Compartment, BM = basal membrane, PMC = peritubular myoid cell layer)

Adapted from Sharpe, 1994.

1.3.2.1 Somatic cells

Within the seminiferous epithelium there are two major types of somatic cell; the Sertoli cell and peritubular myoid cells.

The Sertoli cells are columnar cells (75-100µm in height), located within the germinal epithelium of the testis. These cells are attached to the basement membrane and their apex reaches into the lumen of the tubule. The cytoplasm of Sertoli cells contains a complex cytoskeleton and a range of organelles (as in most somatic cells).

Each Sertoli cell is found in association with a number of germ cells (as shown in Figure 1.1), which are dependent on that Sertoli cell for their survival and development. Therefore, it is the number of Sertoli cells that defines the testicular volume and rate of production of spermatozoa. The number of germ cells each Sertoli cell can support varies between species (Weinbauer, *et al.*, 2001).

Sertoli cells provide structural support for the developing germ cells, maintaining their position in the seminiferous epithelium. Though the precise molecular mechanisms of this process are not fully understood, it has been shown that Sertoli cell factors are passed to the germ cells via germ cell membrane receptors and endocytic processes junctional complexes (Jegou, 1993). In addition to supporting developing germ cells, it is the role of the Sertoli cells to recruit early meiotic spermatocytes from the basal to the adluminal compartment and to release mature spermatozoa from the epithelium (spermiation) (Russell, *et al.*, 1993).

The Sertoli cells form a blood-testis barrier, creating two distinct regions within the seminiferous tubule; the basal compartment and the adluminal compartment (See figure 1.2). Tight junctions connect adjacent Sertoli cells enabling strict regulation of the transport of substances between these two compartments. The blood-testis barrier may serve two important purposes; the protection of the haploid (and thereby antigenic) germ cells from the immune system, and the maintenance of the specific milieu (nutrients, fluid, ions etc.) required for spermatogenesis (Weinbauer, *et al.*, 2001).

Due to the blood-testis barrier, the germ cells (located in the adluminal compartment) are dependent on the Sertoli cells for their nutritional requirements

(sugars, lipids, amino acids, metallic elements etc). Again, the precise molecular mechanisms behind this process are not fully understood, however, it has been shown that Sertoli cells are able to extend processes containing nutrients into the cytoplasm of elongated spermatids. These processes are then incorporated into digestive vacuoles and the nutrients absorbed by the spermatids (Russell, *et al.*, 1993).

In addition to the transfer of nutrients to the developing germ cells, Sertoli cells also secrete a wide range of substances (including proteins and water) into both the seminiferous (in the form of seminiferous tubule fluid; STF) and interstitial compartments (Richburg, *et al.*, 1994).

Finally, Sertoli cells have an endocytic function; the phagocytosis and elimination of cytoplasmic bodies discarded by the spermatozoa before spermiation, and the pinocytosis of fluid from the lumen (Russell, *et al.*, 1993).

Peritubular myoid cells are stratified around the seminiferous tubules in concentric layers and the number of layers is species-specific (6 in humans, 2-4 in other mammals). These cells are poorly differentiated myoid cells, which contain actin and are capable of spontaneous contraction. The resulting peristaltic contractions of the seminiferous tubules are believed to facilitate the movement of mature spermatozoa through the tubules to the vas deferens (Weinbauer, *et al.*, 2001).

1.3.2.2 Germ cells and Spermatogenesis

Spermatogenesis is generally divided into three phases; mitotic proliferation, meiotic division and spermiogenesis (See Figure 1.3). The first phase, mitotic proliferation includes the proliferation of the spermatogonia (A, intermediate (In) and B spermatogonia). The second phase, meiosis, involves major changes in the DNA of the developing spermatocytes (preleptotenes (P), leptotenes (L), zygotenes (Z) and meiosis (M) spermatocytes). The final phase of spermatogenesis within the testis is spermiogenesis, this phase involves the differentiation of the spermatids into

spermatozoa. The stages and cell types involved in spermatogenesis are described in more detail in section 1.3.2.3.

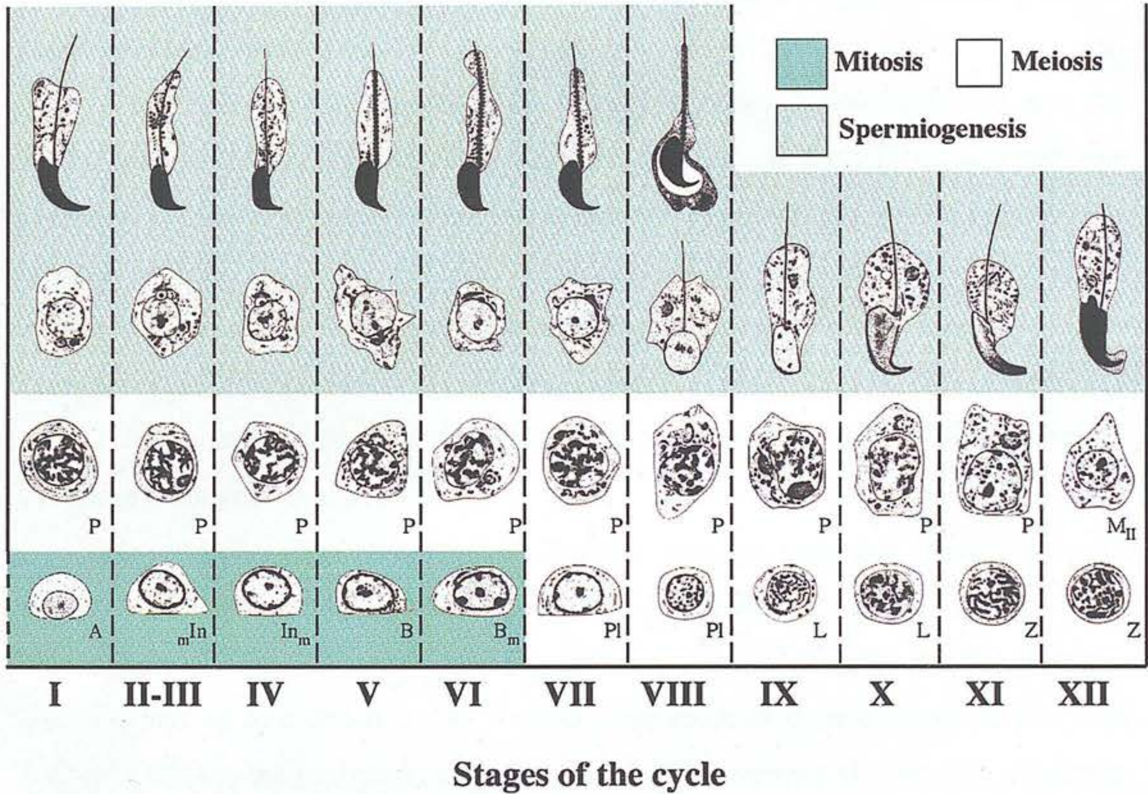


Figure 1.3 Stage diagram of spermatogenesis in the mouse, indicating the three phases of spermatogenesis; mitosis, meiosis and spermiogenesis

Adapted from Russell (1990) and Sharpe (1994).

1.3.2.2.1 Mitosis

Pro-spermatogonial germ cells located in the basal region of the tubule (See Figure 1.2) enter mitosis at puberty and become self-regenerating (via mitosis) spermatogonial stem cells. From these stem cells, two types of A spermatogonia form. The first of these do not normally proliferate and will undergo mitosis only if the spermatogonial population is severely depleted for any reason (e.g. following exposure of the testis to radiation). The second enters spermatogenesis, undergoing further mitoses to form B spermatogonia.

1.3.2.2.2 Meiosis

During the meiotic prophase, several important events occur; the recombination of genetic material, the reduction in chromosome number and the development of spermatids.

At the final mitotic division, the B spermatogonia differentiate and give rise to the preleptotene spermatocytes. These cells duplicate their DNA and are then recruited by the Sertoli cells into the adluminal compartment where they enter a lengthy meiotic prophase (Sharpe, 1994). During this meiotic prophase, the morphology of the cells changes gradually by progressive differentiation. These morphological changes allow the recognition and classification of the different cell types (leptotene, zygotene and pachytene primary spermatocytes) within the meiotic prophase (Johnson, *et al.*, 1995).

In addition to morphological changes, dramatic changes occur within the nuclei of the cells during meiotic prophase. Chromatin condenses and becomes filamentous in the leptotenes and in each zygotene, homologous chromosomes thicken, pair-up and attach to the nuclear membrane at their extremities (forming loops). Within the pachytene cell, the paired chromosomes shorten and condense, and nuclear and cytoplasmic volume increases. The sister chromatid strands on the paired homologous chromosomes meet and form synaptoneal contacts, the chromatids break, exchange segments of their genetic material and then rejoin (Johnson, *et al.*, 1995). Within the diplotene cell, the homologous chromosomes separate to the opposite ends of the cell along the meiotic spindle. Cytokinesis results in the production of two secondary spermatocytes from each diplotene, each containing a single set of chromosomes (two chromatids joined at the centromere) (Johnson, *et al.*, 1995). The two secondary spermatocytes enter the second meiotic division, during which their chromatids separate and move to the opposite ends of the second meiotic spindle before dividing to yield a total of four early round spermatids (Sharpe, 1994;Johnson, *et al.*, 1995).

1.3.2.2.3 Spermiogenesis

Spermiogenesis is the process by which the spermatids differentiate into mature spermatozoa. This process is outlined in Figure 1.4. Spermiogenesis occurs in a series of sequential steps and developing spermatids are classified according to their step number.

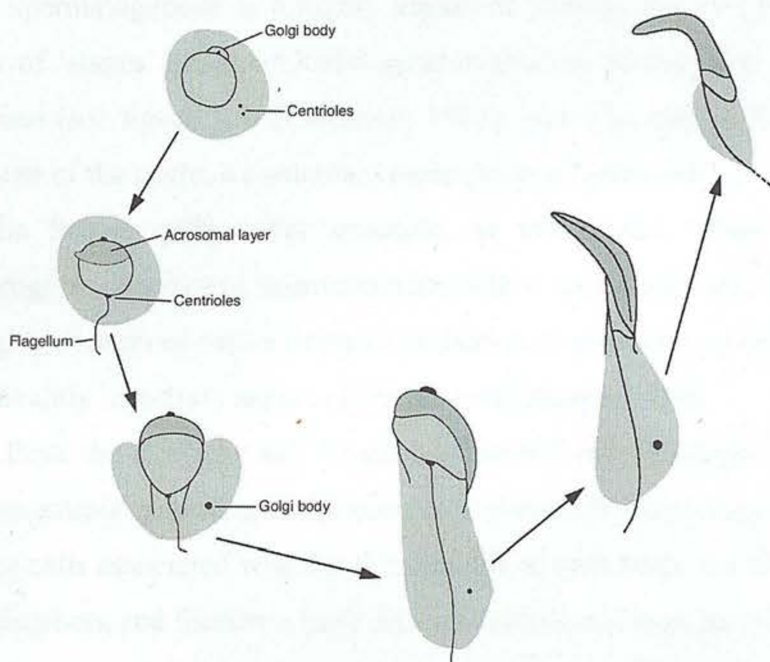


Figure 1.4 The process of spermiogenesis

Adapted from Johnson (1995)

Spermiogenesis commences with the Golgi body producing glycoprotein-rich granules, which come together to form the acrosomal layer. This gradually grows over the surface of the nucleus and a sub-acrosomal region forms between the acrosomal cap and the nucleus. The centrioles of the spermatid migrate to the opposite pole of the nucleus and form the point of origin of the spermatozoa tail and the neck, which connects the spermatozoa head with the tail. The nucleus and acrosomal cap migrate towards the cytoplasmic membrane and the spermatid begins

to lengthen. The condensation of the chromatin begins, the species-specific shape of the spermatozoa head is established and the spermatozoa loses its Golgi apparatus and cytoplasm (Johnson, *et al.*, 1995).

1.3.2.3 Functional organisation of the seminiferous tubules

Spermatogenesis is a highly organised process that has been divided into a number of 'stages' based on histological evaluation of testicular sections (eg. 12 in the mouse (see figure 1.5) (Clermont, 1972), and 6 in man (Clermont, 1966)). At each stage of the cycle, a particular combination of germ cells is found in association with the Sertoli cells. For example, in the mouse, Stage I consists of A spermatogonia, pachytene spermatocytes, step 1 spermatids and step 13 spermatids. The highly conserved nature of the organisation of spermatogenesis suggests that it is a significantly important aspect of the process (Sharpe, 1994).

Both the structure and function of Sertoli cells changes with each stage of spermatogenesis according to the germ cell types with which they are associated. The different cells associated with the Sertoli cells at each stage are all at different stages of development and therefore have different nutritional requirements. It is likely that each cell type controls certain aspects of Sertoli cell function in order to obtain the combination of nutrients it requires.

The organisation of spermatogenesis along the seminiferous tubule (the spermatogenic wave) differs amongst species. In the rodent, spermatogenesis follows a longitudinal pattern. Serial transversal sections through rat tubules show that whole segments are at the same stage of the cycle and adjacent segments are either one stage ahead or one stage behind in the cycle (Johnson, *et al.*, 1995). In the human, and some monkeys, the organisation of the spermatogenic wave is somewhat different. Serial transversal sections through human tubules show a number of different stages simultaneously. It has therefore been proposed by some investigators that the spermatogenic wave in the human is organised in a helical pattern within the tubule (Weinbauer, *et al.*, 2001).

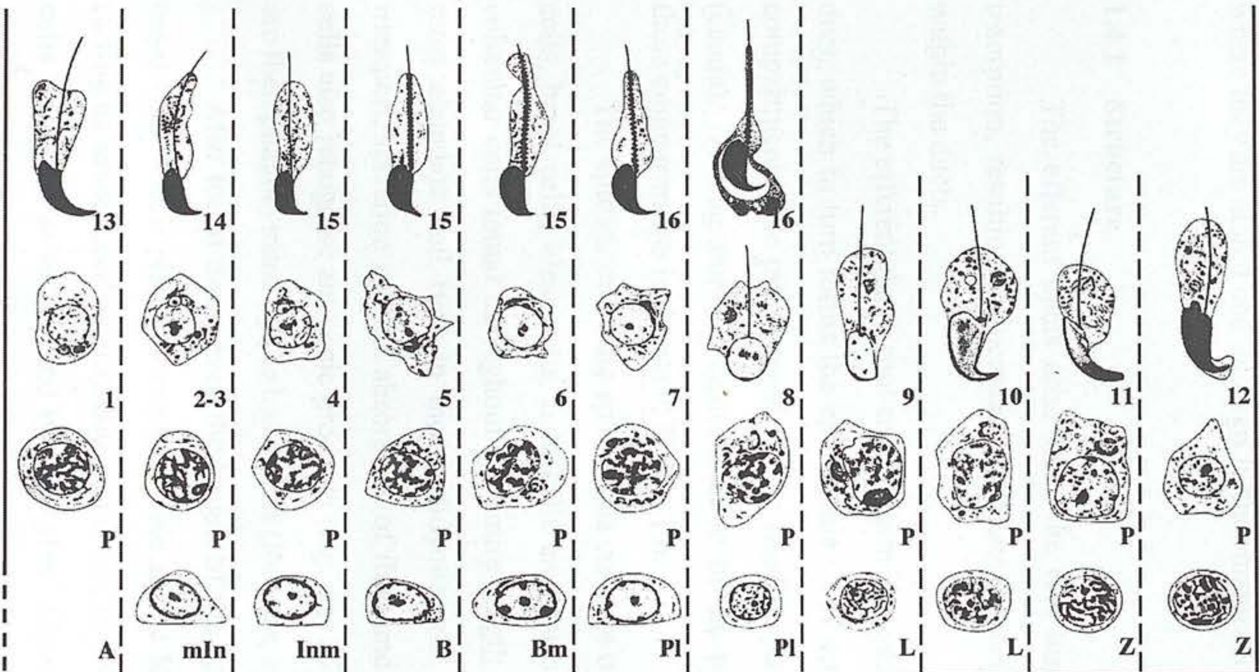


Figure 1.5 Spermatogenic cycle of the mouse

Adapted from Russel, 1990

1.4 Epididymis

Upon completion of spermatogenesis, spermatozoa leave the testis via the rete testis and then the efferent ducts (*ductus efferentes*) and enter the epididymis where they are stored and undergo further maturation before release.

1.4.1 Structure

The efferent ducts arise from the rete testis and are a major site of fluid resorption, resulting in increased spermatozoa density and decreased fluid content within the ducts.

The efferent ducts converge to form a single, very long and highly convoluted duct, which in turn forms the epididymis. The epididymis consists of four distinct compartments: the initial segment, the head (caput), the body (corpus) and the tail (cauda). A diagrammatic representation of the testis and epididymis highlighting these compartments is shown in Figure 1.6.

The epithelium of the epididymis consists of five distinct cell types: principal cells, basal cells, clear cells, halo cells and narrow cells. Principal cells are tall, columnar cells found throughout the entire length of the epididymis. They are the most abundant cell type in the epididymis, and are believed to be involved in transport, secretion and the absorption of fluid and matter from the lumen. Principal cells also recognise antigenic products (e.g. due to spermatozoa degradation), which are then phagocytosed by the basal cells (Robaire, *et al.*, 1988).

Also found throughout the length of the epididymis are the small elongated basal cells which play a scavenging role in the local immune defence mechanism (acting as tissue-fixed macrophages) and may be involved in detoxification, and halo cells which act as mono- and lymphocytes. Clear cells span the epithelium of the epididymis and are found in all compartments except for the initial segment and are involved in the removal of unwanted materials from the lumen. Narrow cells are found in the initial segment only (Robaire, *et al.*, 1988;Yeung, *et al.*, 1994).

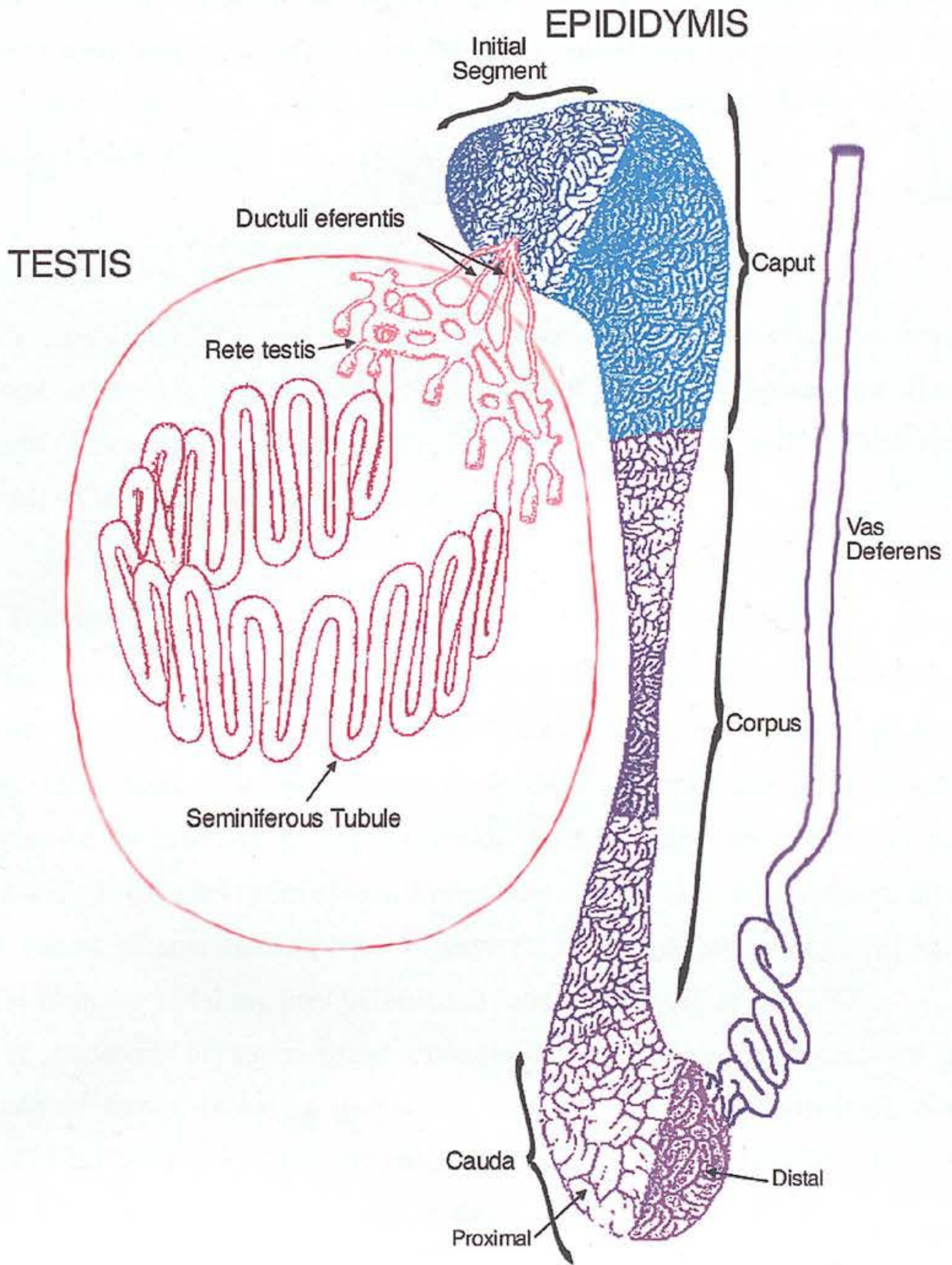


Figure 1.6 The mammalian testis and epididymis.

Figure indicates the various compartments of the epididymis; the initial segment, the caput, the corpus and the cauda.

Adapted from Robaire, 1988.

The luminal compartment of the epididymis is a constantly changing environment containing spermatozoa and the many various substances required for its storage and maturation. These include ions, proteins, glycoproteins, organic molecules and water (Robaire, *et al.*, 1988).

1.4.2 Function

The functions of the epididymis are the transportation of spermatozoa from the efferent ducts to the vas deferens, the maturation of spermatozoa (i.e. acquirement of increased chromatin stability, fertilising ability and motility) and the storage and protection of mature spermatozoa.

1.4.2.1 Transport

The time taken for spermatozoa to travel the length of the epididymis (epididymal transit time) has been established by a variety of methods for a number of species. (See Figure 1.7) For example, the epididymal transit time of the mouse was determined by labelling spermatozoa with ^{35}S-methionine and following the progression of the labelled spermatozoa through the epididymis. It was shown that an entire cohort of spermatozoa takes 7 days to travel the entire length of the epididymis from the initial segment to the distal cauda (Cornwall, *et al.*, 1990).

The transport of spermatozoa through the epididymis is driven by a combination of forces, including hydrostatic pressure, muscular contractions, the movement of epithelial cell cilia in the epididymis and the flow of fluid entering the epididymis from the testis (Robaire, *et al.*, 1988).

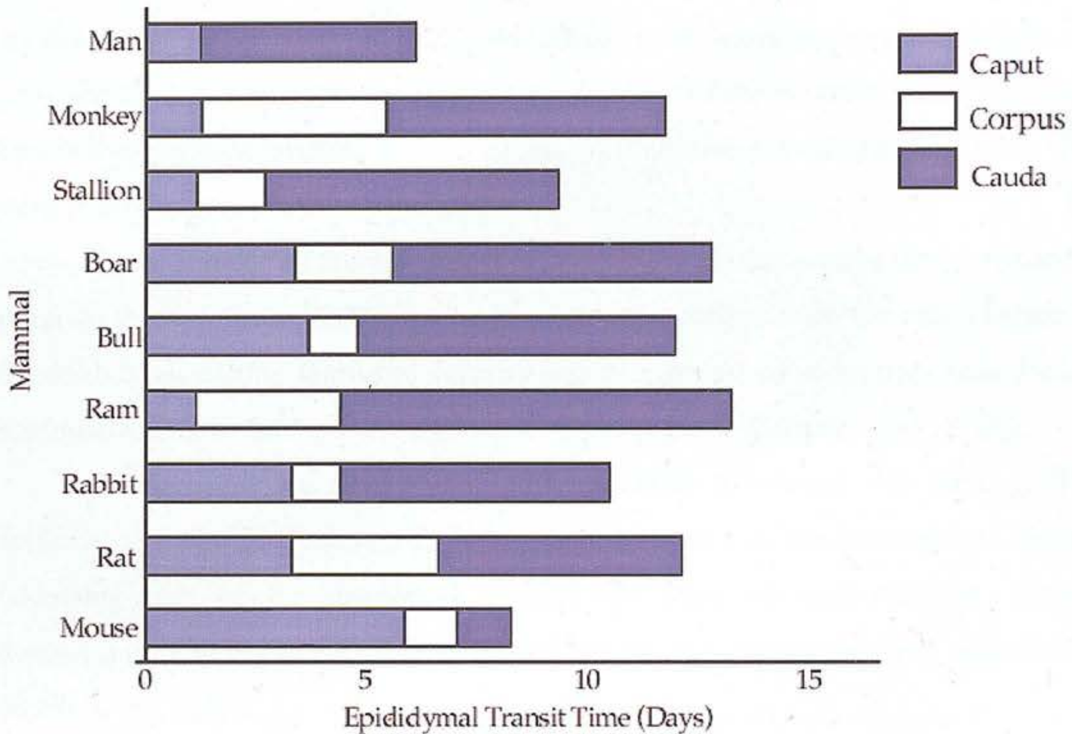


Figure 1.7 The epididymal transit time of certain mammals

Note: For man, the darker shaded area represents the combined transit time for the corpus and cauda epididymis.

Adapted from Robaire, 1988.

1.4.2.2 Maturation

Spermatozoa must undergo further maturation in the epididymis to become motile and to be able to fertilise eggs (Cooper, *et al.*, 2000).

Motility is acquired as the spermatozoa progress through the epididymis though the precise mechanisms behind the acquisition of motility by spermatozoa in the epididymis are unclear. It has been suggested that development of motility is regulated by changes in intracellular pH, cAMP and Ca^{2+}, and the action of protein phosphatases (Lindemann, *et al.*, 1989;Yeung, 1995;Vijayaraghavan, *et al.*, 1996;Turner, *et al.*, 1998).

However, spermatozoa remain immotile until released from the epididymis. As discussed earlier (section 1.4.2), within the epididymis Na+ concentrations are high and K^+ concentrations are low and the motility of the spermatozoa is promoted by a Na^+-dependant increase in intracellular pH which occurs during ejaculation or *in vitro* (Cooper, *et al.*, 2000).

Co-ordination of the movement of the flagellum improves as the spermatozoa progress through the epididymis. As a result, spermatozoa from the cauda region of the epididymis exhibit increased velocity and straightness of swim path compared to spermatozoa retrieved from the caput and corpus regions (Cooper, *et al.*, 2000).

The spermatozoa must also acquire the ability to interact with the egg. The epididymal epithelium secretes proteins which interact with the spermatozoa surface becoming part of the membrane and/or modifying existent residues, thereby developing or activating sites concerned with gamete recognition and capacitation (Krull, *et al.*, 1993).

1.4.2.3 Storage and protection of spermatozoa

Spermatozoa can be stored in the cauda epididymis for long periods of time, though the fertilising ability and motility of the spermatozoa do gradually decrease over time. The ability of the cauda epididymis to store spermatozoa for extended periods is due to increased oxygen availability within the cauda epididymis and the decreased respiration rate of spermatozoa at scrotal temperatures (Djakiew, *et al.*, 1986;Mieusset, *et al.*, 1992). Furthermore, it has been suggested that the necessity to store spermatozoa at reduced temperature was one of the reasons for the migration of the male reproductive tract from the abdomen to the scrotum (Mieusset, *et al.*, 1992).

The epididymis protects the spermatozoa in a number of ways. The blood-epididymis barrier is composed of tight junctions and allows only selective entry of substances into the epididymal lumen while the epithelial cells of the epididymis ensure the rapid elimination of harmful by-products and exogenous toxic substances from the lumen (Robaire, *et al.*, 1988).

Many proteins are secreted into the lumen of the epididymis and protect the spermatozoa from specific threats. For example, it has been demonstrated that a number of antioxidant enzymes are expressed in a region-specific manner within the epididymis of both rats and humans (Zini, *et al.*, 1997;Potts, *et al.*, 1999). Other specific threats to spermatozoa within the epididymis are proteolysis (due to the premature release of acrosome proteins) and complement-mediated cell lysis. The precise mechanisms in place to protect spermatozoa from these threats are yet to be determined, though it is believed that certain secretory proteins may be involved (Hinton, *et al.*, 1995).

1.5 Spermatozoa

1.5.1 Structure

The components of mature spermatozoa are the head, the flagellum (tail) and the neck (which connects the head and the tail) as shown in Figure 1.8.

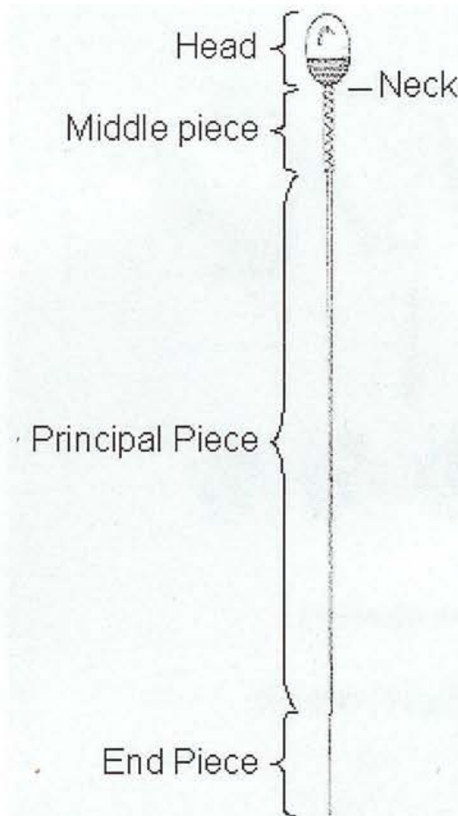


Figure 1.8 Structure of human spermatozoa showing main regions

1.5.1.1 Head

The head of the spermatozoa contains the highly compacted and inactive DNA which will be passed on to the oocyte during fertilisation.

The packaging of DNA in mammalian spermatozoa is very different to that of somatic cells. If spermatozoal DNA was packaged into nucleosomes in the same way as somatic cell DNA, it would require double the space available in the spermatozoa head. In the spermatozoa nucleus, the DNA is bound to protamines and then organised into linear, side-by-side arrays. This arrangement allows a much larger amount of DNA to be packaged into a small space (See Figure 1.9) (Ward, *et al.*, 1991).

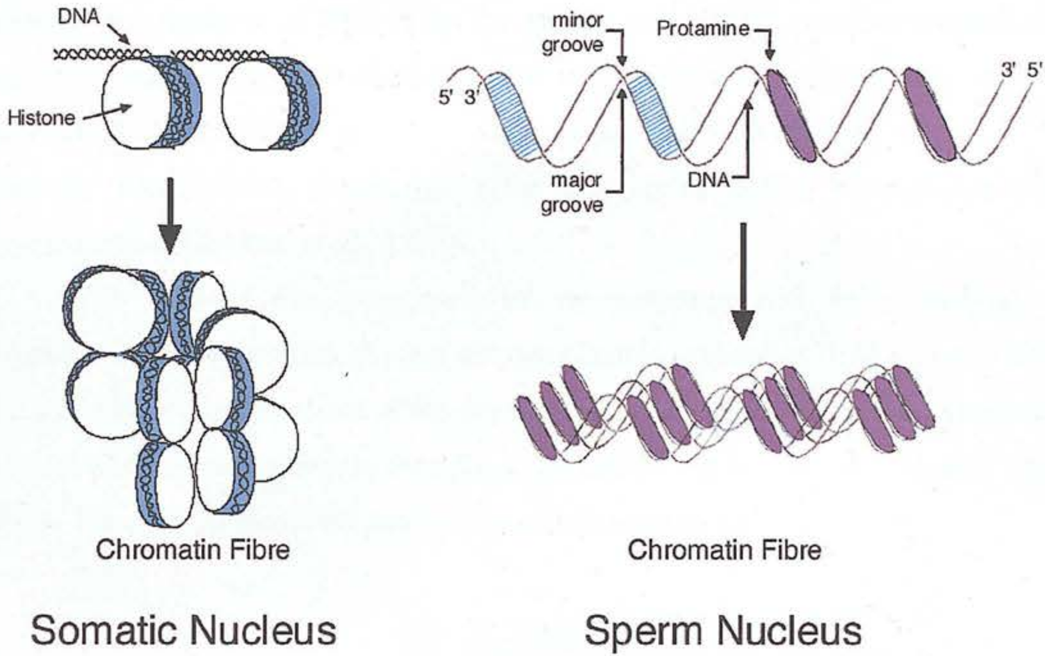


Figure 1.9 Comparison of the packaging of DNA in somatic versus spermatozoan nuclei

Adapted from Ward (1991)

The sequential substitution of somatic histones by testis specific histones, transition proteins, and finally by protamines, the condensation of the chromatin and the formation of disulphide bonds occurs during the transition from round to elongated spermatid (Hecht, *et al.*, 1987). This process continues in the proximal region of the epididymis with the further condensation of the chromatin and the formation of further S-S cross-links between thiol groups of TSP cysteine residues (Calvin, *et al.*, 1971).

Chromatin condensation ceases and spermatozoa heads reach their final size in the caput epididymis. The stabilisation of chromatin occurs in the vas deferens but this process does not alter chromatin superstructure (Manfredi Romanini, *et al.*, 1986).

It has been shown that endogenous DNA nicks and strand breaks occur in the DNA of developing spermatozoa cells during spermatogenesis (Sakkas, *et al.*,

1995;Smith, *et al.*, 1998). These DNA strand breaks and endogenous nicks may facilitate the transition of DNA from the somatic cell histone complex to the tightly packed protamine complex of the mature spermatozoa by relieving the torsional stress created during this process (Ward, *et al.*, 1991;McPherson, *et al.*, 1992). Normally these strand breaks and nicks are ligated before the completion of spermiogenesis (Sakkas, *et al.*, 1995).

The head is also composed of the acrosome and small amounts of cytoskeleton and cytoplasm, though not cytoplasmic organelles (Eddy, *et al.*, 1994). The majority of the cytoplasm of the developing spermatid is lost via the cytoplasmic droplet/residual body, which is then phagocytosed by the Sertoli cell (Johnson, *et al.*, 1995). Figure 1.10 shows the head of a murine spermatozoa.

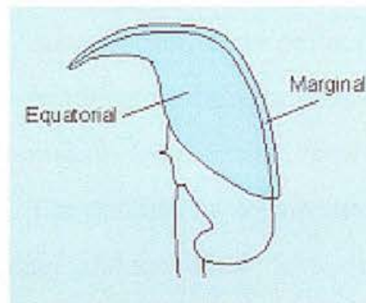


Figure 1.10 Diagrammatic representation of the head of the murine spermatozoa showing the equatorial and marginal regions of the acrosome

The acrosome contains the many enzymes required for the penetration of the oocyte. The release of these enzymes may be triggered by a number of species-specific signals, often reactive oxygen species (Eddy, *et al.*, 1994;de Lamirande, *et al.*, 1997).

1.5.1.2 Tail

The main section of the spermatozoa, the tail, consists of a central axoneme, surrounded by outer dense fibres which form the cytoskeleton of the tail. Again, the tail contains little cytoplasm, however, this region of the spermatozoa is abundant

with mitochondria which provide the energy required for motility (Eddy, *et al.*, 1994).

1.5.2 Function

In the male reproductive tract and lower female reproductive tract, spermatozoa are prevented from undergoing precocious capacitation by decapacitation factors. During the passage of spermatozoa through the female reproductive tract these factors are removed and the spermatozoa are able to undergo capacitation. Upon reaching the ampulla of the oviduct, the spermatozoa can then hyperactivate, interact with the oocyte, undergo the acrosome reaction and penetrate the egg (Cooper, 2001).

Spermatozoa must produce reactive oxygen species (ROS) in order to undergo normal hyperactivation, capacitation, zona pellucida binding and acrosome reaction. The enzymatic system resulting in the production of ROS in spermatozoa is unknown. Spermatozoal capacitation is an oxidative process which requires low concentrations of ROS to occur. The conditions within the female reproductive tract may provide these ROS or may induce their formation by the spermatozoa themselves. Oxidative conditions may also be required for the spermatozoa to bind to the zona pellucida and it has been shown that hydrogen peroxide is required for the acrosome reaction to take place. (de Lamirande, *et al.*, 1997)

1.5.3 Susceptibility to insult

Spermatozoa are generally regarded as being less susceptible to a number of endogenous insults including heat and oxidative stress, radiation and cytotoxic agents, than somatic cells. To date, there have been few studies which have compared the susceptibility to insult of spermatozoa from different species.

Haines *et al* studied the effects of *in vitro* irradiation on both human and murine spermatozoa (Haines, *et al.*, 1998). Following irradiation with increasing levels of gamma radiation, both murine and human spermatozoa exhibited increasing

levels of DNA damage as measured by the neutral Comet assay. However, the high levels of gamma radiation required to produce measurable amounts of DNA damage in spermatozoa reflects the high resistance of spermatozoa to gamma radiation compared to somatic cells. The authors suggest that this difference may be due to the differences in DNA packaging and conformation in spermatozoa compared to somatic cells.

Estop *et al* incubated murine spermatozoa in Tyrode's T6 fertilization media and found that this resulted in chromosome structural abnormalities and changes in sperm chromatin structure which could in turn lead to breaks in the spermatozoal DNA (Estop, *et al.*, 1993). Similarly, Twigg *et al* have demonstrated that DNA damage can be induced during the preparation of human spermatozoa for assisted reproduction techniques (Twigg, *et al.*, 1998).

Further study is required in order to determine the comparative susceptibility of spermatozoa from different species before direct correlations between animal models and humans can be made.

1.6 Maintenance of normal spermatogenesis/epididymal function

1.6.1 Hormonal support

1.6.1.1 Testis

As discussed previously (Section1.1.1), testosterone is produced in testicular Leydig cells under the control of luteinizing hormone (LH) and is essential for the maintenance and regulation of spermatogenesis. Testosterone may then be metabolized to either 5 α-dihydrotestosterone (DHT) or to 17 β-estradiol.

Testosterone and DHT are essential for the differentiation of the external genitalia and accessory sex organs. 17 β-estradiol is required for differentiation of sexual dimorphic nuclei in the brain (Chan, *et al.*, 1989) (McLachlan, 2000).

Results from experiments using mouse models suggest that the role of follicle-stimulating hormone (FSH) in spermatogenesis is the regulation of Sertoli

cell proliferation, the synthesis of secretory proteins (including transferring) which are involved in the transfer of nutrients to germ cells and, ultimately, the size and spermatogenic capacity of the testis (Griswold, 1993;Heckert, *et al.*, 2002).

FSH, DHT and testosterone are also involved in the process of spermiation (the release of mature elongated spermatids from the testis) and disruption of this process via alteration of steroid levels has been proposed as a main feature of male steroidal contraceptives (O'Donnell, *et al.*, 1996;McLachlan, *et al.*, 2002).

1.6.1.2 Epididymis

The epididymis is dependant on testicular androgens for regulation, in particular DHT and 5α-androstan-3 α, 17β-diol (3 α-diol), which are both synthesised from testosterone in the epididymis (Robaire, *et al.*, 1995).

It has been known for over 60 years that testosterone is required for the maintenance of normal epididymal histology. Within the epididymis, androgens are required for the transport of ions, inositol and carnitine across the epididymal epithelium, the regulation of intermediary metabolism, the synthesis and secretion of many epididymal glycoproteins, and the regulation of enzymatic activity. In addition, dihydrotestosterone is required for the acquisition of fertilising potential by spermatozoa in the epididymis (Robaire, *et al.*, 1988).

Androgens are not the only hormones required by the epididymis. Estrogen receptors are expressed within the epididymis and estrogen is required for normal epididymal function, in particular fluid transport and reabsorption (as reviewed by (Hess, *et al.*, 2001). Receptors for a number of other hormones and substances have been identified in the epididymis including aldosterone (Hinton, *et al.*, 1985), prolactin (Hair, *et al.*, 2002) and vitamins A (Porter, *et al.*, 1985) and D (Kidroni, *et al.*, 1983).

1.7 Stress responses in the testis/epididymis

1.7.1 Apoptosis

The many rounds of mitosis which occur during spermatogenesis results in excessive numbers of germ cells which the Sertoli cells are unable to support (Sakkas, *et al.*, 1999). These excess germ cells are removed from the testes by selective apoptosis (Tapanainen, *et al.*, 1993;Blanco-Rodriguez, *et al.*, 1996;Yamamoto, *et al.*, 2000).

Apoptosis occurs throughout spermatogenesis at specific stages of the cycle; I, VII, VIII, XIV and XII (Blanco-Rodriguez, *et al.*, 1996). The process of apoptosis is an important regulator of cell density in the testis and is regulated by a number of pro-survival and pro-apoptosis genes.

1.7.1.1 Genes involved in the regulation of apoptosis

Bax is a promoter of apoptosis and is normally expressed in the testis of the mouse where it acts as a regulator of germ cell density (Beumer, *et al.*, 2000;Yamamoto, *et al.*, 2000). Loss of Bax expression in the testis results in disordered and increased apoptosis (Knudson, *et al.*, 1995). It has been suggested that Bax plays an important role in the regulation of the meiotic cycle but it is unclear if it is involved in the induction of apoptosis following DNA damage (Knudson, *et al.*, 1995;Beumer, *et al.*, 2000).

The Bcl family is intrinsic to the regulation of apoptosis in the testis and Bcl-w is highly expressed in the basal regions of the testis. The expression of Bcl-w is highest in spermatogonia but is also observed to a lesser degree in spermatocytes, round spermatids, and Sertoli cells. In most tissues, Bcl-w is dispensable, with loss of expression having no major effects. However, in the testis, loss of Bcl-w expression leads to germ and Sertoli cell loss via apoptosis and subsequently infertility (Print, *et al.*, 1998). Bcl-2 and Bcl-x_s do not play a role in the regulation of spermatogonial apoptosis while Bcl-x_l regulates spermatogonial apoptosis following DNA damage (Beumer, *et al.*, 2000).

p53 belongs to a class of genes known as 'tumor suppressor genes'. When functioning normally, these genes act to inhibit the development and spread of cancer. There are two mechanisms by which p53 is thought to act. Both involve the activation of specific target genes. However, one mechanism leads to a temporary stop in cell growth, possibly allowing repair genes to be activated, while the other leads to the death of the affected cell. It has been demonstrated that p53 is expressed in the testis and is involved in the regulation of spermatogenesis (Almon, *et al.*, 1993).

Cells which normally express p53, primary spermatocytes, increase their expression of this protein following exposure to γ-irradiation (in the rat; (Sjoblom, *et al.*, 1996)) and experimentally induced cryptorchidism (in the mouse; (Yin, *et al.*, 1998)). The increased expression of p53 may lead to the arrest of the cell cycle to allow DNA repair, or to apoptosis (Basu, *et al.*, 1998;Sasagawa, *et al.*, 2001).

1.7.2 Antioxidant enzymes

A number of antioxidant enzymes have been identified within the testis. These include superoxide dismutase (SOD), catalase, glutathione (GSH), and glutathione reductase (GR), S-transferase (GST) and peroxidase (GPx). (Bauche, *et al.*, 1994;Gu, *et al.*, 1996) The expression of these enzymes in the testis is both cell- and region-specific; Sertoli and peritubular cells have high levels of SOD and GSH-dependent enzymes and GSH, pachytene spermatocytes and round spermatids, however, have higher levels of SOD and GSH and low levels of GSH-dependant enzymes. Spermatozoa have high levels of SOD, low levels of GSH-dependant enzymes and no GSH (Bauche, *et al.*, 1994).

The ability of the epididymis to protect spermatozoa from oxidative stress is dependent on antioxidant enzymes. The antioxidant expression in the epididymis was studied via the levels of antioxidant present in the semen of normozoospermic and vasectomised men (Potts, *et al.*, 1999). The levels of the antioxidant enzymes ascorbate, urate and thiols in semen from vasectomised men were found to be significantly lower than in normozoospermic men indicating that the epididymis is

the major contributor to the antioxidants within human seminal plasma. In addition it was also demonstrated that these antioxidants are most likely expressed in a region-specific manner within the epididymis in order to provide the most efficient protection for the spermatozoa against oxidative stress (Potts, *et al.*, 1999).

Furthermore, a study of the antioxidant expression within the rat epididymis has determined that cellular glutathione peroxidase, secretory epididymal glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, copper-zinc superoxide dismutase (SOD), secretory epididymal superoxide dismutase and catalase are all expressed in a region-specific manner within the epididymis (Zini, *et al.*, 1997).

1.8 Impact of heat stress on testicular and epididymal function

The normal temperature of the scrotum and testis is lower than body temperature in most mammals, including humans (Kitayama, 1965). This lower temperature is required for normal spermatogenesis to occur, though the reasons for this are not yet understood (Setchell, 1998). Two thermoregulatory systems are employed to maintain a lower scrotal and testicular temperature. The scrotum itself has a large surface area, no subcutaneous fat and thin skin, enabling efficient heat loss either directly or indirectly via the evaporation of sweat (Waites, 1976;Waites, 1991;Candas, *et al.*, 1993). Secondly, counter-current exchange of heat from arterial to venous blood occurs at the pampiniform plexus, which is located in the spermatic cord (Glad Sorensen, *et al.*, 1991). This system results in the cooling of arterial blood before it even reaches the testis and has been shown to be a very efficient regulator of testicular temperature. (Mieusset, *et al.*, 1995)

1.8.1 Causes of raised scrotal temperature in man

There are a number of factors (including clothing, posture, occupation, lifestyle and season) which may affect scrotal temperature in man, and may, in turn affect fertility.

1.8.1.1 Clothing

It was first observed that clothing affects scrotal temperature in 1965 (Rock, *et al.*, 1965). Since then, a number of studies have confirmed this observation and in a several studies, a link between the wearing over certain types of underwear, increased scrotal temperature and impaired semen parameters/fertility has been established (Brindley, 1982;Zorgniotti, *et al.*, 1982).

Bedford (1994) proposed that the insulating effects of clothing on the scrotum of men are equivalent to those of experimentally-induced raised scrotal temperatures in the rabbit (Bedford, 1994).

1.8.1.2 Posture

A number of studies have demonstrated that posture affects scrotal temperature. Lowest scrotal temperatures were observed in normal, naked men whilst standing. Sitting with legs either apart or together, or lying in a supine position, raised scrotal temperatures significantly (Rock, *et al.*, 1965;Brindley, 1982;Jockenhovel, *et al.*, 1990). It is not been established whether the increases in scrotal temperature observed in these different postures are pathological (i.e. likely to be of clinical significance) or remain within the physiological range (Mieusset, *et al.*, 1995).

1.8.1.3 Occupation

It has been shown that the fertility of men in certain occupations may be affected by exposure to high temperatures. In particular, studies of welders and ceramic workers suggest that these occupations, which involve exposure to very high temperatures, may play a role in the increased incidence of infertility reported by these workers (Figa-Talamanca, *et al.*, 1992;Bonde, 1993).

Occupations involving long periods in a seated position (office workers, professional drivers etc.) have also been implicated as causing increased scrotal temperatures.

It has been demonstrated that, in normal volunteers, scrotal temperature can increase by up to 2.2°C following 2 hours of driving compared to measurements taken while walking. This increase in scrotal temperature was shown not to be associated with increase in ambient temperature within the car (Bujan, *et al.*, 2000).

In a study of men attending a fertility clinic in Hungary, it was observed that a high proportion of these men worked as professional drivers (Sas, *et al.*, 1979). Furthermore, it was found that men who had worked as drivers for extended periods exhibited greater numbers of abnormal spermatozoa.

More recent studies have also demonstrated that workers in the transport and communications industries are more likely to exhibit reduced numbers of spermatozoa and reduced motility than workers in other industries (Henderson, *et al.*, 1986;Chia, *et al.*, 1994).

1.8.1.4 Lifestyle

There is conflicting evidence as to whether or not lifestyle factors such as saunas, hot baths, extended periods of sleep/sitting or the use of electric blankets etc. have an effect on scrotal temperature and/or fertility.

The most studied of these so-called lifestyle factors is sauna bathing. One study, using advanced temperature measurement techniques has demonstrated that exposure to sauna can result in the scrotum reaching body temperature within 10 minutes of exposure (Jockenhovel, *et al.*, 1990).

Studies of spermatozoa parameters following single or multiple exposures to sauna baths have demonstrated that this increase in scrotal temperature can lead to a reversible decrease in the spermatozoa number and/or movement parameters of healthy, fertile volunteers (Procope, 1965;Brown-Woodman, *et al.*, 1984;Saikhun, *et al.*, 1998).

It is important to note that the factors mentioned above may not cause infertility in all men. It is likely that those men who do become infertile due to heat stress are for some reason more susceptible to this problem than others.

1.8.1.5 Medical conditions

There are a number of medical conditions which have been shown to lead to increased scrotal temperature and/or infertility.

Varicocele is caused by a contralateral scrotal veinous drainage abnormality and has been shown to be an important factor in male infertility (Mieusset, *et al.*, 1995). This condition results in increased testicular temperature and/or decreased testicular blood flow (Zorgniotti, 1980;Goldstein, *et al.*, 1989;Lerchl, *et al.*, 1993). A number of studies have concluded that infertility associated with varicocele may be a direct consequence of elevated testis temperature in these patients (Zorgniotti, 1980;Mieusset, *et al.*, 1987;Goldstein, *et al.*, 1989;Lerchl, *et al.*, 1993).

