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Sperm Chromatin (DNA) Instability and Reproductive Wastage

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1.0 Abstract

During the final stages of sperm production (spermiogenesis) the chromatin (DNA) in the sperm nucleus undergoes profound changes. At the start of spermiogenesis the chromatin in sperm is found in association with histone nucleoproteins which gives it a nucleosome core particle structure similar to the structure of chromatin in all other cells of the body. During spermiogenesis the histone proteins are replaced by transition proteins which are then replaced by protamines. As protamines progressively bind to sperm chromatin, the chromatin undergoes conformational changes including a major condensation which is thought to be a mechanism that protects chromatin in sperm from damage until the sperm reaches the egg for fertilisation. The integrity and stability of the protamine-chromatin association in sperm can be measured using the sperm chromatin structure assay (SCSA). Studies primarily in men have indicated that sperm chromatin instability, as determined by the SCSA, does not influence fertilisation but is associated with early pregnancy failure. One of the aims in the present study was to ascertain the prevalence of sperm chromatin instability in bulls in northern Australia. This would serve as a first step in evaluating whether sperm chromatin instability is a contributing factor to reproductive wastage in cattle. The SCSA yields a value for the DNA fragmentation index (DFI) and in men a DFI > 27% is associated with early embryonic mortality. A relationship between DFI and embryonic mortality has yet to be established in bulls and in the current project a DFI > 27% was arbitrarily chosen as indicative of substantial chromatin instability for bulls. The SCSA was conducted on semen samples from a total of 565 bulls at 14 locations and representing 7 genotypes. When samples were exposed to the SCSA for 0.5 minutes 4.9% of bulls had a DFI > 27% and this increased to 11.5% of bulls with DFI > 27% when samples were exposed to the SCSA for 5 minutes. The vast majority of bulls (513/565, 91%) had a DFI < 15% at 0.5 min SCSA and it could be concluded that sperm chromatin is stable in most bulls. Location appeared to have a greater influence on DFI than genotype although this information was based primarily on Brahman. For 149 bulls, most (136/149, 91%) had a DFI < 15% on 2 occasions and for 21 bulls, 17/21 (81%) had a DFI < 15% on 4 occasions. These results suggested that the measure of sperm chromatin stability is repeatable; however, there were very few bulls in the 15-27% and > 27% DFI categories. Seventeen bulls with DFI ranging from 0.8 to 8.6% all sired calves and 4 bulls with DFI > 27% had also sired calves in the preceding 1 to 6 years. For half-sib bulls, 82/87 (94%; comprised of smaller half-sib groups) had a DFI < 15% and the odd progeny, amongst different sires, had a 15-27% DFI (4/87, 5%) or > 27% DFI (1/87, 1%). Six sires had a DFI ranging from 0.8 to 3.1% and of their progeny 41/45 (91%) had < 15% DFI, 3/45 (7%; different sires) had 15-27% DFI and 1/45 (2%) had > 27% DFI. In summary, based on the 3 categories of sperm chromatin status adopted in the project, < 15% DFI (stable), 15-27% DFI (moderately stable) and > 27% (unstable), it would appear that the majority of bulls in northern Australia have a stable sperm chromatin structure and no relationship with reproductive performance was identified. Whilst it would appear that sperm chromatin structure may have a genetic component, the majority of bulls had a DFI < 15% with only small numbers of bulls with a DFI of 15-27% or > 27%. Larger numbers of bulls in the latter 2 categories will need to be monitored before the heritability of sperm chromatin structure can be determined.

2.0 Main Research Report

2.1 Background and Industry Context

One of the primary constraints to cattle production in tropical and subtropical regions of Australia is the common occurrence of extended conception periods in naturally mated beef herds. The cattle industry has achieved major progress in the management of breeder females (in particular replacement heifers) so that they have an appropriate live weight and body condition at the time of joining. Likewise, herd bulls are subjected to a breeding soundness evaluation (BSE) during purchase and/or before mating. Yet, cumulative conception patterns after the start of mating often occur at a slower rate than would be predicted based on a high incidence of oestrous cycles amongst females and the use of reproductively sound bulls. The consequence is extended calving periods which have a negative impact on herd performance and profitability due to the longer-term effects on growth and reproductive performance in calves born relatively late in the season. It is possible that a proportion of females in oestrus are not mated because of poor bull dispersal, or other unidentified factors. Recent evidence, however, suggested that repeat incidences of early pregnancy failure with re-conception may contribute to extended conception periods in extensive beef herds in northern Australia (R Holroyd and G Fordyce, unpublished results).