The failure of the testes to descend to the scrotum (cryptorchidism) is a frequent and increasing pathology in childhood. It has long been established that untreated cryptorchidism is associated with increased testis temperature, decreased testis size and decreased spermatozoa output (Kitayama, 1965).

Even following treatment by medical or surgical means, cryptorchidism may result in raised scrotal temperature and infertility in adult life (Mieusset, *et al.*, 1995). The increase in testicular temperature associated with cryptorchidism could be either a concomitant or a main factor in the infertility of cryptorchid patients (Mieusset, *et al.*, 1993).

Figure 1.11 outlines the potential causes and effects of raised temperature of the scrotum and/or testis.

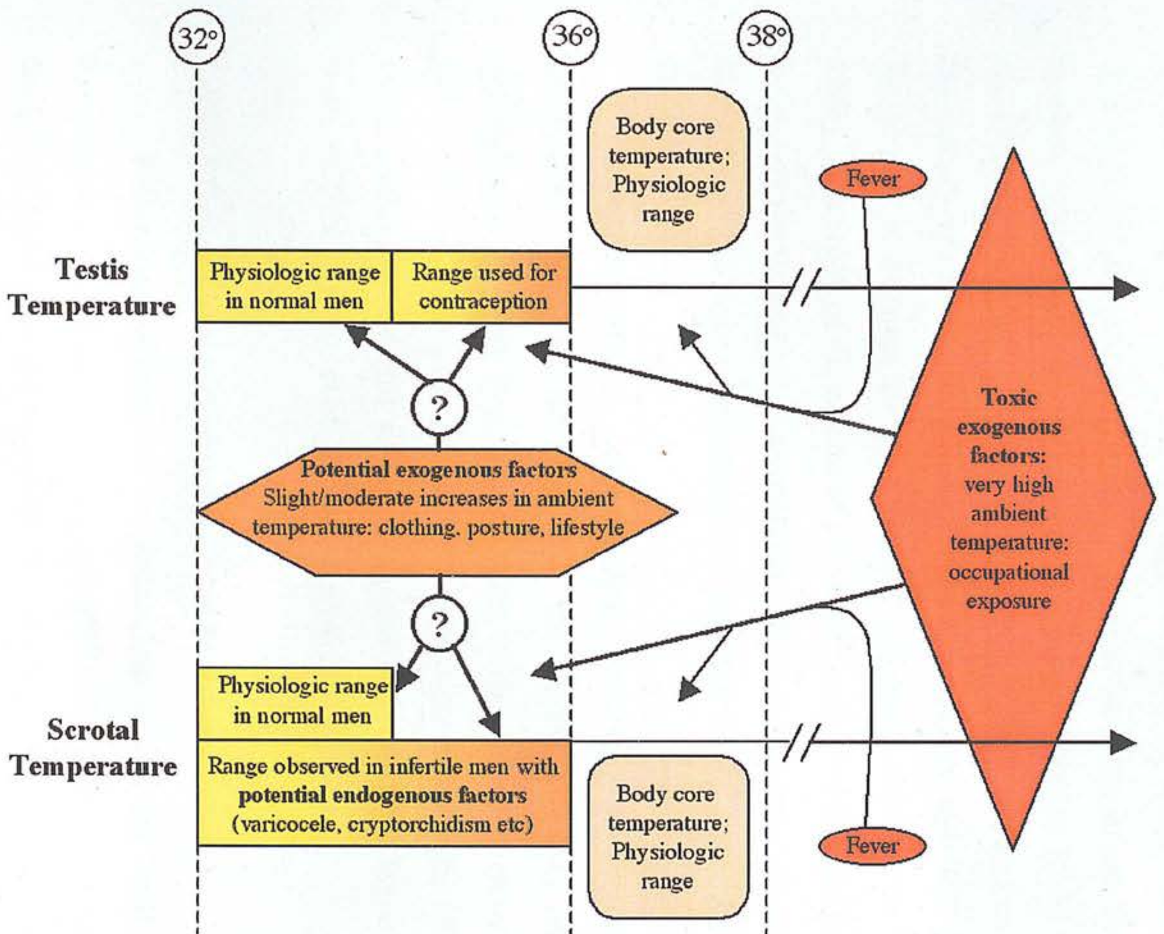


Figure 1.11 Causes and consequences of raised scrotal temperatures in man.
Figure shows possible changes induced by potential exogenous and endogenous factors on testicular and scrotal temperature in humans.

Adapted from Mieusset, 1995

1.8.2 Animal models of heat stress

Heat stress has been induced in animal models by two methods; a single transient exposure of the testis to heat or surgically-induced cryptorchidism.

In rodents, a single exposure of heat stress may be administered by the emersion of the scrotal region, hind legs and tail of the animal in a water bath for the desired period of time. In a number of studies, the testes of rats have been subjected to temperatures in the range of 39-43°C for periods between 15-30 minutes. (Galil, *et al.*, 1988;Setchell, *et al.*, 1988;Blanco-Rodriguez, *et al.*, 1998;Lue, *et al.*, 2000). In the mouse, effects of temperatures within the range 39 and 42°C have been studied for periods of between 20 and 30 minutes (Jannes, *et al.*, 1998;Nishiyama, *et al.*, 1998). The study of the effects of prolonged heat stress (longer than 30 minutes) is limited by the constraints of anaesthesia. Other studies have examined the effects of increased whole-body temperature and humidity on the testes and fertility of mice (Bellve, 1972).

Surgically-induced cryptorchidism involves the translocation of the reproductive tract from the scrotum to the abdominal cavity and ligation of the inguinal canal, thereby exposing the testis to abdominal temperatures for extended periods of time. Using this procedure, it is possible to study the effects of heat stress on the testis or epididymis alone, or together (Glover, 1960;Jegou, *et al.*, 1983;Yin, *et al.*, 1997;Nishiyama, *et al.*, 1998;Yin, *et al.*, 1998).

1.8.3 Effects of heat stress on testicular function

Following experimental cryptorchidism in the rat, testis weight dropped significantly within 4 days, reaching a plateau at 2 weeks. This weight loss is due to the loss of germ cells and decreased fluid production by Sertoli cells. Heat-induced Sertoli cell dysfunction may also result in the altered function of Leydig cells (Leydig cell hypertrophy) (Jegou, *et al.*, 1983).

Significantly increased levels of apoptosis observed in stages II-III, XI, XII, XIII and XIV in the rat following heat stress. Cell types most susceptible to heat-

induced apoptosis are primary pachytene spermatocytes, primary metaphase spermatocytes and secondary spermatocytes, and to a lesser degree, round spermatids and A2-A4 spermatogonia (Lue, *et al.*, 1999). A1, intermediate and B spermatogonia appear resistant to heat-induced apoptosis (Blanco-Rodriguez, *et al.*, 1998). In the mouse, it has been shown that germ cell loss (via apoptosis) following heat stress is mediated via p53-dependant and -independent pathways (Yin, *et al.*, 1997;Yin, *et al.*, 1998).

Further studies in which cell loss has been quantified have identified pachytene spermatocytes and early spermatids as being susceptible to heat stress (Yin, *et al.*, 1997). In addition, two critical periods in spermatogenesis (leptotene-pachytene and maturation division) have been identified through which cells were unable to progress following heating (Collins, *et al.*, 1969;De-Vita, *et al.*, 1990).

Following heating, the expression of a number of genes in the testis is down regulated. In particular, decreased expression of oxidative stress response genes following heating may leave cells more susceptible to oxidative DNA damage. Genes involved in DNA repair and recombination are down-regulated by heat stress, therefore it is possible that heated germ cells may be less capable of repairing heat-induced strand breaks/lesions. Other genes affected by heat stress include many involved in cell cycle regulation, glutathione metabolism and stress response (Barroso, *et al.*, 2000;Rockett, *et al.*, 2001).

Heat stress results in decreased vascular resistance and increased blood flow in the testis of the rat. Vascular resistance in the testis is decreased significantly following exposure to 43°C (Setchell, *et al.*, 1995).

Heat stress may affect the DNA integrity of spermatozoa in a direct or indirect manner. It has been shown that heat stress leads to abnormal chromatin packaging in spermatozoa, which may in turn cause DNA damage (Sailer, *et al.*, 1997).

As discussed previously (section 1.5.1.1), DNA strand breaks occur during spermiogenesis to facilitate the process of chromatin condensation. The disruption of spermiogenesis (i.e. following heat stress) may result in the failure of the

spermatids to ligate these strand breaks which could persist into the mature spermatozoa (Manicardi, *et al.*, 1995).

1.8.4 Effects of heat stress on epididymal function

The effects of raised temperature on the epididymis have been observed in a number of animal models and studies have shown that spermatozoa located within the epididymis at the time of heating are affected.

Following heat stress, the epididymis loses its ability to store and maintain viable spermatozoa, resulting in the gradual and progressive accumulation of dead, decapitated and immotile spermatozoa (Glover, 1960;Jegou, *et al.*, 1983;Mieusset, *et al.*, 1992;Bedford, 1994).

The cauda appears to be the region of the epididymis most affected by heat stress, decreasing in size, and therefore storage capacity, resulting in decreased numbers of viable spermatozoa in the ejaculate (Glover, 1960;Bedford, 1978;Bedford, 1991).

Heat stress has been shown to affect the epididymis in a number of ways. As discussed earlier, oxygen is required in the epididymis to successfully sustain and store spermatozoa and raised temperatures would reduce the amount of oxygen available to the epididymis, thereby decreasing its storage capacity. (Djakiew, *et al.*, 1986)

The water and ion transport mechanisms of the epididymis, in particular the resorption of Na^+ and Cl^- and the secretion of K^+, are affected by raised temperature resulting in altered ionic composition of the epididymal fluid (Bedford, 1991)

The biosynthesis of secretory proteins in the epididymis is temperature regulated. Following heating, several characteristic proteins disappear from the lumen and the synthesis of proteins required for the efficient maturation and storage of spermatozoa is altered (Esponda, *et al.*, 1990;Regalado, *et al.*, 1993).

Exposure of the epididymis to abdominal temperature alters the cellular composition of the epididymal epithelium. In particular, the overall number of basal

cells in the caput and corpus regions increases, with many of these basal cells exhibiting increased macrophage expression (Seiler, *et al.*, 2000).

In addition, the epididymal transit time in cryptepididymal rabbits decreases from 10 to 3-4 days and the spermatozoa produced exhibit poor morphology and motility (Bedford, 1978). It has not been shown whether this occurs in other species.

1.8.5 Effects of heat stress on fertilising ability/embryonic development

Bellve (1979) first demonstrated that fertilization and embryonic development was adversely affected by the heat treatment of mice. Male and female mice were subjected to whole-body heat treatment (34.5°C, 65% relative humidity for 24 hours). Pre- and post-implantation loss of embryos was observed in both treated and non-treated females mated to treated males, indicating that heat treatment of the male mice alone was sufficient to cause embryo loss. Spermatozoa parameters of heat-treated males were not measured in this study.

More recently, a number of studies have demonstrated a direct association between raised scrotal temperature and fertilisation failure/poor embryonic development.

It has been demonstrated that heated male rats fail to impregnate normal females despite the production of adequate numbers of spermatozoa, and furthermore, litter sizes from females which were successfully impregnated by heated males were significantly reduced (Setchell, *et al.*, 1988).

Jannes, *et al* (1998) demonstrated that mild scrotal heating (42°C for 20 minutes) of the male mouse results in decreased fertilisation rates and embryo weight (Jannes, *et al.*, 1998) and similar effects of scrotal heating have been observed in the ram. Scrotal insulation (resulting in temperatures 1.4-2.2°C above controls) of rams for 16 hours a day for 21 consecutive days, resulted in normal fertilisation rates but increased embryonic mortality in non-treated ewes (Mieusset, *et al.*, 1992).

1.9 Male infertility

In a study of publications from the period 1938-1991 and the evaluation of data from nearly 15,000 men, Carlsen *et al* concluded that there had been a decrease in spermatozoa production over the 50-year period studied (Carlsen, *et al.*, 1992). More recently, this data has been re-evaluated (corrected for specimen collection method, abstinence time, age and percent of men with proven fertility), and the number of publications included in the study increased to include data from the period 1934-1994. The findings of Carlsen *et al* were confirmed and it was concluded that a significant decline in the density of spermatozoa has occurred in this period (Swan, *et al.*, 2000).

A number of reports have reviewed the findings of Carlsen, Swan and others (Hull, *et al.*, 1985;Auger, *et al.*, 1995), and have concluded that further, more detailed and controlled prospective studies are required in order to obtain a true representation of the status of male fertility (Jouannet, *et al.*, 2001;Multigner, *et al.*, 2002). Furthermore, following the Carlsen paper, Bromwich *et al* published a report claiming that the findings of Carlsen *et al* were based on the wrong form of statistical analysis of the data, and that the decline in spermatozoa counts observed was in fact due to a statistical artefact (Bromwich, *et al.*, 1994). This debate was then continued in a letter to the British Medical Journal by Irvine (1994) who proposed that the statistical analysis methods employed by Bromwich *et al* were also inappropriate, leading to misinterpretation of their results. Irvine then goes on to discuss the results of a study which assessed the data on 3729 semen samples submitted by healthy volunteers over the period 1940-1969. Data was grouped according to the year of the donors' birth and it was found that the spermatozoa concentration of the men born in the 1940's was significantly greater than that of the men born in the late 1960s. Irvine concluded that though Carlsen's statistical analysis may not have been entirely representative of the facts, the evidence presented for the fall in spermatozoa concentrations is not unconvincing (Irvine, 1994).

A number of studies have clearly refuted the findings of Carlsen *et al* and have demonstrated no fall in semen quality. Paulsen *et al* studied semen volume,

sperm concentration, spermatozoa number per ejaculate and morphology in 510 healthy men between 1972 and 1993. The authors concluded that there had been no decline in semen quality in this population of healthy men over the 21 year period studied (Paulsen, *et al.*, 1996). Handelsman reviewed the existing evidence for falling spermatozoa counts in 2001. The author concluded that there was not sufficient evidence to support a general deterioration in spermatozoa counts and that it is highly unlikely that any deterioration in male fertility could be linked to exposure to environmental estrogens (Handelsman, 2001).

In summary, it is clear that much debate exists regarding the deterioration (or lack of it) of male reproductive health. One of the contributing factors to this debate is the lack of agreement on which outcomes should be measured, which populations should be studied, and perhaps most importantly, how the data obtained should be analysed. Until agreement is reached on these issues, groups from around the world will continue to report conflicting findings which cannot be directly compared against one another.

1.9.1 Assisted reproduction techniques (ART)

The use of assisted reproductive techniques such as in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of couples with so-called male factor infertility.

1.9.2 Selection of spermatozoa for ART

The success of assisted reproduction techniques is dependent on the selection of morphologically normal and motile spermatozoa (Mansour, *et al.*, 1995). Clinical data indicates that the use of poor quality spermatozoa in ART can result in reduced fertilisation rates, fewer blastocysts, and poor embryonic development (Ron-el, *et al.*, 1991;Parinaud, *et al.*, 1993;Janny, *et al.*, 1994;Lopes, *et al.*, 1998).

Loss of DNA integrity within sperm does not affect the ability of sperm to fertilize the oocyte following intracytoplasmic sperm injection (ICSI) (Irvine, *et al.*,

2000). However, it has been shown that DNA abnormalities in spermatozoa can be passed to the offspring following ICSI/IVF treatment. For example, in a number of studies of the spermatozoa and offspring of smoking fathers, it has been demonstrated that smoking can cause oxidative DNA damage in the form of DNA adducts and strand breaks (which can in turn lead to deletions), and that this DNA damage can be passed from the father to the offspring (Ji, *et al.*, 1997;Shen, *et al.*, 1997;Potts, *et al.*, 1999;Zenzes, *et al.*, 1999;Zenzes, 2000).

With the increasing use of ICSI in the treatment of male factor infertility, the risk of oocytes being fertilized with genetically-impaired sperm is greatly increased. The DNA integrity of spermatozoa used in assisted reproduction techniques is not currently assessed.

There are a number of assays available for the detection of DNA damage in spermatozoa (including the single cell gel electrophoresis (Comet) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP- nick end labelling (TUNEL) assays, the sperm chromatin structure assay (SCSA), 8-Hydroxydeoxyguanosine (8-OhdG) measurement, *in situ* nick translation (ISNT) and enzyme-linked immunosorbant assay (ELISA)) (Sailer, *et al.*, 1995;Hughes, *et al.*, 1999;Irvine, *et al.*, 2000;Shen, *et al.*, 2000).

These assays have all been used with success in a number of studies of DNA integrity of spermatozoa from fertile and infertile men. However, these assays can be costly and time-consuming, and it has yet to be proven that their use in the selection of spermatozoa for use in ART is warranted.

1.10 Aims of the project

The main objectives of the work presented in this thesis were:

1. To develop an assay for the study of DNA damage in murine spermatozoa.

2. To study DNA damage in mature, motile spermatozoa from a number of genetically-modified mouse models for male infertility.
   The genetically modified mouse models to be studied were:
   a. Mice deficient in a protein known to be associated with male infertility (dazl).
   b. Mice deficient in a DNA-repair protein known to be highly expressed in the testis (Ercc-1).
   c. Mice found to be infertile due to deficiency in proteins with no known role in the testis/fertility (PrP and PrnD).

3. To study the effects of in vivo heating on developing germ cells and mature spermatozoa with particular emphasis on:
   a. The stress response of the testis and epididymis following scrotal heating.
   b. The number and DNA integrity of motile spermatozoa located within the epididymis at the time of heating.
   c. The number and DNA integrity of motile spermatozoa developed from germ cells present within the testis at the time of heating.

Chapter 2 Materials and methods

2.1 Animals and animal welfare

All animals used in these studies were maintained and treated in accordance with Government guidelines as stated in the Animals Scientific Procedures Act, 1986.

2.1.1 *Dazl* mice

Dazl is an autosomal homologue of the Y chromosome gene *DAZ* which has been implicated in infertility in the human (Yen, *et al.*, 1996;Chang, *et al.*, 1999;Krausz, *et al.*, 1999). In the mouse, the dazl protein is expressed in the cytoplasm of germ cells (Ruggiu, *et al.*, 1997) and has been shown to be essential for the differentiation of these cells (Cooke, *et al.*, 1996;Venables, *et al.*, 2001). Males entirely lacking this protein are infertile and produce no spermatozoa while heterozygous males are fertile but exhibit reduced numbers of spermatozoa with high incidence of morphological abnormality (Ruggiu, *et al.*, 1997). The animals used in this study were sexually mature male +/+ and +/- *dazl* MF1 mice. The genotype of these mice has been described previously by Ruggiu *et al* (1997).

2.1.2 Ercc-1

Ercc-1 (excision repair cross-complementing gene 1) is a gene involved in the nucleotide excision repair (NER) pathway which is responsible for the recognition and repair of DNA damage (Wood, 1999). Within this pathway, *Ercc-1* forms a complex with *xeroderma pigmentosum* factor (XPF; also known as *Ercc-4*). This complex is a structure-specific endonuclease, which incises the damaged DNA strand at the 5' side of the lesion. *Ercc-1* has also been shown to be involved in the process of mitotic recombination (Biggerstaff, *et al.*, 1993).

In *Ercc-1* knockout mice, the NER pathway is lost and mitotic recombination is impaired. The organs most affected by the loss of *Ercc-1* are the liver, kidney and spleen. Death occurs in *Ercc-1* (-/-) mice before the first wave of spermatogenesis is

complete due to liver failure. To allow studies on the role(s) of *Ercc-1* in other organs the liver phenotype has been corrected by introduction of an *Ercc-1* transgene under the control of a liver-specific promoter into the *Ercc-1* null background (Selfridge, *et al.*, 2001).

2.1.3 Prion mice

The animals used were sexually mature male PrP and PrnD mice. The Prion diseases (Scrapie, Creutzfeldt-Jakob Disease) are fatal neurodegenerative disorders which may be either inherited or acquired.

Mice deficient in the Prion protein (PrP) develop ataxia and exhibit altered circadian rhythms (Tobler, *et al.*, 1996) and electrophysiological disorders (Collinge, *et al.*, 1994). PrP variants are expressed both in the testis and the mature spermatozoa of mice (Shaked, *et al.*, 1999;Li, *et al.*, 2000). PrP deficient male mice exhibit normal testicular/epididymal function. However, there have been conflicting findings regarding the fertility of these mice, for example Shaked *et al* have reported that these mice are fertile (Shaked, *et al.*, 1999) while Melton *et al.,* have observed complete infertility (Melton DW, personal communication).

The PrP-related gene doppel (*PrnD*) encodes a Doppel protein (Dpl), which is normally expressed at low levels in the brain and is highly expressed in the testis of the sheep, cow and mouse (Silverman, *et al.*, 2000;Tranulis, *et al.*, 2001). In the mouse, Dpl is expressed during the late stages of spermiogenesis and sperm from Dpl deficient (*PrnD -/-*) male mice have been shown to be immotile, malformed and unable to fertilise eggs *in vitro* (Behrens, *et al.*, 2002).

2.1.4 Animal accommodation

All animals were housed at University of Edinburgh Animal Facilities (George Square or King's Buildings) and were maintained under standard conditions of 12 hour (h) light/ 12 h dark cycle, in an ambient temperature of 20-25°C. Animals had access to food (Standard Mouse Diet) and water (domestic supply) *ad libitum*.

2.1.5 Animal genotyping

2.1.5.1 Dazl mice

Genotyping of the *dazl* mice was performed by Ms Joanne McVerry (MRC Human Reproductive Sciences Unit, Edinburgh).

DNA was prepared from tail tips taken from mice after weaning (at 21 days). A small section (2mm) of tail was placed in a 500μl Eppendorf tube with 100μl NaOH/0.2mM EDTA, and incubated at 95°C for 20 minutes (min) before adding 100μl 40mM Tris-HCl. Tubes were then vortexed before storing at –20°C.

The primers used for the PCR reaction were as follows:

dazl U660	CAGTGGCTTTTGGAAATTATCA	22 bases
dazl U661	GCTTCCTCTTGCAAAACCAC	20 bases
dazl U662	CCTCCTCCACCACAGTTCCA	20 bases
dazl U663	TGATTTCAGCTTAGCATAAACAGC	24 bases

Each PCR reaction tube contained 5μl 10x PCR Gold Reaction Buffer (750mM Tris/HCl (pH 9.0), 200 mM $(NH_4)_2SO_4$, 0.1% TWEEN-20, 15mM $MgCl_2$; Hybaid), 8μl dNTP mix (Hybaid), 0.6μl U660.U661 primer, 0.6μl U662.U663 primer, 31.55μl H2O, 4μl MgCl, 0.25μl Ampli Taq Gold (Perkin-Elmer LOCATION), and 5μl DNA from tail digest. Tubes were placed in an OmniGene PCR machine and subjected to the following programme:

95°C for 10 min x1
94°C for 4 min x1

94°C for 1 min
60°C for 45 seconds } x35 cycles
72°C for 1 min

72°C for 10 min x1

The PCR product (with Orange G loading buffer; Promega, UK) was run on an agarose gel (2%, TBE buffer, 90 volts) until the dye front had travelled 2.5cm. Results were interpreted as shown in Figure 2.1.

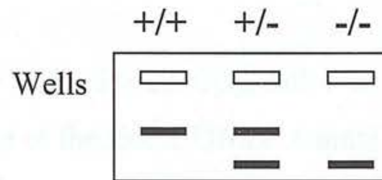


Figure 2.1 Diagram of agarose gel showing location of bands following genotyping.

2.1.5.2 Ercc-1 mice

Genotyping of *Ercc-1* mice was performed by Ms Carolanne MwEwan (Institute of Cell and Molecular Biology, University of Edinburgh). A triple PCR method was used to identify *Ercc-1* wild-type and knock-out animals possessing the *Ercc-1* transgene which restored liver-function in these animals, thereby increasing their lifespan into sexual maturity.

The ERCC1 genotypes were identified using the following primers:

ERCC1 gene	= CCAGTGTTGAAGTTTGTGCG	20 bases
Transgene	= CGAAGGGCGAATTCTTCCCC	20 bases

2.1.5.3 Prion mice

Genotyping of the PrP and PrnD mice was performed by Derek Paisley (Institute of Cell and Molecular Biology, University of Edinburgh). A standard PCR reaction was performed for the identification of both genotypes (see above).

The PrP null animals were identified using the following primers:

PrP intron 2	= AATCGCCACCTGCATTAGGG	20 bases
PrP exon 9	= AGCCTACCCTCTGGTAGATTGTCG	24 bases

The PrnD null animals were identified using the following primers:

Prnd exon 1	= GATGCTAGGAGCCTGCTCATTCC	23 bases
PrP exon 9	= AGCCTACCCTCTGGTAGATTGTCG	24 bases

2.1.6 Sacrifice of animals

Animals were killed by inhalation of CO_2, followed by cervical dislocation in accordance with Schedule One of the Home Office Animal Act.

2.2 Treatments

2.2.1 Anaesthetic

Animals were anaesthetised with an intraperitoneal injection of Hypnorm® (fentanyl citrate 0.315mg/ml, fluanisone 10mg/ml; Janssen, UK) and Hypnovel® (midazolam hydrochloride; Roche, UK). Both were diluted 1:1 with distilled water and the resulting dilutions mixed 1:1 to give the final anaesthetic. All animals were weighed and the amount of anaesthetic given was determined by body weight as shown in Table 2.1.

Animal Weight (g)	Volume of Anaesthetic (ml)
<45	0.28
45-50	0.29
>50	0.30

Table 2.1 Volume of anaesthetic given to animals before testicular heating.

2.2.2 Testicular heating method

A circulating water bath was cleaned and filled with fresh water before each use and the water heated to 42°C (+/- 0.5°C). Following anaesthesia, the lower part of the body (i.e. the hind legs, tail and scrotum) of each animal was passed through a hole in a polystyrene 'raft' (Figure 2.1, 2.2) and the animal was secured using a piece of rubber band and 2 needles (Figure 2.3). The raft was placed in the water bath for 30 min. The animals were removed from the raft, dried and returned to their cages which were placed on a warm mat to facilitate the recovery of the animals from the anaesthetic.

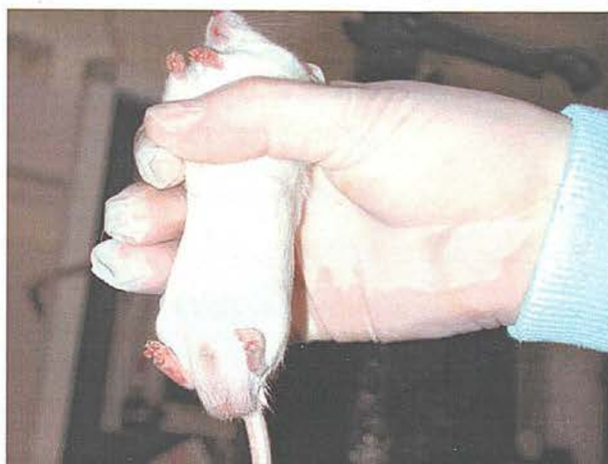


Figure 2.2 Anaesthetised mouse showing exposed scrotum

Figure 2.3 Position of mouse on polystyrene raft

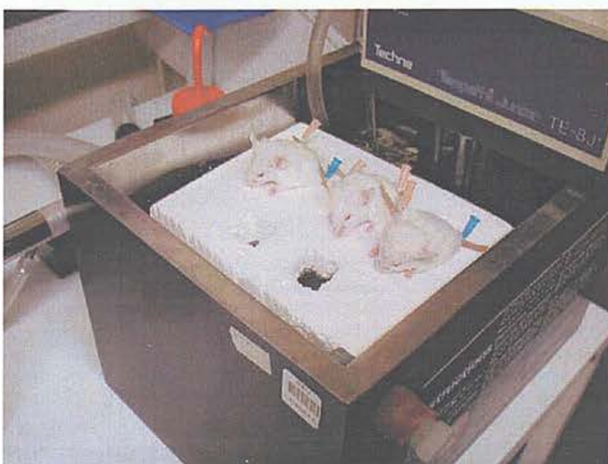


Figure 2.4 Position of mice on polystyrene raft in water bath showing rubber bands and needles holding them in position

2.3 Methods for tissue fixation, processing and staining

2.3.1 Tissue fixation for paraffin embedding

Testes and epididymides were carefully removed and placed in Bouin's fixative (500ml 40% v/v formaldehyde, 100ml acetic acid and 2 l saturated picric acid) for 10 h and 6 h respectively and then transferred into 70% ethanol until embedding in paraffin wax. The tissue was orientated so that horizontal cross sections of the testis and longitudinally cross sections of the epididymis could be achieved.

2.3.2 Tissue processing and sectioning of paraffin blocks

Using a 17.5 h automated cycle on a Leica TP-1050 processor (Leica UK Ltd, Milton Keynes, UK), tissue was dehydrated through a series of graded alcohols before being saturated and embedded in paraffin wax. To increase the adherence of the tissues to the microscope slides prior to immunohistochemistry, the slides were dipped twice in (v/v) 3-aminopropyl triethoxysaline (TESPA, Sigma, Nottinghamshire, UK) made 4% (v/v) in acetone (BDH, Poole, UK), washed in acetone, rinsed in double distilled water and dried overnight. Paraffin sections (5μm) were cut using a hand-operated microtome (Jung RM2035; Leica) with disposable blades. Sections were floated in a heated water bath (containing distilled water) at approximately 50°C. The sections were transferred onto slides and dried in a 50°C oven overnight before use.

2.3.3 Haematoxylin and eosin (H&E) staining

Harris's Haematoxylin was prepared by dissolving 50g potassium allum in 500 ml warm distilled water and combining with 2.5g haematoxylin (BDH) previously dissolved in 25 ml absolute alcohol. The resulting mixture was rapidly brought to the boil and 1.25 g mercuric oxide (BDH) added. The solution was cooled by plunging the flask into a sink containing cold water and ice. 20 ml acetic acid was added and the stain was then filtered before use. A 1 % (w/v) Eosin Y (BDH) solution was prepared using distilled water to which 0.5 ml/l acetic acid was added.

2.4 Immunohistochemistry

Immunohistochemistry was used to identify and localise specific proteins within cells using antibodies raised against the protein under investigation. In brief, an enzyme-linked secondary antibody was used to amplify the signal of the primary antibody bound to the target antigen allowing visualisation under a microscope.

2.4.1 Immunohistochemistry protocol for paraffin sections

Paraffin wax was removed from sections by washing in 2 changes (5 min each change) of xylene (BDH). Sections were rehydrated by washes in decreasing concentrations of ethanol (100%, 95%, 75% (v/v) and finally water) and then incubated at room temperature for 30 min in a bath of methanol/hydrogen peroxide. (methanol 270 ml: H_2O_2 30 ml, Sigma). If antigen retrieval was required to make epitopes available, slides were pressure-cooked in either citrate (0.01M, pH 6.0, Sigma) or glycine (0.05M, pH 3.5, Sigma) buffer for 3 min, followed by cooling for 20 min (Norton, *et al.*, 1994). Slides were washed briefly in tap water then in 2 changes of Tris Buffered Saline (TBS, Sigma; 5 min each change). Slides were removed individually from TBS and any excess fluid carefully removed using paper towels. Slides were placed in a humidity chamber containing a small amount of dH_2O to prevent the sections from drying out during incubation.

Sections were covered with 50-100 µl (depending on the size of the section) blocking serum (16 ml TBS, 4 ml normal rabbit serum; Diagnostics Scotland, Carluke, UK, 1 g bovine serum albumin; Sigma) and incubated for 30 min at room temperature. Slides were again washed twice in TBS and then returned to the humidity chamber. 50-100 µl primary antibody at optimum dilution (See relevant chapters for details) in blocking solution was added to each section and the humidity box sealed and incubated at 4°C overnight. Negative control slides were incubated with either species-specific control serum or pre-immune serum.

To visualise bound primary antibody, slides were washed in TBS as before and then returned to the humidity box. Biotinylated second antibody was added at the appropriate dilution (See relevant chapters for details) in blocking serum and the

slides were incubated at room temperature for 30 min. Following further washes in TBS, Avidin-Biotin Complex (ABC; Dako Ltd, Cambridgeshire, UK) was added to each section and again incubated at room temperature for 30 min. Slides were washed for the final time in TBS before the addition of 3,3'-diaminobenzidine tetrachlorideH_2O_2 (DAB; Dako).

Sections were studied under a microscope until the coloured reaction product (brown) appeared. Over-development of the colour reaction was prevented by placing slides in tap water. Sections were then counter-stained by placing in Harris's haematoxylin for 2-5 min, followed by acid alcohol and finally Scott's Tap water, rinsing between each step in tap water. Slides were dehydrated through a series of alcohols and cleared in xylene for 5 min before being mounted using Pertex mounting medium (Cell Path, Hemmel Hempstead, UK) and cover-slipped.

2.4.2 Procedure for detection using fluorescence

Sections were de-paraffinised and re-hydrated as previously described and then washed twice in phosphate buffered saline (PBS: 5 min each wash). Incubation with methanol/hydrogen peroxide was followed by further washes in PBS and antigen retrieval if required (as previously described). Again a humidity box was used to prevent sections from drying out during incubations. Sections were covered with 50-100 μl (depending on size of section) of blocking serum (See above) and incubated for 1 h at room temperature.

The blocking serum was replaced with primary antibody at the appropriate dilution (approximately 10x stronger than in DAB detection protocol) and the slides were either incubated for 2 h at room temperature or overnight at 4°C. The slides were again washed in PBS before the addition of the appropriate fluorescent secondary antibody (1:50 in blocking serum) and incubated for 1 h at room temperature in a humidity box. The slides were again washed in PBS and mounted using Citifluor mounting solution (Citifluor Ltd, London, UK) under glass cover slips.

2.4.3 TUNEL

Sections were de-waxed in xylene, rehydrated through a series of alcohols to 70% ethanol and blocked with methanol/hydrogen peroxide as in the immunohistochemistry method (2.4.1). Slides were washed twice (5 min each wash) in PBS (0.01M, pH 7.4) and then incubated with 20 μg/ml Proteinase K (Anachem Ltd, Bedfordshire, UK) in buffer (0.1M Tris, 0.01M EDTA, pH 8) for 10 min at room temperature. Slides were again washed with PBS and placed on an ice-cold tray.

The reaction mix (30mM TRIS/HCl, pH7.2, 140mM Na Cacodylate, 1.5mM CoCl) containing 1μl/ml terminal d-transferase (TdT; Roche), 5μl/ml digoxigenin (Dig; Roche) was added and sections sealed under cover slips with cow gum/hexane then incubated at 37°C for 30 min. The reaction mix added to control slides (1 control slide included in each run) did not include TdT. Following removal of the cover slips and further washes in PBS, sections were blocked with 20% NRS in PBS for 10 min at room temperature. Sheep anti-Dig IgG (1:100 in NRS/PBS) was added and the slides incubated at room temperature for 90 min before further washes in PBS. Rabbit anti-sheep IgG biotinylated (1:500 in NRS/TBS) was added for 30 min at room temperature before washing twice in TBS (5 min each wash).

As in the ABC method, bound antibodies were visualised using ABC conjugated to HRP which was added to the slides and incubated for 30 min at room temperature; sections were washed twice in TBS (5 min each wash) before the addition of DAB. The resulting colour reaction was stopped with water. Sections were counterstained with Mayer's haematoxylin, dehydrated through a series of alcohols ending in xylene, mounted in Pertex and coverslipped.

2.5 Image analysis

2.5.1 Digital photomicroscopy

Tissue sections were examined using an Olympus Provis microscope (Olympus Optical, London, UK) and images captured using a digital Kodak DCS420 camera (Eastman Kodak, Rochester, NY, USA). Captured images were stored on a G4 Apple MacIntosh computer (Apple Computer, CA, USA) and compiled using Photoshop 6.0 (Adobe Systems Inc., CA, USA).

2.5.2 Fluorescent photomicroscopy

Tissue sections were examined using a Zeiss 510 Laser scanning confocal microscope (Carl Zeiss) connected to a G4 Apple MacIntosh computer. Images were captured using LSM 510 software (Zeiss) and compiled using Photoshop 6.0.

2.6 Methods for the preparation of spermatozoa

2.6.1 Preparation of Biggers, Whitten and Whitingham solution

Biggers, Whitten and Whitingham (BWW; Biggers, 1971) solution was prepared fresh every other day and stored at 4°C. The solution was made to the following recipe; 90mM NaCl (BDH), 4.5mM KCL (BDH), 1.6mM $CaCl_2$ (BDH), 1.1mM KH_2PO_4 (BDH), 1.1mM $MgSO_4.7H_2O$ (BDH), 25mM $NaHCO_3$ (BDH), 5.6mM glucose (Sigma), 55μM sodium pyruvate (Sigma), 0.2% sodium lactate (Sigma), 20000 IU penicillin/streptomycin (Calbiochem, UK), 20mM Hepes buffer (Gibco, Life Technologies, UK), 0.3% human albumin solution (Immuno Ltd, Kent, UK) and then corrected to pH 7.6.

2.6.2 Preparation of human spermatozoa (centrifugation)

Semen was allowed to liquefy at room temperature for 20 min and then diluted (1:9) with BWW before centrifugation at 500g (1900rpm) for 5 min. The supernatant was removed and discarded and the pellet resuspended in 7-10ml BWW

before a further centrifugation at 500g (1900rpm) for 5 min. This process was repeated and the pellet resuspended in a known volume of BWW.

2.6.3 Preparation of human spermatozoa (percoll gradient)

A percoll gradient was prepared by placing 3ml 100% Percoll (10ml 10x Earle's balanced salts solution (Flow labs, Irvine, UK), 90ml Percoll (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK), 1.5ml 20% Abuminar (Armour Pharmaceutical Company, Eastbourne, UK), 3mg sodium pyruvate (Sigma), 0.37ml sodium lactate (Sigma), 200mg $NaHCO_3$ (BDH), and 1ml penicillin/streptomycin (Gibco)), followed by 3ml 50% Percoll (100% Percoll diluted 1:1 in BWW) in a tube, taking care not to mix the layers. A further layer of 2ml semen sample was added and the gradient centrifuged for 20 min at 1900 rpm (500g).

The layer of seminal plasma was removed and stored as required. Using a Pasteur pipette, 50% quality spermatozoa was carefully removed from the 50%/100% interface and the 100% quality spermatozoa removed from the bottom of the tube. The samples of spermatozoa were then diluted (in 7-10ml BWW) separately and centrifuged for 5 min at 1900rpm (500g). The supernatant was removed and discarded, and the spermatozoa pellets resuspended in a known volume of BWW.

2.6.4 Preparation of murine spermatozoa

Whole epididymides were carefully dissected out and placed in BWW taking care to avoid contamination with blood and fat. The tissue was placed in a 500μl eppendorf tube, minced using a pair of fine scissors and incubated at 30°C for 30 min to allow the tissue debris, dead and immotile spermatozoa to sink to the bottom of the tube and motile spermatozoa to 'swim-up'. The buffer containing motile spermatozoa was carefully removed and diluted to a total volume of 1ml in BWW.

2.6.5 Storage of murine spermatozoa

Diluted spermatozoa were aliquoted (100μl per aliquot) and stored at –20°C. Before use, aliquots were slowly defrosted at room temperature and shaken gently to

redistribute spermatozoa. Each aliquot was used a maximum of 2 times to prevent damage to the spermatozoa by repeated freeze-thawing.

2.6.6 Study of concentration of spermatozoa

Samples were diluted 1:10 with spermatozoa diluting fluid (SDF; 50g $NaHCO_3$, 10ml Formalin, dH_2O to 1L) and 10µl diluted sample was placed into the chamber of an improved Neubauer haemocytometer (BDH). The number of spermatozoa located within 5 squares of the haemocytometer grid was carefully counted. If the number of spermatozoa in 5 squares was not equal to or greater than 100, 10 or 25 squares were counted. From this value, the number of spermatozoa in 1ml was calculated using the following equation:

$$\text{Concentration} = \text{No. spermatozoa counted} \div \frac{(4 \times \text{No. Squares})}{\text{Dilution Factor}}$$

2.6.7 Study of morphology of spermatozoa (DiffQuik staining)

For each sample to be studied, 2 microscope slides were prepared by washing in 100% ethanol, then water, and drying in an oven. 10µl sample was pipetted onto the first slide and the second was used to drag the sample across the slide, creating a smear which was then allowed to air-dry. This process was repeated a total of 3 times in order to obtain a high concentration of spermatozoa on each slide. The slides were fixed in ether/alcohol fixative (70% alcohol, 30% ether) and allowed to air-dry.

Each slide was dipped 10 times into Diffquick fixative and the excess solution removed by placing the edge of each slide onto an absorbent tissue and then wiping the underside. Each slide was then dipped 20 times into Diffquick I solution, drained of excess solution as before, dipped 20 times into Diffquick II solution, again drained of excess solution, and finally dipped in clean water to remove any remaining solution. Slides were allowed to air-dry before mounting using Pertex and a cover slip.

2.7 *In vitro* heating of spermatozoa

A water bath was heated to 42°C. An aliquot (100ml in an Eppendorf) of each sample was carefully sealed, placed in a float and incubated in the water bath for 10 min. Following heating, each aliquot was used once (for the Comet assay; see below) and then discarded.

2.8 Single cell gel electrophoresis (Comet) assay

2.8.1 Comet assay (Kit method)

Spermatozoa samples were defrosted at room temperature and 5μl each sample was mixed with 25μl LMA (37°C; Trevigen). This gel/sample mix was then dropped onto a CometSlide (Trevigen) and covered with a clean, warm (37°C) cover slip. Slides were placed horizontally in a box and incubated at 4°C until the gels were set (~10 min). Cover slips were carefully removed and the slides submerged in lysis buffer (pH13.5, 0.75% SDS, Lysis buffer; 2.5M NaCl, 100mM EDTA, 10mM Tris, 1% sodium lauryl sarcosinate, 0.01% Triton X-100; Trevigen) for 3h at 37°C.

The slides were removed from the lysis buffer and placed in a horizontal gel electrophoresis tank. Alkaline electrophoresis buffer (3M NaOH, 1mM EDTA, pH12.3) was carefully added to a depth of 0.5cm above the slides and left for 20 min at room temperature. Current was applied at 25V, 300mA for 10 min. The slides were then transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min). The slides were then allowed to dry overnight at room temperature.

2.8.2 Comet analysis

The slides were stained with 50μl ethidium bromide (1:1000 in dH_2O; Sigma) and viewed using a Zeiss Axiostar microscope (Zeiss, Germany).

For each sample, 50-200 cells (see later chapters for details) were analysed using the Komet Image Analysis system, version 4.0 (Kinetic Imaging Ltd). The percentage of head DNA, tail DNA and the Comet Moment was calculated for each

cell. Comet Moment is a measurement that takes into account both the length of the Comet tail and the amount of DNA present in the tail and is expressed as an arbitrary unit (the greater the value, the higher the level of DNA damage).

Chapter 3

Development of a Comet assay for use with murine spermatozoa

3.1 Introduction

DNA damage present in spermatozoa may be passed on to the next generation, in the form of mutations, leading to congenital abnormalities and developmental problems. For example, it is known that oxidative damage and DNA adducts are found in the spermatozoa DNA of smokers (Shen, *et al.*, 1997;Potts, *et al.*, 1999). It has also been shown that genetic abnormalities can be passed on to the oocyte leading to developmental abnormalities and increased risk of childhood cancers in the offspring of cigarette-smoking fathers (Ji, *et al.*, 1997;Zenzes, *et al.*, 1999).

It has been demonstrated that loss of DNA integrity within spermatozoa does not affect the ability spermatozoa to fertilize the oocyte following intracytoplasmic sperm injection (ICSI) (Irvine, *et al.*, 2000) and, with the increasing use of ICSI in the treatment of male factor infertility, the chances of oocytes being fertilized with genetically-impaired spermatozoa are greatly increased. It has been claimed that embryos conceived by ICSI are more at risk from pre-clinical and clinical pregnancy loss then those conceived by conventional IVF (Bar-Hava, *et al.*, 1997).

However, there is conflicting evidence regarding the risk of congenital abnormalities and developmental problems in children conceived via ICSI. Kurinczuk *et al* (1997) proposed that infants conceived by ICSI are twice as likely to have major birth defects and 50% more at risk of minor defects than naturally-conceived infants (Kurinczuk, *et al.*, 1997). Bowen *et al* (1998) found that though infants conceived via ICSI were healthy, they were more at risk of developmental deficiencies than infants conceived either naturally or by IVF (Bowen, *et al.*, 1998). However, there have been a number of studies which have found that infants conceived by ICSI are at no greater risk of congenital or developmental

abnormalities than those conceived naturally or by IVF (Bonduelle, *et al.*, 1999;Sutcliffe, *et al.*, 2001). This conflicting evidence suggests the need for further research into the long-term safety of ICSI. Meanwhile, it would seem prudent to make every effort to ensure that spermatozoa with defective DNA are not used in assisted reproduction techniques and that counseling is provided to individuals with high levels of baseline DNA damage before undergoing ICSI treatment.

At present the genetic integrity of spermatozoa selected for use in assisted reproduction techniques is not assessed, though a number of methods are available for the study of DNA in spermatozoa. A summary of these assays, including the number of cells and time required, the forms of DNA damage detected and the main advantages and disadvantages of each assay are presented in Table 3.1.

Generally these methods can be divided into two groups; methods which measure DNA abnormalities (e.g. adducts, chromatin packaging) and methods which measure DNA damage (e.g. nicks, fragments).

3.1.1 Measures of DNA abnormalities

3.1.1.1 8-Hydroxydeoxyguanosine (8-OhdG)

8-OhdG is an oxidative DNA adduct which is the most commonly studied biomarker for oxidative DNA damage. Levels of 8-OhdG were first measured in the DNA of human spermatozoa by Fraga and collegues (Fraga, *et al.*, 1991). To date, 8-OhdG levels in spermatozoa DNA from a number of populations (control patients, smokers, non-smokers, infertile patients) have been studied.

Higher levels of 8-OhdG are present in the spermatozoa-DNA of fertile compared to infertile patients (Kodama, *et al.*, 1997;Shen, *et al.*, 1999), and the level of 8-OhdG in spermatozoa-DNA correlates with semen parameters (positive correlation with the number of head abnormalities, negative correlation with normal morphology, motility, total spermatozoa number and spermatozoa density) (Ni, *et al.*, 1997;Shen, *et al.*, 1999).

Briefly, spermatozoa membranes are removed by incubating with DTT, proteinase K and SDS, the DNA is extracted with chloroform isoamyl alcohol,

digested with ribonuclease A and then dissolved in Tris-HCL for enzymatic DNA digestion with a number of enzymes including alkaline phosphatase and nuclease P1.

The levels of 8-OhdG in the digested DNA from spermatozoa are then measured using either gas chromatography-mass-spectrometry (GC/MS) or, more commonly, high performance liquid chromatography (HPLC) (Shen, *et al.*, 2000).

3.1.1.2 Spermatozoa chromatin structure assay (SCSA)

The SCSA assay is used to study the susceptibility of spermatozoa chromatin to heat- or acid-induced denaturation in vitro. Following denaturation, the ratio of double- to single-stranded DNA is altered. This is quantified using the metachromatic dye acridine orange (AO), which differentially intercalates into double- (fluorescing green) and single-stranded DNA (fluorescing red). This method was first described by Evenson in 1989 (Evenson, 1989) and has since been used in a number of studies of DNA integrity in mammalian spermatozoa. In particular, this assay has been used to assess the genetic integrity of spermatozoa in infertile patients (Evenson, *et al.*, 1999), smokers (Potts, *et al.*, 1999), and cancer patients (Fossa, *et al.*, 1997;Kobayashi, *et al.*, 2001).

3.1.2 Measures of DNA integrity

3.1.2.1 Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay is an immunochemical method for the quantitative detection of DNA damage in a population of cells and has been used to study the induction and repair of DNA at different cellular stages of spermatogenesis of the hamster (Van Loon, *et al.*, 1991) and also in mature human spermatozoa (Hughes, *et al.*, 1999). In this method, single- and double-strand breaks form initiation points for partial unwinding of the cellular DNA under alkaline conditions. The degree of unwinding is then determined by the use of a specific monoclonal antibody which binds to single-stranded DNA. Though this has been shown to be a highly sensitive and effective assay, the need for expensive, specialist equipment for quantification of results is a major drawback (Table 3.1).

Table 3.1 Comparison of several methods used for the detection of DNA damage in spermatozoa (d=days, h=hours) Adapted from Shen *et al* (2000) with additional information from Hughes *et al* (1999 - ELISA), Irvine *et al* (2000 - ISNT) and Evenson *et al* (1999 - SCSA).

Method	No. of Cells Required	Time Required	DNA abnormality Detected	Main Advantages	Main Disadvantages
8-OhdG Analysis	3×10^7	2 d	Base Modifications	High Specificity Quantitative Correlation with spermatozoa function Association with infertility	Large sample Artifacts Special equipment No standard protocols
SCSA	1×10^6	1-2 h	Abnormal chromatin structure single strand breaks	Association with infertility Independent of semen parameters	Non-specific
TUNEL assay	3×10^6	4-5 h	Single strand breaks Double strand breaks	Simple and fast High sensitivity Indicative of apoptosis Correlation with seminal parameters Association with infertility Commercial kits	Special equipment Expensive
ISNT	10×10^6	2-3 h	Single strand breaks Double strand breaks	Correlation with seminal parameters High sensitivity Quantitative	Expensive
ELISA	3×10^5	2-3 d	Single strand breaks Double strand breaks	Simple Quantitative Commercial kits	Special equipment Expensive Time-consuming
Comet Assay	2×10^4	1-2 d	Single strand breaks Double strand breaks	Simple and inexpensive High sensitivity Qualitative Quantitative Correlation with seminal parameters Small sample required Commercial kits	No standard protocols

3.1.2.2 In situ nick translation (ISNT)

The presence of endogenous nicks in the DNA of spermatozoa was first studied in 1995 using *in situ* nick translation (Sakkas, *et al.*, 1995). This method has been successfully used in both murine (Sakkas, *et al.*, 1995) and human (Twigg, *et al.*, 1998;Irvine, *et al.*, 2000) spermatozoa. As with the majority of methods employed to assess DNA integrity, negative correlations between the amounts of DNA damage detected and semen parameters (total spermatozoa, motility, morphology etc) have been observed.

A number of ISNT protocols have been developed; several have been modified specifically for use in spermatozoa (Sakkas, *et al.*, 1995;Irvine, *et al.*, 2000). An example of one of these modified methods is as follows: in brief, spermatozoa are incubated with DTT before being fixed using ethanol/glacial acetic acid and then placed on a microscope slide and allowed to air-dry. SDS is then added to the slides in order to remove the spermatozoa membranes, thereby exposing the DNA. Endogenous biotin/avidin binding sites within the spermatozoa are blocked using sequential incubations in avidin and biotin. DNA polymerase is used to join biotin-linked 16-dUTP to the sites of DNA nicks. The biotin can then be detected using detection methods such as streptavidin fluoroscein-isothiocyanate (FITC) or streptavidin alkaline phosphatase (Irvine, *et al.*, 2000).

3.1.2.3 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP- nick end labelling (TUNEL) assay

The TUNEL assay is a one of many *in situ* end-labelling methods originally developed to study apoptosis. These methods involve the incorporation and detection of biotinylated nucleotides at the 3'-OH ends of DNA strand breaks (Gavrieli, *et al.*, 1992;Wijsman, *et al.*, 1993). As the name suggests, the incorporation of biotinylated nucleotides in the TUNEL assay is mediated by terminal deoxynucleotidyl transferase (TdT). Commercial kits are available for this assay.

3.1.2.4 Single cell gel electrophoresis (SCGE or 'Comet') assay.

In the present study, DNA damage in murine spermatozoa was to be determined using the Comet assay which has been shown to give highly reproducible results in the study of DNA damage in human spermatozoa (Hughes, *et al.*, 1996;Hughes, *et al.*, 1997;McKelvey-Martin, *et al.*, 1997;Shen, *et al.*, 1997).

The Comet assay has been used in a number of studies of human spermatozoa. These studies include the comparison of spermatozoa from fertile and infertile men (Hughes, *et al.*, 1996;Irvine, *et al.*, 2000;Shen, *et al.*, 2000), spermatozoa retrieved from the epididymis and from the testis (Steele, *et al.*, 1999;Steele, *et al.*, 2000), and the effects of exogenous factors (e.g. preparation and cryopreservation during assisted reproduction methods) (Anderson, *et al.*, 1997;Donnelly, *et al.*, 2000), cancer drugs (e.g. fludarabine) (Chatterjee, *et al.*, 2000) and oestrogens (Anderson, *et al.*, 1997).

In 1978, Rydberg and Johanson first described a method for studying DNA integrity in eukaryotic cells which formed the basis for what is now known as the single cell gel electrophoresis assay (SCGE, or the 'Comet assay') (Rydberg, *et al.*, 1978). This method involved embedding cells in agarose, subjecting them to mild alkaline conditions and then studying the ratio of green (indicating double stranded DNA; dsDNA) to red (indicating single stranded DNA; ssDNA) fluorescence following staining with acridine orange (Rydberg, *et al.*, 1978). The method was later expanded to include an alkaline electrophoresis stage which caused the migration of DNA from the cell nucleus (Ostling, *et al.*, 1984). In addition to improving the sensitivity of the assay, Ostling *et al* also demonstrated the potential use of the assay as a genotoxicity test, using the assay in a study of the dose-dependant effects of gamma ray exposure on the DNA integrity of murine lymphomas cells (Ostling, *et al.*, 1984). In subsequent years, the assay has been modified and optimised for study of many different cell types, most commonly lymphocytes, fibroblasts and other somatic cell types (McKelvey-Martin, *et al.*, 1993).

In the spermatozoa head, DNA is tightly packaged in association with highly basic proteins (protamines) rather than in the looser octamer arrangement encircling histones as seen in somatic cells. (Ward, *et al.*, 1991) (See section 1.3.2.2.4). These differences have made it necessary to modify the Comet assay specifically for this cell type, most notably by the introduction of a proteinase K digestion stage to remove the protamines and expose the DNA for study (Hughes, *et al.*, 1996).

When this project was initiated, a Comet assay for murine spermatozoa was not available. Therefore the aim of the experiments described in this chapter was to devise a sensitive and reliable Comet assay protocol to allow evaluation of spermatozoa DNA integrity in the mouse.

3.2 Materials and Methods

3.2.1 Preparation of spermatozoa

Full methods for the preparation of human and murine spermatozoa are described in section 2.6.

Human spermatozoa samples were used as positive controls during the development of the murine Comet assay. Semen samples were obtained from 3fertile donors and the spermatozoa prepared using either the Percoll gradient or centrifugation methods as described in sections 2.6.1 and 2.6.2. The samples from 3 donors were pooled together and then divided into aliquots for storage.

Murine spermatozoa were obtained from wild-type mice (*dazl* +/+, normal fertility (Ruggiu, *et al.*, 1997)). Briefly, whole epididymides were removed, cleaned of fatty tissue and placed in 1ml BWW (as previously described – section 2.6.4). The tissue was then minced with fine scissors and incubated at 34°C (95% air, 5% CO_2) for 30 min prior to careful removal of buffer containing the motile spermatozoa. Samples were diluted (to a final volume of 1ml) and stored in BWW at –20°C until required. The samples from 3 mice were pooled together and then divided into aliquots for storage.

3.2.2 Development of a Comet Assay for murine spermatozoa

Three approaches were taken to develop a stable and reproducible Comet assay for use on murine spermatozoa; firstly, the modification of an existing protocol used for the study of human spermatozoa, secondly, the development of a novel method, and finally, the modification of a commercially available Comet assay kit.

3.2.2.1 Modification of the Human Spermatozoa Comet Assay

A Comet assay method used for the study of human spermatozoa had been developed previously in our laboratory (Irvine, *et al.*, 2000) and the initial aim of this work was to adapt this assay protocol for use on murine spermatozoa. The original method for the human spermatozoa Comet assay is described below and the modifications made to this method are detailed in section 3.3.1 of this chapter.

Normal microscope slides were prepared by dipping in 1% normal melt agarose (NMA; Sigma), wiping off any excess and air-drying overnight. Prepared slides were stored in racks contained within an airtight box. Cover slips were placed in racks and washed with 70% ethanol, then water, and dried in an oven overnight.

Each prepared slide was coated with 160μl 0.6 % (w/v) NMA/PBS and then covered with a washed cover slip. This first gel layer was allowed to set by placing the slides on a cool tray (over ice) for ~20 min before carefully removing the cover slips. Spermatozoa samples were adjusted to a concentration of ~6.6 million/ml by diluting in BWW medium. 10μl (approximately 6.6×10^3 cells) of each corrected sample was then mixed with 75μl of 0.5% low melt agarose in PBS (LMA; Sigma) at 37°C and the mixture added to the slide. A new cover slip was carefully added to spread the agarose/spermatozoa suspension across the slide forming a second gel layer. The slides were again chilled on a cool tray to allow the new gel/cell layer to set (~5 min).

The cover slip was removed before the addition of the third and final gel layer (100μl LMA/PBS, 37°C). A fresh cover slip was added and the slides chilled on a cool tray (~5 min) before incubation at 4°C for 1 h to allow the gels to set fully. Thereafter, the cover slips were once again removed and the slides placed in a bath

of chilled (4°C) complete lysis solution (2.5M NaOH, 0.25M EDTA, 10mM Tris, 10% DMSO, 1% Triton X, pH 10) for 1 h at 4°C. The lysis buffer was then carefully drained from the slides and replaced with 0.1mg/ml proteinase K (PK) in decondensation buffer (2.5M NaOH, 0.25M EDTA, 10mM Tris, 10% DMSO, pH 7.4); the slides were then incubated in this solution overnight (16 h) at 37°C.

The next day, the slides were removed from the decondensation buffer and placed in a horizontal gel electrophoresis tank (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK). Alkaline electrophoresis buffer (3M NaOH, 1mM EDTA, pH 12.3) was carefully added to a depth of 0.5cm above the slides and allowed to sit undisturbed for 20 min at room temperature. The purpose of this alkaline stage was to allow the DNA to unwind and the DNA strands to separate. Current was applied at 300mA, 25V (0.66V/cm) for 4 min resulting in the migration of fragmented DNA from the cell nucleus. The slides were removed from the electrophoresis tank and neutralised by placing them in a coplin jar filled with 400 mM Tris (pH 7.4) buffer for 5 min, replacing this with fresh buffer a total of 3 times. The slides were transferred to a new Coplin jar filled with ethanol for 5 min, then removed, drained and allowed to dry overnight at room temperature. Staining with ethidium bromide enabled the visualisation of the DNA under the microscope.

3.2.2.2 Development of novel Comet assay for murine spermatozoa

Data presented by Qiu *et al* (1995) described a method for the preparation of demembranated rat spermatozoa for use in decondensation studies following cyclophosphamide treatment. In order to lyse the spermatozoa, they incubated the cells in lysis buffer (50mM Tris, 1mM EDTA, pH 7.4) containing 1% SDS for 10 min at 21°C. In contrast to Triton X (a non-ionic detergent which leaves the cell nucleus intact), which was used in the human spermatozoa Comet assay (see above), Qiu *et al* used SDS, an ionic detergent that lyses both the outer cell membrane and the cell nuclei, releasing the DNA.

In addition to developing a lysis protocol, Qiu *et al* also reported the conditions required for the decondensation of rat spermatozoal DNA. Spermatozoal DNA is not supercoiled in the manner of somatic cell DNA but instead is packaged

in linear, side-by-side arrays as a result of its association with protamines. This protamine-DNA complex is further stabilised by the presence of intermolecular and intramolecular covalent disulphide bonds between the protamines (Ward, *et al.*, 1991). Qiu *et al* determined that complete decondensation of rat spermatozoal DNA occurred only after the reduction of the disulphide bonds between the protamine molecules, followed by the progressive degradation of the protamines molecules themselves. Their protocol involved incubation of the spermatozoa in Tris buffer (50mM, pH7.4) containing PK (0.1mg/ml) and dithiothreitol (DTT, 10mM) for 1 h at 21°C (Qiu, *et al.*, 1995). The DTT present in the buffer specifically reduces disulphide bonds between proteins present within the spermatozoa nucleus which are normally tightly-packaged around the DNA.

We considered that, by combining the lysis and decondensation methods described by Qiu *et al* with the alkali electrophoresis methods used in the human spermatozoa Comet assay, a novel Comet assay for the study of murine spermatozoa could be developed. The initial assay conditions tested are described in full below and the modifications made to this method are presented in section 3.3.2 of this chapter.

As in the human spermatozoa Comet assay previously described (section 3.2.2.1), spermatozoa were embedded in an agarose gel layer on a normal microscope slide. Incubation in a lysis solution (50mM Tris Buffer, 1mM EDTA, 1% SDS, pH 10) for 10 min at 21°C was followed by 3 washes in 50mM Tris (5 min each wash) and then incubation in 50mM Tris (pH7.4) containing 0.1mg/ml proteinase K and 10mM DTT for 1 h at 21°C. Again slides were placed in alkaline electrophoresis buffer for 20 min at 21°C before undergoing electrophoresis followed by dehydration. Staining with ethidium bromide enabled the visualisation of the DNA under the microscope.

3.2.2.3 Modification of method supplied with a Comet assay kit

During the course of the present study, a Comet assay kit designed for use on somatic cells became available from R&D Systems Europe (Abingdon, Oxon, UK). The advantages of this kit were that it contained pre-prepared lysis buffer and

specially treated CometSlides™, which enhanced the adherence of the LMA gel layer (thereby eliminating the time-consuming and often unreliable method of preparing base layers of agarose). The method described below is as recommended by the manufacturers. The modifications made to this method to improve its application to murine spermatozoa are presented in section 3.3.3.

Spermatozoa samples were defrosted at room temperature and 5μl of each sample was mixed with 25μl LMA (37°C; Trevigen). This gel/sample mix was dropped onto each well of a CometSlide (Trevigen) which was then placed horizontally in a sealed box and incubated at 4°C until the gels were set (~10 min). The slides were submerged in lysis buffer (2.5M NaCl, 100mM EDTA (pH10), 10mM Tris, 1% sodium lauryl sarcosinate, 0.01% Triton X-100; Trevigen) for 30 min at 4°C.

The slides were removed from the lysis buffer and placed in alkali buffer (300mM NaOH, 1mM EDTA, pH10) for 20 min at 21°C. The slides were then placed in a horizontal gel electrophoresis tank and covered with alkaline electrophoresis buffer (300mM NaOH, 1mM EDTA, pH10). Current was applied at 300mA, 25V for 20 min. The slides were transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min) and allowed to dry overnight at room temperature. The slides were then stained with ethidium bromide and studied under a fluorescent microscope.

3.2.3 Comet analysis

Following completion of the comet assay, slides were stained with ethidium bromide which intercalated into the DNA released from the spermatozoa cells. Generally, the cell nuclei (Comet "head") retained the greatest proportion of DNA (which therefore fluoresced very brightly) compared to the fragmented DNA which had migrated away from the nucleus, forming the Comet "tail" (see Figure 3.1).

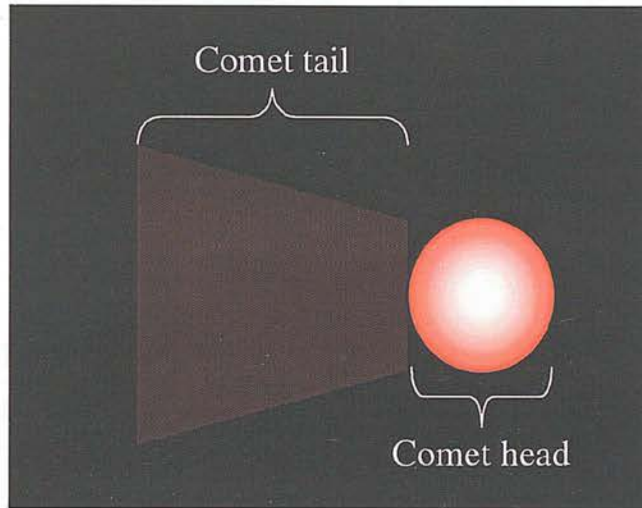
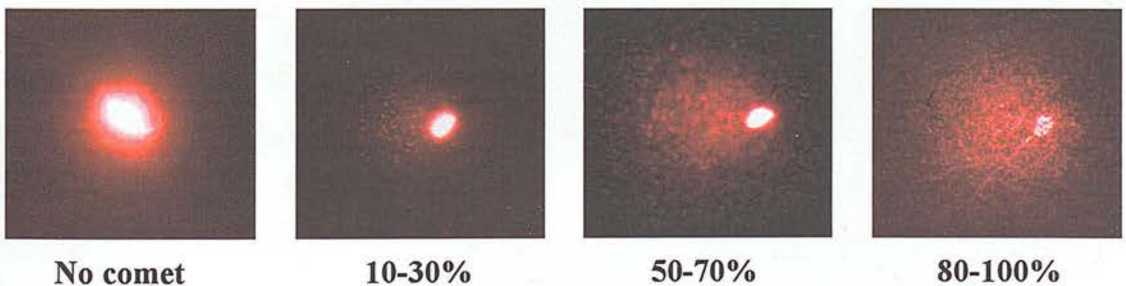


Figure 3.1 Diagram of a comet indicating the head and tail regions

Comet analysis was performed using the Komet Image Analysis system, version 4.0 (Kinetic Imaging Ltd) as discussed in section 2.8.2. Briefly, 100-200 cells (calculation of the number of cells required for analysis is described in section 3.3.2) per sample were analysed. The percentage of DNA (as determined by the degree of fluorescence) in the comet head and tail of each cell was calculated and an average obtained for the population. Examples of comets showing an approximate range of DNA damage (% tail DNA) can be seen in Figure 3.2.



No comet **10-30%** **50-70%** **80-100%**

Figure 3.2 Examples of comets indicating approximate levels of DNA damage (% tail DNA)

3.2.4 Requirements of the Comet assay

The purpose of the assay was to compare the levels of DNA damage in spermatozoa from control animals with that of treated and/or genetically modified animals. Therefore the assay was optimised using spermatozoa from control animals

to obtain a low level of baseline DNA damage (average ~15-20% DNA in the comet tail) within a tight range (0-50%). These levels of baseline DNA damage would enable the measurement of much higher levels of DNA damage in spermatozoa from the test animals.

3.3 Results

3.3.1 Modification of human spermatozoa Comet assay for use on murine spermatozoa

A number of assay conditions were tested using spermatozoa from the pooled samples of 3 mice as previously described. A summary of these conditions and the results obtained can be seen in Table 3.2.

Successful lysis of the spermatozoa membrane was classified by the loss of the characteristic 'hook' shape of the spermatozoa head and the loss of the spermatozoa tail. The lysis conditions used successfully on human spermatozoa (Trials 1-2; Table 3.2) had no visible effects on murine spermatozoa (Figure 3.3) nor did initial modifications of these conditions (Trials 3-11; Table 3.2, Figures 3.4-3.8). These initial modifications included the addition of increasing concentrations (0.1-1mM) of DTT to the decondensation buffer while leaving the lysis stage and incubation times/temperatures unaltered. Murine spermatozoa cells exposed to these conditions retained both their shape and their tails and did not release their DNA for electrophoresis. Modification of the method by increasing the period of incubation in lysis buffer resulted in the successful lysis of the spermatozoa and, together with the addition of low doses of DTT (0-0.25mM) to the PK decondensation buffer, comets were detected after electrophoresis (Trials 12-16; Table 3.2, Figures 3.9 and 3.10).

However, the use of higher concentrations of Triton X (2-5%) and/or DTT (0.5-1mM) did not improve the results but resulted in comets that consisted mainly of tail DNA with very little DNA remaining in the cell nucleus (Figures 3.11-3.22).

The most successful assay conditions (resulting in complete lysis with low levels (< 30%) of DNA observed in the comet tail) were identified as 1% Triton X in

the Lysis buffer with a 2 h Lysis incubation time, followed by 10 h decondensation in a buffer containing 0.25mg/ml proteinase K and 0-0.25mM DTT.

Table 3.2 Summary of assay conditions and results obtained during modification of human spermatozoa Comet assay for use on murine spermatozoa

Trial No.	Lysis: Triton X (%)	Lysis: Lysis Time (h)	Lysis: DTT (mM)	Decondensation: Proteinase K (mg/ml)	Decondensation: Decondensation (h)	Result	Picture
1	0	1	0	0	10	Cells Intact	
2	0	1	0	0.25	10	Cells Intact	Fig 3.3
3	0	2	0	0	10	Cells Intact	
4	0	2	0	0.25	10	Cells Intact	Fig 3.4
5	1	1	0	0.25	10	Cells Intact	Fig 3.5
6	1	1	0.1	0.25	10	Cells Intact	
7	1	1	0.15	0.25	10	Cells Intact	
8	1	1	0.2	0.25	10	Cells Intact	
9	1	1	0.25	0.25	10	Cells Intact	Fig 3.6
10	1	1	0.5	0.25	10	Cells Intact	Fig 3.7
11	1	1	1	0.25	10	Cells Intact	Fig 3.8
12	1	2	0	0.25	10	Comets	Fig 3.9
13	1	2	0.1	0.25	10	Comets	
14	1	2	0.15	0.25	10	Comets	
15	1	2	0.2	0.25	10	Comets	
16	1	2	0.25	0.25	10	DNA damage too high*	Fig 3.10
17	1	2	0.5	0.25	10	DNA damage too high	Fig 3.11
18	1	2	1	0.25	10	DNA damage too high	Fig 3.12
19	2	1	0	0.25	10	DNA damage too high	Fig 3.13
20	2	1	0.25	0.25	10	DNA damage too high	Fig 3.14
21	2	1	0.5	0.25	10	DNA damage too high	Fig 3.15
22	2	1	1	0.25	10	DNA damage too high	Fig 3.16
23	2	2	0	0.25	10	DNA damage too high	Fig 3.17
24	2	2	0.1	0.25	10	DNA damage too high	
25	2	2	0.15	0.25	10	DNA damage too high	
26	2	2	0.2	0.25	10	DNA damage too high	
27	2	2	0.25	0.25	10	DNA damage too high	Fig 3.18
28	2	2	0.5	0.25	10	DNA damage too high	Fig 3.19
29	2	2	1	0.25	10	DNA damage too high	Fig 3.20
30	5	1	0	0.25	10	DNA damage too high	Fig 3.21
31	5	2	0	0.25	10	DNA damage too high	Fig 3.22
32	5	2.5	0	0.25	10	DNA damage too high	

**Comets assessed visually and observed to have levels of DNA damage greater than 50% (Approx.) See Figure 3.2.*

3.3.1.1 Reproducibility of the assay

The optimum assay conditions were tested approximately 20 times over a 3-month period. The assay was tested using both fresh and frozen spermatozoa with a

control sample present in each run (same control sample used in each run) and fresh buffers made up regularly. Despite strict adherence to the assay protocol, considerable variation was observed in the degree of lysis and/or decondensation of the cells on any one slide, and also between duplicate slides. The variation in comets produced was clearly visible under the microscope (see Figure 3.2), therefore analysis of the slides using the Komet Image Analysis system was deemed to be inappropriate.

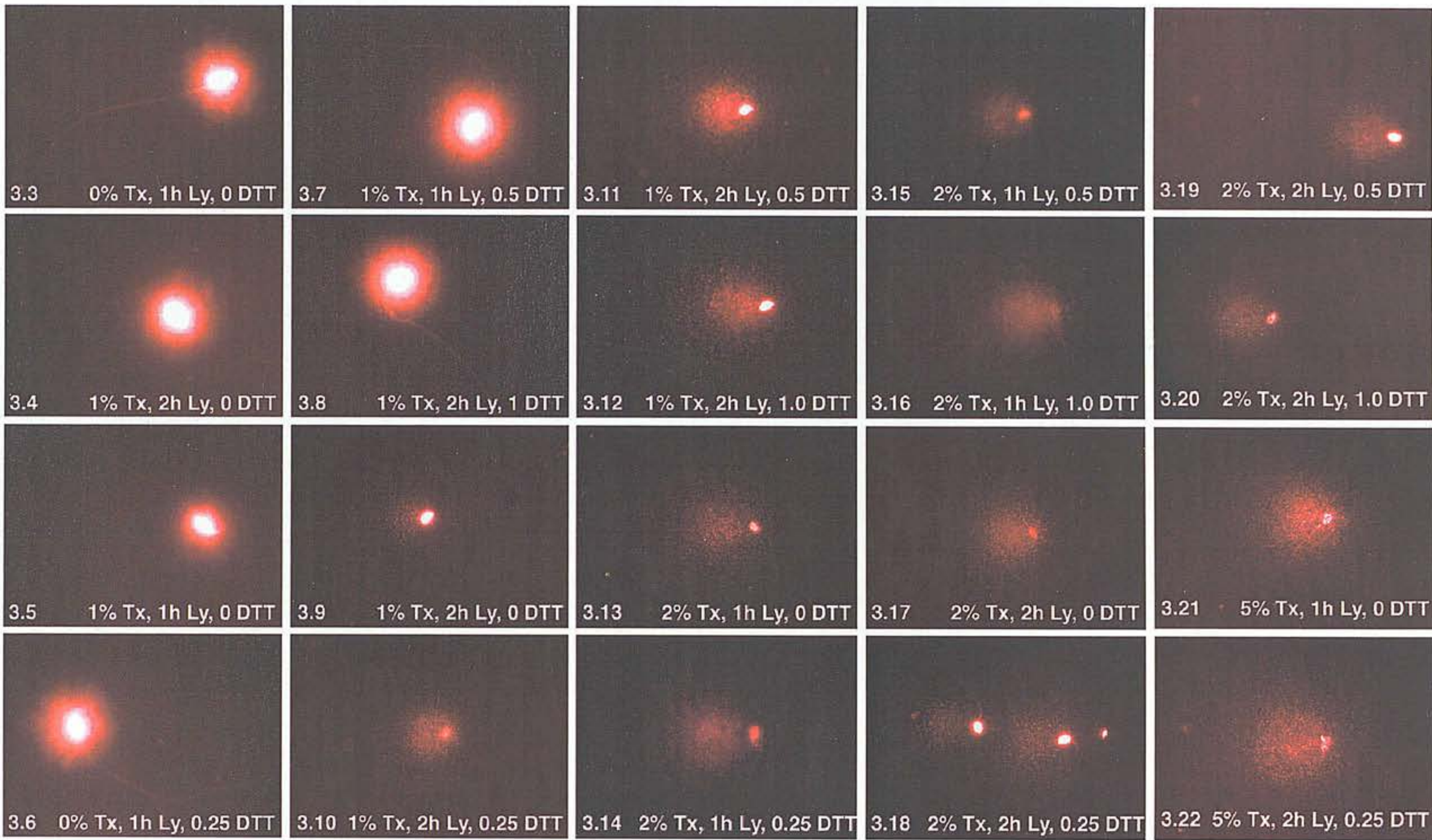
It was concluded that modification of the human spermatozoa Comet assay was unlikely to result in a reliable and reproducible assay for use on murine spermatozoa.

3.3.2 Development of a novel Comet assay for study of murine spermatozoa

The method used by Qiu *et al* (1995) to lyse the membranes and decondense the DNA of rat spermatozoa was combined with the alkaline electrophoresis method previously used in the human spermatozoa Comet assay to create a novel Comet assay method for use on murine spermatozoa. This combination of conditions resulted in disintegration of the spermatozoa and comets were not visible on the slides following electrophoresis. Staining of a number of slides at earlier points in the assay confirmed that the spermatozoa were present in a highly fragmented condition on the slides prior to electrophoresis.

This result demonstrated that the conditions suitable for rat spermatozoa (1% SDS, 0.1mg/ml PK, 10mM DTT) were too harsh for murine spermatozoa. Therefore a number of alternative assay conditions were tested and a summary of these conditions and the results obtained can be seen in Table 3.3.

A concentration of 0.6% SDS and an incubation time of 30 min was found to be sufficient to lyse the spermatozoa membranes, though loss of spermatozoa head shape was not always complete (Trial 2; Figure 3.21). Higher concentrations of SDS (0.9-1.0%) resulted in very high levels of DNA damage (Trials 5, 8 and 10; Figures 3.22-3.24) while lower concentrations (0.5%) and increased incubation times (20-30 min) did not result in satisfactory lysis of the cell membranes (Trials 6 and 9; Figures 3.25-3.26)

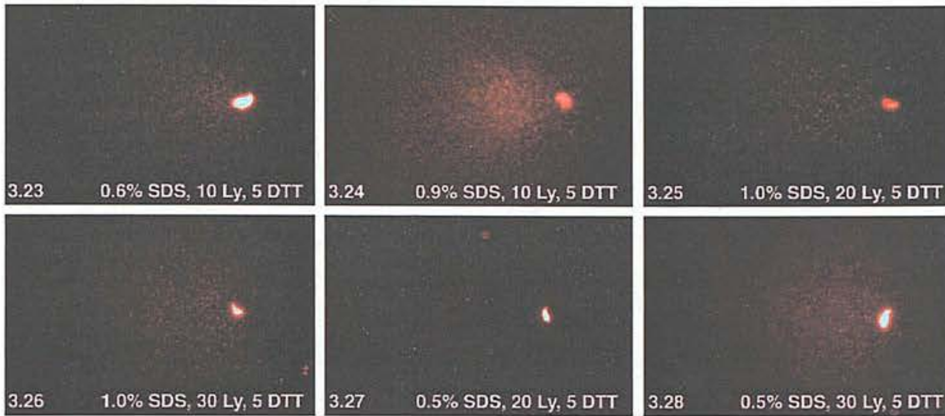


Figures 3.3-3.22. Examples of comets obtained using range of assay conditions. Figures 3.3-3.9 = x 100 magnification Figures 3.9-3.22 = x 40 magnification, Tx = Triton X, ly = Lysis incubation (min), DTT = mM DTT

Table 3.3 Summary of assay conditions and results obtained during development of novel Comet assay for use on murine spermatozoa

	Lysis Stage		Decondensation stage				
Trial No.	**SDS (%)**	**Lysis Time (min)**	**DTT (mM)**	**PK (mg/ml)**	**Decondensation (h)**	**Result**	**Picture**
1	0.5	10	5	0.1	1	Incomplete Lysis	
2	0.6	10	5	0.1	1	DNA damage too high*	**Fig 3.23**
3	0.7	10	5	0.1	1	DNA damage too high	
4	0.8	10	5	0.1	1	DNA damage too high	
5	0.9	10	5	0.1	1	DNA damage too high	**Fig 3.24**
6	0.5	20	5	0.1	1	Incomplete Lysis	**Fig 3.27**
7	0.75	20	5	0.1	1	DNA damage too high	
8	1	20	5	0.1	1	DNA damage too high	**Fig 3.25**
9	0.5	30	5	0.1	1	Incomplete Lysis	**Fig 3.28**
10	1	30	5	0.1	1	DNA damage too high	**Fig 3.26**
11	0.75	30	1	0.1	1	Incomplete Comet	
12	0.75	30	2.5	0.1	1	Incomplete Comet	
13	0.75	30	2.75	0.1	1	Incomplete Comet	
14	0.75	30	2.8	0.1	1	Comet	
15	0.75	30	2.9	0.1	1	Comet	**Fig 3.29, 3.30**
16	0.75	30	3.0	0.1	1	Comet	
17	0.75	30	3.1	0.1	1	Comet	
18	0.75	30	3.2	0.1	1	Comet	
19	0.75	30	3.25	0.1	1	Comet	
20	0.75	30	3.3	0.1	1	Comet	
21	0.75	30	3.4	0.1	1	Comet	
22	0.75	30	3.5	0.1	1	DNA damage too high	
23	0.75	30	3.6	0.1	1	DNA damage too high	
24	0.75	30	3.7	0.1	1	DNA damage too high	
25	0.75	30	3.75	0.1	1	DNA damage too high	
26	0.75	30	3.8	0.1	1	DNA damage too high	
27	0.75	30	3.9	0.1	1	DNA damage too high	
28	0.75	30	4	0.1	1	DNA damage too high	
29	0.75	30	5	0.1	1	DNA damage too high	
30	0.75	30	5	0.1	1	DNA damage too high	
31	1.0	30	5	0.1	1	DNA damage too high	

**Comets assessed visually and observed to have levels of DNA damage greater than 50% (Approx.) See Figure 3.2.*



Figures 3.23-3.28. Examples of comets obtained using a range of assay conditions x40 magnification, Ly = lysis incubation (min), DTT = DTT (mM)

The assay conditions which appeared to result in appropriate levels of lysis and decondensation, consisted of an SDS concentration of 0.75%, a lysis incubation time of 30 min and a DTT concentration in the range 2.8 – 3.4mM DTT. These assays were repeated and DNA damage in 100 cells (maximum number cells analysed following Comet assay of human spermatozoa) from each assay analysed using Kinetic Imaging software. A summary of the results obtained is presented in Table 3.4.

Table 3.4. Summary of results obtained following analysis of 100 comets from assays using a range of DTT concentrations

Trial Number	DTT (mM)	Average % Tail DNA	Range % Tail DNA
14	2.8	18.46	0.17 - 91.86
15	2.9	14.65	0.48 - 45.14
16	3.0	14.97	0.00 - 82.83
18	3.2	30.11	2.09 - 82.03
21	3.4	41.81	1.15 - 78.19

A DTT concentration in the range of 2.8-3.0mM was found to result in an average % tail DNA within the range of ~15-20%, the low level of baseline DNA damage that should be expected in control spermatozoa. However, within the population of comets analysed, there was a considerable degree of variation in the levels of DNA damage measured (0.17-91.86%).

Further testing of these assay conditions was undertaken in order to improve technique and reproducibility.

3.3.2.1 Cumulative cell analysis

In order to obtain an accurate representation of the DNA damage in a population of cells, an appropriate number of cells must be analysed. In order to keep analysis time as short as possible, it is necessary to determine the minimum number of cells to analyse in order to obtain an accurate representation of the whole population. This number is calculated using cumulative cell analysis as follows; the comet assay was performed and 100 cells from a single slide were analysed as previously described. The average % tail DNA was calculated for cell 1, cell 1+2, cell 1+2+3, and so on until a cumulative average was obtained for each number of cells analysed. Table 3.5 provides an example of the method of calculating the cumulative averages.

Table 3.5 Example of cumulative average calculation

Cell Number	% Tail DNA	Calculation	Cumulative Average
1	32	32 / 1	32.00
2	25	(32+25) / 2	28.50
3	28	(32+25+28) / 3	28.33
4	34	(32+25+28+34) / 4	29.75

These cumulative averages were then plotted on a graph. An example of a cumulative cell analysis graph is presented in Figure 3.29. The point at which the graph reaches a stable value is identified (as indicated on Figure 3.29). This point indicates the number of cells required for analysis in order to obtain an accurate representation of a population, i.e. analysis of 75 cells (as shown in the example presented in Figure 3.29) would provide the same result as analysis of 100+ cells.

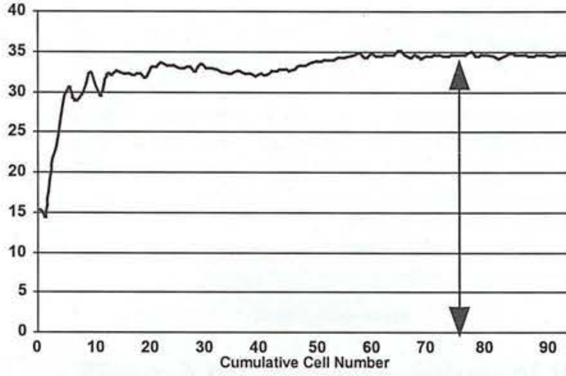


Figure 3.29 Example of a cumulative cell analysis graph showing the approximate point at which the data reaches a stable value indicating number of cells required

Cumulative cell analysis was performed for each assay using a range of DTT concentrations (2.8, 2.9, 3.0, 3.2 and 3.4 mM DTT). Previous studies of human spermatozoa analysed 50 cells per sample (Irvine, *et al.*, 2000), therefore 100 cells were used for the cumulative cell analysis of the mouse assay in order to identify the appropriate number of cells required for accurate analysis (Figures 3.30-3.34).

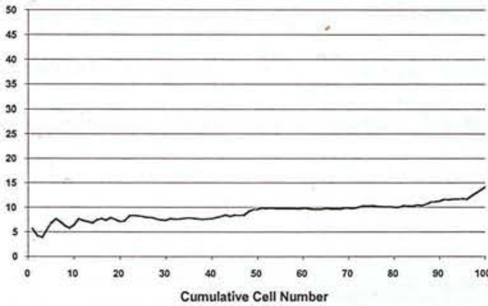


Figure 3.30 Cumulative analysis of 100 comets from Trial 14 (2.8mM DTT)

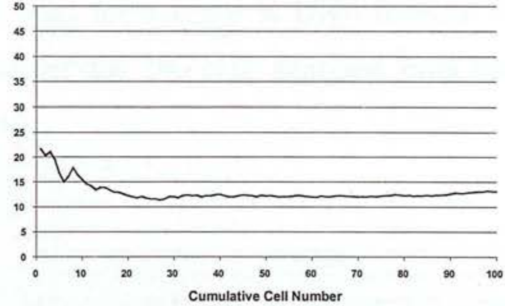


Figure 3.31 Cumulative analysis of 100 comets from Trial 15 (2.9mM DTT)

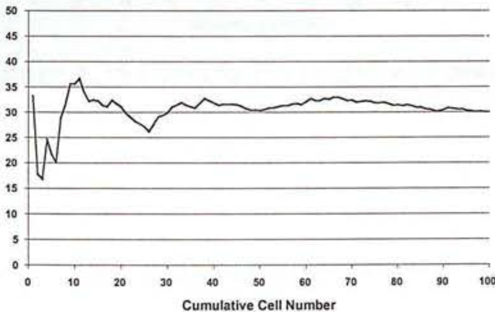


Figure 3.32 Cumulative analysis of 100 comets from Trial 16 (3.0mM DTT)

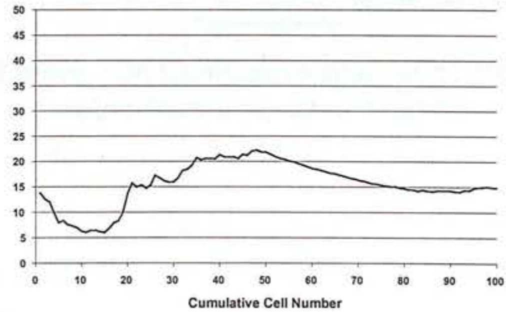


Figure 3.33 Cumulative analysis of 100 comets from Trial 18 (3.2mM DTT)

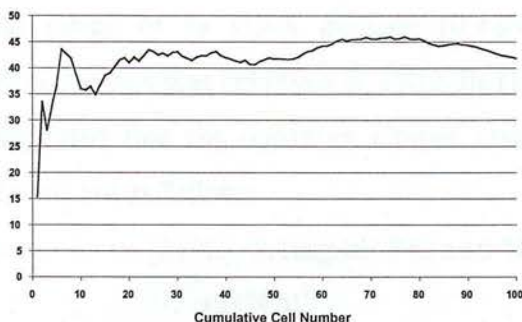


Figure 3.34 Cumulative analysis of 100 comets from Trial 21 (3.4mM DTT)

The data from analysis of 100 cells from each assay indicated that assay conditions with a concentration of 2.8 - 2.9mM DTT would produce a suitable range of DNA damage in control spermatozoa (summarised in Table 3.4). While 2.8mM DTT resulted in a lower average % Tail DNA, the failure of the cumulative average graph to reach a satisfactory plateau using less than 100 cells indicated that analysis of this number might not be sufficient to predict the average % DNA damage in a population (Figure 3.30). Assays were repeated and 200 cells analysed from each, the results are shown in Figures 3.35 and 3.36.

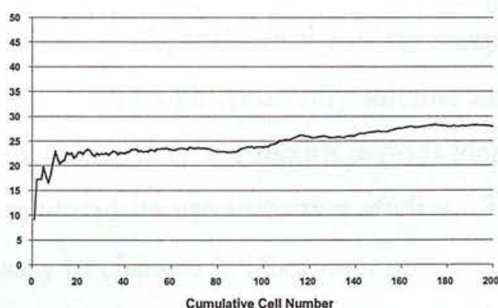


Figure 3.35 Cumulative analysis of 200 comets from a repeat of Trial 14 (2.8mM DTT)

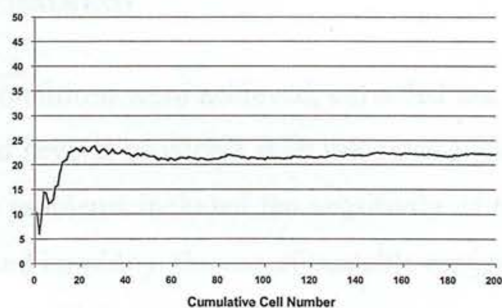


Figure 3.36 Cumulative analysis of 200 comets from a repeat of Trial 15 (2.9mM DTT)

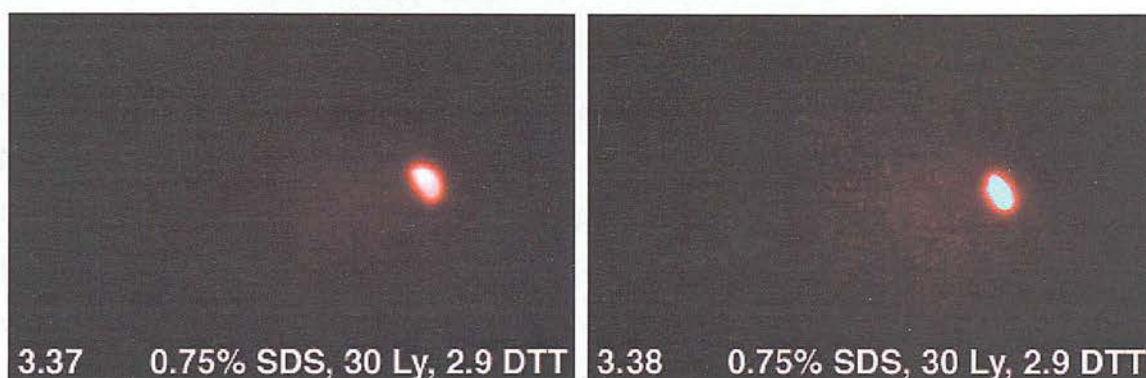
The results obtained indicate that although the use of 2.8mM DTT gives a good average and range of DNA damage in control spermatozoa, over 200 cells would need to be analysed in order to achieve an accurate representation of the population (Figure 3.35). In contrast, the use of 2.9mM DTT not only resulted in an

appropriate average and range of % DNA damage (0.48-45.14%), but that a representational population average was achieved at 150 cells (Figure 3.36).

These results suggested that the optimum Comet assay conditions for the study of murine spermatozoa are as follows:

0.75% SDS, 30 minutes Lysis (21°C), 0.1mg/ml PK and 1h decondensation (21°C)

Examples of comets obtained using these conditions are shown in Figures 3.37 and 3.38.



Figures 3.37 and 3.38 Examples of comets obtained using optimum assay conditions (0.75% SDS, 30 minutes lysis, 0.1mg/ml PK and 1h decondensation)

3.3.2.2 Reproducibility of the assay

Although apparently suitable assay conditions were achieved, extended use of this assay (over a 4 month period) identified several problems with the assay which prohibited its use in further studies. These problems included the sensitivity of the assay to changes in laboratory temperature and humidity, the use of unstable reagents (i.e. PK and DTT; potency changed with age and between batches) and human error. In addition, the tendency of the agarose gel layers (containing the spermatozoa cells) to detach from the slides together with the need to analyse 150 cells per slide, meant that a great deal of repetition was required to obtain reliable results. It was therefore concluded that this Comet assay protocol might not prove robust enough to produce the consistent results required.

3.3.3 Modification of a Comet assay kit for use on murine spermatozoa

Initial trials of this kit following the protocol recommended by the manufacturers resulted in intact murine spermatozoa which had failed to lyse or decondense. In addition, it was found that agarose gels created without the use of coverslips (as recommended by the manufacturer) had an uneven surface and were difficult to study under the microscope. Therefore, a number of modifications were introduced, the first of which was to introduce the use of cover slips to level the gels as they set. The modifications made to the lysis and decondensation buffers and incubation times are presented in Table 3.7.

The kit assay method did not include the use of SDS, DTT or PK, all of which were found to enhance results using the Comet methods discussed in the previous section (3.3.2). Therefore, SDS, DTT and PK were introduced at the appropriate stages in the concentrations determined previously (3.3.2). In addition, the pH of the electrophoresis buffer was increased to 12.3 and the electrophoresis time reduced to 10 min. Lysis at 4°C was not successful due to the failure of SDS to remain in solution. Lysis of the cells was not achieved unless the incubation temperature was adjusted to 37°C (Table 3.7). However, though successful lysis was achieved, it was found that the decondensation conditions were too harsh, resulting in very high baseline levels of DNA damage. It was concluded that following adequate lysis of the cells, the DTT and PK in the decondensation buffer had better access to the DNA, and rather than simply causing the DNA to unwind, actually caused further DNA damage. Therefore additional modifications to the assay decondensation conditions were undertaken; firstly concentrations of DTT and PK were reduced until eventually they were no longer used, the incubation time was reduced to 20 min and the temperature to 21°C. Removing the PK and DTT resulted in cells which failed to lyse properly but were beginning to form comets. Analysis of these cells indicated that the average % Tail DNA was very low and that the running average was unsettled, failing to reach a stable value by 100 cells (See Figure 3.39).

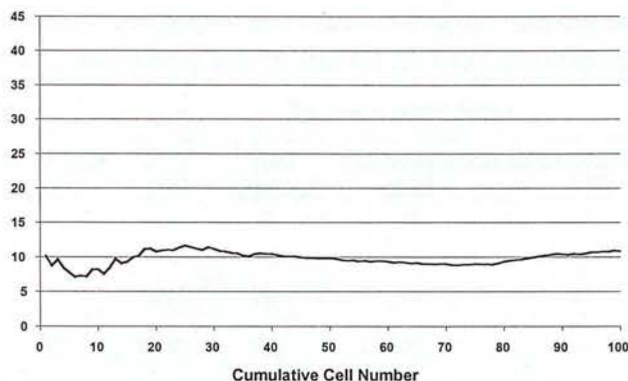


Figure 3.39 Cumulative analysis of 100 comets following 30 min lysis

It was decided that in order to achieve complete lysis of the cells it was necessary to increase the lysis incubation period further (60 min, 120 min, 180 min). A summary of the results obtained using the different lysis incubation times is presented in Table 3.6.

Table 3.6 Summary of results obtained following analysis of 100 comets from assays using a range of Lysis incubation periods

Lysis Time (min)	Average % Tail DNA	Range % Tail DNA
30	10.85	0.54 – 27.36
60	16.06	0.96 – 44.31
120	16.84	0.25 – 37.89
180	16.34	0.48 – 45.14

Table 3.7 Summary of assay conditions and results obtained during the modification of a Comet assay kit for use on murine spermatozoa

Trial No.	Lysis Stage			Decondensation Stage				Results
	SDS (%)	Lysis Time (min)	Lysis Temp (°C)	DTT (mM)	Prot K(mg/ml)	Decondensation (min)	Decondensation (°C)	
1	0.5	30	4	0.015	60	60	21	Cells Intact
2	0.5	30	21	0.015	60	60	21	Cells Intact
3	0.5	30	37	0.015	60	60	21	DNA damage too high
4	0.5	25	37	0.015	60	30	21	DNA damage too high
5	0.5	25	37	0.01	60	30	21	DNA damage too high
6	0.5	25	37	0.005	60	30	21	DNA damage too high
7	0.5	25	37	0	60	30	21	Cells Intact
8	0.75	30	4	0.015	60	60	21	Cells Intact
9	0.75	30	21	0.015	60	60	21	Cells Intact
10	0.75	30	37	0.015	60	60	21	Incomplete Lysis
11	0.75	30	37	0.015	60	30	21	Incomplete Lysis
12	0.75	30	37	0.01	60	30	21	Incomplete Lysis
13	0.75	30	37	0.005	60	30	21	Incomplete Lysis
14	0.75	30	37	0	60	30	21	Incomplete Lysis
15	0.75	30	37	0	60	30	21	Incomplete Lysis
16	0.75	30	37	0	60	30	37	Incomplete Lysis
17	0.75	30	37	0	60	35	21	Incomplete Lysis
18	0.75	30	37	0	60	35	37	Incomplete Lysis
19	0.75	30	37	0	60	40	21	Incomplete Lysis
20	0.75	30	37	0	60	40	37	Incomplete Lysis
21	0.75	30	37	0	40	30	21	Poor Comets
22	0.75	30	37	0	40	30	37	Poor Comets
23	0.75	30	37	0	20	30	21	Poor Comets
24	0.75	30	37	0	20	30	37	Poor Comets
25	0.75	30	37	-	-	-	-	Poor Comets
26	0.75	60	37	-	-	-	-	Comets
27	0.75	120	37	-	-	-	-	Comets
28	0.75	180	37	-	-	-	-	Comets

**Comets assessed visually and observed to have levels of DNA damage greater than 50% (Approx.) See Figure 3.2.*

These results suggest that increasing lysis incubation time does not increase the average % DNA damage in the cells. In addition, the low level of damage after 30 min incubation confirms the lack of lysis occurring after this short incubation period.

Progressive increases in incubation times in lysis buffer resulted in improved comets until, after 180 min, complete lysis of the cells occurred and satisfactory comets were achieved. Figures 3.40 – 3.42 show the running averages of % Tail DNA achieved with each of these extended incubation periods.

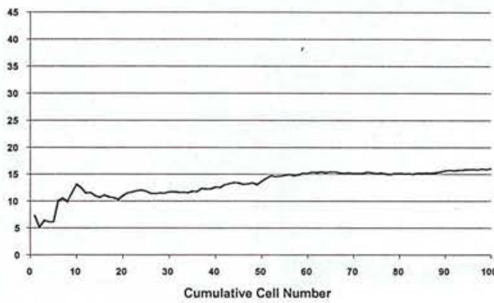


Figure 3.40 Cumulative analysis of 100 comets following 60 min Lysis

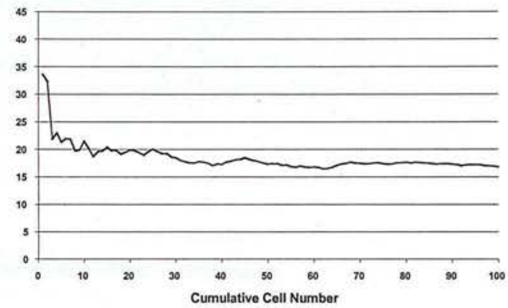


Figure 3.41 Cumulative analysis of 100 comets following 120 min Lysis

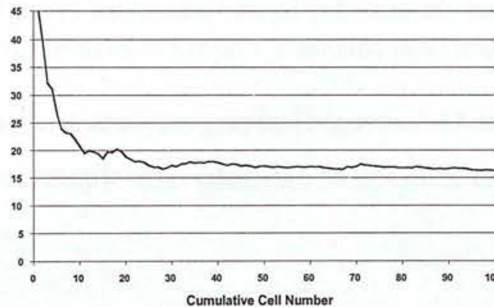


Figure 3.42 Cumulative analysis of 100 comets following 180 min Lysis

These results indicated that a lysis time of 180 minutes resulted in an appropriate average % tail DNA of 16.35% and a range of 0.48 - 45.14%. In addition, it was observed that at a count of 100 cells, the running average appeared to have settled, indicating that analysis of 100 cells was adequate to predict the population average. To confirm this finding, the assay was repeated using the same conditions and 200 cells were analysed each time (Figures 3.43 and 3.44).

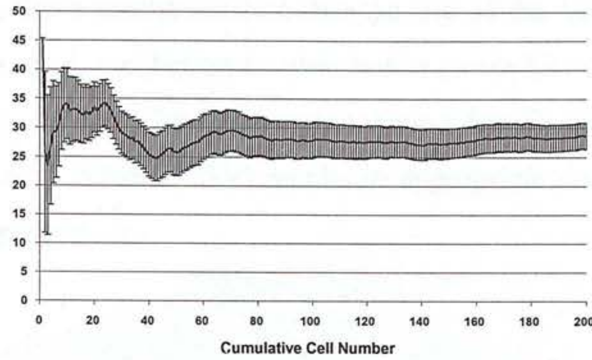


Figure 3.43 Repeated cumulative analysis of 200 comets following 180 min Lysis (cumulative average ± standard deviation)

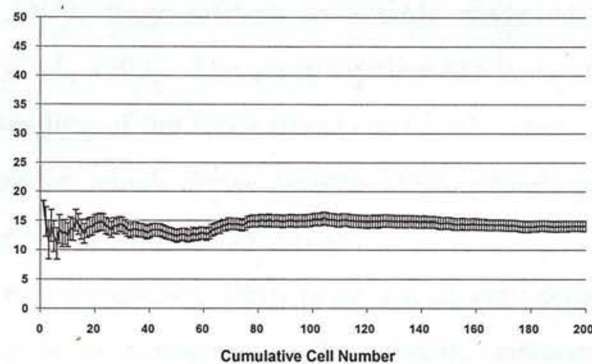


Figure 3.44 Repeated cumulative analysis of 200 comets following 180 min Lysis (cumulative average ± standard deviation)

These cumulative cell analysis graphs (Figures 3.43 and 3.44), confirmed that analysis of 100 cells per sample was adequate to obtain a true representation of the population.

3.3.3.1 *Reproducibility of the assay*

The optimum assay conditions were tested for reproducibility a number of times over a period of time (1-2 months). A total of 100 cells was analysed and the mean % tail DNA was calculated for each sample tested (n=20). A summary of the results obtained for these 20 samples is presented in Table 3.8.

Table 3.8 Summary of results for optimum assay reproducibility

Factor	Value
Mean	16.682
Standard Error	0.133
Standard Deviation	0.595
Coefficient of Variation (%)	3.569

In summary, these results indicate that the use of the CometKit slides, gels levelled using cover slips, incubation in lysis buffer (from kit) containing 1% SDS for 180 min (37°C), followed by alkaline incubation for 20 min (21°C) and then electrophoresis (10 min, 300mA, 25V) results in appropriate and consistent Comet analysis of murine spermatozoa.

3.4 Discussion

The Comet assay is a highly sensitive and reproducible assay commonly used to detect levels of DNA fragmentation in a wide range of somatic cell types (McKelvey-Martin, *et al.*, 1993). The assay involves the lysis of the cell membrane followed by the unwinding of the DNA (most commonly under alkaline conditions) and then electrophoresis which draws broken DNA strands away from the cell nucleus, forming a characteristic 'comet' image.

In mammalian spermatozoa, DNA is packaged very differently from that of somatic cells. During spermiogenesis, the somatic histones are replaced by arginine/cysteine-rich protamines and the DNA is restructured into a highly organised nucleoprotamine complex (Gatewood, *et al.*, 1987). This complex is further stabilised by the formation of disulphide (-SS-) bridges during transit of the spermatozoa through the epididymis (Bedford, 1979;Cooper, *et al.*, 1986). The arrangement of the DNA in mammalian spermatozoa is specifically designed to protect it from exogenous insult, therefore in order to study the integrity of the DNA it must first be exposed without causing further damage. The initial modification of the standard somatic cell Comet assay for use on human spermatozoa included the introduction of a proteinase K digestion step to remove the tight protamine links present in the spermatozoa nucleus (Hughes, *et al.*, 1996).

The Comet assay has been successfully used in a number of studies of DNA integrity in human spermatozoa. A negative relationship between semen quality and DNA damage has been reported by a number of laboratories (Sun, *et al.*, 1997;Irvine, *et al.*, 2000), and the increased susceptibility of spermatozoa from infertile men to exogenous DNA damage has been demonstrated by others (Hughes, *et al.*, 1997). In comparison with other methods used for spermatozoa analysis, such as the enzyme-

linked immunosorbant (ELISA) and the TUNEL assays (Hughes, *et al.*, 1999;Shen, *et al.*, 2000), the Comet assay appears to be both sensitive and reliable and has the benefit of the ability to assess DNA integrity of individual cells and then combine data from many cells to assess a population (Hughes, *et al.*, 1996).

At the time this project started, there were no publications of a Comet assay used for the study of DNA integrity in murine spermatozoa. However, during the present study an *in vitro* study of the effects of radiation on the integrity of DNA in both human and murine spermatozoa was published by Haines *et al* (1998). They used both neutral and alkaline versions of the Comet assay to study the DNA integrity of human and murine spermatozoa. In both assays, slides were submerged in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris HCl; pH10) containing 1% Triton and 40mM DTT for 1h (21°C) followed by a further 3h incubation (37°C) in the same buffer with the addition of 0.1mg/ml proteinase K. Electrophoresis (20 min, 10mA, 25V) was performed using either Tris-Borate EDTA (TBE) buffer (neutral assay), or alkali (0.05M NaOH, 1mM EDTA) buffer (alkali assay). The authors did not state the exact pH of the buffers used for electrophoresis. Analysis of 50 cells per sample was performed using the Komet 3.0 image analysis system and the results from the alkali Comet assay indicated an average % Tail DNA of 90.8% (± 6.4) in the control (untreated) spermatozoa rising to 94.3% (± 2.5) in the spermatozoa subjected to the greatest radiation dose (100 Gy). The high concentration of DTT (40mM) in the lysis buffer is the most likely cause of this very high level of baseline DNA damage in the control spermatozoa resulting in a very narrow upward range available for measuring damage in the treated spermatozoa. The results presented in this study suggest that the assay used was not optimised for use on murine spermatozoa and could not provide a true comparison between the DNA integrity of the control and treated spermatozoa – the DNA damage induced by the conditions of the assay appeared to be so high that it 'masked' any damage induced by the radiation treatment. In addition, the analysis of 50 cells per sample may not have been sufficient to provide an accurate representation of the population.

In the present study, three approaches were taken to develop an appropriate assay; the modification of an existing protocol used for the study of human

spermatozoa, the development of a novel method, and the modification of a commercially available Comet assay kit.

The modification of the human assay did not result in an assay suitable for use on murine spermatozoa. It was found that the assay conditions tested did not produce satisfactory comets. Human and murine spermatozoa differ in a number of ways, including membrane construction and DNA packaging (Evenson, *et al.*, 1980;Haaf, *et al.*, 1995). These differences may account for the failure of the human assay to work on murine spermatozoa.

In 1995, a paper published by Qiu *et al* outlined a method used for the lysis and decondensation of rat spermatozoa (Qiu, *et al.*, 1995). It was decided that due to the morphological similarities between rat and mouse spermatozoa, conditions which lysed and decondensed rat spermatozoa might be successfully used in a Comet assay for use on mouse spermatozoa. The main difference between the lysis method used with rat spermatozoa and that used with spermatozoa in the human Comet assay was the presence of the ionic detergent SDS instead of the non-ionic detergent Triton X (Hughes, *et al.*, 1996). SDS is a powerful ionic denaturant which solubilises proteins by producing conformational changes in the molecules and disrupting non-covalent protein interactions (such as -SS- bridges). Previous studies have reported that the use of SDS, in addition to removing the outer membrane of rabbit spermatozoa, also removes the nuclear membrane surrounding the DNA (Calvin, *et al.*, 1971). In addition to proteinase K, the decondensation buffer also contained DTT, a reducing agent which acts specifically on the -SS- bridges (Rodriguez, *et al.*, 1985;Tateno, *et al.*, 1999) and has been used in the decondensation of rabbit spermatozoa (Calvin, *et al.*, 1971).

Both SDS and DTT were tested in the lysis and decondensation buffers of a novel Comet assay for murine spermatozoa and an assay protocol was determined (0.75% SDS, 30 min Lysis (21°C), 0.1mg/ml PK and 1h decondensation (21°C)) which resulted in acceptable comets following electrophoresis.

However, after repeated tests of this protocol, it was found that results were inconsistent and that the assay was very sensitive to changes in either laboratory temperature or humidity. Furthermore, the efficiency of both the proteinase K and

the DTT appeared to alter over time. A possible explanation for the changing effectiveness of the proteinase K is the presence of SDS in the lysis buffer. It has been shown that SDS enhances the effects of proteinase K (Hilz, *et al.*, 1975), and it is possible that any traces of SDS remaining in the agarose gels after washing could have had an effect on the potency of the proteinase K in the decondensation buffer.

In addition to the observed inconsistencies of this assay, the tendency of the agarose gel layers (containing the spermatozoa cells) to detach from the slides together with the need to analyse 150 cells per slide, resulted in the need to repeat the analysis of any one spermatozoa sample a number of time in order to be confident in the results. It was therefore decided not to continue with the use of this assay .

The introduction of a Comet assay kit by R&D Systems presented another opportunity to improve the previous assay methods. This kit was originally intended for use on somatic cells but, following modifications based on findings during the modification of the human spermatozoa assay and the development of the novel method, an assay was developed which produced acceptable and consistent results when used on murine spermatozoa. The final assay method produced using the kit as a basis included an extended lysis incubation period (180 min at 37°C), the use of SDS (0.75%) in the lysis buffer, the removal of both proteinase K and DTT from the alkali decondensation stage, and an extended electrophoresis time (10 min). The removal of the proteinase K and DTT resulted in a more reliable assay, as these were the factors identified as being responsible for the inconsistencies observed in the novel method.

In contrast to the previous assays, which involved a short lysis incubation and then a long decondensation incubation with proteinase K and DTT, the method based on the kit reagents involves a long lysis incubation followed by a short alkali decondensation incubation without the use of either proteinase K or DTT. It is believed that allowing the cells to lyse fully under the influence of SDS is sufficient to expose the DNA and enable it to unwind and decondense due to the effects of the alkali conditions alone. Removal of both the proteinase K and the DTT reduces the risk of inducing DNA damage during the assay, thereby improving the sensitivity of

the assay to DNA damage already present in the spermatozoa. In addition, any assay variability due to variation between batches of proteinase K was removed.

Other benefits of the Comet assay kit included the use of the specially treated slides which reduced the both the preparation time of the slides and the risk of gel loss, the reduction in the time required to perform the assay and the reduced number (100) of cells required for accurate analysis.

In summary, the assay developed using the Comet assay kit produced reliable and consistent results, and was more efficient in terms of time spent performing the assay and analysing the slides. The Comet assay developed from this work was then used for the studies of murine spermatozoa DNA integrity described in this thesis.

Chapter 4

DNA integrity in motile spermatozoa from genetically-altered mouse models of male infertility:

Levels of endogenous DNA damage and susceptibility to heat-induced DNA damage

4.1 Introduction

4.1.1 Mouse models for male infertility

A number of mouse models have been developed which demonstrate various degrees/types of male infertility. In many cases, male infertility is described merely as a "side-effect" following deletion of particular genes, for example, the *Ercc-1* knockout mouse was originally developed to study the role of this gene in the repair of UV-induced DNA damage. After the introduction of an *Ercc-1* transgene, which extended the lifespan of these animals beyond sexual maturity, it was discovered that the male animals were infertile (Selfridge, *et al.*, 2001, Melton personal communication). In other cases, genes normally expressed in the testis/reproductive tract, or genes known to be expressed in germ cells (e.g. *dazl,* mHR6B), were deleted/altered in order to study their role in spermatogenesis/spermatozoa maturation (Cooke, *et al.*, 1996;Grootegoed, *et al.*, 1998).

The mice studied in this chapter contained targeted ablation of genes encoding *dazl, Ercc-1* or Prion/Prion-related proteins.

4.1.1.1 Deleted in azoospermia (DAZ) and daz-like autosomal (DAZL)

The Y chromosome encoded gene *DAZ*, and the autosomal gene *DAZL* have been implicated in human male infertility (Yen, *et al.*, 1996;Chang, *et al.*, 1999;Krausz, *et al.*, 1999). Mice do not have a *daz* gene on their Y chromosome (Cooke, *et al.*, 1996;Reijo, *et al.*, 1996), however, an autosomal homologue of

human DAZ, known as *dazl*, has been isolated from mouse chromosome 17 and is expressed in the cytoplasm of germ cells (Cooke, *et al.*, 1996;Ruggiu, *et al.*, 1997).

Expression of *dazl* in murine germ cells is essential for the differentiation of these cells in both the ovary and testis (Cooke, *et al.*, 1996;Shen, *et al.*, 1997;Venables, *et al.*, 2001). It has been suggested that, due to the presence of an RNA binding domain, and the cytoplasmic location of the protein, *dazl* may play a role in translational control via the packaging or localisation of mRNAs (Ruggiu, *et al.*, 1997).

Dazl-deficient mice (-/-) are infertile, and males fail to produce any mature spermatozoa; heterozygous males are fertile but exhibit reduced numbers of spermatozoa with high incidence of morphological abnormality (Ruggiu, *et al.*, 1997).

The animals used in this study were sexually mature male +/+ and +/- *dazl* mice on an MF1 genetic background. The preparation of these mice has been described previously by Ruggiu *et al* (1997).

4.1.1.2 Excision repair cross-complementing gene 1 (Ercc-1)

Ercc-1 is a gene involved in the nucleotide excision repair (NER) pathway which is responsible for the recognition and repair of DNA damage (McWhir, *et al.*, 1993). Within this pathway, *Ercc-1* forms a complex with xeroderma pigmentosum factor (XPF; also known as *Ercc-4*). This complex is a structure-specific endonuclease which incises the damaged DNA strand at the 5' side of the lesion. *Ercc-1* is also thought to be involved in the process of mitotic recombination which is required for the repair of DNA cross-links (Adair, *et al.*, 2000;Sargent, *et al.*, 2000).

Ercc-1-knockout mice were first described by McWhir *et al* in 1993. The authors noted that these mice exhibited disturbed (runted) growth, reduced life span and abnormalities of liver nuclei (McWhir, *et al.*, 1993). These findings were then confirmed by Weeda *et al* (1997) who also described kidney, spleen and cutaneous deficits, as well as performing a detailed analysis of cross-link repair, cell cycle arrest, NER and replicative senescence in these mice. The liver and kidney

malfunctions in *Ercc-1* knockout mice have been attributed to the prominent nuclear abnormalities (e.g. polyploidy) which have been observed in these organs. *Ercc-1* knockout mice exhibit several signs of premature senescence, including disturbed growth, reduced weight and reduced lifespan, possibly caused by an early block to cellular proliferation (Weeda, *et al.*, 1997).

In *Ercc-1* knockout mice, the NER pathway is lost and mitotic recombination is impaired. Death normally occurs in *Ercc-1*-deficient mice on or around day 22, before the first wave of spermatogenesis is complete, and is due to liver failure. To allow studies on the role(s) of *Ercc-1* in other organs the liver phenotype has been corrected by introduction of an *Ercc-1* transgene under the control of a liver-specific promoter into the *Ercc-1* null background (Selfridge, *et al.*, 2001).

4.1.1.3 Prion protein (PrP) and Prion protein-like protein Doppel (PrnD)

The Prion diseases (Scrapie, Creutzfeldt-Jakob Disease) are fatal neurodegenerative disorders which may be either inherited or acquired. It has been shown that the fundamental mechanism of these genes is the altered structural configuration of the Prion protein (Prusiner, 1998).

Mice deficient in the Prion protein (PrP) develop ataxia and exhibit altered circadian rhythms (Tobler, *et al.*, 1996) and electrophysiological disorders (Collinge, *et al.*, 1994). PrP variants are expressed both in the testis and the mature spermatozoa of mice (Shaked, *et al.*, 1999;Li, *et al.*, 2000). It has been shown that spermatozoa from PrP-deficient mice are more susceptible to high copper concentrations than spermatozoa from control mice, suggesting a protective role for PrP against copper toxicity in the testis (Shaked, *et al.*, 1999). While PrP deficient male mice exhibit normal testicular/epididymal function, there have been conflicting findings regarding the fertility of these mice, for example Shaked *et al* have reported that these mice are fertile (Shaked, *et al.*, 1999) while Melton *et al.*, have observed complete infertility (Melton DW, personal communication).

The PrP-related gene doppel (*PrnD*) encodes a Doppel protein (Dpl), which is normally expressed at low levels in the brain. However, in PrP-deficient mice, Dpl is over-expressed and may provoke neurodegeneration in these animals (Moore, *et*

al., 1999). It has been speculated that Dpl may play a role in angiogenesis, in particular blood-brain barrier maturation in the central nervous system (Li, *et al.*, 2000). In addition, it has been reported that Dpl protein is highly expressed in the testis of the sheep, cow and mouse (Silverman, *et al.*, 2000;Tranulis, *et al.*, 2001). In the mouse, Dpl is expressed during the late stages of spermiogenesis and spermatozoa from Dpl deficient (*PrnD* -/-) male mice have been shown to be immotile, malformed and unable to fertilise eggs *in vitro* (Behrens, *et al.*, 2002).

In the current investigation, spermatozoa from both *PrP* -/- and *PrnD* -/- were studied.

4.1.2 Aims of this chapter

Mouse models of infertility have generally been studied in respect of their ability to produce motile and fertile spermatozoa, and the numbers of live offspring produced either naturally or via IVF techniques.

As previously discussed (section 3.1), DNA damage present in spermatozoa has been shown to result in congenital and developmental abnormalities, and increased risk of childhood cancers in the offspring (Ji, *et al.*, 1997;Shen, *et al.*, 1997;Potts, *et al.*, 1999;Zenzes, *et al.*, 1999).

The purpose of the present investigation was to study the following in several mouse models of infertility:

a. Numbers of motile spermatozoa
b. Morphology of motile spermatozoa
c. Levels of endogenous DNA damage in motile spermatozoa
d. Susceptibility of DNA of motile spermatozoa to exogenous insult

Baseline levels of DNA damage in spermatozoa from these mice will be determined using the murine spermatozoa Comet assay. The susceptibility of the spermatozoa DNA to exogenous insult will also be investigated via *in vitro* heat treatment.

4.2 Methods

4.2.1 Genotyping of mice

Full methods for the genotyping of all animals used in this study are described in section 2.15. Briefly, tail tips were taken from each mouse and digested to retrieve the DNA. Using specific primers for the genes in question, PCR reactions were performed to determine the genotype of each mouse.

4.2.2 Preparation of murine spermatozoa

Full methods for the preparation of murine spermatozoa are described in section 2.6. The animals used in this study were from the following genotypes: *dazl* (wt and +/-), *Ercc-1* (129wt. +/-, and -/-) and Prion (129wt, *Prp* -/- and *PrnD* -/-). Briefly, whole epididymides were removed, cleaned of fatty tissue and placed in 1ml BWW. The tissue was then minced with fine scissors and incubated at 34°C (95% air, 5% CO_2) for 30 min prior to careful removal of buffer containing the motile spermatozoa. Samples were diluted (to a final volume of 1ml) and stored in BWW at –20°C until required.

4.2.3 Study of concentration of spermatozoa

Samples were diluted 1:10 with spermatozoa diluting fluid (SDF; 50g $NaHCO_3$, 10ml Formalin, dH_2O to 1L) and 10µl diluted sample was placed into the chamber of an improved Neubauer haemocytometer (BDH). The number of spermatozoa located within 5 squares of the haemocytometer grid was carefully counted. If the number of spermatozoa in 5 squares was not equal to or greater than 100, 10 or 25 squares were counted. From this value, the number of spermatozoa in 1ml was calculated using the following equation:

$$\text{Concentration (millions/ml)} = \frac{\text{No Spermatozoa Counted}}{(4 \times \text{No Squares}) \div \text{Dilution}}$$

4.2.4 Study of morphology of spermatozoa (DiffQuik staining)

For each sample to be studied, 2 microscope slides were prepared by washing in 100% ethanol, then water, and drying in an oven. 10μl sample was dropped onto the first slide and the second was used to drag the sample across the slide, creating a smear which was then allowed to air-dry. This process was repeated a total of 3 times per slide in order to obtain a high concentration of spermatozoa on each slide.

Once completely dry, each slide was dipped 10 times into Diffquick fixative and the excess solution removed by placing the edge of each slide onto an absorbent tissue and then wiping the underside. Each slide was then dipped 20 times into Diffquick I solution, drained of excess solution as before, dipped 20 times into Diffquick II solution, again drained of excess solution, and finally dipped in clean water to remove any remaining solution. Slides were allowed to air-dry before mounting using Pertex and a cover slip.

Morphologically-normal spermatozoa were defined as having crescent-shaped heads – pointing forwards, away from the mid-piece, no residual cytoplasm, straight mid-pieces (with no folds or bends) and straight tails (with no folds, loops or "lasso-like" coils).

4.2.5 *In vitro* heating of spermatozoa

A circulating water bath was heated to 42°C. An aliquot of each sample (in BWW) was carefully sealed, placed in a float and incubated in the water bath for 10 min. Following heating, each aliquot was used once (for the Comet assay; see below) and then discarded.

4.2.6 Comet assay

As discussed in Chapter 3, a commercially-available Comet assay kit was modified for use on murine spermatozoa. Briefly, 5μl spermatozoa samples were added to 25μl LMA (37°C). This gel/sample mix was then dropped onto a CometSlide (Trevigen) and covered with a clean, warm (37°C) cover slip. The slides were incubated at 4°C until the gels were set (~10 min) and the cover slips were

carefully removed. The slides were then incubated in lysis buffer (0.75% SDS, Lysis buffer; 2.5M NaCl, 100mM EDTA, 10mM Tris, 1% sodium lauryl sarcosinate, 0.01% Triton X-100) for 3h at 37°C.

The slides were transferred to a horizontal gel electrophoresis tank into which alkaline electrophoresis buffer (3M NaOH, 1mM EDTA, pH 12.3) was added to a depth of 0.5cm above the slides. Following a 20 min incubation at room temperature, current was applied at 25V, 300mA for 10 min. The slides were then transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min) and allowed to dry overnight at room temperature.

4.2.7 Comet analysis

Following completion of the comet assay, slides were stained with ethidium bromide which intercalated into the DNA released from the spermatozoa cells. Comet analysis was performed using the Komet Image Analysis system (version 4.0; Kinetic Imaging Ltd) as discussed in section 2.8.2. Briefly, 100-200 cells (calculation of the number of cells required for analysis is described in section 3.3.2) per sample were analysed. The percentage of DNA (as determined by the degree of fluorescence) in the comet head and tail of each cell was calculated and an average obtained for the population.

4.2.8 Statistical Analysis

Statistical analysis was limited due to the small size of the samples available for study. The results obtained for the heated animals were compared against those for the control animals using the Kruskal Wallis non-parametric test. However, it is acknowledged that the significance of this statistical test is diminished when used with small sample sizes ($n<10$).

4.3 Results

4.3.1 Dazl

4.3.1.1 Spermatozoa counts

The number of motile spermatozoa retrieved from the epididymides of each wt and *dazl* +/- mouse was calculated (Figure 4.1; black points). From these figures, the average number of motile spermatozoa for both genotypes was determined (Figure 4.1; red points).

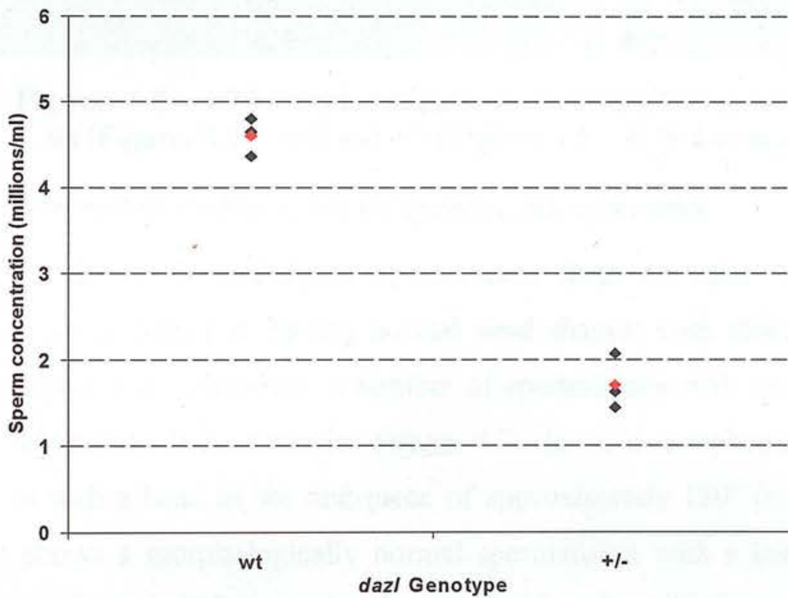
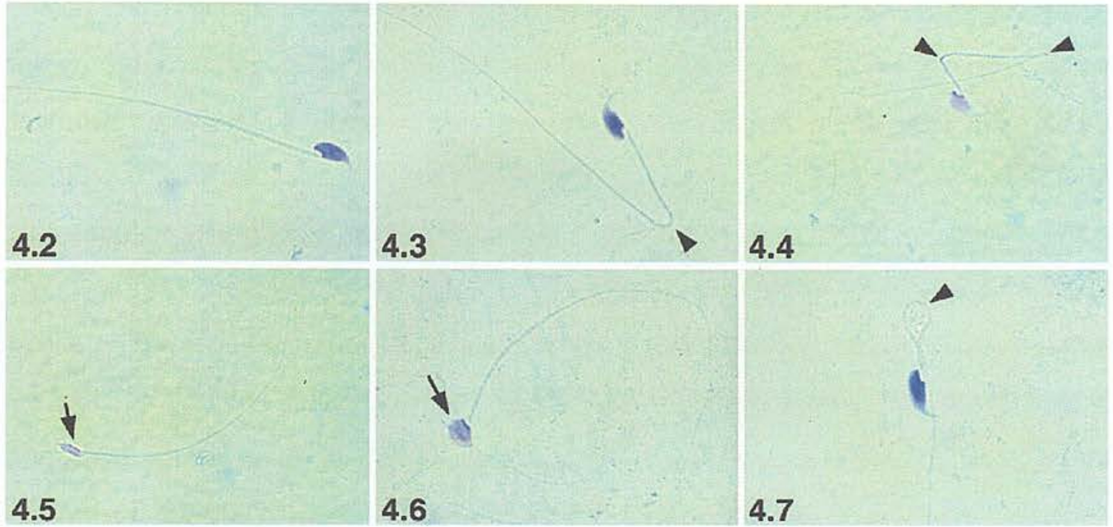


Figure 4.1 Spermatozoa concentrations of *dazl* genotypes (n=3 for both genotypes, red marker = average)

Statistical analysis revealed that the average number of motile spermatozoa retrieved from the epididymides of wt mice (4.6 million/ml; range = 4.2 - 4.8 million/ml), was significantly higher ($p<0.05$) than the average number of motile spermatozoa retrieved from the epididymides of *dazl* +/- mice (1.71 million/ml; range = 1.5 - 2.1 million/ml).

4.3.1.2 Spermatozoa morphology



Figures 4.2 – 4.7 Examples of spermatozoa morphologies from wt (Figures 4.2 – 4.4) and +/- (Figures 4.5 – 4.7) *dazl* mice

4.3.1.2.1 Morphologies observed in the spermatozoa of wt mice

The majority of epididymal spermatozoa from wt mice appeared to be morphologically normal (i.e. having normal head shapes) with straight mid-pieces and tails (Figure 4.2). However, a number of spermatozoa with tail abnormalities were also observed. For example, Figure 4.3 shows a morphologically normal spermatozoa with a bend in the mid-piece of approximately 180° (arrow head) and Figure 4.4 shows a morphologically normal spermatozoa with a bend in the mid-piece of approximately 90° (arrow head) and a bend in the tail of approximately 180° (arrow head). As expected, amongst the motile spermatozoa population of wt mice, the occurrence of abnormal head morphologies was rare. The percentage of abnormal spermatozoa in the wt spermatozoa population is estimated to be in the region of 15-20% (based on observations made during the determination of spermatozoal concentration - no formal calculation of percentage was made and therefore statistical analysis was not possible).

4.3.1.2.2 Morphologies observed in the spermatozoa of +/- dazl mice

Morphologically abnormal spermatozoa were observed more frequently in the epididymal population in the *dazl* +/- mice compared to that of wt mice. These included examples of both head and tail abnormalities. For example, in the epididymal spermatozoa of +/- mice, spermatozoa heads were more often found to be abnormal in size and/or shape (Figures 4.5 and 4.6) compared to wt mice. Other abnormalities observed in the motile spermatozoa population of +/- mice included bends in the mid-piece or tail (as seen in the wt population), often in combination with the presence of retained cytoplasm (Figure 4.7, arrow head). The percentage of abnormal spermatozoa in the +/- spermatozoa population is estimated to be in the region of 50-60% (based on observations made during the determination of spermatozoal concentration).

4.3.1.3 Endogenous DNA damage

For both wt and *dazl* +/- genotypes, 100 spermatozoa from each mouse (n=3) were analysed to determine the level of endogenous DNA damage present (See Figure 4.8).

Statistical analysis revealed that the average level of DNA damage in motile spermatozoa retrieved from the epididymides of wt mice (19.4% Tail DNA; range = 14.5-25%) was significantly lower ($p<0.05$) than the average level of DNA damage in motile spermatozoa retrieved from the epididymides of *dazl* +/- mice (39% Tail DNA; range = 32.2 -44.4%).

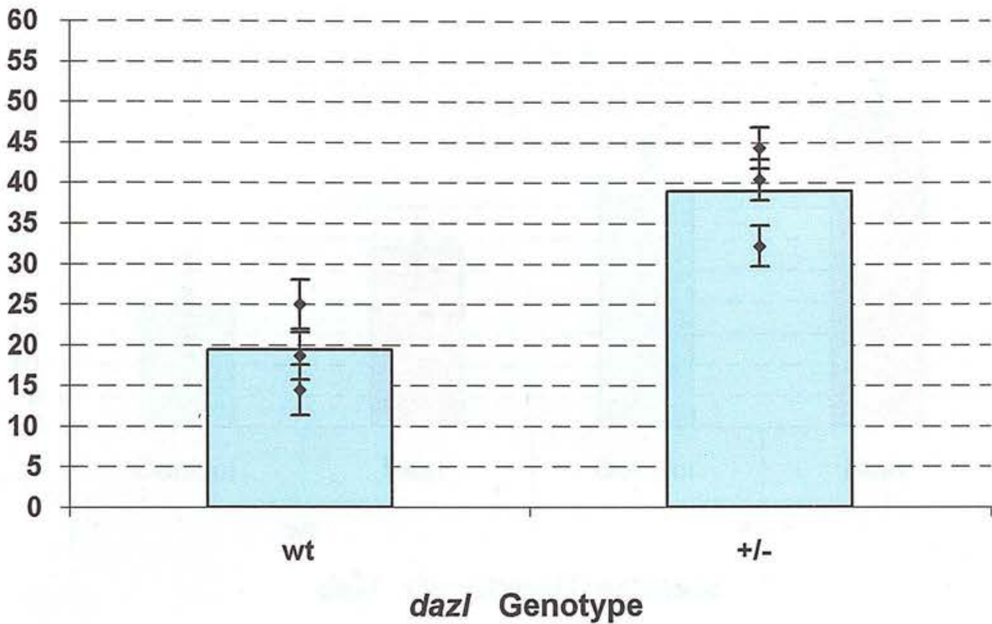


Figure 4.8 Levels of endogenous DNA damage in spermatozoa from *dazl* mice

(n=3 for both genotypes, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 3 mice)

4.3.1.4 DNA damage after heating

Following 10 min *in vitro* heat treatment (42°C), the level of DNA damage in spermatozoa from each genotype (wt and +/-) was again determined via the Comet assay and the results plotted against the endogenous levels of DNA damage for comparison (see Figure 4.9).

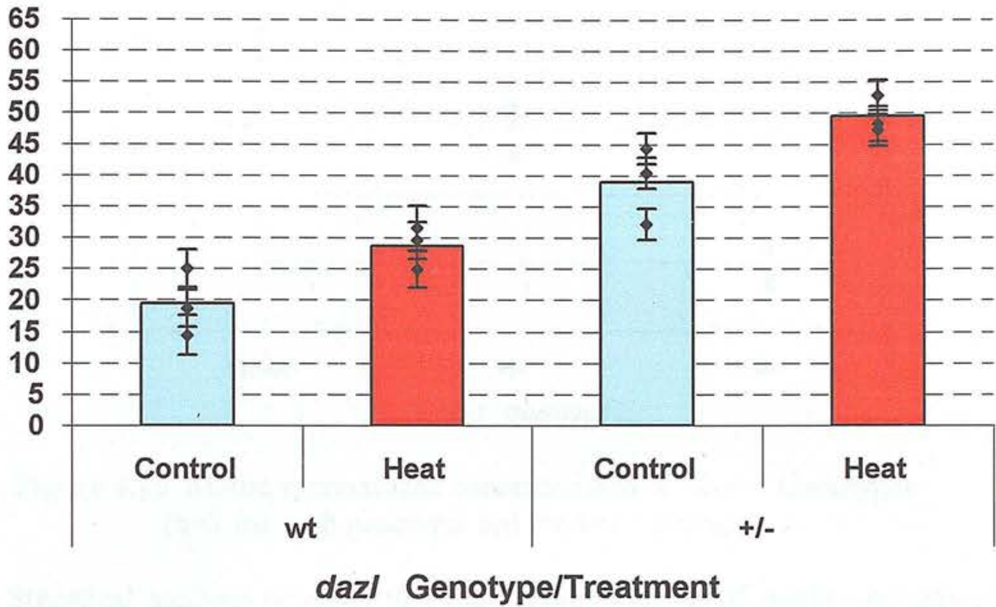


Figure 4.9 Levels of DNA damage before and after *in vitro* heat treatment in motile spermatozoa from wt and *dazl* +/- mice
(n=3 for both genotypes, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 3 mice)

Following *in vitro* heat treatment, DNA damage in the spermatozoa of wt mice increased significantly ($p<0.05$) from 19.4% (endogeous level) to 28.7% (24.9–31.6%). Likewise, in the spermatozoa of +/- *dazl* mice, DNA damage increased significantly ($p<0.05$) following *in vitro* heating from 39% (endogenous level) to 49.6% (47.3 – 52.9%).

4.3.2 *Ercc-1*

4.3.2.1 Spermatozoa counts

The number of motile spermatozoa retrieved from the epididymides of each 129wt, *Ercc-1* +/- and *Ercc-1* -/- mouse was calculated (Figure 4.10; black points). From these figures, the average number of motile spermatozoa for each genotype was determined (Figure 4.10; red points).

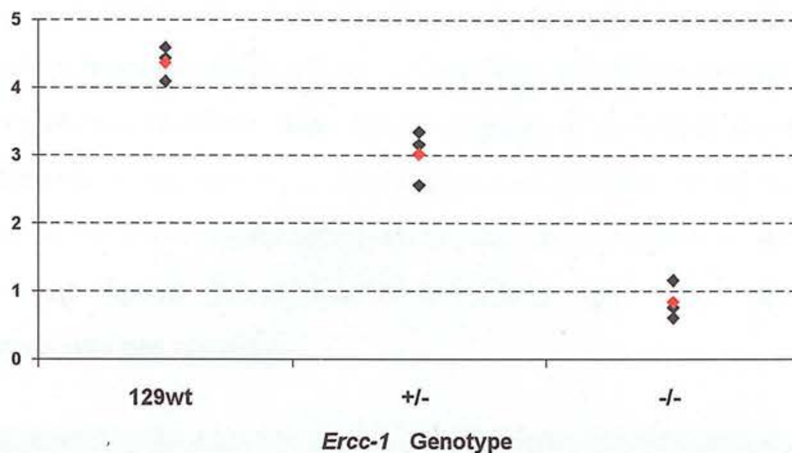


Figure 4.10 Motile spermatozoa concentrations of *Ercc-1* Genotypes (n=3 for each genotype, red marker = average)

Statistical analysis revealed that the average number of motile spermatozoa retrieved from the epididymides of 129wt mice (4.4 million/ml; range = 4.1 - 4.6 million/ml) was significantly higher than the average number of motile spermatozoa retrieved from the epididymides of *Ercc-1* +/- mice (3 million/ml; range = 2.6 – 3.4 million/ml; $p<0.05$) and *Ercc-1* -/- mice (0.8 million/ml; range = 0.6 – 1.2 million/ml; $p<0.05$). In addition, *Ercc-1* -/- mice also had significantly fewer ($p<0.05$) motile spermatozoa than *Ercc-1* +/- mice.

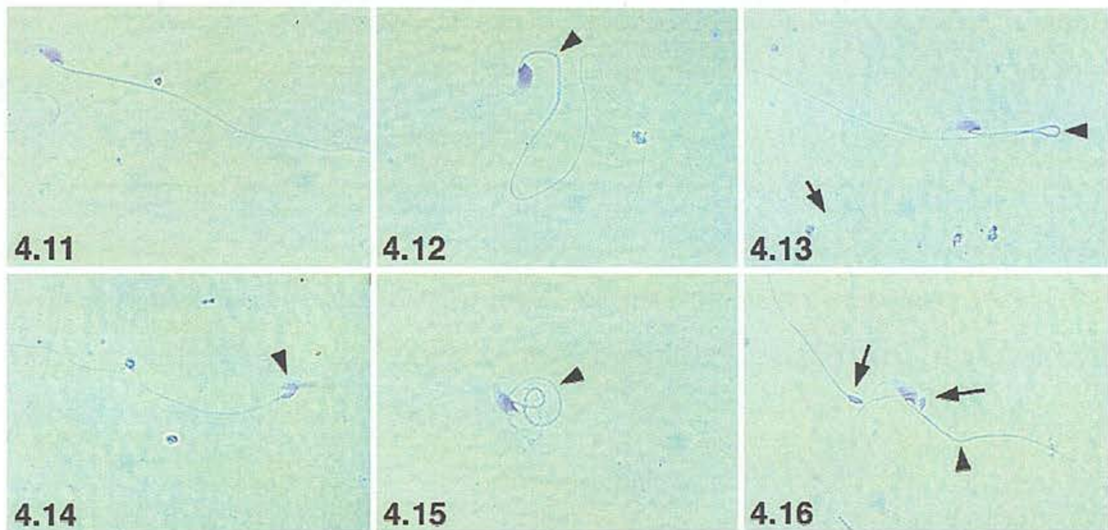
4.3.2.2 Spermatozoa morphology

Examples of morphologies observed in the spermatozoa of 129wt and *Ercc-1* -/- mice are shown in Figures 4.11 – 4.16.

4.3.2.2.1 Morphologies observed in the spermatozoa of 129wt mice.

It was found that, as observed in wt mice (section 4.3.1.2), the majority of motile spermatozoa from the 129wt mice tended to be morphologically normal, with morphologically normal heads, straight mid-pieces and tails (Figure 4.11). However, as with the wt mice, 129wt mice also appeared to produce a number of morphologically abnormal spermatozoa. The most common examples of these abnormalities included 90° bends (Figure 4.12; arrowhead) and 180° bends (Figure 4.13; arrowhead) in the mid-piece. In addition to these mid-piece and tail

abnormalities, examples of very small, morphologically abnormal and underdeveloped spermatozoa (Figure 4.13; arrow) were also found among the motile spermatozoa population of 129wt mice. The percentage of abnormal spermatozoa in the motile spermatozoa population of 129wt mice was estimated to be in the region of 15-25% (based on observations made during the determination of spermatozoal concentration - no formal calculation of percentage was made and therefore statistical analysis was not possible).



Figures 4.11 – 4.16 Examples of spermatozoa morphologies from 129wt (Figures 4.11 – 4.13) and *Ercc-1* -/- (Figures 4.14 – 4.16) mice

4.3.2.2.2 Morphologies observed in the spermatozoa of Ercc-1 -/- mice.

The percentage of abnormal spermatozoa from *Ercc-1* -/- mice was estimated to be in the region of 60-70% (based on observations made during the determination of spermatozoal concentration - no formal calculation of percentage was made and therefore statistical analysis was not possible). The majority of abnormalities were found to be in the mid-pieces and tails of the spermatozoa. However, examples of head abnormalities were observed (Figure 4.14; arrow head). Figures 4.15 and 4.16 both show spermatozoa with normal head morphologies but with abnormal mid-pieces and tails; in Figure 4.15 the tail and mid-piece have coiled up to form a 'lasso' while Figure 4.16 shows a spermatozoon with a relatively mild ($< 90^{o}$) bend in the

mid-piece. In addition, Figure 4.16 also shows 2 very small, morphologically abnormal and underdeveloped spermatozoa (arrows) as observed within the wt spermatozoa population (Figure 4.13; arrow).

4.3.2.3 Endogenous DNA damage

For each genotype (129wt, *Ercc-1* +/- and *Ercc-1* -/-), 100 spermatozoa from each mouse (n=3) were analysed to determine the level of endogenous DNA damage present (See Figure 4.17).

Statistical analysis revealed that the average level of DNA damage in motile spermatozoa retrieved from the epididymides of 129wt mice (average = 17.3% Tail DNA; range = 15.6 - 18.6%), was significantly lower ($p<0.05$) than the average level of DNA damage in motile spermatozoa retrieved from the epididymides of +/- *Ercc-1* mice (average = 26.2% Tail DNA; range = 22.8 - 29.2%). The spermatozoa from -/- *Ercc-1* mice were found to have levels of DNA damage (average = 35.9% Tail DNA; range = 34.6 - 37.1%) which were significantly higher than those of both 129wt ($p<0.01$) and *Ercc-1* +/-($p<0.05$) mice.

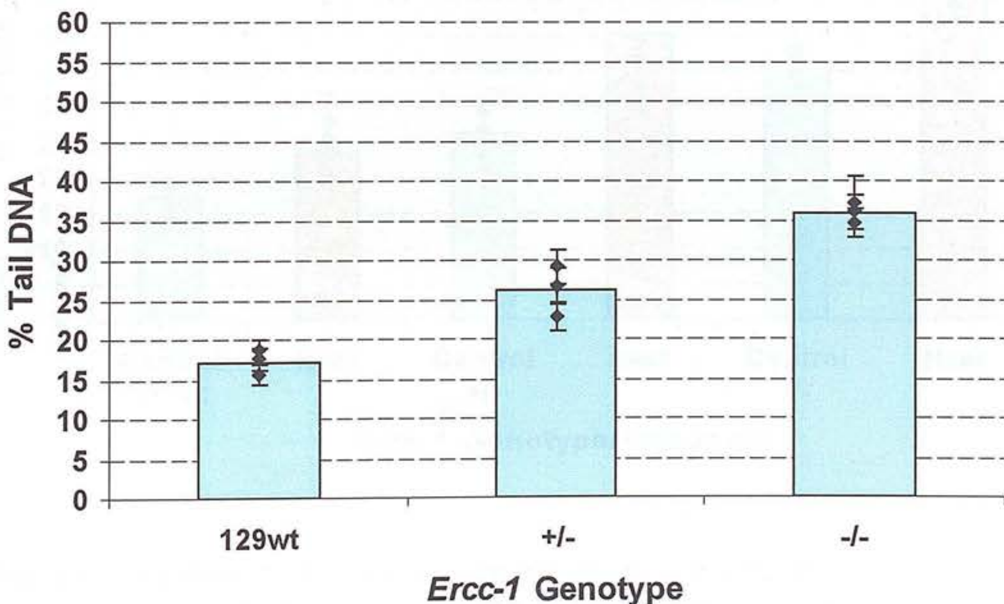


Figure 4.17 Levels of endogenous DNA damage in spermatozoa from *Ercc-1* mice (n=3 for each genotype, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 3 mice)

4.3.2.4 DNA damage after heating

Following 10 min *in vitro* heat treatment (42°C), the level of DNA damage in spermatozoa from each genotype (129wt, *Ercc-1* +/- and *Ercc-1* -/-) was again determined via the Comet assay and the results plotted against the endogenous levels of DNA damage for comparison (see Figure 4.18).

Following *in vitro* heat treatment, DNA damage in the spermatozoa of 129wt mice increased significantly ($p<0.05$) from 17.3% (endogeous level) to 23.6% (20.7 – 27.8%). Levels of DNA damage in the spermatozoa of *Ercc-1* +/- mice also increased (not statistically significant) following in vitro heat treatment, from 26.2% (endogenous level) to 40.1% (32.9 – 53%). Finally, levels of DNA damage in spermatozoa from *Ercc-1* -/- mice also increased significantly ($p<0.05$) following *in vitro* heat treatment, from 35.9% (endogenous level) to 49.6% (44.4 – 56.4).

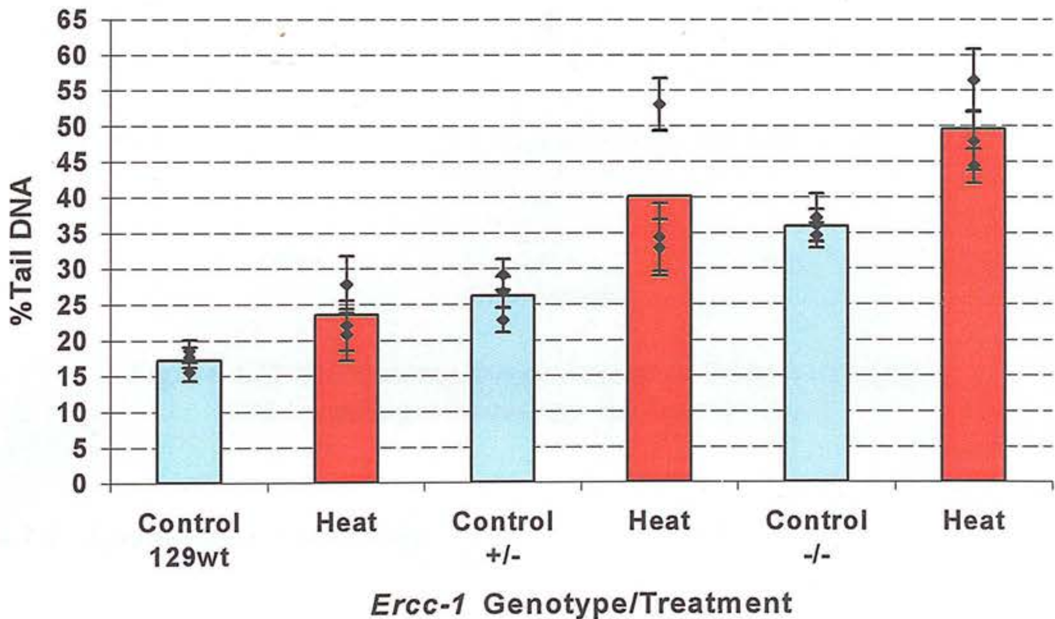


Figure 4.18 Levels of DNA damage before and after *in vitro* heat treatment in spermatozoa from 129wt, *Ercc-1* +/- and *Ercc-1* -/- mice
(n=3 for each genotype, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 3 mice)

4.3.3 PRION

4.3.3.1 Spermatozoa counts

The average number of motile spermatozoa retrieved from the epididymides of Prion +/+ mice (1.2 million/ml; range = 1.0 – 1.3 million/ml) did not appear to differ from the average number of motile spermatozoa retrieved from the epididymides of *PrP* -/- mice (1.1 million/ml; range = 1.1 – 1.2 million/ml) and *PrnD* -/- mice (1.5 million/ml; range = 1.5 – 1.6 million/ml). However, due to the low number of mice studied (n=2), statistical analysis was not possible. See Figure 4.19.

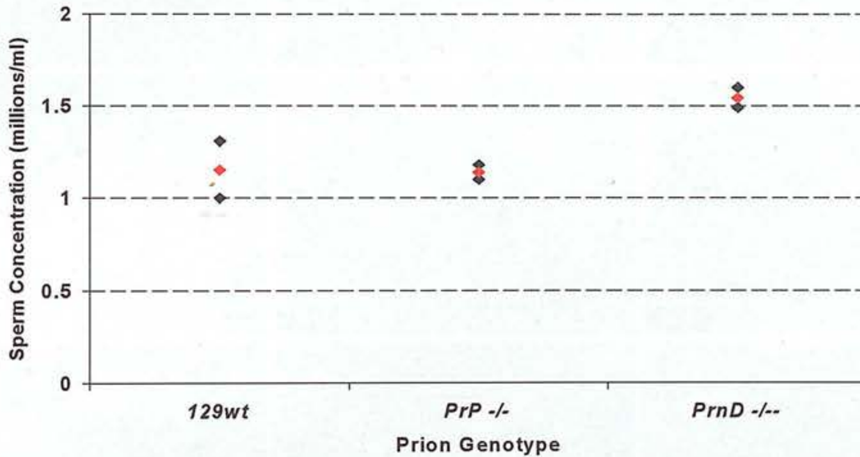


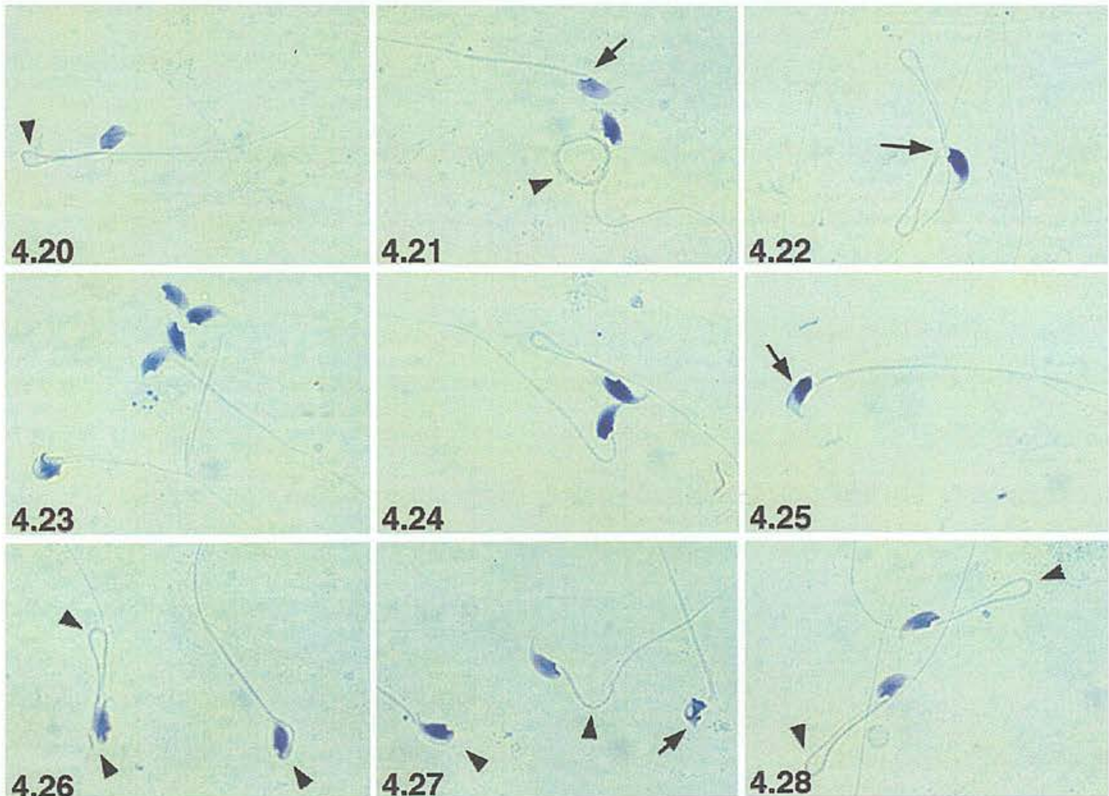
Figure 4.19 Spermatozoa concentrations of Prion Genotypes
(n=2 for both genotypes, red marker = average)

4.3.3.2 Spermatozoa morphology

4.3.3.2.1 Morphologies observed in the spermatozoa of 129wt mice.

Within the motile spermatozoa population retrieved from 129wt mice, the percentage of abnormal spermatozoa was estimated to be in the region of 10-20% (based on observations made during the determination of spermatozoal concentration – no formal calculation of percentage was made and therefore statistical analysis was not possible). These abnormalities included bends to the mid-piece and/or tail,

abnormal (club-shaped) heads and spermatozoa with 2 tails. Figure 4.20 shows a spermatozoon with an abnormal (club-shaped) head and a bend (arrow head) in the mid-piece. Figure 4.21 shows a morphologically normal spermatozoa (arrow) and a spermatozoon with a tail coiled into a lasso shape (arrow head). Figure 4.22 shows a spermatozoon with a morphologically normal head which appears to have 2 tails originating from the same site (arrow), both of which have severe bends.



Figures 4.20 – 4.28 Examples of spermatozoa morphologies from 129wt (Figures 4.20 – 4.22), *PrnD -/-* (Figures 4.23 – 4.25) and *PrP -/-* (Figures 4.26 – 4.28) mice.

4.3.3.2.2 Morphologies observed in the spermatozoa of PrnD -/- mice.

Compared to the 129wt mice, abnormal head morphologies were more common in the motile spermatozoa population retrieved from the *PrnD -/-* mice. In addition, abnormalities of the mid-piece and tail were also more common in *PrnD -/-* mice. Figure 4.23 shows a spermatozoon with a severely abnormal head and a

number of spermatozoa with bends of 180° in their mid-pieces, resulting in the spermatozoa head lying back against the mid-piece in the wrong direction. Figure 2.24 shows 2 spermatozoa with severe bends (~180°) in their mid-pieces and Figure 2.25 shows a further example of a spermatozoon with an abnormal (club-shaped) head. Within the motile spermatozoa population retrieved from *PrnD -/-* mice, the percentage of abnormal spermatozoa was estimated to be in the region of 35-45% (based on observations made during the determination of spermatozoal concentration – no formal calculation of percentage was made and therefore statistical analysis was not possible).

4.3.3.2.3 Morphologies observed in the spermatozoa of PrP -/- mice.

Severe (~180°) and/or multiple bends in the mid-pieces and tails were observed in the population of motile spermatozoa from *PrP -/-* mice studied (arrowheads, Figures 4.26-4.28). In addition, head abnormalities were also common (arrow, Figure 4.27). Within the motile spermatozoa population retrieved from *PrP -/-* mice, the percentage of abnormal spermatozoa was estimated to be in the region of 60-70% (based on observations made during the determination of spermatozoal concentration – no formal calculation of percentage was made and therefore statistical analysis was not possible).

4.3.3.3 Endogenous DNA damage

For each genotype (129wt, *PrP -/-* and *PrnD -/-*), 100 spermatozoa from each mouse (n=2) were analysed to determine the level of endogenous DNA damage present (See Figure 4.29).

Due to the low numbers of mice available for study (n=2), statistical analysis of the levels of endogenous DNA damage in the motile spermatozoa from the Prion genotypes was not possible. However, from the results obtained, it appeared that the levels of endogenous DNA damage in motile spermatozoa from both *PrnD -/-* (average = 47.4%) and *PrP -/-* mice (average = 55%) are considerable higher than those of wt mice (average =27.2) from the same genetic background (129wt).

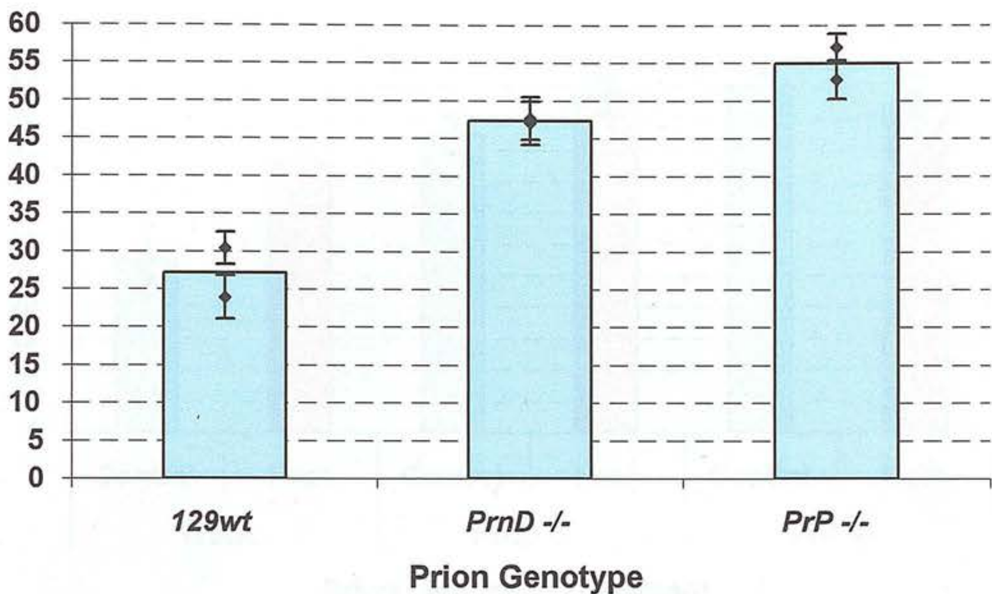


Figure 4.29 Levels of endogenous DNA damage in motile spermatozoa retrieved from the epididymides of 129wt, *PrnD -/-* and *PrP -/-* mice (n = 2 for each genotype, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 2 mice)

4.3.3.4 DNA damage after heating

Following 10 min *in vitro* heat treatment (42°C), the level of DNA damage in spermatozoa from each Prion genotype (129wt. *PrP -/-* and *PrnD -/-*) was again determined via the Comet assay and the results plotted against the endogenous levels of DNA damage for comparison (see Figure 4.30).

Following *in vitro* heat treatment, DNA damage in the spermatozoa of 129wt mice increased from 27.2% (endogenous level) to 43% (41.3 – 44.6%). Similarly, levels of DNA damage in the spermatozoa of *PrnD -/-* mice also increased following *in vitro* heat treatment, from 47.4% (endogenous level) to 53.7% (53.7 – 53.8%). In contrast to both the 129wt mice and *PrnD -/-* mice, the average level of DNA damage in spermatozoa from *PrP -/-* mice did not increase following *in vitro* heat treatment, instead decreasing from 55% (endogenous level) to 52.2%. Again, due to the low numbers of animals available for study, statistical analysis was not possible.

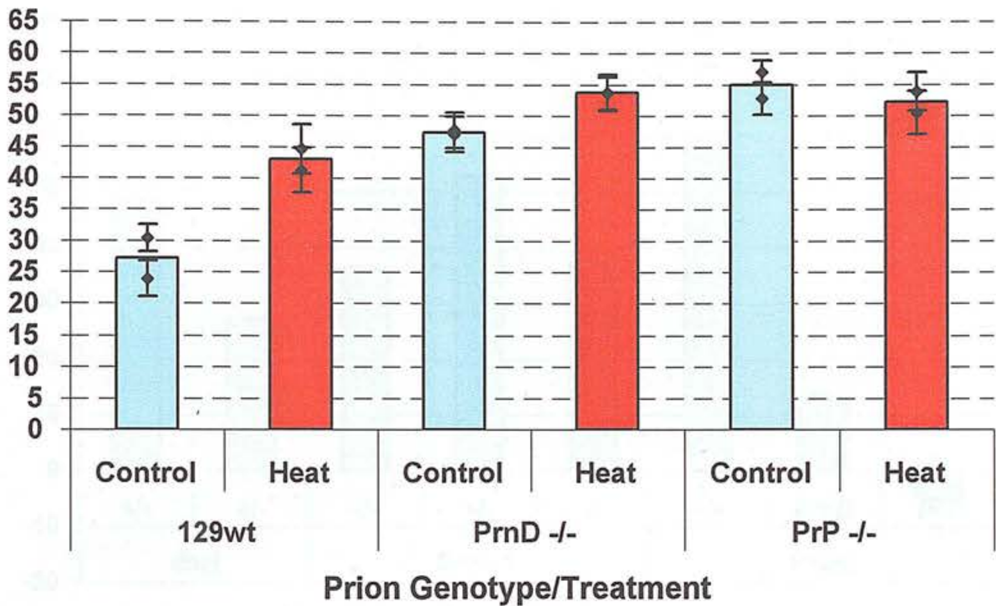


Figure 4.30 Levels of DNA damage before and after *in vitro* heat treatment, in motile spermatozoa retrieved from 129wt, *PrnD* -/- and *PrP* -/- mice (n=2 for each genotype, point = average % Tail DNA for 100 cells ± 95% confidence interval, bar = average % Tail DNA for 2 mice)

4.3.4 Comparison between all mouse strains

In order to compare the susceptibility of spermatozoa from the various mouse strains (*dazl*, *Ercc-1* and Prion/Prion-related) to exogenous DNA damage, the percentage increase/decrease in DNA damage following *in vitro* heat treatment was calculated (see Figure 4.31).

Motile spermatozoa from both +/+ and +/- *dazl* mice were susceptible to heat-induced DNA damage with average increases in DNA damage after heat treatment of 47.9% and 26.7% respectively compared to endogenous levels.

Similarly, the motile spermatozoa from all *Ercc-1* genotypes (+/+, +/- and -/-) were also found to be susceptible to heat-induced DNA damage with average increases of 36.2%, 52.7% and 37.9% respectively.

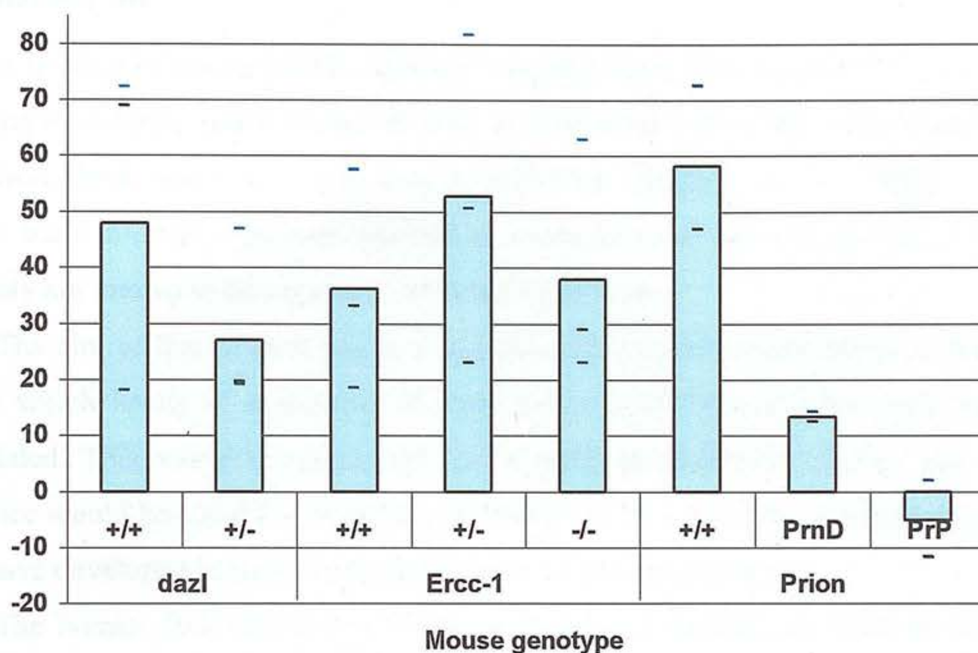


Figure 4.31 Comparison of % change in spermatozoa DNA damage following *in vitro* heating in all mouse genotypes studied

Motile spermatozoa from +/+ Prion mice were also found to be susceptible to heat-induced DNA damage with an average increase in DNA damage of 58.2%. Motile spermatozoa from -/- PrP and -/- PrnD mice were found to be least susceptible to heat-induced DNA damage of all the genotypes. Motile spermatozoa from -/- PrP mice were found to be slightly susceptible to heat-induced DNA damage with an average increase in DNA damage of 13.4%. Unlike all other genotypes, levels of DNA damage in motile spermatozoa from -/- PrP mice did not increase following heating and in fact an average decrease in DNA damage of 5.1% was observed.

The response of the spermatozoa from the different genotypes studied was varied and appeared to follow no particular pattern. The spermatozoa from -/- PrnD and -/- PrP mice appeared to be least susceptible to heat-induced DNA damage. In fact, the level of DNA damage in heat-treated spermatozoa was found to be lower than in control spermatozoa. In all other genotypes, the average levels of DNA damage in spermatozoa increased following *in vitro* heating.

4.4 Discussion

A number of mouse models for male infertility have been developed by either modifying or deleting genes known to play specific roles within the testis or genes which have been associated with human infertility (Matsuk, *et al.*, 2002). In addition, male infertility has been reported as a side-effect of deleting/altering genes previously not known to be associated with male infertility.

The aim of this chapter was to study the motile spermatozoa retrieved from mice in which levels of expression of *dazl*, *Ercc-1* and Prion proteins had been manipulated. This research was carried out in order to determine whether any of these mice would be suitable models for the study of DNA integrity of spermatozoa which have developed in testes with disturbances of gene expression.

The human *DAZ* (Deleted in AZoospermia) gene encodes an RNA binding protein which is exclusively expressed in the germ cells of the testis. The *DAZ* gene has been shown to be deleted in a high proportion of azoospermic/oligozoospermic men, and has been proposed as possible candidate for the so-called 'azoospermia factor' (AZF) (Yen, *et al.*, 1996). *DAZ* shares strong homology with the *Drosophila* male infertility gene *boule*. The boule protein has been shown to be a binding protein for all cell-cycle regulators, making it essential in the process of spermatogenesis (Eberhart, *et al.*, 1996). A homologue of *DAZ* has not been identified on the murine Y chromosome. Instead, an autosomal *DAZ*-like gene (*dazl*) which shares a degree of homology with both *DAZ* and *boule* has been identified and has been shown to be required for normal spermatogenesis in the mouse (Cooke, *et al.*, 1996;Ruggiu, *et al.*, 1997).

It has been shown previously that mice entirely lacking the autosomal gene *dazl* fail to produce mature spermatozoa and are therefore infertile. Heterozygote animals were reported to produce reduced numbers of motile spermatozoa with increased numbers of morphological abnormalities but remained fertile (Ruggiu, *et al.*, 1997). Similar results were obtained in the current study; reduced numbers of motile spermatozoa and increased numbers of morphologically abnormal

spermatozoa were observed in the epididymal spermatozoa population of heterozygote animals compared to wild-types.

In the present study, levels of endogenous DNA damage in motile epididymal spermatozoa from heterozygous mice were also studied and were found to be significantly higher than that of wild-type mice.

There appears to be no clear explanation as to why the deletion of *dazl* would affect the DNA integrity of spermatozoa. However, taking into consideration the homology between *dazl* and *boule*, it is possible that *dazl,* like *boule*, may play an important role in cell cycle regulation. Based on this, as yet unproven hypothesis, partial deletion of *dazl* may result in the disruption of spermatogenesis, thereby leading to impaired DNA integrity in the resulting spermatozoa.

Alternatively, the deletion of *dazl* might have a more direct effect on the DNA integrity of developing germ cells. It is known that *dazl* plays an important role in RNA binding in developing germ cells within the testis (Venables, *et al.*, 2001). It is possible that *dazl* could be involved in the binding of mRNA which codes for proteins involved in DNA packaging and/or repair. Disruption of the DNA packaging and repair mechanisms within the testis would most likely result in the production of spermatozoa with imperfect DNA.

Following *in vitro* heating, levels of DNA damage in motile spermatozoa from both heterozygotes and wild-type mice increased significantly. However, it was found that spermatozoa from wild-type mice were more susceptible to heat-induced DNA damage than spermatozoa from heterozygote mice. This finding contradicts the hypothesis that spermatozoa with higher levels of endogenous DNA damage would be more susceptible to further DNA damage.

The findings of the current study suggest that the genetic integrity of spermatozoa of men with *DAZ* deletions should be tested, and appropriate counselling given, before proceeding with assisted reproductive techniques such as IVF or ICSI. Future work might also involve the study of the susceptibility of spermatozoa from both mouse models and patients with *DAZ* deletions, to other forms of insult, e.g. chemical/mechanical.

The *Ercc-1* gene is involved in the nucleotide excision repair pathway which is known to be involved in 3 human disorders; *xeroderma pigmentosum* (XP), Cockayne's syndrome (CS) and trichothiodystrophy (Nunez, *et al.*, 2000). *Ercc-1* -deficient mice were first developed in order to study the role of this gene in these disorders. However, it has also been shown that *Ercc-1* is involved in recombinational repair pathways (Adair, *et al.*, 2000) and is highly expressed in the testis (Cheng, *et al.*, 1999). For these reasons, spermatogenesis in *Ercc-1* knockout mice has been studied (Melton, submitted) and the current investigation into the DNA integrity in spermatozoa from these animals was prompted. *Ercc-1* knockout mice usually die around 21 days of age, before the first wave of spermatogenesis is completed due to liver failure (McWhir, *et al.*, 1993). The animals used in this study remained viable beyond 21 days because of the introduction of an *Ercc-1* transgene under the control of a liver-specific promoter (Selfridge, *et al.*, 2001). Prior to the current study, investigators had observed that male *Ercc-1* knockout mice appeared to be subfertile, failing to produce many offspring (Melton, personal communication).

In the present study, it was found that compared to wild-type and heterozygote animals, *Ercc-1* knockout mice produced significantly fewer motile spermatozoa ($p<0.01$). In addition, it was observed that abnormal morphologies, in particular abnormal spermatozoa heads, appeared to be more common amongst motile spermatozoa from *Ercc-1* knockout mice. It has been shown that mean testis weight for *Ercc-1* knockout mice is on average only 60% of wild-type (Selfridge, *et al.*, 2001). Histological analysis has shown that the testes of Ercc-1 knockout mice contain fewer germ cells than those of wild-type littermates, in the knockout mice seminiferous tubules are generally reduced in diameter, and that there were also tubules completely devoid of germ cells (Hsia, *et al.*, 2003). These reduced numbers of germ cells observed within the seminiferous tubules of *Ercc-1* knockout mice would account for the reduced numbers of motile spermatozoa produced by these animals.

Levels of endogenous DNA damage within the motile spermatozoa population of *Ercc-1* knockout mice were also found to be significantly higher than that of both wild-type and heterozygous mice. Following *in vitro* heating, levels of DNA damage increased in all *Ercc-1* genotypes. Knockout and wild-type *Ercc-1* mice appeared to have similar susceptibility to heat-induced DNA damage, with similar increases in DNA damage. *Ercc-1* heterozygotes appeared to be most susceptible to heat-induced DNA damage, with the greatest increase in mean % DNA damage following *in vitro* heating.

As previously discussed (section 1.1.5.1) DNA stand breaks and nicks occur during the process of spermatogenesis to facilitate the remodelling of DNA from the somatic arrangement in association with histones, to the tightly compacted arrangement of DNA in association with protamines observed in spermatozoa (Sakkas, *et al.*, 1995). In the absence of *Ercc-1*, strand breaks and nicks in the DNA of developing germ cells in the testis could accumulate and persist to the mature spermatozoa.

The reduced numbers of motile spermatozoa, together with the increased numbers of morphological abnormalities and increased levels of DNA damage in the motile spermatozoa population may in part explain the failure of these mice to produce expected numbers of offspring.

The role of Prion and Prion-related proteins in the testis has not been determined. However, it has been shown that these proteins are highly expressed in the testis and it has been suggested their role in the testis might be similar to their role in the brain – i.e. maintaining the blood-brain/blood-testis barrier (Li, *et al.*, 2000). Previous investigators have noted the very poor fertility of Prion deficient mice (Melton DW, personal communication). In particular, male PrP- and Dpl-deficient mice, while demonstrating normal sexual behaviour, have both been found to be infertile. The fertility of the female mice with which these males were mated has been proven and the overall morphology of the testes of these mice appears normal without obvious germ cell depletion (Behrens, *et al.*, 2002)(Melton DW, personal communication).

In the present study, all Prion genotypes tested (wild-type, PrP and PrnD-deficient) were found to have very few motile spermatozoa compared to the other mice genotypes studied. All genotypes, including wild-type, produced motile spermatozoa with a range of morphological abnormalities, in particular abnormalities of the spermatozoa head. In addition, levels of endogenous DNA damage in these mice were also very high in comparison with the other strains tested. Following *in vitro* heating, levels of DNA damage in motile spermatozoa from wild-type spermatozoa increased considerably (more than any population studied in this research) while motile spermatozoa from PrP and PrnD-deficient mice appeared to be unresponsive to heat-treatment.

It should be noted that though the levels of endogenous damage in these mice is high, the upper limit of DNA damage measured by the assay was not reached and therefore if the *in vitro* heat treatment had affected the integrity of these spermatozoa, it would have been detected.

The low numbers of mice available for this study (n=2 for each genotype) resulted in the inability to perform statistical analysis on the results obtained. Therefore, in order to verify these results, future studies involving higher numbers of animals are necessary.

There is no obvious reason why a lack of PrP protein would result in reduced numbers of spermatozoa, or a loss of DNA integrity in surviving spermatozoa. It has been proposed that Dpl is required for the correct development of the head and acrosome during spermiogenesis, a period when a great deal of DNA remodelling occurs (Behrens, *et al.*, 2002). Further work on determining the roles of these proteins, particularly in relation to DNA packaging, repair etc., within the testis is required.

The purpose of this study was to determine whether the presence of impaired DNA integrity in the spermatozoa of 3 mouse models for male infertility is a contributory factor to their infertility.

It should be noted that limited numbers of animals were available for use in the current study. Furthermore, due to the subjective nature of the analysis of DNA

damage in the motile spermatozoa, it would have been preferable to analyse the results in a 'blind' manner. However, the results obtained suggest that future study into the genetic integrity of spermatozoa from these mouse models is warranted.

It has been shown that it is difficult to produce adequate numbers of both *Ercc-1* and Prion mice and this would prove impractical if these mice were to be used in large studies requiring high numbers of subjects for statistical analysis. The low numbers of motile spermatozoa and high numbers of morphologically abnormal spermatozoa retrieved from heterozygous and knock-out *dazl*, *Ercc-1* and Prion mice suggest that these mice may not be suitable models for studying levels DNA damage in 'healthy' spermatozoa.

However, this study has shown that the DNA of motile spermatozoa is susceptible to heat-induced damage. The role of heat stress in infertility has already been demonstrated in a number of studies. Wild-type mice produce large numbers of motile, morphologically normal spermatozoa and are capable of producing large numbers of offspring. Endogenous levels of DNA damage in spermatozoa from these mice are low but have been shown to be susceptible to heat-induced damage. It is proposed that wild-type mice may be a suitable model for the study of male infertility via heat-induced DNA damage in motile spermatozoa.

Chapter 5

The effect of scrotal heating on the testis and spermatozoa developed from heated germ cells

5.1 Introduction

5.1.1 Heat stress and the testis

In most mammals, scrotal temperature is 2-8°C lower than body temperature and this lower temperature is required for normal spermatogenesis (Harrison, *et al.*, 1948;Ulberg, 1958;Bishop, *et al.*, 1960).

The effects of raised scrotal temperature have been studied to varying degrees in a number of species (including man) and general observations in the rat (Lue, *et al.*, 2000), mouse (Jannes, *et al.*, 1998) and human (Mieusset, *et al.*, 1987) include decreased testis weight and poor spermatozoa viability, morphology and motility.

Animal studies have shown that raised scrotal temperature results in reduced testicular blood flow and altered vasomotion as observed in the rat (Setchell, *et al.*, 1995) and the ram (Mieusset, *et al.*, 1992). In addition, studies in the rat have shown that protein synthesis by Sertoli cells and androgen production by Leydig cells can be adversely affected by the subjection of the testis to abdominal temperature which may in turn have an adverse affect on germ cell function (Jegou, *et al.*, 1983). Direct effects of heating on the developing germ cells in the mouse may include altered DNA, RNA and protein synthesis, protein denaturation and abnormal chromatin packing (Hand, *et al.*, 1979;Steinberger, 1991;Sailer, *et al.*, 1997).

Previous studies have determined the effects on the mouse testis of a range of temperatures (38-42°C) and exposure times (20 min - 1 hour). In an extensive study by De Vita *et al* (1990), it was shown that even exposure to mild heat stress (38°C) for a short time (20 min) caused cytotoxic effects in some germ cell types and that these effects were increased with higher temperatures and longer exposure times (De-Vita, *et al.*, 1990).

5.1.1.1 Clinical consequences

In man, raised scrotal temperature may occur as a result of occupational exposure, lifestyle or clinical disorder, such as varicocele or cryptorchidism (Zorgniotti, 1980;Mieusset, *et al.*, 1987). In particular, it has been shown that men with a past history of cryptorchidism or current varicocele are likely to exhibit scrotal temperatures above the normal range and are often sub- or infertile. It has not yet been clearly established whether raised scrotal temperature is the cause of infertility in these men or simply a concomitant symptom of these disorders. (Ali, *et al.*, 1990;Mieusset, *et al.*, 1995).

In infertile men with elevated scrotal temperatures, an increased incidence of abnormalities such as tapered/elongated forms and immature spermatozoa in the ejaculate has been observed and a correlation between temperature and degree of abnormality has been inferred (Mieusset, *et al.*, 1987;Ali, *et al.*, 1990;Mieusset, *et al.*, 1995).

5.1.2 Aims of this chapter

It is now common to treat couples with male-factor infertility using techniques such as *in vitro* fertilisation (IVF) and intracytoplasmic spermatozoa injection (ICSI). Clinical data indicates that poor spermatozoa quality (e.g. motility and DNA integrity) may result in reduced fertilisation rates, fewer blastocysts, and poor embryonic development following assisted reproduction techniques (Lopes, *et al.*, 1998). At present, DNA integrity is not routinely used in the selection of spermatozoa for use in these techniques.

The aim of this study was to investigate the effects of heat stress on the process of spermatogenesis, and to identify the cell types in the testis susceptible to heat stress which may then develop into DNA-damaged spermatozoa.

5.1.3 Experimental outcomes to be studied in this chapter

5.1.3.1 Expression of stress markers

In order to confirm the response of the testis to heat stress, a number of stress proteins were used as markers.

Cold-inducible RNA-binding protein (Cirp) is constitutively expressed in the germ cells of the testis (Nishiyama, *et al.*, 1997). Following experimental cryptorchidism or heat stress (42°C), decreased expression of Cirp in the testis has been observed within 6h (Nishiyama, *et al.*, 1998). Cirp protein expression was used to confirm that the temperature of the testis had been raised following scrotal heating.

It has been demonstrated that heat shock protein (HSP) 105 is specifically expressed in the germ cells of the rat testis and that heat stress *in vivo* (via experimental cryptorchidism) results in the translocation of HSP105 from the cytoplasm to the nuclei of germ cells. The precise role of HSP105 in the testis has not yet been defined, however, it has been proposed that HSP105 may be involved in the regulation of p53 (a pro-apoptotic protein) in the testis (Wakatsuki, *et al.*, 1998;Ishihara, *et al.*, 2000).

Bax is a pro-apoptotic regulator of apoptosis and is expressed in the testis of a number of species, including mouse, rat and human. *In vivo* heat stress results in the altered expression of Bax in the testis of the mouse, rat and human (Penault-Llorca, *et al.*, 1998;Xu, *et al.*, 2000;Yamamoto, *et al.*, 2000).

For example, in the rat, scrotal heating reportedly results in the redistribution of Bax from a cytoplasmic to perinuclear localization in all germ cells prior to activation of apoptosis in these cells (Yamamoto, *et al.*, 2000). Experimental cryptorchidism in the mouse also leads to altered expression of Bax and increased apoptosis (Xu, *et al.*, 2000).

In the current study, HSP105 and Bax expression was studied in order to determine whether or not a stress response within the testis had been triggered following scrotal treatment.

5.1.3.2 Detection of apoptotic cells

The presence of cells exhibiting apoptotic characteristics (i.e. DNA strand breaks, altered morphology) was also used as an indicator of a stress response in the testis caused by *in vivo* heat treatment. Possible apoptotic cells were identified using a modified Terminal UdTP nick end-labelling (TUNEL) method.

5.1.3.3 Study of spermatozoa

It has been well documented that increased scrotal temperature results in the decreased production of motile spermatozoa in both humans and animal models (Brown-Woodman, *et al.*, 1984; Figa-Talamanca, *et al.*, 1992;Wang, *et al.*, 1997; Jannes, *et al.*, 1998; Saikhun, *et al.*, 1998;Setchell, 1998).

In the present study, the number of motile spermatozoa which had developed from specific types of germ cells subjected to scrotal heating was calculated in order to determine the relative susceptibility of these cell types to raised testicular temperature.

The effects of raised scrotal temperature on integrity of spermatozoa DNA have yet to be determined. In the current study, the DNA integrity of motile spermatozoa developed from germ cells exposed to raised scrotal temperatures was measured using the Comet assay. This assay has been used in a number of studies of mammalian spermatozoa (human and animal models), and has been shown to be a sensitive and reproducible method for the detection of DNA strand breaks in speratozoa (Hughes, *et al.*, 1996;Anderson, *et al.*, 1997;Steele, *et al.*, 1999;Donnelly, *et al.*, 2000;Irvine, *et al.*, 2000;Shen, *et al.*, 2000;Steele, *et al.*, 2000).

5.2 Materials and Methods

5.2.1 Animals

The animals used were sexually mature male wild-type (+/+) *dazl* mice which were maintained under standard conditions of a 12L:12D cycle, in an ambient temperature of 20-25°C with access to food and water *ad libitum*.

5.2.2 Scrotal Heating

Adult male mice were anaesthetised with a 1:1 mix of Hypnorm and Hypnovel via an i.p. injection. Following anaesthesia, the lower third (hind legs, tail and scrotum) of each male was passed through a hole in a polystyrene 'raft' which was then placed in a circulating water bath for 30 min at 42°C. Control animals received anaesthetic only. All animals were returned to their cages, which were placed on a warm mat (20-25°C) to maintain body temperature, until fully recovered from the anaesthetic.

5.2.3 Time-points

For each time-point studied, 3 mice underwent *in vivo* heat treatment and 3 mice were used as controls. All mice were matched for age and weight.

Animals were sacrificed at 1 hour (h), 2h, 4h, 6h, 24h, 7 days (d), 14d, 21d, 24d, 28d, and 32d post-treatment. Each time-point was chosen to allow the study of the initial response of the testis to heat stress and a particular population of cells within the testis at the time of heating. Calculations were based on the stage durations of mouse spermatogenesis as defined by Oakberg (1956) and Russell (1990) (Oakberg, 1956; Russell, 1990), and a 7-day epididymal transit time as defined by Cornwall (1990). Table 5.1 indicates the cell types represented by the motile spermatozoa in the epididymis at the chosen time-points after in vivo scrotal heating.

At each time-point, testes were removed and fixed in Bouins for 10h then transferred to 70% ethanol and processed into paraffin wax for immunohistochemical analysis. At the later time-points (7-32d), epididymides were removed and placed in ~1ml BWW containing 20mM HEPES and 0.3% HSA.

Table 5.1 Cell types represented by the population of motile spermatozoa retrieved at the chosen time-points after scrotal heating.

Time Point	Cell Type at Time of Treatment
1h, 2h, 4h, 6h, 24h,	Mature Spermatozoa in Epididymis
7 Days	Step 11 Spermatid – Mature Spermatozoa
14 Days	Step 1 Spermatid – Step 11 Spermatid
21 Days	Stage IV Pachytene – Step 1 Spermatid
24 Days	Stage XII Pachytene – Stage X Pachytene
28 Days	Preleptotene - Stage III Pachytene
32 Days	A Spermatogonia - Zygotene

5.2.4 Immunohistochemistry

Bouins-fixed, paraffin-embedded tissues were sectioned at 5µm. Paraffin wax was removed from sections by washing in 2 changes of xylene (5 min each) and the sections were rehydrated in decreasing concentrations of ethanol (100%. 95%, 75% and finally water).

5.2.4.1 Diaminobenzidine (DAB) staining

Endogenous peroxidase activity was blocked by incubation in H_2O_2/methanol as previously described followed by washing twice (5 min each) in Tris-buffered saline (pH 7.4). Slides were pre-incubated with serum block (20% NSS, 5% BSA in TBS) at room temperature for 30 min before addition of the primary antibody and overnight incubation at 4°C. Slides were washed twice in TBS (5 min each) before addition of the secondary antibody and incubation at room temperature for 30 min. Slides were again washed twice in TBS (5 min each). ABC conjugated to HRP (DAKO) was added to the slides and incubated for 30 min at room temperature and the slides were again washed twice in TBS before the addition of DAB. The

resulting colour reaction was stopped with water and the slides were counterstained with Mayer's Haematoxylin.

5.2.4.2 Fluorescent staining

Slides were washed twice in PBS (5 min each) before incubation in a bath of 3% hydrogen peroxide (H_2O_2) in methanol at room temperature for 30 min. After further washes in PBS, slides were blocked with normal swine serum (NSS; 5% BSA, 10% swine serum; Diagnostics Scotland in PBS) for 1h at room temperature. The anti-Cirp antibody was added at a 1:2000 dilution (in NSS) for 2h at room temperature before washing in PBS as before. Slides were then incubated with swine anti-rabbit FITC (1:50 in NSS; DAKO) for 1h at room temperature. Slides were again washed in PBS before mounting using Permafluor (Coulter).

5.2.4.3 Detection of apoptotic cells

Slides were deparraffinised, rehydrated and blocked with H_2O_2/methanol as previously described. Slides were washed in water, then PBS (5 min each) before digestion of the tissue with 20μg/ml Proteinase K in buffer (3.5ml 1M Tris, pH 8, 0.7ml 0.5M EDTA, pH 8, made up to 35ml with dH_20). Slides were washed twice in PBS (5 min each). The reaction mix (30mM Tris/HCl, pH 7.2, 140mM Na Cacodylate, 1.5mM CoCl) containing 1μl/ml Terminal d-Transferase (TdT; Roche), 5μl/ml Digoxigenin (DIG; Roche) was added followed by cover-slips which were sealed with cow gum/hexane before incubation at 37°C for 30 min.

Following the removal of cover-slips and washes in PBS, sections were blocked with 20% normal rabbit serum (NRS; Diagnostics Scotland) in PBS for 10 min at room temperature. Sheep anti-DIG IgG (1:100 in NRS/PBS) was added and the slides incubated at room temperature for 90 min before further washes in PBS. Rabbit anti-sheep IgG Biotinylated (1:500 in NRS/TBS) was added for 30 min at room temperature before washing twice in TBS (5 min each). As in the ABC method, ABC conjugated to HRP was added to the slides and incubated for 30 min at

room temperature and the slides were again washed twice in TBS (5 min each) before the addition of DAB. The resulting colour reaction was stopped with water before the slides were counterstained with Mayer's Haematoxylin.

For each time-point (control and heated animals), the number of TUNEL-positive cells in 3 random fields were counted and the average value calculated to obtain an estimate of the degree of apoptosis occurring.

5.2.5 Preparation of murine spermatozoa

Full methods for the preparation of murine spermatozoa are described in section 2.6. Briefly, whole epididymides were removed, cleaned of fatty tissue and placed in 1ml BWW. The tissue was then minced with fine scissors and incubated at 34°C (95% air, 5% CO_2) for 30 min prior to careful removal of buffer containing the motile spermatozoa. Samples were diluted to a final volume of 1ml in BWW and stored at -20°C until required.

In the current chapter, spermatozoa samples from mice sacrificed at the later time-points (7d – 32d) were studied (representing cells within the testis at the time of heating). Spermatozoa samples from the earlier time-points (1h – 7d) are studied in Chapter 6 which will examine the effects of scrotal heating on spermatozoa present within the epididymis at the time of heating (See Chapter 6).

5.2.6 Study of concentration of spermatozoa

Samples were diluted 1:10 with SDF and 10μl diluted sample was placed into the chamber of an improved Neubauer haemocytometer. The number of spermatozoa located within 5 squares of the haemocytometer grid was carefully counted. If the number of spermatozoa in 5 squares was not equal to or greater than 100, 10 or 25 squares were counted. From this value, the number of spermatozoa in 1ml was calculated using the following equation:

$$\text{Concentration (millions/ml)} = \frac{\text{No Spermatozoa Counted}}{(4 \times \text{No Squares}) \div \text{Dilution}}$$

5.2.7 Comet Analysis of spermatozoa

DNA integrity of motile spermatozoa from the epididymis was studied using a modified Comet assay (See Chapter 3). Briefly, spermatozoa samples were defrosted at room temperature and 5µl each sample was mixed with 25µl LMA (37°C). This gel/sample mix was dropped onto a CometSlide (Trevigen) and covered with a clean, warm (37°C) cover-slip. Slides were then placed horizontally in a box and incubated at 4°C until the gels were set. Cover-slips were carefully removed and the slides submerged in lysis buffer (Trevigen) containing 0.75% SDS, for 3 h at 37°C. The slides were removed from the lysis buffer and placed in a horizontal gel electrophoresis tank. Alkaline electrophoresis buffer (3M NaOH, 1mM EDTA, pH 12.3) was carefully added to a depth of 0.5cm above the slides and left for 20 min at room temperature. Current was applied at 25V, 300mA for 10 min. The slides were transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min). The slides were allowed to dry overnight at room temperature before staining with 50µl ethidium bromide (1:1000 in dH_2O). For each sample, 100 cells were analysed using the Komet Image Analysis system. The percentage of head DNA, tail DNA and the Comet Moment was calculated for each cell. Comet Moment is a measurement that takes into account both the length of the Comet tail and the amount of DNA present in the tail and is expressed as an arbitrary unit (the greater the value, the higher the level of DNA damage).

5.2.8 Statistical Analysis

Statistical analysis was limited due to the small size of the samples available for study. The results obtained for the heated animals were compared against those for the control animals using the Kruskal Wallis non-parametric test. However, it is acknowledged that the significance of this statistical test is diminished when used with small sample sizes ($n<10$).

5.3 Results

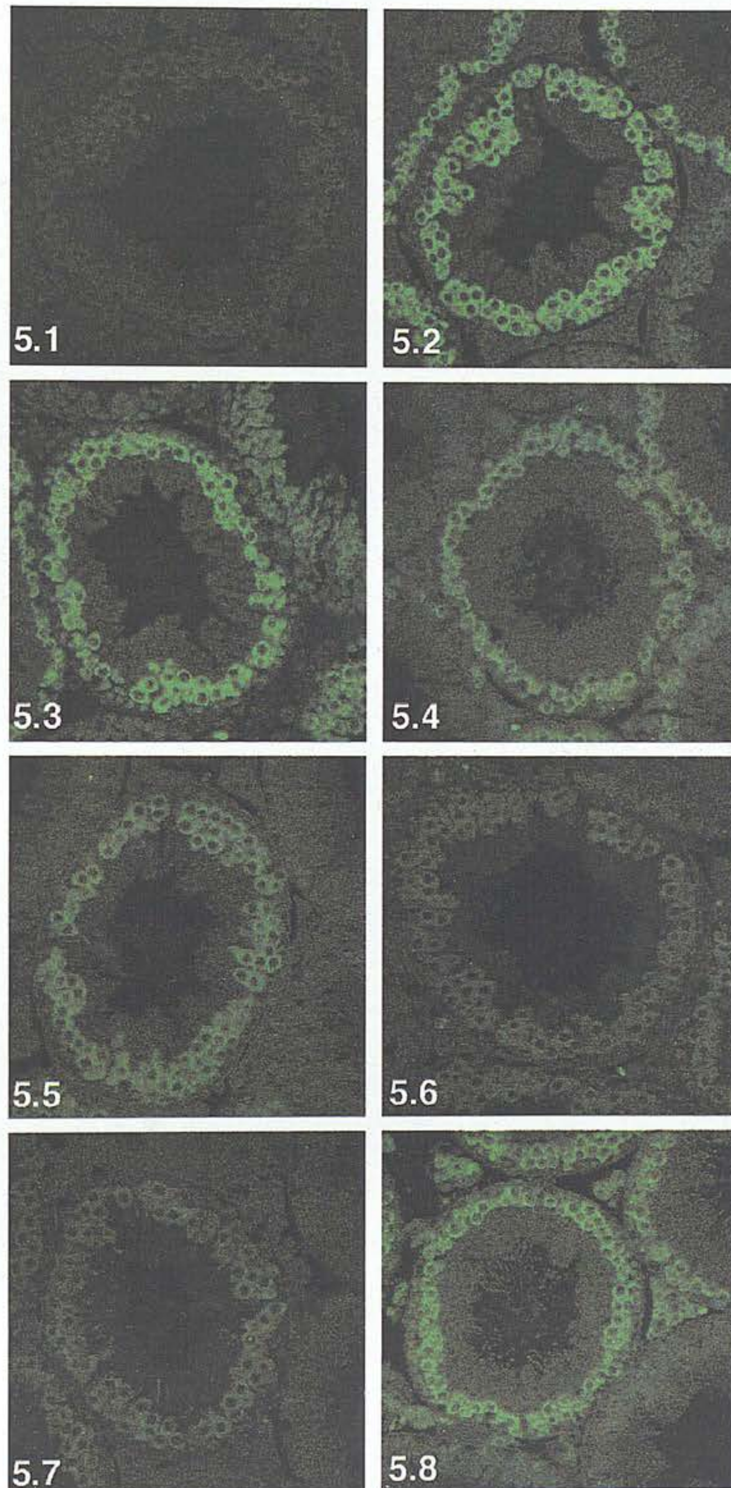
5.3.1 Immunohistochemistry

The purpose of the immunohistochemical analysis was to confirm that the temperature of the testis had increased and to study the stress response of the testis following this increase in temperature.

5.3.1.1 Response of the testis to heat stress

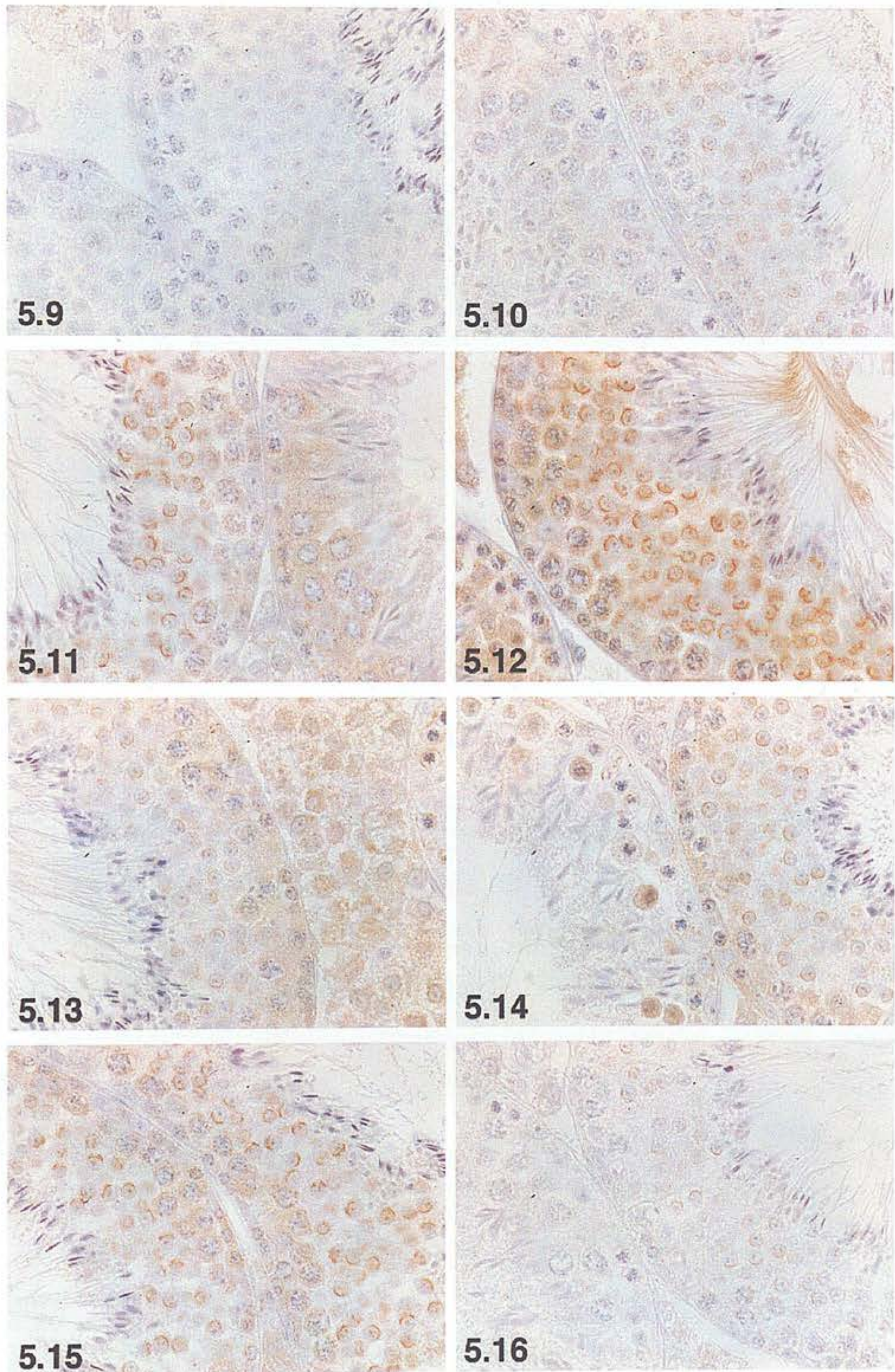
Cirp protein was detected predominantly in the pachytene spermatocytes in the control testis (Figure 5.1). Following *in vivo* heat treatment, normal levels of Cirp were detected in the heated testis at the 1 and 2h time-points (Figures 5.2-5,3). However, at the 4h time-point (Figure 5.4), the level of detection of Cirp protein decreased and did not recover (Figures 5.5-5.7) until 7d after treatment (Figure 5.8).

In the control testis, low levels of HSP105 expression were detected in pachytene spermatocytes and early spermatids (Figure 5.10). Within 1h of heat treatment, the levels of HSP expression detected were much higher, particularly in the early spermatids (Figure 5.11). The highest levels of HSP105 expression were detected 4h after heating with strong expression in both the pachytene and developing spermatids (Figure 5.12). Expression of HSP105 decreased at 6h (Figure 5.13) and at 24h staining was evident in a number of cells with the appearance of apoptotic cells (Figure 5.14). Expression of HSP105 decreased further at 24 h post heating (Figure 5.15) and at 7 d had returned to control levels (Figure 5.16).



Figures 5.1-5.8 Cirp expression in the testis following in vivo heat treatment.

Figures show staining for the Cirp protein in the negative control (5.1), the control testis (5.1) and the heated testis after 1h (5.3), 2h (5.4), 4h (5.5), 6h (5.6), 24h (5.7) and 7d (5.8).



Figures 5.9-5.16 HSP105 expression in the testis following in vivo heat treatment.

Figures show staining for the HSP105 protein in the negative control (5.9), the control testis (5.10) and the heated testis after 1h (5.11), 2h (5.12), 4h (5.13), 6h (5.14), 24h (5.15) and 7d (5.16)

A summary of the observed levels of expression of Cirp and HSP105 in the testis following in vivo heat treatment is shown in Table 5.2.

Table 5.2 Levels of expression of Cirp and HSP105 protein in the testis following scrotal heating. (- = low expression, +, ++, and +++ indicate increasing levels of expression)

	Control	1h	2h	4h	6h	24h	7d	14d	21d	24d	28d	32d
Cirp Expression	+	+	+	-	-	-	+	+	+	+	+	+
HSP105 Expression	-	+	++	+++	++	+	-	-	-	-	-	-

5.3.1.2 Stress responses in the testis

In the control testis, low levels of Bax expression were detected in germ cells (Figure 5.18). Bax expression increased within 1h of heating (Figure 5.19), remained high at 2 and 4h (Figures 5.20 and 5.21) and then gradually returned to low levels of expression at 6, 24h and 7 d (Figures 5.22-5.24) after heating.

The number of TUNEL-positive (+ve) cells in the control testes was consistently low in the animals examined (Figure 5.17). Isolated +ve cells were occasionally observed in tubules (Figures 5.26-5.28). At 2, 4 and 6h after heating, significantly higher numbers of +ve cells were observed, often in clusters of +ve cells in neighbouring tubules (Figure 5.29) but with the majority of tubules containing few (Figure 5.31) or no (Figure 5.30) +ve cells. The highest numbers of +ve cells were observed at the 14d time-point (Figure 5.17). In addition to high numbers of +ve cells there was also evidence of cell loss in the tubules 14d after heating, with obvious gaps in the seminiferous tubules where germ cells had once been (Figures 5.32 – 5.34). After 14d, the numbers of +ve cells in the tubules decreased until, at 32d, control levels were reached (Figure 5.17).

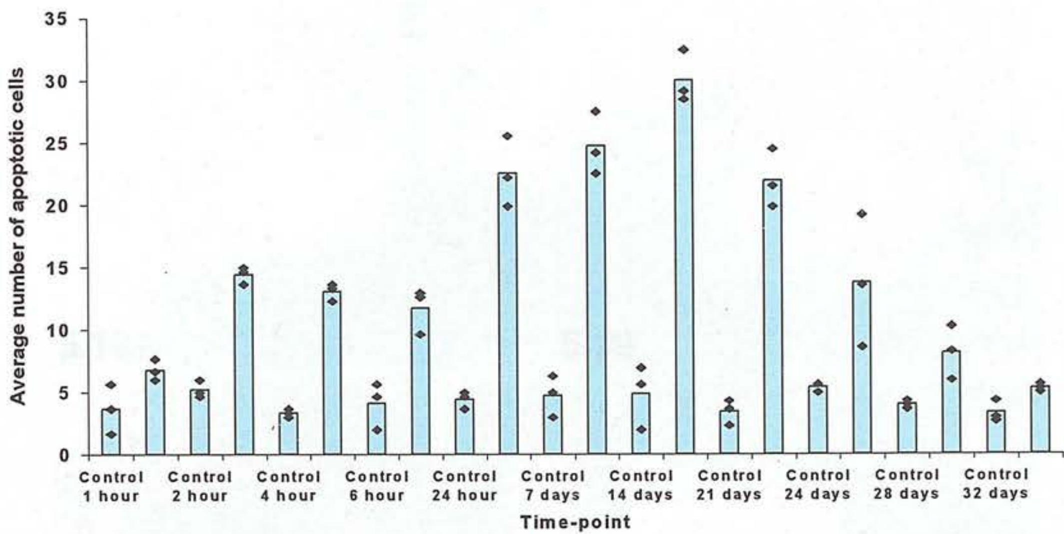
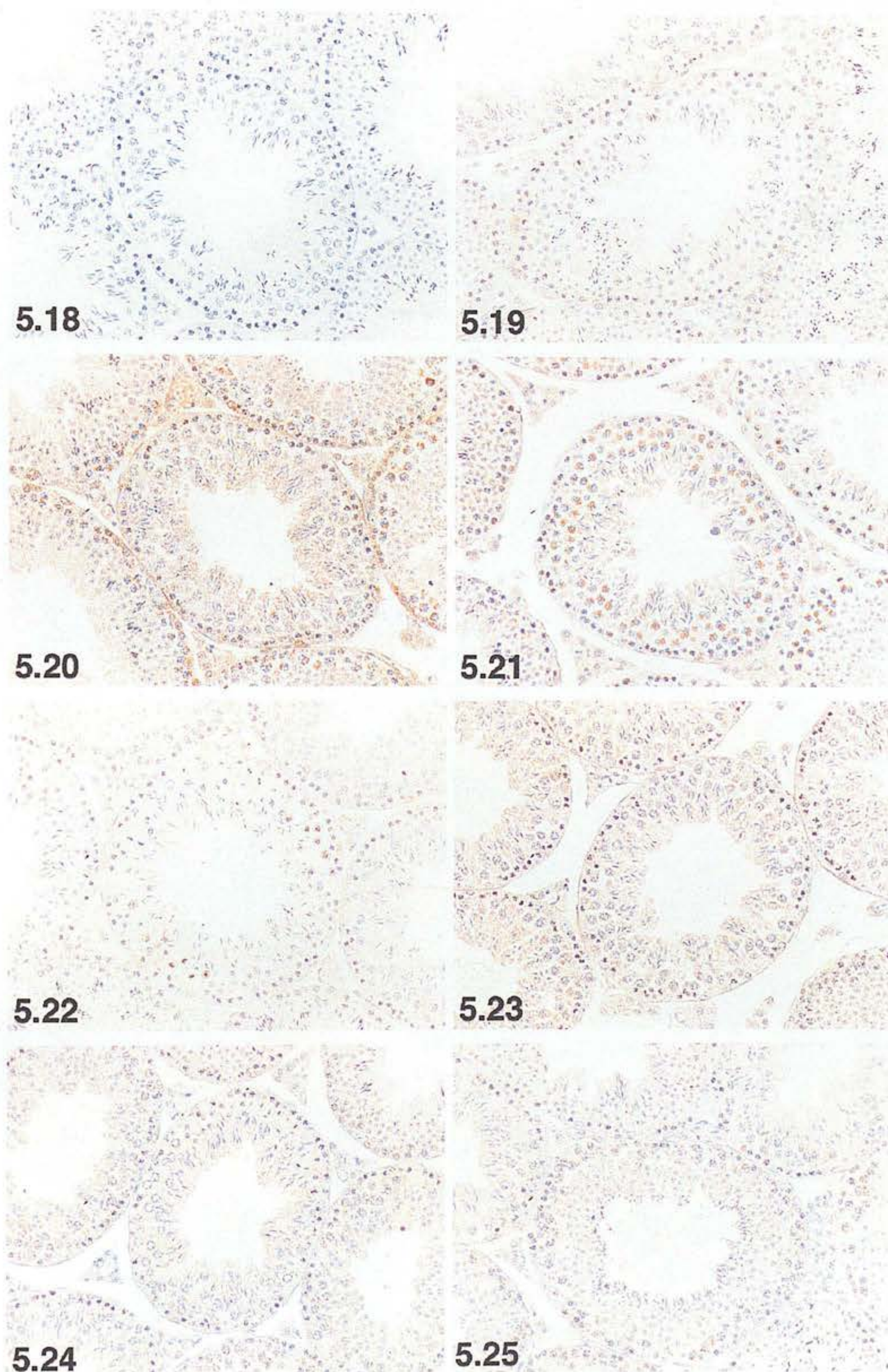
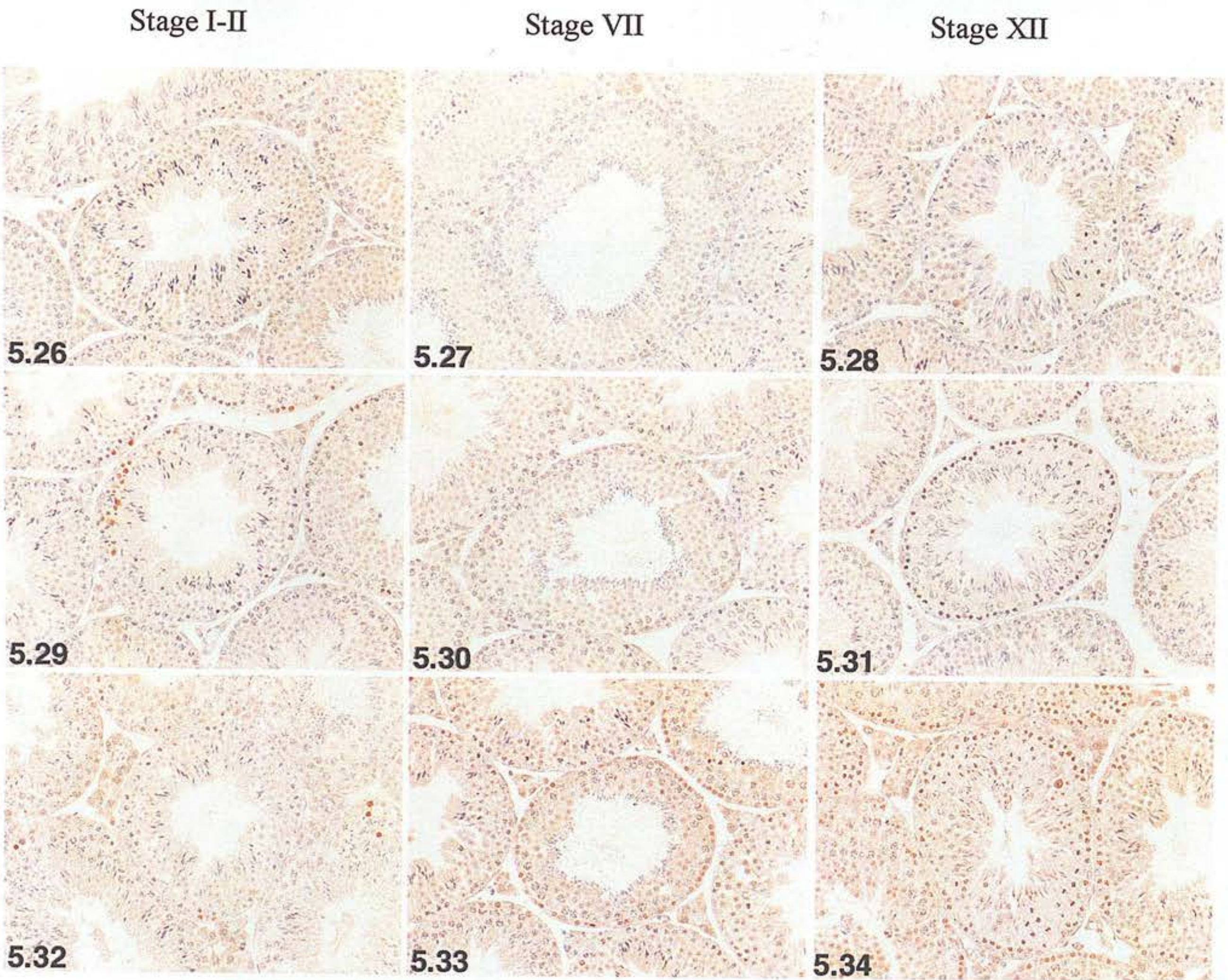


Figure 5.17 Average number of TUNEL +ve cells counted in the testis following scrotal heating. (Points represent average values for individual mice, bar indicates average for group).



Figures 5.18-5.25 Bax expression in the testis following *in vivo* heat treatment. Figures show staining for the Bax protein in the negative control (5.18), the control testis (5.19) and the heated testis after 1h (5.20), 2h (5.21), 4h (5.22), 6h (5.23), 24h (5.24) and 7d (5.25)

Stage I-II | Stage VII | Stage XII



Figures 5.26-5.34 Detection of TUNEL +ve cells in the testis following in vivo heat treatment. Control testis at stages I-II, VII and XII (figures 5.26-5.28), heated testis (6h) at stages I-II, VII and XII (figures 5.29-5.31) and heated testis (14d) at stages I-II, VII and XII (figures 5.32-5.34)

5.3.2 Number of Motile Spermatozoa

The concentration of motile spermatozoa retrieved from the epididymis of control animals was in the range of 5.25 – 7.2 x 10^6/ml (average 6.4 x 10^6/ml). The number of motile spermatozoa present within the epididymis of heated animals at 7 days post-heating had dropped to 1.68 x 10^6/ml ($p<0.05$), recovered slightly at 14d (2.12 x 10^6/ml; $p<0.05$) and then remained below 1.5 x 10^6/ml ($p<0.05$) for the duration of the experiment (Figure 5.35).

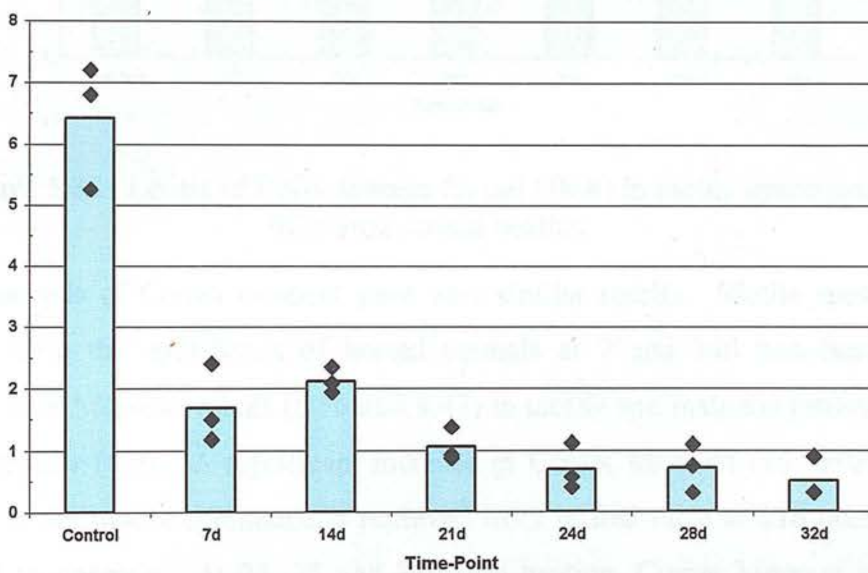


Figure 5.35 Number of motile spermatozoa in the epididymis after scrotal heating

5.3.3 DNA integrity of Motile Spermatozoa

DNA damage in motile spermatozoa, as measured by the Comet assay, was expressed either as % DNA in Comet tail (Figure 5.36) or as Comet moment (Figure 5.37). Compared to the controls (25.3% DNA in Comet tail), the level DNA damage in the motile spermatozoa of heated animals retrieved 7 and 14d after heating were similar to control levels (22.35% and 22.55% respectively). However, at 21d post-heating, the level of DNA damage within the motile spermatozoa had increased significantly (34.9%; $p<0.05$) compared to control levels. This elevated level of DNA damage in motile spermatozoa was observed at each of the later time-points post-heating, reaching a peak at 28d (38.86%; $p<0.05$).

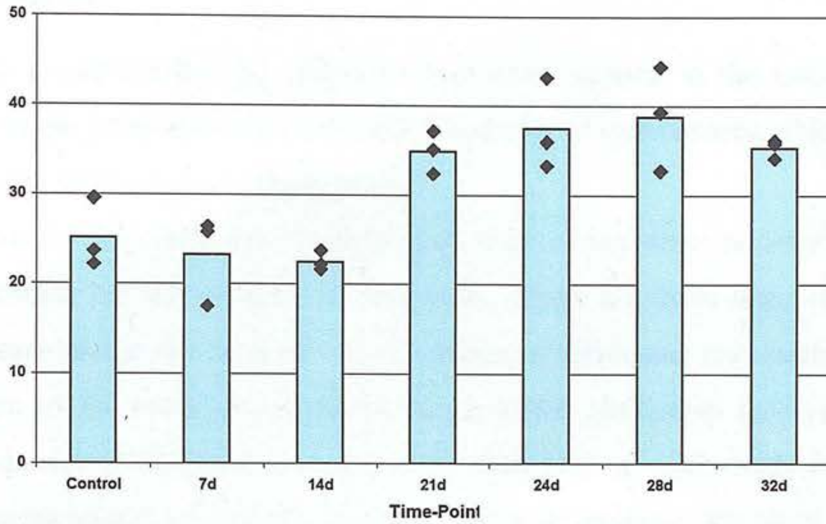


Figure 5.36 Levels of DNA damage (% tail DNA) in motile spermatozoa following scrotal heating

Analysis of Comet moment gave very similar results. Motile spermatozoa retrieved from the epididymis of heated animals at 7 and 14d post-heating had similar Comet Moment values (6.56 and 8.47) to motile spermatozoa retrieved from control animals (6.9). A significant increase in Comet Moment (15; $p<0.05$) was observed in the motile spermatozoa retrieved from heated mice at 21d post-heating compared to controls. At 24, 28 and 32d post-heating, Comet Moment remained significantly higher than controls.

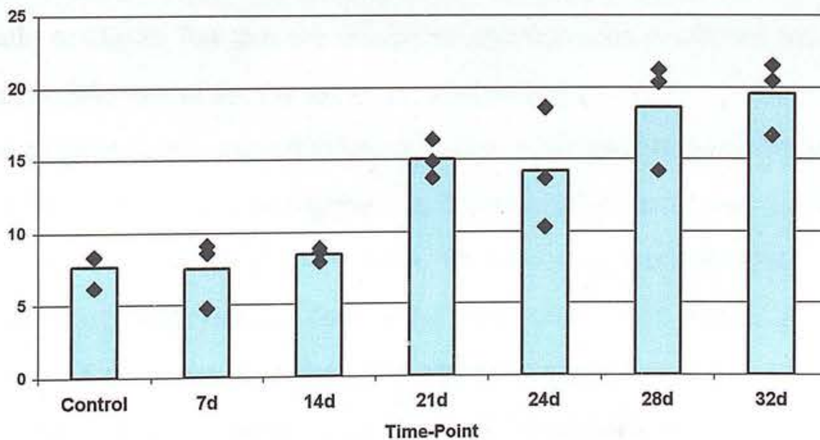


Figure 5.37 DNA damage (Comet moment) in motile spermatozoa following scrotal heating

5.4 Discussion

In the present study, the effects of heat stress applied to the testis on gene expression in the germ cells and on the DNA integrity of spermatozoa which develop from surviving germ cells have been studied.

The aim of the study was to subject the testis to heat stress in order to disrupt spermatogenesis, but not to block it altogether. There are three main methods of inducing heat stress in the testis of animal models; experimental cryptorchidism (the translocation of the testis and/or epididymis into the abdominal cavity), a single transient exposure of the scrotum to heat via a water bath or whole-body exposure to increased temperatures for extended periods. Of these methods, the most controlled and easily performed in the mouse is the immersion of the scrotal region, hind legs and tail of the animal in a water bath at the appropriate temperature for the desired period of time.

In the mouse, effects of temperatures within the range 39 and 42°C have been studied for periods of between 20 and 30 minutes (Jannes, *et al.*, 1998;Nishiyama, *et al.*, 1998). The heating regime (30 min at 42°C) used in the current study was based on that used by Jannes *et al.* They found that subjecting wild type mice to scrotal heating at a temperature of 42°C for 20 min resulted in the production of poor quality spermatozoa, reduced embryo weight *in vivo*, and reduced fertilisation rates *in vitro*. Their results suggested that spermatogenesis in animals subjected to this heating regime would continue, but that the quality of spermatozoa produced would be sub-optimal – a suitable model for the study of sub-fertility.

In the present study, immunohistochemistry was used to confirm the response of the testis to the mild heating regime. In control (anaesthetic only) animals, Cirp expression was found in most germ cells, in particular, preleptotenes, leptotenes, zygotenes and early pachytenes. Following heat stress, Cirp expression decreased within 4 hours of treatment and did not recover to control levels until 7 days after treatment. These findings agree with those of Nishiyama *et al* (1998) who first characterised this protein, proposed its role in the regulation and co-ordination of

mitosis and meiosis during spermatogenesis, and determined the effects of heat stress on its expression in the human and mouse testis (Nishiyama, *et al.*, 1998).

The expression of HSP105 in the testis was also altered following scrotal heating. HSP105 is known to be expressed in the germ cells of the rat testis in a temperature-dependant manner. It has been shown that following heat stress (via experimental cryptorchidism) the HSP105 protein translocates from the cytoplasm to the nuclei of the germ cells where it is believed to play a role in the regulation of p53 (a pro-apoptotic protein (Wakatsuki, *et al.*, 1998;Ishihara, *et al.*, 2000). In the present study, expression of HSP105 was observed in the cytoplasm of germ cells in the mouse testis. Following heat treatment, the location of HSP105 within the cells altered, moving from the cytoplasm to the nuclei, as previously observed in the rat.

It has been previously shown that heat stress (via experimental cryptorchidism and scrotal heating) results in increased apoptosis of germ cells in the testis (Yin, *et al.*, 1997;Lue, *et al.*, 1999;Yamamoto, *et al.*, 2000). In the present study, both the expression of the pro-apototic marker Bax and the number of TUNEL-positive cells were used to identify the apoptotic response of germ cells to the heating regime. Following heat stress, Bax expression was increased and some redistribution of the protein from the cytoplasm to the perinucleus of the germ cells was observed. In the testis of control animals, few apoptotic cells were identified using the TUNEL method. Following heat stress, increased numbers of apoptotic cells appeared within 2 hours of treatment and the average number of apoptotic cells in the testis remained significantly higher than that of the control testis up to 21 days after treatment (data not shown). Again, these findings agree with previous studies in rodents using both experimental cryptorchidism and scrotal heating (Yin, *et al.*, 1997;Lue, *et al.*, 1999;Yamamoto, *et al.*, 2000).

The immunohistochemical analysis confirmed that the testes had been affected by the heating regime (altered expression of Cirp, HSP105), spermatogenesis had been disrupted (evidence of increased apoptosis; Bax expression, TUNEL-positive cells) but that germ cells were surviving and continuing through the process of spermatogenesis to become mature spermatozoa.

Based on the duration of spermatogenesis and epididymal transit time in the mouse, it was possible to determine the effects of heat stress on specific groups of developing germ cells by studying motile spermatozoa retrieved at specific time-points following treatment. In the present study, attention was focused on the motile spermatozoa developed from heat-treated germ cells. Motile spermatozoa were studied as these are most equivalent to the spermatozoa preferentially used for IVF/ICSI in the treatment of male infertility.

The number of motile spermatozoa retrieved from the epididymis at 7d post-heating was significantly decreased compared to control levels and this was seen at each of the time-points tested post-heating. The reduced numbers of motile spermatozoa retrieved the epididymis at these later time-points correspond to reduced numbers of germ cells within the testis following heat treatment as demonstrated by the increased numbers of TUNEL-positive cells observed within the spermatogenic tubules following heat treatment.

The DNA integrity of motile spermatozoa was studied using a modified alkaline Comet assay. This assay was originally developed to study the DNA integrity of somatic cells and more recently has been adapted for use in spermatozoa (Singh, *et al.*, 1988;Haines, *et al.*, 1998;Irvine, *et al.*, 2000).

Motile spermatozoa retrieved from the epididymis at the later time-points (7d – 32d) originated from cells located within the testis at the time of heating (Table 5.1). Each cohort of spermatozoa retrieved represented a 7-day period in spermatogenesis (based on a 7-day epididymal transit time). Therefore, the number of motile spermatozoa and level of DNA damage in each cohort of spermatozoa must be applied to a 7-day period of spermatogenesis. Figure 5.38 shows a summary of the number of motile spermatozoa retrieved from the epididymis and the level of DNA damage in these motile spermatozoa developed from different cell types in the testis at the time of heating.

These data suggest that, following heating, cells in the pre-meiotic stages of spermatogenesis develop into spermatozoa with higher levels of DNA damage than

other stages and that the DNA integrity in spermatozoa developed from cells undergoing spermiogenesis at the time of heating is comparable with that of controls. Previous studies in which cell loss has been quantified have identified pachytene spermatocytes and early spermatids as being susceptible to heat stress (Collins, *et al.*, 1969;De-Vita, *et al.*, 1990). During the meiotic phase of spermatogenesis, a great deal of rearrangement of the DNA occurs within the nuclei of the leptotene, zygotene and pachytene spermatocytes (Johnson, *et al.*, 1995). The sequential changes to the configuration of the chromatin within these cells makes them particularly vulnerable to stress during this period.

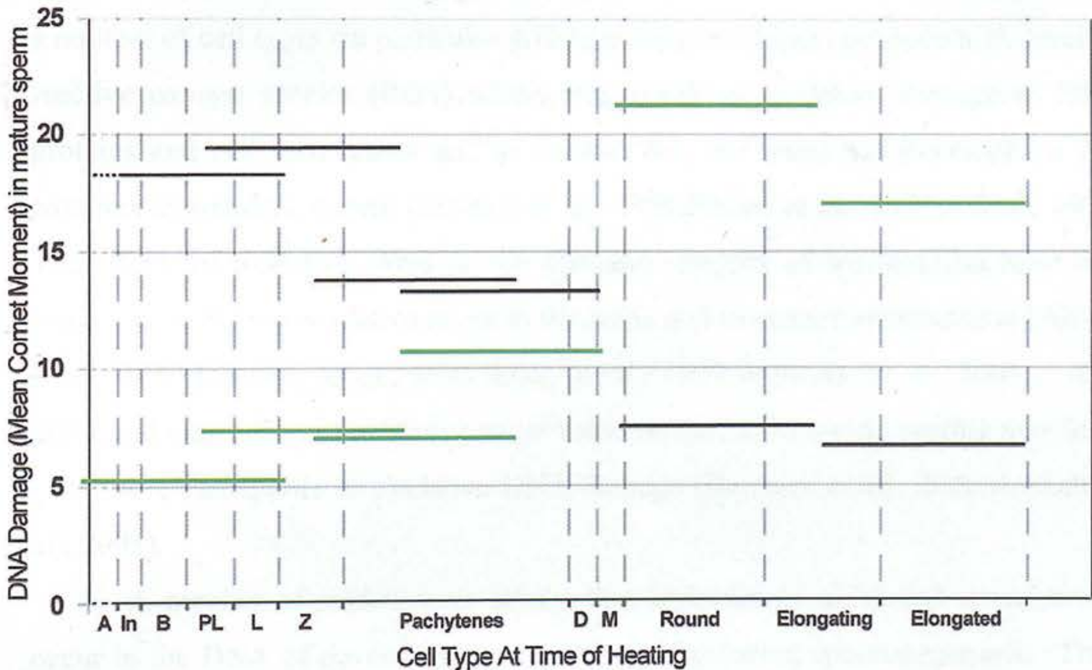


Figure 5.38 Summary graph showing number of motile spermatozoa (green bars) and levels of DNA damage (black bars) in motile spermatozoa developed from different cell types present in the testis at the time of heating.

Two critical periods in spermatogenesis (leptotene-pachytene and maturation division) have been identified through which cells were unable to progress following heating (Collins, *et al.*, 1969). These data identifies cells in the earlier (premieotic) stages of spermatogenesis which, though affected by heating, have progressed

through these critical periods in germ cell differentiation to develop into DNA-damaged spermatozoa.

It is proposed that the loss of DNA integrity in mature spermatozoa resulting from heated germ cells may be explained in two ways; DNA damage occurs in the cells at the time of heating and is not repaired during spermatogenesis, or the physiology of the testis is disrupted by heat stress resulting in sub-optimal support for germ cell development which is detected as a loss of DNA integrity in mature spermatozoa.

It has been shown that raised temperature can lead to oxidative stress within the rat testis, this in turn can trigger apoptosis (Ikeda, *et al.*, 1999). Within the testis a number of cell types (in particular differentiating cell types) produce high levels of reactive oxygen species (ROS) which may result in oxidative damage to DNA, proteins and cell membranes and to combat this, the testis has developed a very complex antioxidant system (Bauche, *et al.*, 1994;Fisher, *et al.*, 1997;Aitken, 1999). The effects of oxidative stress on the genomic integrity of spermatozoa have been studied by inducing oxidative stress in the testis and in mature spermatozoa (Aitken, *et al.*, 1989;Lucesoli, *et al.*, 1995;Ikeda, *et al.*, 1999;Wellejus, *et al.*, 2000). Also, decreased expression of oxidative stress response genes following heating may leave cells more susceptible to oxidative DNA damage (Barroso, *et al.*, 2000;Rockett, *et al.*, 2001).

A number of studies have shown that endogenous nicks and strand breaks occur in the DNA of developing spermatozoa cells during spermatogenesis. These DNA strand breaks and nicks may facilitate the transition of DNA from the somatic cell histone complex to the tightly packed protamine complex of the mature spermatozoa by relieving the torsional stress created during this process (Ward, *et al.*, 1991;McPherson, *et al.*, 1992). Normally these strand breaks and nicks are ligated before the completion of spermiogenesis (Sakkas, *et al.*, 1995), however, if spermiogenesis were disrupted (i.e. due to heat stress), residual strand breaks could persist into the mature spermatozoa (Manicardi, *et al.*, 1995).

A number of DNA repair mechanisms are employed during spermatogenesis (van Loon, *et al.*, 1993;Shen, *et al.*, 1997;Kuraoka, *et al.*, 2000;Richardson, *et al.*, 2000;Richardson, *et al.*, 2000;Aguilar-Mahecha, *et al.*, 2001;Aitken, *et al.*, 2001;Rockett, *et al.*, 2001). These mechanisms are responsible for the repair of the DNA strand breaks known to occur unintentionally during spermatogenesis, particularly during meiosis, and also those nicks and breaks induced intentionally to facilitate DNA packaging as described above. It is known that a number of these mechanisms may be disrupted by exogenous insult, such as heat stress, resulting in the inefficient repair of DNA strand breaks and nicks of the developing germ cells. Following heating, the expression of a number of genes in the testis is down-regulated, these genes include many involved in DNA repair and recombination, cell cycle regulation, glutathione metabolism and stress response (Rockett, *et al.*, 2001). Finally, the level of DNA damage induced by heat stress may simply overwhelm the repair mechanisms in the testis, and while a certain amount of DNA damage is repaired, some may persist into the mature spermatozoa. Any or all of these factors may contribute to the possibility that heated germ cells may be less capable of repairing heat-induced strand breaks/lesions.

The ability of spermatozoa to remain motile and morphologically normal (observation made during calculation of motile sperm number), while containing high levels of DNA damage may have implications in the treatment of male infertility. It is generally accepted that spermatozoa quality is a good indicator of success in both IVF and ICSI. The findings of this study suggest the possibility that motile and morphologically normal spermatozoa may not contain intact DNA and therefore are not necessarily good candidates for use in IVF and ICSI. Furthermore, these findings suggest that DNA quality of donor spermatozoa should be taken into consideration before being used in these procedures.

It should be noted that limited numbers of animals were available for use in the current study. Furthermore, due to the subjective nature of the analysis of DNA damage in the motile spermatozoa, it would have been preferable to analyse the results in a 'blind' manner. However, the results obtained suggest the need for

larger, more in-depth studies to examine the effects of raised scrotal temperature on spermatozoal number and genetic integrity.

Chapter 6
The effect of scrotal heating on the epididymis and mature spermatozoa

6.1 Introduction

6.1.1 Structure and Function of the epididymis

The epididymis consists of four distinct compartments: the initial segment, the head (caput), the body (corpus) and the tail (cauda). The epithelium of the epididymis consists of five distinct cell types: principal cells, basal cells, clear cells, halo cells and narrow cells (Robaire, *et al.*, 1988;Yeung, *et al.*, 1994).

The luminal compartment of the epididymis is a constantly changing environment containing spermatozoa and a variety of substances required for their storage and maturation. These include ions, proteins, glycoproteins, organic molecules and water (Robaire, *et al.*, 1988).

The functions of the epididymis are the transportation of spermatozoa from the efferent ducts to the vas deferens, the maturation of spermatozoa (i.e. acquirement of increased chromatin stability, fertilising ability and motility) and the storage and protection of mature spermatozoa.

It has been demonstrated that the epididymal transit time of the mouse is around 7 days. This is the time it takes an entire cohort of spermatozoa to travel the entire length of the epididymis from the initial segment to the distal cauda (Cornwall, *et al.*, 1990). During their journey through the epididymis, spermatozoa must undergo further maturation in order to be able to fertilise eggs. Perhaps the most important maturation process to occur in the epididymis is the acquisition of motility. The precise mechanism of motility acquisition is unclear. However, it has been noted that the more time spent in the epididymis, the more coordinated the movement of the flagellum becomes, resulting in increased velocity and straightness of swim path (Cooper, *et al.*, 2000).

The spermatozoa must also acquire the ability to interact with the egg. The epididymal epithelium secretes proteins which interact with the spermatozoa surface becoming part of the membrane and/or modifying existent residues, thereby developing or activating sites concerned with gamete recognition and capacitation (Krull, *et al.*, 1993).

6.1.2 Storage and protection of spermatozoa

Spermatozoa can be stored in the cauda epididymis for long periods of time (depending on species) although following extended periods, the fertilising ability and motility of the spermatozoa gradually decreases. The cauda epididymis is able to store spermatozoa due to the increased oxygen availability and decreased respiration rate of spermatozoa at scrotal temperatures (Djakiew, *et al.*, 1986;Mieusset, *et al.*, 1992). The epididymis protects the spermatozoa in a number of ways. The blood-epididymis barrier allows only selective entry of substances into the epididymal lumen while the epithelial cells of the epididymis ensure the rapid elimination of harmful by-products and exogenous toxic substances from the lumen (Hinton, *et al.*, 1995).

Certain proteins secreted in the lumen protect spermatozoa from specific threats, for example, proteolysis (due to the premature release of acrosome proteins) and complement-mediated cell lysis (Hinton, *et al.*, 1995).

6.1.3 Heat stress and the epididymis

Following heat stress, the epididymis is reported to lose its ability to store and maintain viable spermatozoa, resulting in the gradual and progressive accumulation of dead, decapitated and immotile spermatozoa (Glover, 1960;Jegou, *et al.*, 1983;Mieusset, *et al.*, 1992;Bedford, 1994).

6.1.3.1 Effects on epididymal function

The cauda appears to be the region of the epididymis most affected by heat stress, decreasing in size and therefore storage capacity. The net result of these changes is a decrease in the number of viable spermatozoa in the ejaculate (Glover, 1960;Bedford, 1978;Bedford, 1991).

Raised temperature has also been shown to have a direct effect on epididymal function. Resulting in changes in oxygen levels, water and ion transport mechanisms, protein biosynthesis and secretion, and the cellular structure of the epididymal epithelium itself (Djakiew, *et al.*, 1986;Esponda, *et al.*, 1990;Bedford, 1991;Regalado, *et al.*, 1993;Seiler, *et al.*, 2000). These changes result in an epididymal environment which is unable to efficiently maintain and store viable spermatozoa.

6.1.3.2 Effects on the spermatozoa

It has also been reported that increasing scrotal temperature adversely affects the spermatozoa within the epididymis. Fertilisation rates are decreased and developmental failure of the foetuses occurs, resulting in significantly decreased litter sizes (Setchell, *et al.*, 1988).

6.1.4 Aims of this chapter

The aims of this work were to monitor the response of the epididymis to heat stress by the study of stress proteins, the number of motile spermatozoa and the DNA integrity of the motile spermatozoa in the heated epididymis. Additionally, we aimed to investigate whether the DNA integrity of spermatozoa within the epididymis was affected by heat stress, even though they are packaged in a condensed state.

6.2 Methods

6.2.1 Animals

The animals used were sexually mature male wild-type MFI mice (*dazl* strain) (Ruggiu, *et al.*, 1997) which were maintained under standard conditions of a 12L:12D cycle, in an ambient temperature of 20-25°C with access to food and water *ad libitum*.

6.2.2 Scrotal Heating

Adult male mice were anaesthetised with a 1:1 mix of Hypnorm and Hypnovel via an intraperitoneal (i.p.) injection. Following anaesthesia, the lower third (hind legs, tail and scrotum) of each male was passed through a hole in a polystyrene 'raft' which was then placed in a circulating water bath for 30 min at 42°C. Control animals received anaesthetic only. All animals were returned to their cages, which were placed on a warm mat (20-25°C) to maintain body temperature, until fully recovered from the anaesthetic.

6.2.3 Time-points

For each time-point studied, 3 mice underwent *in vivo* heat treatment and 3 mice were used as controls. All mice were matched for age and weight.

Animals were sacrificed at 6 hours (h), 14 days (d), and 28d post-treatment. Each time-point was chosen to allow the study of a particular population of cells within the epididymis at the time of heating. Calculations were based on a 7-day epididymal transit time (Cornwall, *et al.*, 1990).

Epididymides were removed and fixed in Bouins for 6h then transferred to 70% ethanol and processed into paraffin wax for histological analysis. or placed in ~1ml BWW containing 20mM HEPES and 0.3% HSA.

6.2.4 Immunohistochemistry

Bouins-fixed, paraffin-embedded tissues were sectioned at 5μm. Paraffin wax was removed from sections by washing in 2 changes of xylene (5 min each) and the sections were rehydrated in decreasing concentrations of ethanol (100%. 95%, 75% and finally water).

6.2.4.1 Haematoxylin and Eosin Staining (H&E)

Standard H&E staining was performed.

6.2.4.2 Stress Markers

In order to confirm the response of the epididymis to heat stress, two stress proteins were used as markers. Cold-inducible RNA-binding protein (Cirp) is constitutively expressed in the germ cells of the testis. Following experimental cryptorchidism or heat stress (42°C), decreased expression of Cirp in the testis is observed within 6 h (Nishiyama, *et al.*, 1998); Chapter 5). To date, the expression of Cirp in the epididymis has not been studied. Bax is a pro-apoptotic regulator of apoptosis and it has been shown in the rat that testicular heating results in redistribution of Bax expression from a cytoplasmic to perinuclear localization in all germ cells (Yamamoto, *et al.*, 2000). It has been shown that basal cells in the epididymis have the ability to behave as macrophages in response to increased numbers of dead/immotile spermatozoa and stress (Seiler, *et al.*, 1998;Seiler, *et al.*,

2000). Expression of the macrophage antigen CD68 was studied as an indication of the number of basal cells acting as macrophages in response to heat stress.

Briefly, endogenous peroxidase activity was blocked by incubation in H_2O_2/methanol as previously described followed by washing twice (5 min each) in Tris-buffered saline (pH 7.4). Slides were pre-incubated with serum block (20% NSS, 5% BSA in TBS) at room temperature for 30 min before addition of the primary antibody and overnight incubation at 4°C. Slides were washed twice in TBS (5 min each) before addition of the secondary antibody and incubation at room temperature for 30 min. Slides were again washed twice in TBS (5 min each). ABC conjugated to HRP (DAKO) was added to the slides and incubated for 30 min at room temperature and the slides were again washed twice in TBS before the addition of DAB. The resulting colour reaction was stopped with water and the slides were counterstained with Mayer's Haematoxylin.

6.2.5 Preparation of murine spermatozoa

Spermatozoa samples studied in this chapter were taken from the mice sacrificed at the earlier time-points (1h, 2h. 4h, 6h, 24h and 7d) as described previously in Chapter 5.

Full methods for the preparation of murine spermatozoa are described in section 2.6. Briefly, whole epididymides were removed, cleaned of fatty tissue and placed in 1ml BWW. The tissue was then minced with fine scissors and incubated at 34°C (95% air, 5% CO_2) for 30 min prior to careful removal of buffer containing the motile spermatozoa. Samples were diluted to a final volume of 1ml in BWW and stored at -20°C until required.

6.2.6 Study of concentration of spermatozoa

Samples were diluted 1:10 with SDF and 10μl diluted sample was placed into the chamber of an improved Neubauer haemocytometer. The number of spermatozoa located within 5 squares of the haemocytometer grid was carefully

counted. If the number of spermatozoa in 5 squares was not equal to or greater than 100, 10 or 25 squares were counted.

From this value, the number of spermatozoa in 1ml was calculated using the following equation:

$$\text{Concentration (millions/ml)} = \frac{\text{No. Spermatozoa Counted}}{(4 \times \text{No Squares}) \div \text{Dilution}}$$

6.2.7 Comet analysis of spermatozoa

DNA integrity of motile spermatozoa from the epididymis was studied using a modified Comet assay.

Spermatozoa samples were defrosted at room temperature and 5μl each sample was mixed with 25μl LMA (37°C). This gel/sample mix was dropped onto a CometSlide and covered with a clean, warm (37°C) cover-slip. Slides were then placed horizontally in a box and incubated at 4°C until the gels were set. Cover-slips were carefully removed and the slides submerged in Lysis buffer (Trevigen) containing 0.75% SDS, for 3 h at 37°C. The slides were removed from the Lysis buffer and placed in a horizontal gel electrophoresis tank. Alkaline electrophoresis buffer (3M NaOH, 1mM EDTA, pH 12.3) was carefully added to a depth of 0.5cm above the slides and left for 20 min at room temperature. Current was applied at 25V, 300mA for 10 min. The slides were transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min). The slides were allowed to dry overnight at room temperature before staining with 50μl ethidium bromide (1:1000 in dH_2O). For each sample, 100 cells were analysed using the Komet Image Analysis system. The percentage of head DNA, tail DNA and the Comet moment was calculated for each cell. Comet moment is a measurement that takes into account both the length of the Comet tail and the amount of DNA present in the tail and is expressed as an arbitrary unit (the greater the value, the higher the level of DNA damage).

For each time-point, 3 animals were heated and 3 animals were used as controls. The Mann-Whitney U test was used to compare the results from each heated group against the controls.

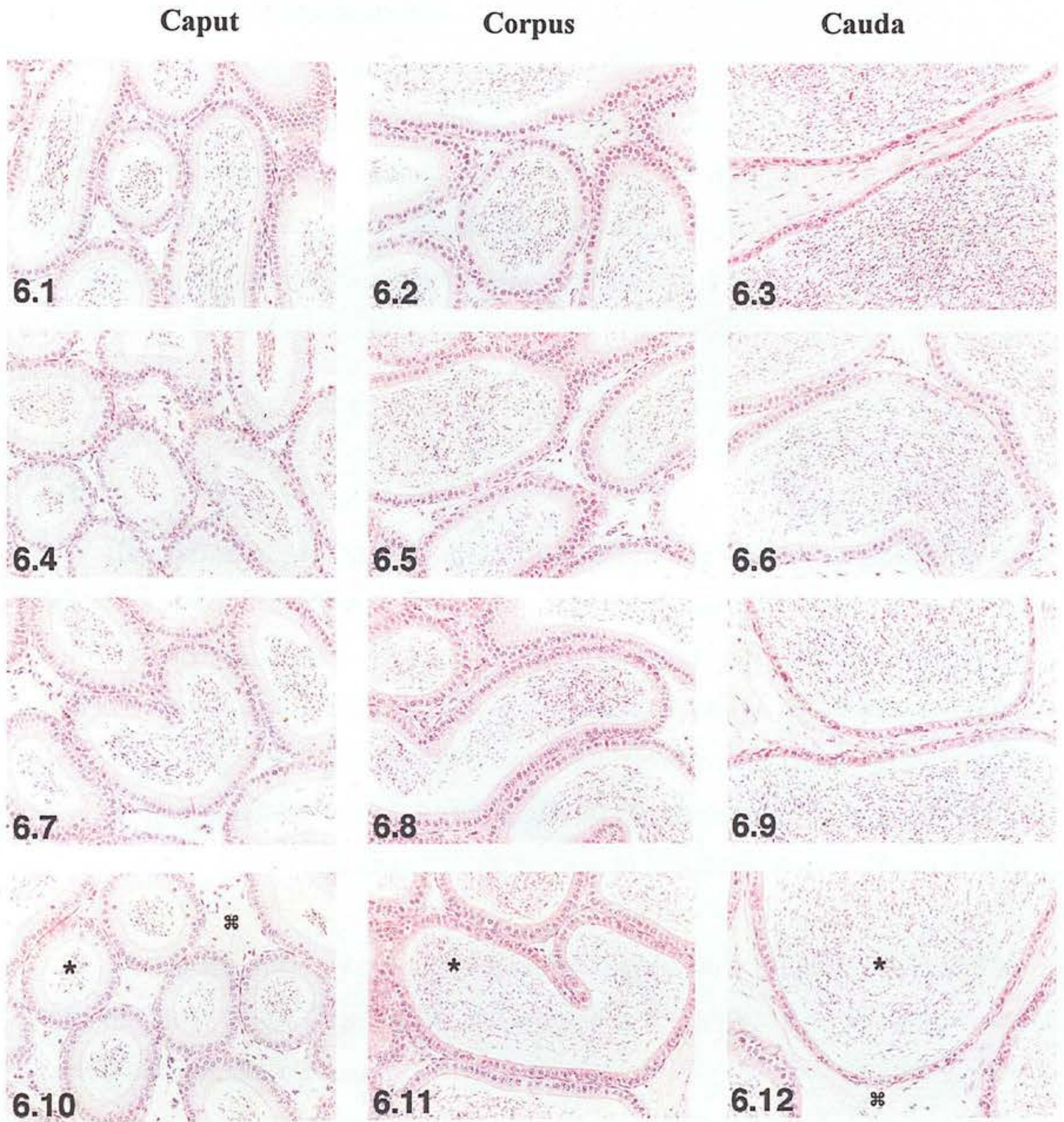
6.2.8 Statistical Analysis

Statistical analysis was limited due to the small size of the samples available for study. The results obtained for the heated animals were compared against those for the control animals using the Kruskal Wallis non-parametric test. However, it is acknowledged that the significance of this statistical test is diminished when used with small sample sizes ($n<10$).

6.3 Results

6.3.1 H&E Staining

Standard H&E staining was performed to study any changes in the morphology of the epididymal tubules and in the density of mature spermatozoa present in the epididymis following scrotal heating. Figures 6.1 - 6.3 show the caput, corpus and cauda regions of the epididymis of a control mouse. Six hours and 14 days after heat treatment (Figures 6.4-6.6 and 6.7-6.9 respectively) there appear to be no obvious changes to either the epididymal epithelium or the luminal compartment. However, 28 days after heating, it appears that the interstitial spaces between the tubules are increased in both the caput (Figure 6.10) and cauda (Figure 6.12) regions, while the density of spermatozoa in the luminal compartments of all 3 regions of the epididymis appears to be decreased.



Figures.6.1 – 6.12 H&E staining of the control and heated epididymis showing the morphology of the caput, corpus and cauda regions of the epididymis in the control (6.1-6.3) and heat-treated mouse at 6h (6.4-6.6), 14d (6.7-6.9) and 28d (6.10-1.12). All figures are x 20 resolution.

- indicates reduced spermatozoa density, ⌘ indicates increased interstitial space.

6.3.2 Immunohistochemistry

Immunohistochemical analysis of the epididymis was performed for both control and heated animals (at 6 hours, 14 days and 28 days post-heating). The purpose of the immunohistochemical analysis was to study the response of the epididymis to heat stress – in particular the altered expression of the heat-responsive protein Cirp, the pro-apoptotic marker Bax, and the macrophage antigen CD68, and to determine the effects of heat stress on the density of spermatozoa within the epididymis.

6.3.2.1 Cirp

Expression of the Cirp protein was studied to determine the response of the epididymis to mild heat stress. The Cirp protein was detected in all regions of the control epididymis. In the caput region, Cirp expression was strongest towards the initial segment (Figure 6.13a) with fainter staining observed in the middle and distal (Figure 6.13b) regions. Following heat treatment however, this pattern of staining in the caput was reversed, with reduced levels of Cirp expressed towards the initial segment (figure 6.14a) and increased expression in the lower region (Figure 6.14b) of the caput.

Within the corpus region of the control epididymis (Figures 6.13c and d), faint expression of the Cirp protein was observed. Following heat treatment, Cirp protein was not detected in this region (Figures 6.14c and d). Similarly, faint expression of the Cirp protein was observed in the cauda region (Figure 6.13e) and towards the vas deferens region (Figure 6.13f) of the control epididymis. Again, expression of the Cirp protein in these regions (Figures 6.14e and f respectively) was not evident following scrotal heating.

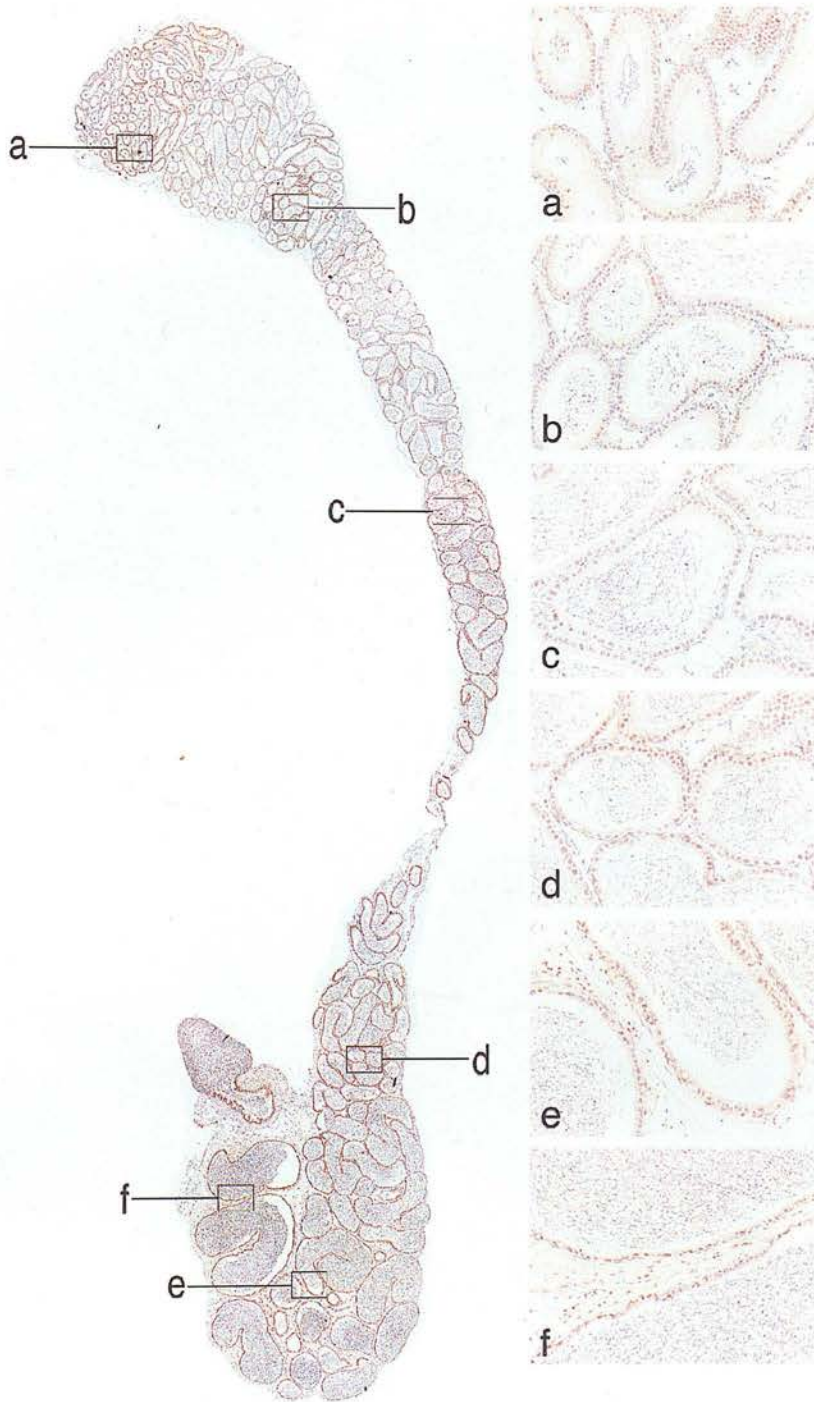


Figure 6.13 Cirp expression in the control epididymis.
Main figure is x4 resolution, figures a-f are x40 resolution.

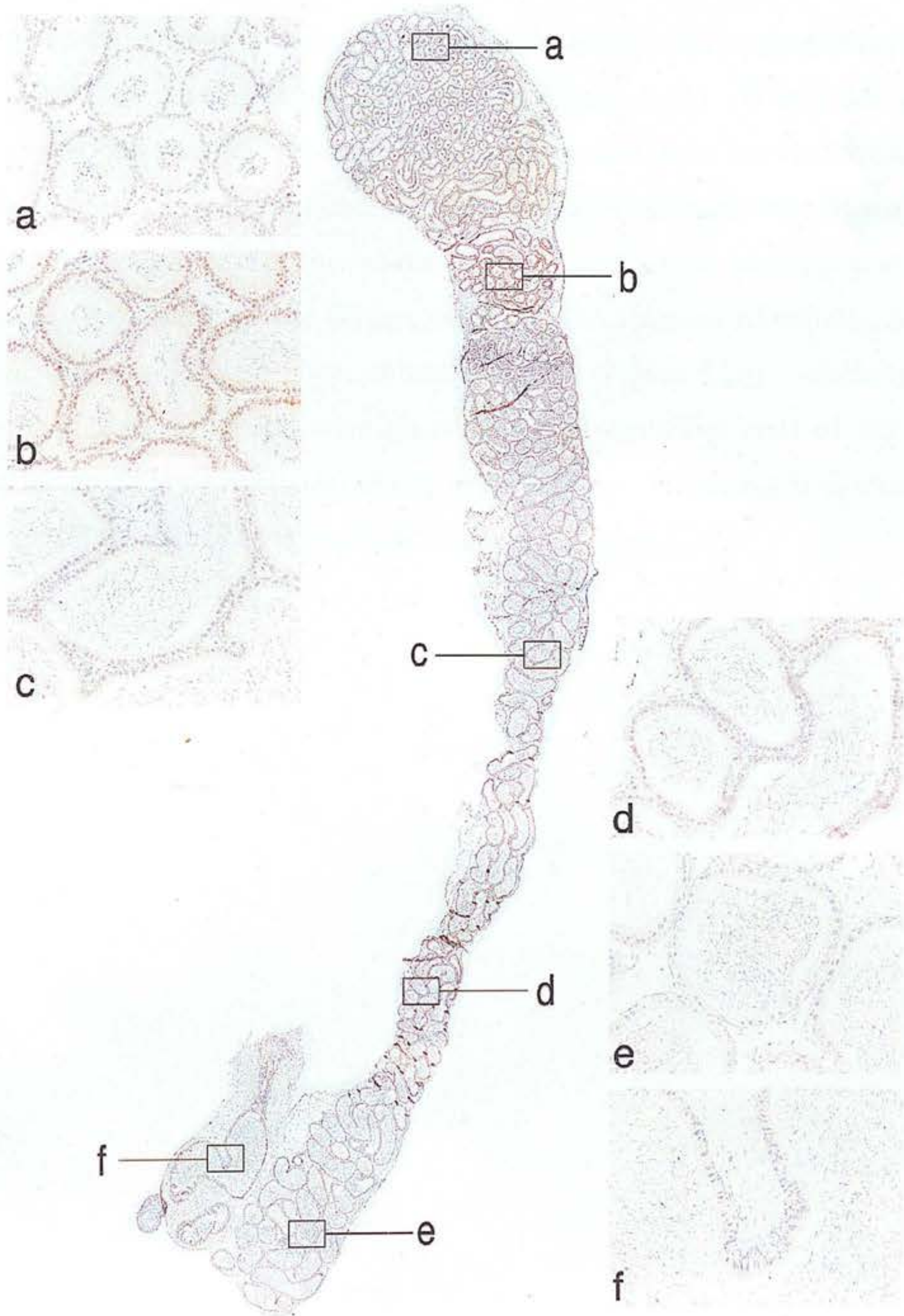
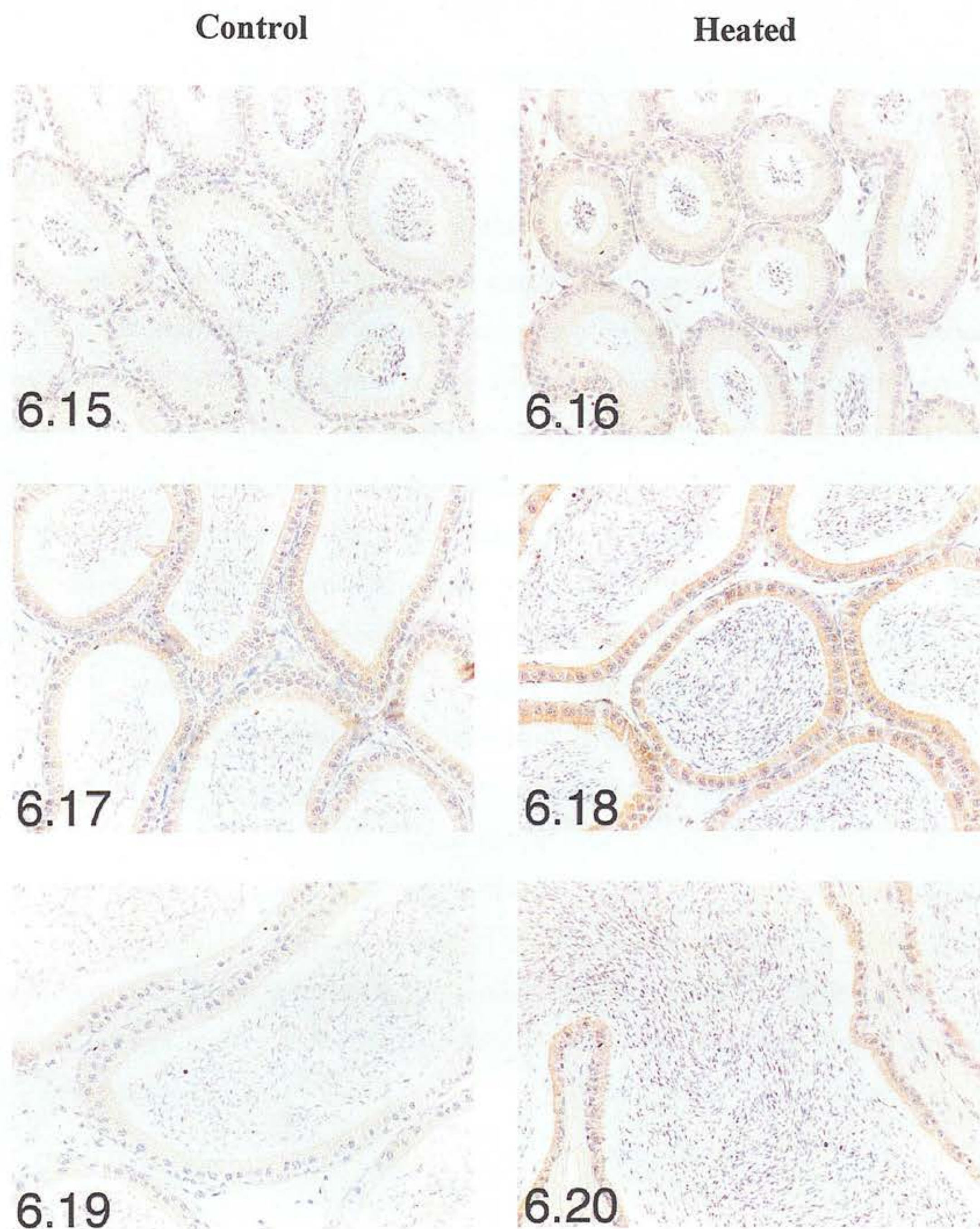


Figure 6.14 Cirp expression in the heated epididymis (+ 6 h). Main figure is x 4 resolution, figures a-f are x40 resolution.

6.3.2.2 Bax

The epithelial cells in the caput region of the epididymis in the control mouse did not express immunodetectable levels of Bax (Figure 6.15). Within this area of the epididymis, heat treatment resulted in a slight increase in Bax expression and faint staining for this protein was observed 6 hours after heat treatment (Figure 6.16).

Bax expression was detected in the epithelial cells of the corpus region of the epididymis in the control mouse (Figure 6.17) and the intensity of staining for this protein was stronger within 6 hours of heat treatment (Figure 6.18). Similarly, very faint levels of Bax expression were observed in the epithelial cells of the cauda region of the epididymis in control mouse (Figure 6.19). Following heat treatment, expression of Bax in these cells was again increased (Figure 6.20).



Figures 6.15-6.20 Bax expression in the epididymis following *in vivo* heat treatment.

Expression of Bax in the caput (6.15 and 6.16), corpus (6.17 and 6.18) and cauda (6.19 and 6.20) regions of the epididymis of control (6.15, 6.17, 6.19) and heated (+ 6 h) animals (6.16, 6.18, 6.19).

6.3.2.3 CD68

Figures 6.21 and 6.22 show that expression of CD68 was tightly regulated and region-specific throughout the epididymis of both the control and heat-treated mouse (+ 6 hours). Levels of expression within specific regions of the epididymis are also shown at higher magnification (x 40).

Within the caput region of the control epididymis, CD68 expression is localised within the epithelial cells of a particular group of tubules in the distal caput (Figure 6.19a). CD68 expression is also present in the caput region of the heated epididymis (Figure 6.20a). The increased size of the group of tubules expressing CD68 may be due to differing orientation of the epididymides, or may be an indication that heat treatment has altered expression of CD68 within the caput region.

Moving down towards the corpus region of the epididymis, Figure 6.19b shows that fewer cells are expressing CD68 in this region of the control epididymis, while the intense staining shown in Figure 6.20b demonstrates the increased expression of CD68 in this region following heat treatment. Within the upper corpus region of the control epididymis, very few cells exhibit CD68 expression (Figure 6.19c) compared with the same region in the heated epididymis (Figure 6.20c). Epithelial cells in the mid-corpus region of both control (Figure 6.19d) and heated (Figure 6.20d) epididymis do not appear to express significant levels of CD68, with only a few cells in the heated epididymis exhibiting faint staining. CD68 expression was not evident in the cauda regions of either the control (Figure 6.19e) or heated (Figure 6.20e) epididymis.

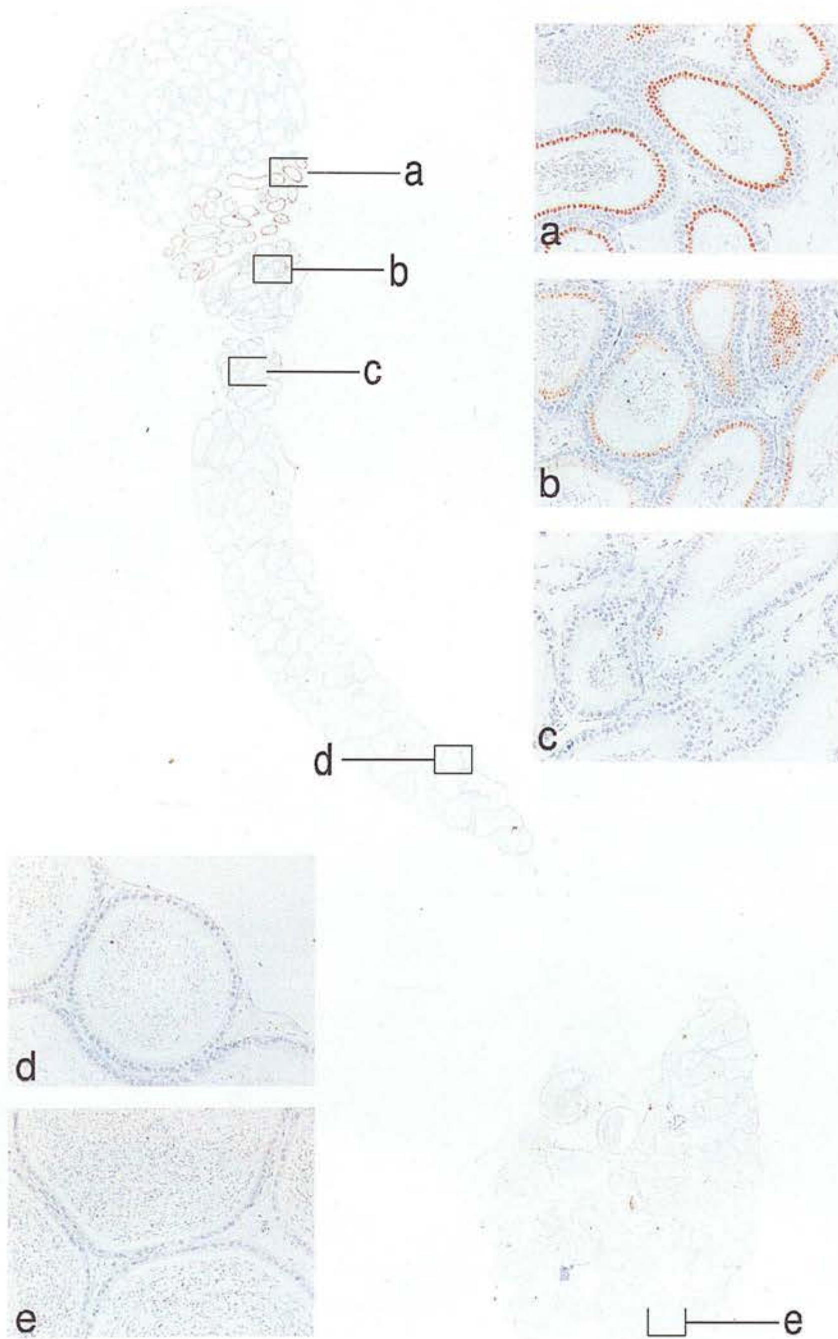


Figure 6.21 CD68 expression in the control epididymis
Main figure is x 4 resolution, figures a-e are x 40 resolution

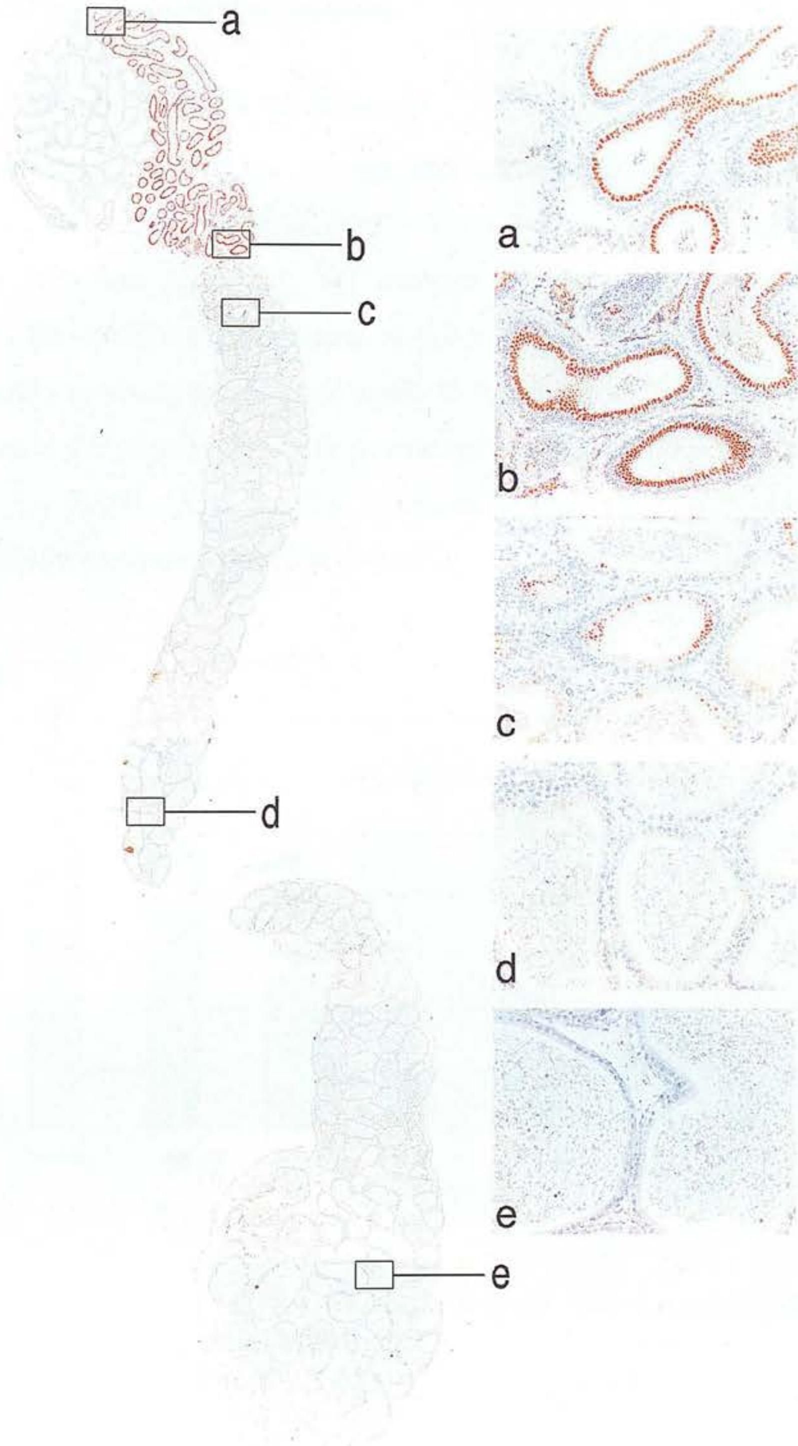


Figure 6.22 CD68 expression in the heated epididymis (+6h)
Main figure is x 4 resolution, figures a-e are x 40 resolution

6.3.3 Analysis of motile spermatozoa

6.3.3.1 Number of motile spermatozoa

The concentration of motile spermatozoa retrieved from the epididymides of control animals was in the range of 5.25 – 7.2 x 10^6/ml (average 6.4 x 10^6/ml). Following *in vivo* heat treatment, the average number of motile spermatozoa retrieved from the epididymides dropped to 4.9 x 10^6/ml within 1 h. The average number of motile spermatozoa dropped again to 2.5 x 10^6/ml ($p < 0.05$) within 2 h, and was lowest at 4 h post-heating with an average number of motile spermatozoa of 1.1 x 10^6/ml ($p < 0.05$). The number of motile spermatozoa retrieved from the epididymis had not recovered after a period of 7d.

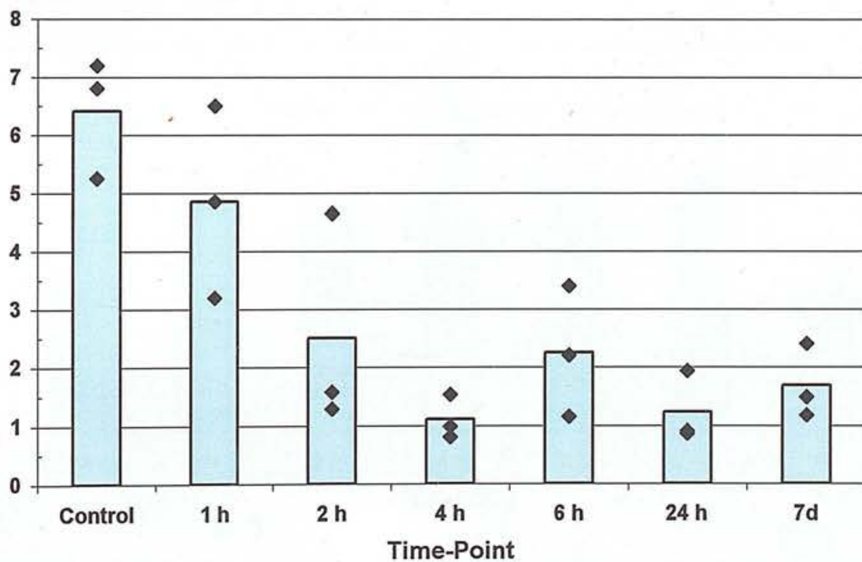


Figure 6.23 Number of motile spermatozoa retrieved from the epididymides of wild-type mice following *in vivo* heat treatment (n=3 for both genotypes, bar = average)

6.3.3.2 Levels of DNA damage in motile spermatozoa

DNA damage in motile spermatozoa, as measured by the Comet assay, is expressed either as % DNA in Comet tail (Figure 6.24) or as Comet moment (Figure 6.25).

Compared to the controls (average = 25.3% DNA in Comet tail), DNA damage in the motile spermatozoa of heated animals increased significantly within 1h of heating (average = 37.8%; $p<0.05$). This elevated level of DNA damage in motile spermatozoa was observed over the initial 24 h period after heating, reaching a peak at 4h (57.8%; $p<0.05$). At 7 d after treatment, the level of DNA damage in the motile spermatozoa had recovered to control levels (22.3%).

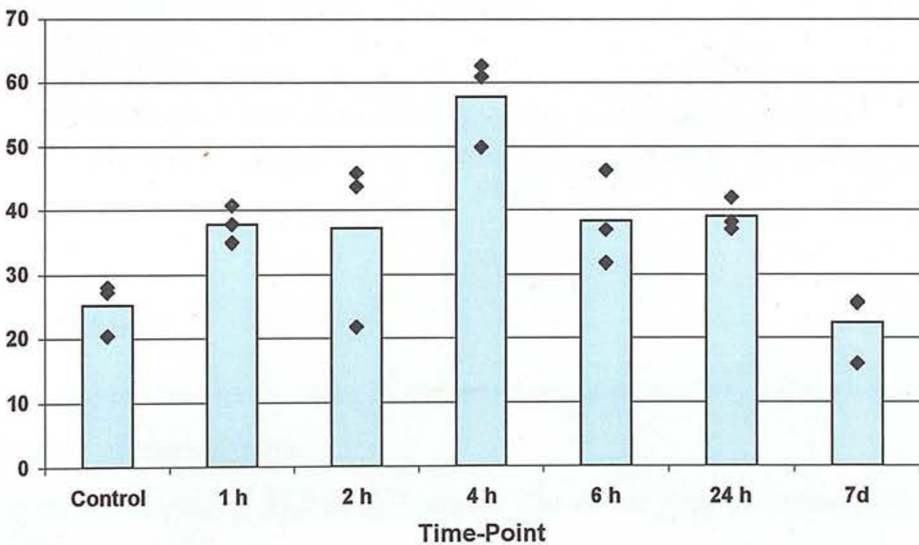


Figure 6.24 Levels of DNA damage (% tail DNA) in motile spermatozoa retrieved from wild-type mice following *in vivo* heat treatment.
(n=3 for each time-point, point = average % Tail DNA for 100 cells, bar = average)

Analysis of Comet moment gave very similar results (Figure 6.25). Within 1h of heating, average Comet moment had increased from 6.9 (control) to 15.8. Again, a peak was observed at 4h (27.7) with Comet moment value remaining high at 24h (13.8) and recovery to control levels seen at 7d (6.6).

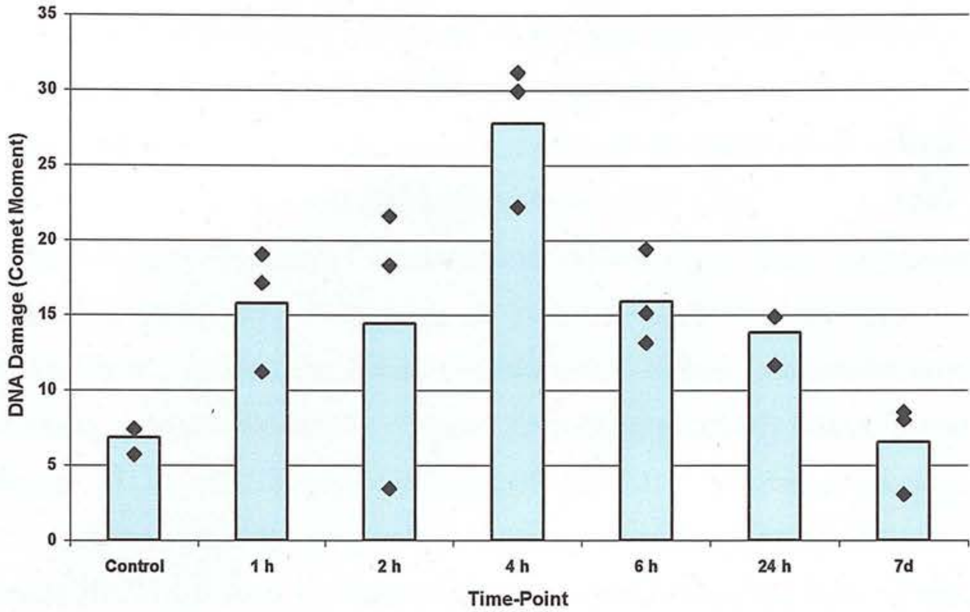


Figure 6.25 Levels of DNA damage (Comet moment) in motile spermatozoa retrieved from wild-type mice following *in vivo* heat treatment. (n=3 for each time-point, point = average Comet moment for 100 cells, bar = average)

6.4 Discussion

The epididymis has 4 main functions; the transportation, maturation, storage and protection of spermatozoa.

In most mammals, including humans, the epididymis is located within the scrotum which is generally maintained at several degrees C lower than body temperature (depending on species) (Bedford, 1978).

The aims of the present study were to subject the epididymis to a mild heat stress, to determine the response of the epididymal epithelial cells to heat stress, to study the ability of the epididymis to maintain viable and motile spermatozoa following heat stress, and to study the effect of heat stress on the genetic integrity of motile spermatozoa within the epididymis.

As in the testis, heat stress can be induced in the epididymis by several methods (see Section 5.4). In the present study, the scrotal temperature of wild-type

male mice was raised by immersion of the lower third of the animals in a water bath at 42°C for a period of 30 minutes, the aim being to determine the effects of a mild heat stress on epididymal function and the integrity of spermatozoa DNA.

The response of the epididymis to raised scrotal temperature was studied using a number of stress and heat response markers.

As previously discussed, Cirp is a protein which is constitutively expressed in the cells of the testis and is inducible at 32°C in mouse somatic cells *in vitro* (Nishiyama, *et al.*, 1997). The presence of an amino-terminal consensus-sequence RNA-binding domain (CS-RBD; a major RNA-binding motif) suggests that the protein may play a role in post-transcriptional regulation of gene expression. In addition, Cirp has been shown to share a sequence similarity with RNA-binding motif gene (RBM), a gene expressed on the Y chromosome, the loss of which is associated with some cases of human male infertility (Nishiyama, *et al.*, 1997).

In the current study, immnohistochemistry has revealed for the first time that Cirp is expressed in the epididymis, where expression is region-specific. Following scrotal heating, the expression of Cirp in the epididymis was altered, with decreased expression in most regions and a slight increase in expression in others. Altered expression of Cirp indicates that scrotal heating had induced an increase in epididymal temperature.

In the context of the present study, the expression of Cirp in the epididymis was studied as a means of confirming the alteration of the temperature within the epididymis following scrotal heating. The region-specific nature of Cirp expression in the epididymis suggests that this protein may also play a role in the storage and maturation of spermatozoa which should be investigated further.

Bax is a pro-apoptotic protein which has been shown to be highly expressed in a number of tissues, including the epididymis (Penault-Llorca, *et al.*, 1998). In the mouse, experimental cryptorchidism results in increased Bax expression and redistribution of the protein, leading to increased apoptosis of germ cells in the testis (Xu, *et al.*, 2000;Yamamoto, *et al.*, 2000). The effect of heat stress on the expression of Bax in the epididymis had not been studied previously and we observed for the

first time that increased levels of Bax were induced in the epithelial cells of the corpus and cauda regions of the epididymis following scrotal heating.

The basal cells in the epididymal epithelium play a scavenging role in the local immune defence mechanism (acting as tissue-fixed macrophages) and may also be involved in the detoxification of the epididymal epithelium and lumen (Yeung, *et al.*, 1994;Seiler, *et al.*, 1999;Seiler, *et al.*, 2000). The distribution of basal cells expressing macrophage antigen (F4/80) has been studied in the mouse (Seiler, *et al.*, 1999) showing region-specific expression of F4/80 +ve cells in the murine epididymis. It was determined that the number of cells expressing F4/80 was highest in the initial segment and caput regions of the epididymis.

In the present study, basal cells with macrophage properties were identified using antibodies raised against CD68. In concurrence with a previous study (Seiler, *et al.*, 1999), in the control mouse, basal cells in the caput region of the epididymis were identified as having macrophage properties, with high levels of expression of CD68 observed in this region. Faint expression of CD68 was also seen in the upper part of the corpus region. The lower corpus and caudal regions of the control epididymis did not exhibit CD68 expression.

Following scrotal heating, the region of CD68 expression in the caput increased, though it is unclear if this is due to the orientation of the epididymides studied. In addition, the level of CD68 expression in the upper corpus region also increased, with expression of CD68 extending to basal cells lower down the corpus. Again, CD68 expression was absent in the lower corpus and cauda region.

A possible explanation for these findings is that the heat stress induces more basal cells to behave like macrophages in order to deal with an increase in the number of dead/immotile cells resulting from the heat stress. This hypothesis would fit with the findings of Seiler *et al* (Seiler, *et al.*, 1998) who observed that an increase in macrophage antigen expression in the developing epididymis coincided with a period when there are high numbers of morphologically abnormal spermatozoa present (Janca, *et al.*, 1986).

The immunohistochemical analysis of the epididymis following heat treatment confirmed that the temperature within the epididymis had been altered, and that in response to the raised temperature, a stress response had occurred.

Haematoxylin and eosin staining was used to examine the density of spermatozoa in the tubules of the caput, corpus and cauda regions of the epididymis following heat treatment. The appearance of the epididymis following heating was similar to that of the control epididymis, with large numbers of spermatozoa present within the tubules. However, reduced numbers of motile spermatozoa were retrieved from the epididymis within 1h of scrotal heating. These findings suggest that though the epididymis retains normal numbers of spermatozoa following scrotal heating, the majority of these spermatozoa lose their motility.

Previous studies have demonstrated that increased temperature affects the function of the epididymis in a number of ways. Translocation of the epididymis into the abdominal cavity of rabbits resulted in the decreased biosynthesis of epididymal secretory proteins compared to control animals, indicating that the biosynthesis of these proteins is temperature regulated (Regalado, *et al.*, 1993). In addition, the water and ion transport mechanisms of the epididymis are affected by raised temperature resulting in altered ionic composition of the epididymal fluid (Bedford, 1991). It has been proposed that the availability of oxygen in the epididymis is of significant importance in the maintenance and storage of maturing spermatozoa (Volgymayr, *et al.*, 1967;Djakiew, *et al.*, 1986). The solubility of oxygen in epididymal fluid is increased and the respiration rate of spermatozoa is decreased at lower (scrotal) temperatures. In combination, these factors ensure that there is sufficient oxygen available to sustain large numbers of spermatozoa within the epididymis (Djakiew, *et al.*, 1986).

It is possible that the increase in temperature within the epididymis reduces the solubility of the available oxygen and increases the metabolism of the spermatozoa resulting in a shortage of oxygen available to the spermatozoa within the epididymis, causing loss of motility and/or death of the spermatozoa.

The density of spermatozoa within the epididymis did not appear to change significantly until 14 and 28 days after heat treatment. The reduced density of spermatozoa observed in the epididymis at this later time-point corresponds to the increased levels of apoptosis (germ cell loss) in the testis following scrotal heating, as discussed previously in Chapter 5.

DNA integrity in motile spermatozoa was assessed using a modified Comet assay (see section 3). The aim was to assess the quality of the DNA within the spermatozoa which had remained intact and motile following heat treatment. It was found that levels of DNA damage within motile spermatozoa increased within 1h of heat treatment, reaching a peak at 4h and then recovering by 7d. A number of studies have shown that DNA damage can occur as a result of oxidative stress (Shen, *et al.*, 1997;Aitken, *et al.*, 1998;Twigg, *et al.*, 1998). Oxidative stress has been shown to affect spermatozoa in the testis, the epididymis and *in vitro*, and can be caused by a number of factors (e.g. cancer treatments, cigarette smoke) (Hinton, *et al.*, 1995;Shen, *et al.*, 1997;Twigg, *et al.*, 1998;Pagano, *et al.*, 2001).

In the current study, the level of DNA damage in motile spermatozoa from the epididymis increased over time, reaching a peak at 4 hours. It is our hypothesis that increasing levels of oxidative stress in the heated epididymis may induce DNA damage in motile spermatozoa.

The gradual decrease in the level of DNA damage may be explained by the introduction of spermatozoa into the epididymis which, at the time of heating, were located in the testis and therefore not exposed to the same high levels of oxidative stress.

It should be noted that limited numbers of animals were available for use in the current study. Furthermore, due to the subjective nature of the analysis of DNA damage in the motile spermatozoa, it would have been preferable to analyse the results in a 'blind' manner. However, the findings of this study suggest that future examination of the genetic integrity of motile spermatozoa should be undertaken.

In the current study, the response of the epididymis to heat stress was proven by the increased/altered expression of the Cirp, Bax and CD68.

Following heat stress, the density of spermatozoa within the epididymis was unaffected while the proportion of these spermatozoa retaining motility was very low. Over time, the numbers of spermatozoa entering the epididymis from the testis decreased and the dead/immotile spermatozoa were phagocytosed by the basal cells and macrophages. Though at this time there were fewer numbers of spermatozoa in the epididymis, a much greater proportion of these spermatozoa had retained their motility. Finally, it was shown that heat stress in the epididymis resulted in increased levels of DNA damage in mature, motile spermatozoa.

Chapter 7 General Discussion

The use of assisted reproductive techniques such as IVF and ICSI has revolutionized the treatment of couples with so-called male factor infertility. However, there is conflicting data regarding the safety of these procedures. The main areas of concern are reports of increased frequency of birth defects, genetic anomalies and developmental problems in children conceived via IVF/ICSI compared to those conceived normally (Wennerholm, *et al.*, 2000;Ericson, *et al.*, 2001;Hansen, *et al.*, 2002;Van Steirteghem, *et al.*, 2002). Other reports have disputed these findings, stating that the increased risk of these problems following IVF/ICSI treatment is negligible compared to natural conception (Tarlatzis, *et al.*, 1999;Bonduelle, *et al.*, 2002).

However, clinical data indicates that poor spermatozoa quality results in reduced fertilisation rates, fewer blastocysts, and poor embryonic development (Ron_el, *et al.*,1991; Parinaud, *et al.*,1993; Janny, *et al.*, 1994;Lopes, *et al.*, 1998). In addition it has been shown that DNA abnormalities (adducts, deletions etc.) can be passed from the father to the offspring (Ji, *et al.*, 1997;Jiang, *et al.*, 1999;Kamischke, *et al.*, 1999;Potts, *et al.*, 1999;Cram, *et al.*, 2000;Zenzes, 2000;Dohle, *et al.*, 2002).

According to previous research, the success of assisted reproduction techniques is dependent on the selection of morphologically normal and motile spermatozoa (Mansour, *et al.*, 1995) and at the present time the DNA integrity of spermatozoa used in assisted reproduction techniques is not assessed. There are a number of assays available for the detection of DNA damage in spermatozoa (including the Comet and TUNEL assays) (Hughes, *et al.*, 1997;Hughes, *et al.*, 1999;Donnelly, *et al.*, 2000), however, these methods can be costly and time-consuming, and it has yet to be proven that the use of these protocols is warranted.

7.1 Aim of the work

The main objective of the work presented for this thesis was to determine the levels of DNA damage present in mature, motile spermatozoa from a number of mouse models for male infertility.

The models used were:

a) Mice deficient in a protein known to be associated with male infertility (*dazl*).
b) Mice deficient in a DNA-repair protein known to be highly expressed in the testis (*Ercc-1*).
c) Mice found to be infertile due to deficiency in proteins with no known role in the testis/fertility (PrP and PrnD).
d) Wild type mice subjected to a mild scrotal heat stress.

In addition, use of the *in vivo* heat stress model enabled the study of the response of the testis and epididymis to heat stress, and the study of the transmission of DNA damage from developing germ cells to mature spermatozoa.

7.2 DNA Damage in the spermatozoa of genetically modified mouse models

The Comet assay is a highly sensitive and reproducible assay commonly used to detect levels of DNA fragmentation in a wide range of somatic cell types (McKelvey-Martin, *et al.*, 1993). The assay involves the lysis of the cell membrane followed by the unwinding of the DNA (most commonly under alkaline conditions) and then electrophoresis which draws broken DNA strands away from the cell nucleus, forming a characteristic 'comet' image.

In mammalian sperm, DNA is packaged very differently from that of somatic cells (Ward, *et al.*, 1991). The arrangement of DNA in mammalian spermatozoa is specifically designed to protect it from exogenous insult, therefore in order to study the integrity of the DNA it must first be exposed without causing further damage. The Comet assay has been adapted for use on human spermatozoa and used

successfully to demonstrate a positive relationship between poor semen parameters and the presence of DNA damage in human spermatozoa (Hughes, *et al.*, 1997;Sun, *et al.*, 1997;Irvine, *et al.*, 2000). However, attempts to use the same method for the study of murine sperm were unsuccessful due to the differences in DNA packaging between murine and human spermatozoa. A new assay based on a commercially available kit was developed and used for the studies of murine spermatozoal DNA described in this thesis.

The spermatozoa of three different genetically-modified mouse models of infertility were studied:

1) The human gene *DAZ* is located on the Y chromosome within the AZF region and has been implicated in male infertility. To date a murine homologue of *DAZ* has not been found, however, deletion of the *dazl* gene in the mouse results in complete spermatogenic failure while heterozygote animals produce reduced numbers of motile spermatozoa with increased incidence of morphologically abnormal spermatozoa. The role of *dazl* in the testis is not fully understood, however, the homology between *dazl* and the Drosophila gene *boule* (a binding protein for all cell cycle regulators which is essential for spermatogenesis) suggests a role for *dazl* in cell cycle regulation (Eberhart, *et al.*, 1996;Yen, *et al.*, 1996).

2) The *Ercc-1* gene is involved in the nucleotide excision repair and recombinational repair pathways and is highly expressed in the testis (Adair, *et al.*, 2000;Sargent, *et al.*, 2000;Melton DW, *et al*, submitted). Prior to the current study, investigators had observed that male *Ercc-1* knockout mice appeared to be subfertile, failing to produce many offspring (Melton DW, personal communication).

3) The role of Prion and Prion-related proteins in the testis has not been determined. However, it has been shown that these proteins are highly expressed in the testis and it has been suggested their role in the testis might be similar to their role in the brain – i.e. maintaining the blood-brain/blood-testis barrier (Collinge, *et al.*, 1994;Li, *et al.*, 2000;Behrens, *et al.*, 2002). Previous investigators have noted the very poor fertility of Prion deficient mice. In particular, both male PrP- and Dpl-

successfully to demonstrate a positive relationship between poor semen parameters and the presence of DNA damage in human spermatozoa (Hughes, *et al.*, 1997;Sun, *et al.*, 1997;Irvine, *et al.*, 2000). However, attempts to use the same method for the study of murine sperm were unsuccessful due to the differences in DNA packaging between murine and human spermatozoa. A new assay based on a commercially available kit was developed and used for the studies of murine spermatozoal DNA described in this thesis.

The spermatozoa of three different genetically-modified mouse models of infertility were studied:

1) The human gene *DAZ* is located on the Y chromosome within the AZF region and has been implicated in male infertility. To date a murine homologue of *DAZ* has not been found, however, deletion of the *dazl* gene in the mouse results in complete spermatogenic failure while heterozygote animals produce reduced numbers of motile spermatozoa with increased incidence of morphologically abnormal spermatozoa. The role of *dazl* in the testis is not fully understood, however, the homology between *dazl* and the Drosophila gene *boule* (a binding protein for all cell cycle regulators which is essential for spermatogenesis) suggests a role for *dazl* in cell cycle regulation (Eberhart, *et al.*, 1996;Yen, *et al.*, 1996).

2) The *Ercc-1* gene is involved in the nucleotide excision repair and recombinational repair pathways and is highly expressed in the testis (Adair, *et al.*, 2000;Sargent, *et al.*, 2000;Melton DW, *et al*, submitted). Prior to the current study, investigators had observed that male *Ercc-1* knockout mice appeared to be subfertile, failing to produce many offspring (Melton DW, personal communication).

3) The role of Prion and Prion-related proteins in the testis has not been determined. However, it has been shown that these proteins are highly expressed in the testis and it has been suggested their role in the testis might be similar to their role in the brain – i.e. maintaining the blood-brain/blood-testis barrier (Collinge, *et al.*, 1994;Li, *et al.*, 2000;Behrens, *et al.*, 2002). Previous investigators have noted the very poor fertility of Prion deficient mice. In particular, both male PrP- and Dpl-

deficient mice, while demonstrating normal sexual behaviour, have been found to be infertile (Behrens, *et al.*, 2002;Melton DW, personal communication).

In the present study, increased numbers of morphologically abnormal spermatozoa, reduced numbers of motile spermatozoa and increased levels of endogenous DNA damage were observed in the epididymal spermatozoa retrieved from each of the modified mouse strains studied, compared with wild type animals.

In vitro heating of the spermatozoa from these mice was also performed and it was found that, apart from the PrP and PrnD mice which had very high levels of endogenous damage, the DNA in the spermatozoa from these modified mouse models are susceptible to heat-induced DNA damage.

Further work is required to understand the exact mechanisms by which deletion of these 3 different genes results in such similar phenotypes. Based on existing knowledge of the *dazl* gene, current data suggests that deletion of *dazl* may lead to disruption of spermatogenesis, in turn resulting in disordered DNA remodelling/repair. In a process such as spermatogenesis, in which there is a substantial amount of DNA remodelling and repackaging, fully functional DNA repair mechanisms are vital. The deletion of a DNA repair gene such as *Ercc-1* could result in the failure to repair both intentional nicks and DNA strand breaks which are created during spermiogenesis, and unintentional strand breaks which might occur during spermatogenesis. Very little is known about the role of PrP and PrnD in the testis and therefore further study is required before an explanation as to the cause of the poor morphology, motility and DNA integrity of spermatozoa from these mice can be provided.

Each of the genotypes studied exhibited increased levels of DNA damage within the motile spermatozoa population, which may in turn contribute to the reduced fertility of these genotypes.

The low numbers of motile spermatozoa and high numbers of morphologically abnormal spermatozoa retrieved from heterozygous and knock-out *Ercc-1* and Prion mice, suggest that these mice may not be suitable models for studying levels of DNA damage in motile spermatozoa.

7.3 The Effect of mild heat stress on the testis and epididymis

In order to achieve the main aims of the project (to study levels of DNA damage in motile, morphologically-normal spermatozoa), the focus of the work then moved towards using an *in vivo* heated mouse model for infertility. *Dazl* wild-type mice produce large numbers of motile, morphologically normal spermatozoa and are capable of producing large numbers of offspring. Endogenous levels of DNA damage in spermatozoa from these mice are low but in the current study have been shown to be susceptible to heat-induced damage.

It was proposed that the heat-treated wild-type *dazl* mouse might be a suitable model for the study of DNA integrity in motile spermatozoa.

Animals were subjected to scrotal heating at 42°C for a period of 30min. Previous studies have shown that similar regimes cause impaired spermatozoa quality, reduced embryo weight *in vivo*, and reduced fertilisation rates *in vitro* (Bellve, 1972;Jannes, *et al.*, 1998;Rockett, *et al.*, 2001). It was intended that this heating regime would disrupt spermatogenesis, though not block it entirely, in order to study the mature spermatozoa which developed from the affected cells.

Immunohistochemistry was used to confirm the response of the testis and epididymis to the heating regime and altered expression of temperature-regulated proteins Cirp and HSP105 indicated that there had been an increase in temperature within the testis due to scrotal heating. It was found that Cirp expression, which had previously been reported in the testis only (Nishiyama, *et al.*, 1998), is also found in a region-specific manner within the epididymis and that expression in the epididymis is also altered following scrotal heating. Expression of the stress proteins Bax and CD68, and the increased number of TUNEL-positive cells (in the testis) indicated that a temperature-mediated stress response had occurred.

The reduced numbers of motile spermatozoa retrieved and the appearance of the epididymis and testis following heating suggested that, following scrotal heating, an initial loss of motility of spermatozoa in the epididymis occurs followed by a reduction in the number of spermatozoa entering the epididymis from the heat-stressed testis.

Data obtained from the Comet analysis of motile spermatozoa retrieved from the epididymis at specific time-points following heating suggests that cells in the pre-meiotic stages of spermatogenesis develop into spermatozoa with higher levels of DNA damage than other stages and that the DNA integrity in spermatozoa developed from cells undergoing spermiogenesis at the time of heating is comparable with that of controls.

Motile spermatozoa located in the epididymis at the time of heating also exhibited significantly higher levels of DNA damage than those retrieved from control animals. The DNA integrity of motile spermatozoa stored in the heat-treated epididymis deteriorated over time until, at 4 hours after treatment DNA damage had increased 6-fold compared to control samples. Levels of DNA damage then gradually recovered until control values were reached at 7d post-heating. Figure 7.1 shows the numbers of motile spermatozoa, and the level of DNA damage measured in these spermatozoa, for each of the time-points studied.

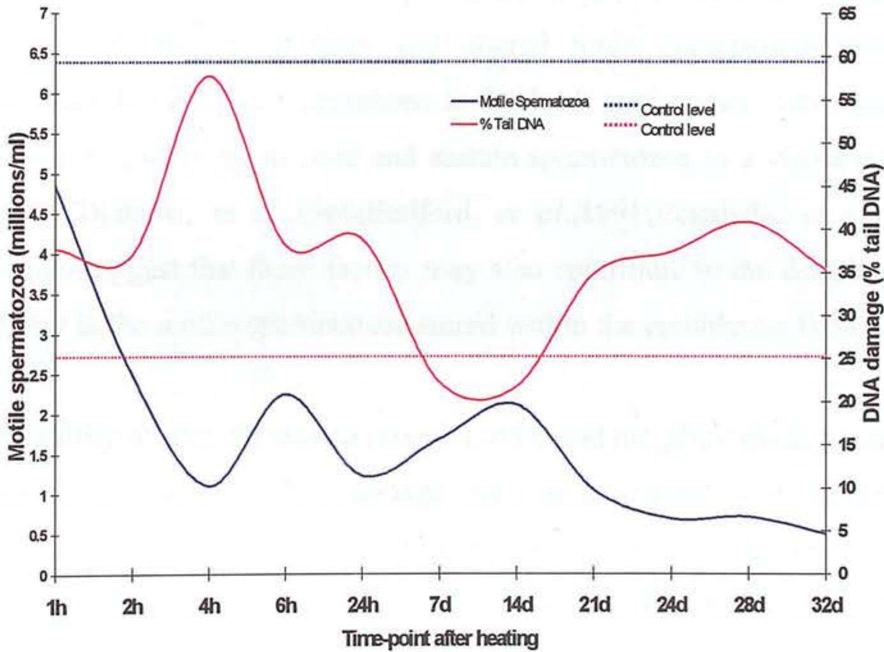


Figure 7.1 Numbers of motile spermatozoa and levels of DNA damage in these spermatozoa at each of the time-points studied.

Previous studies in which cell loss has been quantified have identified pachytene spermatocytes and early spermatids as being susceptible to heat stress (Collins, *et al.*, 1969;De-Vita, *et al.*, 1990). In addition, two critical periods in spermatogenesis (leptotene-pachytene and maturation division) have been identified through which cells were unable to progress following heating (Collins, *et al.*, 1969). The current study has identified cells in the earlier (pre-meiotic) stages of spermatogenesis which, though affected by heating, have progressed through these critical periods in germ cell differentiation to develop into DNA-damaged spermatozoa.

It is proposed that the loss of DNA integrity in mature spermatozoa resulting from heated germ cells may be explained in two ways; DNA damage occurs in the cells at the time of heating and is not repaired during spermatogenesis, or the physiology of the testis is disrupted by heat stress resulting in impaired germ cell function and loss of DNA integrity in mature spermatozoa.

Increased temperature in the epididymis results in altered expression of secretory proteins, reduced oxygen and altered ionic composition within the epididymal fluid. In turn, these alterations to the local environment adversely affect the ability of the epididymis to store and sustain spermatozoa in a viable (alive and motile) state (Djakiew, *et al.*,1986;Bedford, *et al.*,1991;Regalado, *et al.*, 1993). These findings suggest that these factors may also contribute to the deterioration of DNA integrity in the motile spermatozoa stored within the epididymis following heat stress.

The ability of spermatozoa to remain motile and morphologically normal and yet contain high levels of DNA damage may have serious implications in the treatment of male infertility. It is generally accepted that spermatozoa quality is a good indicator of success in both IVF and ICSI. These findings suggest the possibility that these spermatozoa may not contain intact DNA and therefore are not necessarily good candidates for use in IVF and ICSI, indicating that there may be a need for testing DNA quality in spermatozoa to be used in these procedures.

7.4 General conclusions

In conclusion, the current study has demonstrated that mature spermatozoa in the epididymis are not protected from heat-induced DNA damage and that following heat stress the environment of the epididymis itself may contribute to the deterioration of DNA integrity of spermatozoa. Studies involving *in vitro* heating and culturing of spermatozoa and epididymides could be employed to explore this hypothesis further.

Additionally, cell types within the testis which, following heat stress, develop into motile but DNA-damaged spermatozoa have been identified. These findings suggest the need for additional criteria to be taken into consideration when selecting spermatozoa for IVF/ICSI treatment.

Bibliography

Adair, G., Rolig, R., Moore-Faver, D., Zabelshansky, M., Wilson, J. and Nairn, R. 2000. Role of ERCC1 in removal of long non-homologous tails during targeted homologous recombination. *EMBO Journal*, 19, 5552-5561

Aitken, R.J. 1999. The Amoroso Lecture. The human spermatozoon--a cell in crisis? *Journal of Reproduction and Fertility*, 115, 1-7

Aitken, R.J., Clarkson, J.S. and Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of Reproduction*, 41, 183-97

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z. and Irvine, D.S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction*, 59, 1037-46

Ali, J.I., Weaver, D.J., Weinstein, S.H. and Grimes, E.M. 1990. Scrotal temperature and semen quality in men with and without varicocele. *Archives of Andrology*, 24, 215-9

Almon, E., Goldfinger, N., Kapon, A., Schwartz, D., Levine, A.J. and Rotter, V. 1993. Testicular tissue-specific expression of the p53 suppressor gene. *Developmental Biology*, 156, 107-16

Anderson, D., DobrzyÑska, M.M., Yu, T.W., Gandini, L., Cordelli, E. and Spano, M. 1997. DNA integrity in human sperm. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 17, 97-102

Auger, J., Kunstmann, J.M., Czyglik, F. and Jouannet, P. 1995. Decline in semen quality among fertile men in Paris during the past 20 years.PG - 281-5. *New England Journal of Medicine*, 332,

Bar-Hava, I., Ashkenazi, J., Shelef, M., Schwartz, A., Brengauz, M., Feldberg, D., Orvieto, R. and Ben-Rafael, Z. 1997. Morphology and clinical outcomes of embryos after in vitro fertilization are superior to those after intracytoplasmic sperm injection. *Fertility and Sterility*, 68, 653-7

Barroso, G., Morshedi, M. and Oehninger, S. 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa [In Process Citation]. *Human Reproduction*, 15, 1338-44

Basu, A. and Haldar, S. 1998. The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Molecular Human Reproductionrod*, 4, 1099-109

Bauche, F., Fouchard, M.H. and Jegou, B. 1994. Antioxidant system in rat testicular cells. *FEBS Letters*, 349, 392-6

Bedford, J.M. 1978. Influence of abdominal temperature on epididymal function in the rat and rabbit. *American Journal of Anatomy*, 152, 509-21

Bedford, J.M. 1979. Mechanisms of conception: the maturation and interaction of mammalian gametes.PG - 7-17. *Mead Johnson Symp Perinat Dev Med*,

Bedford, J.M. 1994. The status and the state of the human epididymis. *Human Reproduction*, 9, 2187-99

Behrens, A., Genoud, N., Naumann, H., Rulicke, T., Janett, F., Heppner, F.L., Ledermann, B. and Aguzzi, A. 2002. Absence of the prion protein homologue Doppel causes male sterility. *EMBO Journal*, 21, 3652-8

Bellve, A.R. 1972. Viability and survival of mouse embryos following parental exposure to high temperature. *Journal of Reproduction and Fertility*, 30, 71-81

Berthold, A. 1849. Transplantation der Hoden. *Arch Anat ohysiol Wiss Med*, 16, 42-44

Beumer, T.L., Roepers_Gajadien, H.L., Gademan, I.S., Lock, T.M., Kal, H.B. and De_Rooij, D.G. 2000. Apoptosis regulation in the testis: involvement of Bcl-2 family members. *Molecular Reproduction and Development*, 56, 353-9

Biggerstaff, M., Szymkowski, D.E. and Wood, R.D. 1993. Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects in vitro.PG - 3685-92. *EMBO Journal*, 12,

Blanco-Rodriguez, J. and Martinez-Garcia, C. 1996. Induction of apoptotic cell death in the seminiferous tubule of the adult rat testis: assessment of the germ cell types that exhibit the ability to enter apoptosis after hormone suppression by oestradiol treatment. *International Journal of Andrology*, 19, 237-47

Blanco-Rodriguez, J. and Martinez-Garcia, C. 1996. Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. *Cell Proliferation*, 29, 13-31

Blanco-Rodriguez, J. and Martinez-Garcia, C. 1998. Apoptosis pattern elicited by several apoptogenic agents on the seminiferous epithelium of the adult rat testis. *Journal of Andrology*, 19, 487-97

Bonde, J.P. 1993. [Occupational medical research of male reproductive capacity]. *Ugeskr Laeger*, 155, 1029-37

Bonduelle, M., Camus, M., De Vos, A., Staessen, C., Tournaye, H., Van Assche, E., Verheyen, G., Devroey, P., Liebaers, I. and Van Steirteghem, A. 1999. Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Human Reproduction*, 14, 243-264

Bonduelle, M., Liebaers, I., Deketelaere, V., Derde, M.P., Camus, M., Devroey, P. and Van Steirteghem, A. 2002. Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999). *Human Reproduction*, 17, 671-94

Bouin, P. and Ancel, P. 1903. Recherches sur les cellules interstitielles der testicule des mammiferes. *Arch. Zool*, 1, 437-523

Bowen, J.R., Gibson, F.L., Leslie, G.I. and Saunders, D.M. 1998. Medical and developmental outcomes at 1 year for children conceived by intracytoplasmic sperm injection. *Lancet North American Edition*, 315, 1529-34

Brindley, G.S. 1982. Deep scrotal temperature and the effect on it of clothing, air temperature, activity, posture and paraplegia. PG - 49-55. *British Journal of Urology*, 54,

Bromwich, P., Cohen, J., Stewart, I. and Walker, A. 1994. Decline in sperm counts: an atrefact of changed reference range of "normal"? *British Medical Journal*, 309, 19-22

Brown-Woodman, P.D., Post, E.J., Gass, G.C. and White, I.G. 1984. The effect of a single sauna exposure on spermatozoa. *Archives of Andrology*, 12, 9-15

Bujan, L., Daudin, M., Charlet, J.P., Thonneau, P. and Mieusset, R. 2000. Increase in scrotal temperature in car drivers [In Process Citation]. *Human Reproduction*, 15, 1355-7

Calvin, H.I. and Bedford, J.M. 1971. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *Journal of Reproduction and Fertility. Supplement*, 13, Suppl 13:65-75

Candas, V., Becmeur, F., Bothorel, B. and Hoeft, A. 1993. Qualitative assessment of thermal and evaporative adjustments of human scrotal skin in response to heat stress. *International Journal of Andrology*, 16, 137-42

Carlsen, E., Giwercman, A., Keiding, N. and Skakkebaek, N.E. 1992. Evidence for decreasing quality of semen during past 50 years. *British Medical Journal*, 305, 609-13

Chang, P.L., Sauer, M.V. and Brown, S. 1999. Y chromosome microdeletion in a father and his four infertile sons. *Human Reproduction*, 14, 2689-94

Chatterjee, R., Haines, G.A., Perera, D.M., Goldstone, A. and Morris, I.D. 2000. Testicular and sperm DNA damage after treatment with fludarabine for chronic lymphocytic leukaemia. *Human Reproduction*, 15, 762-6

Cheng, L., Guan, Y., Li, L., Legerski, R.J., Einspahr, J., Bangert, J., Alberts, D.S. and Wei, Q. 1999. Expression in normal human tissues of five nucleotide excision repair genes measured simultaneously by multiplex reverse transcription-polymerase chain reaction. *Cancer Epidemiology Biomarkers and Prevention*, 8, 801-7

Chia, S.E., Ong, C.N., Lee, S.T. and Tsakok, F.H. 1994. Study of the effects of occupation and industry on sperm quality. *Annals of the Academy of Medicine Singapore*, 23, 645-9

Clermont, Y. 1966. Spermatogenesis in man. A study of the spermatogonial population. *Fertility and Sterility*, 17, 705-21

Clermont, Y. 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiological Reviews*, 52, 198-236

Collinge, J., Whittington, M.A., Sidle, K.C., Smith, C.J., Palmer, M.S., Clarke, A.R. and Jefferys, J.G.R. 1994. Prion protein is neccesary for normal synaptic function. *Nature*, 370, 295-297

Collins, P. and Lacy, D. 1969. Studies on the structure and function of the mammalian testis. II. Cytological and histochemical observations on the testis of the rat after a single exposure to heat applied for different lengths of time. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 172, 17-38

Cooke, H.J., Lee, M., Kerr, S. and Ruggiu, M. 1996. A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads. *Human Molecular Genetics*, 5, 513-6

Cooper, T.G., Waites, G.M. and Nieschlag, E. 1986. The epididymis and male fertility. A symposium report. *International Journal of Andrology*, 9, 81-90

Cornwall, G., Vreeburg, J., MKHolland and MCOrgebin-Crist. 1990. Interactions of labeled epididymal secretory proteins with spermatozoa after injection of 35S-methionine in the mouse. *Biology of Reproduction*, 43, 121-9

Cram, D.S., Ma, K., Bhasin, S., Arias, J., Pandjaitan, M., Chu, B., Audrins, M.S., Saunders, D., Quinn, F., deKretser, D. and McLachlan, R. 2000. Y chromosome analysis of infertile men and their sons conceived through intracytoplasmic sperm injection: vertical transmission of deletions and rarity of de novo deletions. *Fertility and Sterility*, 74, 909-15

David, K., Dingemanse, E., Freud, J. and Laquer, G. 1935. Ueber krystallinisches mannliches hormon aus hoden (testosteron), wirksamer als aus harn oder aus cholesterin bereites androsteron. *Z. physiol. Chem*, 233, 281-282

de Lamirande, E., Jiang, H., Zini, A., Kodama, H. and Gagnon, C. 1997. Reactive oxygen species and sperm physiology. *Reviews of Reproduction*, 2, 48-54

De_Vita, R., Calugi, A., Chiarantano, C., Forte, D., Mauro, F. and Uccelli, R. 1990. Effects of heat on mouse spermatogenesis monitored by flow cytometry. *International Journal of Hyperthermia*, 6, 543-51

Djakiew, D. and Cardullo, R. 1986. Lower Temperature of the Cauda Epididymis Facilitates the Storage of Sperm by Enhancing Oxygen Availability. *Gamete Research*, 15, 237-245

Dohle, G.R., Halley, D.J., Van Hemel, J.O., van den Ouwel, A.M., Pieters, M.H., Weber, R.F. and Govaerts, L.C. 2002. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. *Human Reproduction*, 17, 13-6

Donnelly, E.T., O'Connell, M., McClure, N. and Lewis, S.E. 2000. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Human Reproduction*, 15, 1552-1561

Eberhart, C.G., Maines, J.Z. and Wasserman, S.A. 1996. Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature*, 381, 783-5

Ericson, A. and Kallen, B. 2001. Congenital malformations in infants born after IVF: a population-based study. *Human Reproduction*, 16, 504-9

Estop, A.M., Munne, S., Jost, L.K. and Evenson, D.P. 1993. Studies on sperm chromatin structure alterations and cytogenetic damage of mouse sperm following in vitro incubation. Studies on in vitro- incubated mouse sperm. *Journal of Andrology*, 14, 282-8

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. 1980. Comparison of human and mouse sperm chromatin structure by flow cytometry. *Chromosoma*, 78, 225-38

Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P. and Claussen, O.P. 1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Human Reproduction*, 14, 1039-49

Figa-Talamanca, I., Dell'Orco, V., Pupi, A., Dondero, F., Gandini, L., Lenzi, A., Lombardo, F., Scavalli, P. and Mancini, G. 1992. Fertility and semen quality of workers exposed to high temperatures in the ceramics industry.PG - 517-23. *Reproductive Toxicology* , 6,

Fisher, H.M. and Aitken, R.J. 1997. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *Journal of Experimental Zoology*, 277, 390-400

Fossa, S., de Angelis, P., Kraggerud, S., Evenson, D., Theodorsen, L. and Clausen, O. 1997. Prediction of posttreatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. *Cytometry*, 30, 192-196

Fraga, C., Motchnik, P., Shigenaga, M., Helbock, H., Jacob, R. and Ames, B. 1991. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 11003-11006

Galil, K.A. and Setchell, B.P. 1988. Effects of local heating of the testes on the concentration of testosterone in jugular and testicular venous blood of rats and on testosterone production in vitro. *International Journal of Andrology*, 11, 61-72

Gatewood, J.M., Cook, G.R., Balhorn, R., Bradbury, E.M. and Schmid, C.W. 1987. Sequence-specific packaging of DNA in human sperm chromatin. *Science*, 236, 962-4

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology*, 119, 493-501

Genissel, C. and Carreau, S. 2001. Regulation of the aromatase gene expression in mature rat Leydig cells. *Molecular Cellular Endocrinology*, 178, 141-6

Glad Sorensen, H., Lambrechtsen, J. and Einer-Jensen, N. 1991. Efficiency of the countercurrent transfer of heat and 133Xenon between the pampiniform plexus and testicular artery of the bull under in-vitro conditions.PG - 232-40. *International Journal of Andrology*, 14,

Glover, T. 1960. Spermatozoa from the isolated cauda epididymis of rabbits and some effects of artificial cryptorchidism. *Journal of Reproduction and Fertility*, 1, 121-129

Goldstein, M. and Eid, J.F. 1989. Elevation of intratesticular and scrotal skin surface temperature in men with varicocele. *Journal of Urology*, 142, 743-5

Grootegoed, J., Baarends, W., Roest, H. and Hoeijmakers, J. 1998. Knockout mouse model and gametogenic failure. *Molecular and Cellular Endocrinology*, 25, 161-166

Gu, W. and Hecht, N.B. 1996. Developmental expression of glutathione peroxidase, catalase, and manganese superoxide dismutase mRNAs during spermatogenesis in the mouse. *Journal of Andrology*, 17, 256-62

Haaf, T. and Ward, D.C. 1995. Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers. *Experimental Cell Research*, 219, 604-11

Haines, G., Marples, B., Daniel, P. and Morris, I. 1998. DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay. *Advances in Experimental Medicine and Biology*, 444, 79-91

Hair, W.M., Gubbay, O., Jabbour, H.N. and Lincoln, G.A. 2002. Prolactin receptor expression in human testis and accessory tissues: localization and function. *Molecular Human Reproduction*, 8, 606-11

Hand, J.W., Walker, H., Hornsey, S. and Field, S.B. 1979. Effects of hyperthermia on the mouse testis and its response to X-rays, as assayed by weight loss. *International Journal of Radiation Oncology, Biology, Physics*, 35, 521-8.

Handelsman, D.J. 2001. Estrogens and falling sperm counts. *Reproduction, Fertility, and Development* , 13, 317-324

Hansen, M., Kurinczuk, J.J., Bower, C. and Webb, S. 2002. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *New England Journal of Medicine*, 346, 725-30

Harrison, R. and Weiner, J. 1948. Abdomino-testicular temperature gradients. *Journal of Physiology*, 18, 256-262

Hecht, N.B. and Penshow, J.D. 1987. In situ localization of mRNAs coding for mouse testicular structural genes. *Experimental Cell Research*, 173, 274-81

Heckert, L.L. and Griswold, M.D. 2002. The expression of the follicle-stimulating hormone receptor in spermatogenesis. *Recent Progress in Hormone Research*, 57, 129-48

Henderson, J., Baker, H.W. and Hanna, P.J. 1986. Occupation-related male infertility: a review. *Clin Reprod Fertil*, 4, 87-106

Hess, R.A., Bunick, D. and Bahr, J. 2001. Oestrogen, its receptors and function in the male reproductive tract - a review. *Molecular and Cellular Endocrinology*, 178, 29-38

Hilz, H., Wiegers, U. and Adamietz, P. 1975. Stimulation of proteinase K action by denaturing agents: application to the isolation of nucleic acids and the degradation of 'masked' proteins. *European Journal of Biochemistry* , 56, 103-8

Hinton, B.T. and Keefer, D.A. 1985. Binding of [3H]aldosterone to a single population of cells within the rat epididymis. *Journal of Steroid Biochemistry and Molecular Biology*, 23, 231-3

Hinton, B.T., Palladino, M.A., Rudolph, D. and Labus, J.C. 1995. The epididymis as protector of maturing spermatozoa. *Reproduction, Fertility, and Development*, 7, 731-45

Hsia, K.T., Millar, M.R., King, S., Selfridge, J., Redhead, N., Melton, D. and Saunders, P.T. 2003. DNA repair gene Ercc1 is essential for normal spermatogenesis and oogenesis and for functional integrity of germ cell DNA in the mouse. *Development*, 130, 369

Hughes, C., Lewis, S., ValerieMcKelvey-Martin and Thompson, W. 1997. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mutation Research*, 374, 261-268

Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J. and Thompson, W. 1996. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Molecular Human Reproduction*, 2, 613-9

Hughes, C.M., McKelvey_Martin, V.J. and Lewis, S.E. 1999. Human sperm DNA integrity assessed by the Comet and ELISA assays. *Mutagenesis*, 14, 71-5

Hull, M.G., Glazener, C.M., Kelly, N.J., Conway, D.I., Foster, P.A., Hinton, R.A., Coulson, C., Lambert, P.A., Watt, E.M. and Desai, K.M. 1985. Population study of causes, treatment, and outcome of infertility. *British Medical Journal (Clinical Research Edition)*, 291, 1693-7

Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y., Murata, M., Kumagai, J. and Tanaka, T. 1999. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heat stress. *Biology of Reproduction*, 61, 393-9

Irvine, D.S. 1994. Falling sperm quality. *British Medical Journal*, 39, 476

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A. and Aitken, R.J. 2000. DNA integrity in human spermatozoa: relationships with semen quality. *Journal of Andrology*, 21, 33-44

Ishihara, K., Yasuda, K. and Hatayama, T. 2000. Phosphorylation of the 105-kDa heat shock proteins, HSP105alpha and HSP105beta, by casein kinase II. *Biochemical and Biophysical Research Communications* , 270, 927-931

Janca, F.C., Jost, L.K. and Evenson, D.P. 1986. Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. *Biology of Reproduction*, 34, 613-23

Jannes, P., Spiessens, C., Van der Auwera, I., D'Hooghe, T., Verhoeven, G. and Vanderschueren, D. 1998. Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Human Reproduction*, 13, 372-5

Janny, L. and Menezo, Y.J. 1994. Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Molecular Reproduction and Development*, 38, 36-42

Jegou, B. 1993. The Sertoli-germ cell communication network in mammals. *International Review of Cytology* , 147, 25-96

Jegou, B., Risbridger, G.P. and de_Kretser, D.M. 1983. Effects of experimental cryptorchidism on testicular function in adult rats. *Journal of Andrology*, 4, 88-94

Ji, B., Shu, X., Linet, M., Zheng, W., Wacholder, S., Gao, Y., Ying, D. and Jin, F. 1997. Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *Journal of the National Cancer Institute* , 89, 238-244

Jiang, M.C., Lien, Y.R., Chen, S.U., Ko, T.M., Ho, H.N. and Yang, Y.S. 1999. Transmission of de novo mutations of the deleted in azoospermia genes from a severely oligozoospermic male to a son via intracytoplasmic sperm injection. *Fertility and Sterility*, 71, 1029-32

Jockenhovel, F., Grawe, A. and Nieschlag, E. 1990. A portable digital data recorder for long-term monitoring of scrotal temperatures.PG - 694-700. *Fertility and Sterility*, 54,

Jouannet, P., Wang, C., Eustache, F., Kold-Jensen, T. and Auger, J. 2001. Semen quality and male reproductive health: the controversy about human sperm concentration decline.PG - 333-44. *Apmis*, 109,

Kamischke, A., Gromoll, J., Simoni, M., Behre, H.M. and Nieschlag, E. 1999. Transmission of a Y chromosomal deletion involving the deleted in azoospermia (DAZ) and chromodomain (CDY1) genes from father to son through intracytoplasmic sperm injection: case report. *Human Reproduction*, 14, 2320-2

Kidroni, G., Har-Nir, R., Menezel, J., Frutkoff, I.W., Palti, Z. and Ron, M. 1983. Vitamin D3 metabolites in rat epididymis: high 24,25-dihydroxy vitamin D3 levels in the cauda region. *Biochemical and Biophysical Research Communications* , 113, 982-9

Kitayama, T. 1965. [Study on testicular temperature in men]. *Hinyokika Kiyo*, 11, 435-65

Knudson, C.M., Tung, K.S., Tourtellotte, W.G., Brown, G.A. and Korsmeyer, S.J. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*, 270, 96-9

Kobayashi, H., Larson, K., Sharma, R., Nelson, D., Evenson, D., Toma, H., Thomas, A. and Agarwal, A. 2001. DNA damage in patients with untreated cancer as measured by the sperm chromatin structure assay. *Fertility and Sterility*, 75, 469-475

Kodama, H., Yamaguchi, R., Fukada, J., Kasai, H. and Tanaka, T. 1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertility and Sterility*, 68, 519-524

Krausz, C., Bussani-Mastellone, C., Granchi, S., McElreavey, K., Scarselli, G. and Forti, G. 1999. Screening for microdeletions of Y chromosome genes in patients undergoing intracytoplasmic sperm injection. *Human Reproduction*, 14, 1717-21

Krull, N., Ivell, R., Osterhoff, C. and Kirchhoff, C. 1993. Region-specific variation of gene expression in the human epididymis as revealed by in situ hybridization with tissue-specific cDNAs. *Molecular Reproduction and Development*, 34, 16-24

Kurinczuk, J.J. and Bower, C. 1997. Birth defects in infants conceived by intracytoplasmic sperm injection: an alternative interpretation. *British Medical Journal*, 315, 1260-5; discussion 1265-6

Lerchl, A., Keck, C., Spiteri-Grech, J. and Nieschlag, E. 1993. Diurnal variations in scrotal temperature of normal men and patients with varicocele before and after treatment. *International Journal of Andrology*, 16, 195-200

Li, A., Sakaguchi, S., Shigematsu, K., Atarashi, R., Roy, B.C., Nakaoke, R., Arima, K., Okimura, N., Kopacek, J. and Katamine, S. 2000. Physiological expression of the gene for PrP-like protein, PrPLP/Dpl, by brain endothelial cells and its ectopic expression in neurons of PrP-deficient mice ataxic due to Purkinje cell degeneration. *American Journal of Pathology*, 157, 1447-52

Lindemann, C.B. and Kanous, K.S. 1989. Regulation of mammalian sperm motility. *Archives of Andrology*, 23, 1-22

Lopes, S., Sun, J.G., Jurisicova, A., Meriano, J. and Casper, R.F. 1998. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertility and Sterility*, 69, 528-32

Lucesoli, F. and Fraga, C.G. 1995. Oxidative damage to lipids and DNA concurrent with decrease of antioxidants in rat testes after acute iron intoxication. *Archives of Biochemistry and Biophysics*, 316, 567-71

Lue, Y., Hikim, A.P., Wang, C., Im, M., Leung, A. and Swerdloff, R.S. 2000. Testicular heat exposure enhances the suppression of spermatogenesis by testosterone in rats: the "two-hit" approach to male contraceptive development. *Endocrinology*, 141, 1414-24

Lue, Y.H., Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A. and Wang, C. 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology*, 140, 1709-17

Manfredi Romanini, M.G., Biggiogera, M., Formenti, D., Fraschini, A., Garagna, S., Pellicciari, C. and Redi, C.A. 1986. Sperm-chromatin maturation in the mouse. A cytochemical approach. *Histochemistry*, 84, 484-91

Manicardi, G.C., Bianchi, P.G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U. and Sakkas, D. 1995. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biology of Reproduction*, 52, 864-7

Mansour, R.T., Aboulghar, M.A., Serour, G.I., Amin, Y.M. and Ramzi, A.M. 1995. The effect of sperm parameters on the outcome of intracytoplasmic sperm injection. *Fertility and Sterility*, 64, 982-6

Matsuk, M.M. and Lamb, D.J. 2002. Genetic dissection of mammalian fertility pathways. *Nature Cell Biology and Nature Medicine*, s41-s49

McKelvey-Martin, V.J., Green, M.H., Schmezer, P., Pool-Zobel, B.L., De Meo, M.P. and Collins, A. 1993. The single cell gel electrophoresis assay (comet assay): a European review. *Mutation Research*, 288, 47-63

McKelvey-Martin, V.J., Melia, N., Walsh, I.K., Johnston, S.R., Hughes, C.M., Lewis, S.E. and Thompson, W. 1997. Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). Human bladder washings and transitional cell carcinoma of the bladder; and (2). Human sperm and male infertility. *Mutation Research*, 375, 93-104

McLachlan, R.I. 2000. The endocrine control of spermatogenesis. *Baillieres Best Pract Res Clin Endocrinol Metab*, 14, 345-62

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., de Kretser, D.M., Pratis, K. and Robertson, D.M. 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Progress in Hormone Research*, 57, 149-79

McPherson, S.M. and Longo, F.J. 1992. Localization of DNase I-hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. *Molecular Reproduction and Development*, 31, 268-79

McWhir, J., Selfridge, J., Harrison, D., Squires, S. and Melton, D. 1993. Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver abnormalities and die before weaning. *Nature Genetics*, 5, 217-224

Mieusset, R. and Bujan, L. 1995. Testicular heating and its possible contributions to male infertility: a review. *International Journal of Andrology*, 18, 169-84

Mieusset, R., Bujan, L., Massat, G., Mansat, A. and Pontonnier, F. 1995. Clinical and biological characteristics of infertile men with a history of cryptorchidism. *Human Reproduction*, 10, 613-9

Mieusset, R., Bujan, L., Mondinat, C., Mansat, A., Pontonnier, F. and Grandjean, H. 1987. Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertility and Sterility*, 48, 1006-11

Mieusset, R., Fouda, P.J., Vaysse, P., Guitard, J., Moscovici, J. and Juskiewenski, S. 1993. Increase in testicular temperature in case of cryptorchidism in boys. *Fertility and Sterility*, 59, 1319-21

Mieusset, R., Quintana Casares, P., Sanchez Partida, L.G., Sowerbutts, S.F., Zupp, J.L. and Setchell, B.P. 1992. Effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *Journal of Reproduction and Fertility*, 94, 337-43

Mieusset, R., Sowerbutts, S.F., Zupp, J.L. and Setchell, B.P. 1992. Increased flow of testicular blood plasma during local heating of the testes of rams. *Journal of Reproduction and Fertility*, 94, 345-52

Moore, R.C., Lee, I.Y., Silverman, G.L., Harrison, P.M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S.H., Chishti, M.A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A.F., Katamine, S., Carlson, G.A., Cohen, F.E., Prusiner, S.B., Melton, D.W., Tremblay, P., Hood, L.E. and Westaway, D. 1999. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *Journal of Molecular Biology*, 292, 797-817

Multigner, L. and Oliva, A. 2002. Secular variations in sperm quality: fact or science fiction? *Cad Saude Publica*, 18, 403-12

Ni, Z.Y., Liu, Y.Q., Shen, H.M., Chia, S.E. and Ong, C.N. 1997. Does the increase of 8-hydroxydeoxyguanosine lead to poor sperm quality? *Mutation Research*, 381, 77-82

Nishiyama, H., Danno, S., Kaneko, Y., Itoh, K., Yokoi, H., Fukumoto, M., Okuno, H., Millan, J.l., Matsuda, T., Yoshida, O. and Fujita, J. 1998. Decreased expression of cold-inducible RNA-binding protein (CIRP) in male germ cells at elevated temperature. *American Journal of Pathology*, 152, 289-296

Nishiyama, H., Higashitsuji, H., Yokoi, H., Itoh, K., Danno, S., Matsuda, T. and Fujita, J. 1997. Cloning and characterisation of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene. *Gene*, 204, 115-120

Nishiyama, H., Itoh, K., Kaneko, Y., Kishishita, M., Yoshida, O. and Fujita, J. 1997. A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *Journal of Cell Biology*, 137, 899-908

Norton, A.J., Jordan, S. and Yeomans, P. 1994. Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. *Journal of Pathology*, 173, 371-9

Nunez, F., Chipchase, M.D., Clarke, A.R. and Melton, D.W. 2000. Nucleotide excision repair gene (ERCC1) deficiency causes G(2) arrest in hepatocytes and a reduction in liver binucleation: the role of p53 and p21. *FASEB Journal*, 14, 1073-82

O'Donnell, L., Stanton, P.G., Wreford, N.G., Robertson, D.M. and McLachlan, R.I. 1996. Inhibition of 5 alpha-reductase activity impairs the testosterone-dependent restoration of spermiogenesis in adult rats. *Endocrinology*, 137, 2703-10

Ostling, O. and Johanson, K.J. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications* , 123, 291-8

Pagano, G., de Biase, A., Deeva, I., Degan, P., Doronin, Y., Iaccarino, M., Oral, R., Trieff, N., Warnau, M. and Korkina, L. 2001. The role of oxidative stress in developmental and reproductive toxicity of tamoxifen. *Life Sciences*, 68, 1735-1749

Papadopoulos, V., Jia, M.C., Culty, M., Hall, P.F. and Dym, M. 1993. Rat Sertoli cell aromatase cytochrome P450: regulation by cell culture conditions and relationship to the state of cell differentiation. *In Vitro Cellular and Developmental Biology – Animal*, 29A, 943-9

Parinaud, J., Labal, B., Miesset, R., Richoilley, G. and Vietez, G. 1993. Influence of sperm parameters on embryo quality. *Fertility and Sterility*, 60, 888-892

Paulsen, C., Berman, N. and Wang, C. 1996. Data from men in greater Seattle area reveals no downward trend in semen quality: further evidence that deterioration in semen quality is not geographicaly uniform. *Fertility and Sterility*, 65, 1015-20

Penault-Llorca, F., Bouabdalla, R., Devilard, E., Vharton-Bain, M., Hassoun, J., Birg, F. and Xerri, L. 1998. Analysis of BAX expression in human tissues using the anti-BAX, 4F11 monoclonal antibody on paraffin sections. *Pathology, Research and Practice*, 194, 457-464

Porter, S.B., Ong, D.E., Chytil, F. and Orgebin-Crist, M.C. 1985. Localization of cellular retinol-binding protein and cellular retinoic acid-binding protein in the rat testis and epididymis. *Journal of Andrology*, 6, 197-212

Potts, R., Newbury, C., Smith, G., Notarianni, L. and Jefferies, T. 1999. Sperm chromatin damage associated with male smoking. *Mutation Research*, 423, 103-111

Potts, R.J., Jefferies, T.M. and Notarianni, L.J. 1999. Antioxidant capacity of the epididymis. *Human Reproduction*, 14, 2513-6

Prevost, J. and Dumas, J. 1824. Nouvelle theorie de la generation. *Ann Sci Nat*, 1, 1-29, 167-87, 274-92

Print, C.G., Loveland, K.L., Gibson, L., Meehan, T., Stylianou, A., Wreford, N., de Kretser, D., Metcalf, D., Kontgen, F., Adams, J.M. and Cory, S. 1998. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 12424-31

Procope, B.J. 1965. Effect of repeated increase of body temperature on human sperm cells. *International Journal of Fertility*, 10, 333-9

Prusiner, S.B. 1998. Prions. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 13363-13383

Qiu, J., Hales, B.F. and Robaire, B. 1995. Effects of chronic low-dose cyclophosphamide exposure on the nuclei of rat spermatozoa. *Biology of Reproduction*, 52, 33-40

Regalado, F., Esponda, P. and Nieto, A. 1993. Temperature and androgens regulate the biosynthesis of secretory proteins from rabbit cauda epididymidis. *Molecular Reproduction and Development*, 36, 448-53

Reijo, R., Seligman, J., Dinulos, M.B., Jaffe, T., Brown, L.G., Disteche, C.M. and Page, D.C. 1996. Mouse autosomal homolog of DAZ, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. *Genomics*, 35, 346-52

Richburg, J.H., Redenbach, D.M. and Boekelheide, K. 1994. Seminiferous tubule fluid secretion is a Sertoli cell microtubule-dependent process inhibited by 2,5-hexanedione exposure. *Toxicol Appl Pharmacol*, 128, 302-9

Robaire, B. and Viger, R.S. 1995. Regulation of epididymal epithelial cell functions. *Biology of Reproduction*, 52, 226-36

Rock, J. and Robinson, D. 1965. Effect of induced intrascrotal hyperthermia on testicular function in man. *American Journal of Obstetrics and Gynecology*, 93, 793-801

Rockett, J.C., Mapp, F.L., Garges, J.B., Luft, J.C., Mori, C. and Dix, D.J. 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biology of Reproduction*, 65, 229-39

Rodriguez, H., Ohanian, C. and Bustos-Obregon, E. 1985. Nuclear chromatin decondensation of spermatozoa in vitro: a method for evaluating the fertilizing ability of ovine semen.PG - 147-58. *International Journal of Andrology*, 8,

Ron-el, R., Nachum, H., Herman, A., Golan, A., Caspi, E. and Soffer, Y. 1991. Delayed fertilization and poor embryonic development associated with impaired semen quality. *Fertility and Sterility*, 55, 338-44

Ruggiu, M., Speed, R., Taggart, M., McKay, S.J., Kilanowski, F., Saunders, P., Dorin, J. and Cooke, H.J. 1997. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature*, 389, 73-7

Saikhun, J., Kitiyanant, Y., Vanadurongwan, V. and Pavasuthipaisit, K. 1998. Effects of sauna on sperm movement characteristics of normal men measured by computer-assisted sperm analysis. *International Journal of Andrology*, 21, 358-63

Sailer, B.L., Jost, L.K. and Evenson, D.P. 1995. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *Journal of Andrology*, 16, 80-7

Sailer, B.L., Sarkar, L.J., Bjordahl, J.A., Jost, L.K. and Evenson, D.P. 1997. Effects of heat stress on mouse testicular cells and sperm chromatin structure. *Journal of Andrology*, 18, 294-301

Sakkas, D., Manicardi, G., Bianchi, P.G., Bizzaro, D. and Bianchi, U. 1995. Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biology of Reproduction*, 52, 1149-55

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzaro, D., Bianchi, P.G. and Bianchi, U. 1999. Origin of DNA damage in ejaculated human spermatozoa. *Reviews of Reproduction*, 4, 31-7

Sargent, G., Meservy, J., Perkins, B., Kilburn, A., Intody, Z., Adair, G., Nairn, R. and Wilson, J. 2000. Role of the nucleotide excision repair gene ERCC1 in formation of recombinant-dependent rearrangements in mammalian cells. *Nucleic Acids Research*, 28, 3771-3778

Sas, M. and Szollosi, J. 1979. Impaired spermiogenesis as a common finding among professional drivers. *Archives of Andrology*, 3, 57-60

Sasagawa, I., Yazawa, H., Suzuki, Y. and Nakada, T. 2001. Stress and testicular germ cell apoptosis. *Archives of Andrology*, 47, 211-6

Seiler, P., Cooper, T.G. and Nieschlag, E. 2000. Sperm number and condition affect the number of basal cells and their expression of macrophage antigen in the murine epididymis. *International Journal of Andrology*, 23, 65-76

Seiler, P., Cooper, T.G., Yeung, C.H. and Nieschlag, E. 1999. Regional variation in macrophage antigen expression by murine epididymal basal cells and their regulation by testicular factors. *Journal of Andrology*, 20, 738-46

Seiler, P., Wenzel, I., Wagenfeld, A., Yeung, C.H., Nieschlag, E. and Cooper, T.G. 1998. The appearance of basal cells in the developing murine epididymis and their temporal expression of macrophage antigens. *International Journal of Andrology*, 21, 217-26

Selfridge, J., Hsia, K.-H., Redhead, N. and Melton, D. 2001. Correction of liver dysfunction in DNA repair-deficient mice with an ERCC1 transgene. *Nucleic Acids Research*, 29, 4541-4550

Setchell, B.P. 1998. The Parkes Lecture. Heat and the testis. *Journal of Reproduction and Fertility*, 114, 179-94

Setchell, B.P., Bergh, A., Widmark, A. and Damber, J.E. 1995. Effect of testicular temperature on vasomotion and blood flow. *International Journal of Andrology*, 18, 120-6
Setchell, B.P., D'Occhio, M.J., Hall, M.J., Laurie, M.S., M.J.Tucker and Zupp, J.L. 1988. Is embryonic mortality increased in normal femal rats mated to subfertile males? *Reproduction and Fertility*, 82, 567-574

Shaked, Y., Rosenmann, H., Talmor, G. and Gabizon, R. 1999. A C-terminal-truncated PrP isoform is present in mature sperm. *Journal of Biological Chemistry*, 274, 32153-8

Shen, H. and Ong, C. 2000. Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. *Free Radical Biology and Medicine*, 28, 529-36

Shen, H.M., Chia, S.E., Ni, Z.Y., New, A.L., Lee, B.L. and Ong, C.N. 1997. Detection of oxidative DNA damage in human sperm and the association with cigarette smoking. *Reproductive Toxicology*, 11, 675-80

Shen, H.M., Chia, S.E. and Ong, C.N. 1999. Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *Journal of Andrology*, 20, 718-23

Silverman, G.L., Qin, K., Moore, R.C., Yang, Y., Mastrangelo, P., Tremblay, P., Prusiner, S.B., Cohen, F.E. and Westaway, D. 2000. Doppel Is an N-Glycosylated, Glycosylphosphatidylinositol-anchored Protein. Expression in testis and ectopic production in the brains of Prnp0/0 mice predisposed to purkinje cell loss. *Journal of Biological Chemistry*, 275, 26834-26841

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175, 184-91

Sjoblom, T. and Lahdetie, J. 1996. Expression of p53 in normal and γ-irradiated rat testis suggests a role for p53 in meiotic recombination and repair. *Oncogene*, 12, 2499-2505

Smith, A. and Haaf, T. 1998. DNA nicks and increased sensitivity of DNA to fluorescence in situ end labeling during functional spermiogenesis. *Biotechniques*, 25, 496-502

Steele, E.K., McClure, N. and Lewis, S.E. 2000. Comparison of the effects of two methods of cryopreservation on testicular sperm DNA. *Fertility and Sterility*, 74, 450-3

Steele, E.K., McClure, N., Maxwell, R.J. and Lewis, S.E.M. 1999. A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. *Molecular Human Reproduction*, 5, 831-835

Sun, J.G., Jurisicova, A. and Casper, R.F. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro.PG - 602-7. *Biology of Reproduction*, 56,

Sutcliffe, A., B Taylor, Saunders, K., Thornton, S., Lieberman, B. and Grudzinskas, J. 2001. Outcome in the second year of life after in-vitro fertilisation by intracytoplasmic sperm injection: a UK case-control study. *Lancet*, 357, 2068-2069

Swan, S.H., Elkin, E.P. and Fenster, L. 2000. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996.PG - 961-6. *Environmental Health Perspectives*, 108,

Tapanainen, J.S., Tilly, J.L., Vihko, K.K. and Hsueh, A.J. 1993. Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. *Molecular Endocrinology*, 7, 643-50

Tarlatzis, B.C. and Grimbizis, G. 1999. Pregnancy and child outcome after assisted reproduction techniques. *Human Reproduction*, 14 Suppl 1, 231-42

Tateno, H. and Kamiguchi, Y. 1999. Dithiothreitol induces sperm nuclear decondensation and protects against chromosome damage during male pronuclear formation in hybrid zygotes between Chinese hamster spermatozoa and Syrian hamster oocytes.PG - 321-7. *Zygote*, 7,

Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., Moser, M., Oesch, B., McBride, P.A. and Manson, J.C. 1996. Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature*, 380, 639-42

Tranulis, M.A., Espenes, A., Comincini, S., Skretting, G. and Harbitz, I. 2001. The PrP-like protein Doppel gene in sheep and cattle: cDNA sequence and expression. *Mammalian Genome*, 12, 376-9

Turner, R.M., Johnson, L.R., Haig-Ladewig, L., Gerton, G.L. and Moss, S.B. 1998. An X-linked gene encodes a major human sperm fibrous sheath protein, hAKAP82. Genomic organization, protein kinase A-RII binding, and distribution of the precursor in the sperm tail. *Journal of Biological Chemistry*, 273, 32135-41

Twigg, J., Fulton, N., Gomez, E., Irvine, D.S. and Aitken, R.J. 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reproduction*, 13, 1429-36

Twigg, J., Irvine, D.S., Houston, P., Fulton, N., Michael, L. and Aitken, R.J. 1998. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Molecular Human Reproduction*, 4, 439-45

Ulberg, L. 1958. The influence of high temperature on reproduction. *Journal of Heredity*, 49, 62-64

Van Loon, A., Den Boer, P., Van der Schans, G., Mackenbach, P., Grootegoed, J., Baan, R. and Lohman, P. 1991. Immunochemical detection of DNA damage induction and repair at different cellular stages of spermatogenesis of the hamster after in vitro or in vivo exposure to ionizing radiation. *Experimental Cell Research*, 193, 303-309

Van Steirteghem, A., Bonduelle, M., Liebaers, I. and Devroey, P. 2002. Children born after assisted reproductive technology. *American Journal of Perinatology*, 19, 59-65

Venables, J.P., Ruggiu, M. and Cooke, H.J. 2001. The RNA-binding specificity of the mouse Dazl protein. *Nucleic Acids Research*, 29, 2479-83

Vijayaraghavan, S., Stephens, D.T., Trautman, K., Smith, G.D., Khatra, B., da Cruz e Silva, E.F. and Greengard, P. 1996. Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. *Biology of Reproduction*, 54, 709-18

Volgymayr, J., Scott, T. and Waites, G. 1967. Metabolism of testicular spermatozoa and characteristics of testicular fluid collected from conscious rams. *Journal of Reproduction and Fertility*, 14, 87-99

Waites, G.M. 1976. Temperature regulation and fertility in male and female mammals. *Israel Journal of Medical Sciences*, 12, 982-93

Waites, G.M. 1991. Thermoregulation of the scrotum and testis: studies in animals and significance for man.PG - 9-17. *Advances in Experimental Medicine and Biology* , 286,

Wakatsuki, T. and Hatayama, T. 1998. Characteristic expression of 105-kDa heat shock protein (HSP105) in various tissues of nonstressed and heat-stressed rats. *Biological and Pharmaceutical Bulletin* , 21, 905-910

Wang, C., McDonald, V., Leung, A., Superlano, L., Berman, N., Hull, L. and Swerdloff, R.S. 1997. Effect of increased scrotal temperature on sperm production in normal men. *Fertility and Sterility*, 68, 334-9

Ward, W.S. and Coffey, D.S. 1991. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biology of Reproduction*, 44, 569-74

Weeda, G., Donker, I., de_Wit, J., Morreau, H., Janssens, R., Vissers, C.J., Nigg, A., van_Steeg, H., Bootsma, D. and Hoeijmakers, J.H. 1997. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Current Biology*, 7, 427-39

Wellejus, A., Poulsen, H.E. and Loft, S. 2000. Iron-induced oxidative DNA damage in rat sperm cells in vivo and in vitro. *Free Radical Research*, 32, 75-83

Wennerholm, U.B., Bergh, C., Hamberger, L., Lundin, K., Nilsson, L., Wikland, M. and Kallen, B. 2000. Incidence of congenital malformations in children born after ICSI. *Human Reproduction*, 15, 944-8

Wijsman, J.H., Jonker, R.R., Keijzer, R., van de Velde, C.J., Cornelisse, C.J. and van Dierendonck, J.H. 1993. A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *Journal of Histochemistry and Cytochemistry*, 41, 7-12

Wood, R.D. 1999. DNA damage recognition during nucleotide excision repair in mammalian cells.PG - 39-44. *Biochimie*, 81,

Xu, J., Xu, Z., Jiang, Y., Qian, X. and Huang, Y. 2000. Cryptorchidism induces mouse testicular germ cell apoptosis and changes in bcl-2 and bax protein expression. *Journal of Environmental Pathology, Toxicology and Oncology*, 19, 25-33

Yamamoto, C.M., Sinha_Hikim, A.P., Huynh, P.N., Shapiro, B., Lue, Y., Salameh, W.A., Wang, C. and Swerdloff, R.S. 2000. Redistribution of Bax is an early step in an apoptotic pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia. *Biology of Reproduction*, 63, 1683-90

Yen, P.H., Chai, N.N. and Salido, E.C. 1996. The human autosomal gene DAZLA: testis specificity and a candidate for male infertility. *Human Molecular Genetics*, 5, 2013-7

Yeung, C.H., Nashan, D., Sorg, C., Oberpenning, F., Schulze, H., Nieschlag, E. and Cooper, T.G. 1994. Basal cells of the human epididymis--antigenic and ultrastructural similarities to tissue-fixed macrophages. *Biology of Reproduction*, 50, 917-26

Yin, Y., Hawkins, K.L., DeWolf, W.C. and Morgentaler, A. 1997. Heat stress causes testicular germ cell apoptosis in adult mice. *Journal of Andrology*, 18, 159-65

Yin, Y., William.C.DeWolf and Morgentaler, A. 1998. Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. *Biology of Reproduction*, 58, 492-496

Zenzes, M. 2000. Smoking and reproduction: gene damage to human gametes and embryos. *Human Reproduction Update*, 6, 122-131

Zenzes, M.T., Puy, L.A., Bielecki, R. and Reed, T.E. 1999. Detection of benzo[a]pyrene diol epoxide-DNA adducts in embryos from smoking couples: evidence for transmission by spermatozoa [In Process Citation]. *Molecular Human Reproduction*, 5, 125-31

Zini, A. and Schlegel, P.N. 1997. Identification and characterization of antioxidant enzyme mRNAs in the rat epididymis. *International Journal of Andrology*, 20, 86-91

Zorgniotti, A., Reiss, H., Toth, A. and Sealfon, A. 1982. Effect of clothing on scrotal temperature in normal men and patients with poor semen. *Urology*, 19, 176-8

Zorgniotti, A.W. 1980. Testis temperature, infertility, and the varicocele paradox. *Urology*, 16, 7-10