The failure to establish a pregnancy has generally been assumed to result from 'female factor' infertility. However, there is evidence, primarily from work in humans, which suggests that 'male factor' infertility can contribute to a higher proportion of embryonic mortalities than was initially recognised (Evenson *et al.* 1999; Larson *et al.* 2000). This information has emerged largely from IVF clinics where a typical scenario involves a couple with a history of early pregnancy failure. Embryos that are apparently normal can be produced by IVF but this is usually followed by relatively early loss after embryo transfer. Analysis of the semen of these men often reveals a relatively high degree of sperm chromatin (DNA) instability (Larson-Cook *et al.* 2003).

Semen quality and its relationship to fertility are of major importance in animal breeding (Peris *et al.* 2004). In Australia, bull semen evaluation is generally restricted to the analysis of conventional semen parameters such as motility, morphology, mass activity (viability), volume and density (Fitzpatrick *et al.* 2002). These tests are relevant as the more intact and functional the spermatozoon are, the greater the likelihood of fertilisation and pregnancy. To date, morphology has been shown to have some relationship to the capacity of bulls to sire calves under multiple-mating in northern Australia (Fitzpatrick *et al.* 2002), although morphology appears to only account for 35-57% (depending on bull genotype) of the variation between bulls in the capacity to sire calves under extensive multiple-sire mating. The apparent lack of a relationship with other conventional parameters of semen quality might be due, in part, to the fact these tests do not provide a measure of chromatin integrity in sperm (Gandini *et al.* 2004).

During the final stages of spermatogenesis (known as spermiogenesis) the chromatin in the sperm nucleus undergoes profound changes. The chromatin in all cells is associated with nucleoproteins which bind to DNA and maintain the integrity of chromatin and also influence gene expression. In typical body cells, chromatin occurs in association with histone nucleoproteins in structures known as nucleosome core

particles (Richmond and Davey, 2003). During spermiogenesis, and before sperm enter the epididymis, histones are replaced on sperm chromatin by transition proteins (TP) (e.g. TP1, TP2) which, in turn, are replaced by protamines (Dadoune, 2003) (see review in Appendix A). As protamines progressively associate with sperm chromatin, the chromatin undergoes a conformational change with the outstanding feature being a major condensation and the formation of toroidal structures (Brewer *et al.* 2003). Chromatin undergoes condensation in the sperm nucleus for two primary reasons. First, the chromatin must compact so that it can fit within a decreasing nuclear volume. Second, the association with protamines and condensation serves to protect chromatin in sperm until the sperm is able to reach the oocyte (unfertilised egg) for fertilisation. The above findings (Evenson *et al.* 1999; Larson *et al.* 2000) of a relationship between chromatin stability (a measure of protamine association and chromatin condensation), and the capacity of embryos to establish a successful pregnancy, suggested that the correct structural conformation of chromatin in sperm is important for paternal gene expression necessary for continued embryonic development and pregnancy establishment and maintenance (Brinkworth, 2000).

During the past two decades several techniques have been developed to measure chromatin integrity (see Section 3, Literature Review; Virro *et al.* 2004). The sperm chromatin structure assay (SCSA) is arguably the most accurate and repeatable method for assessing chromatin stability and it has been shown to be related to male fertility in a number of species, in particular humans as noted above (Larson-Cook *et al.* 2003). The advantages of the SCSA compared with microscopic methods are (1) a considerably larger number of sperm can be evaluated making the SCSA a more powerful and representative test, (2) it is fully automated and therefore an objective rather than a subjective assessment and (3) the automation means that it is repeatable (Evenson *et al.,* 2002).

The SCSA uses flow cytometry and was first described by Evenson and coworkers (1980). SCSA identifies sperm with abnormal chromatin structure (protamine association and condensation) which is measured by the susceptibility to acid-induced chromatin denaturation *in situ* (Bungum *et al.* 2004). The SCSA uses the metachromatic properties of acridine orange to distinguish between denatured chromatin (red fluorescence, single-stranded DNA) and non-denatured chromatin (green fluorescence, double-stranded DNA) after sperm are exposed to a low pH detergent solution (Figure 1). The proportion of sperm with denatured DNA is expressed as the DNA fragmentation index (% DFI). The DNA of spermatozoa with normal chromatin structure is considerably less susceptible to acid denaturation compared with the DNA of sperm in which protamine binding and condensation has not proceeded normally (Evenson *et al.* 1980). The distinction between normal and abnormal chromatin is more readily achieved using the SCSA compared with other sperm chromatin integrity assays such as TUNEL, COMET and Nick Translation (NT) (see Section 3, Literature Review; Evenson and Jost, 2000).

A further advantage of the SCSA over other semen evaluation techniques is the relationship that has been demonstrated with fertility outcomes (Evenson *et al.* 1980, 1999; Larson-Cook *et al.* 2003). From studies primarily in men it has been concluded that % DFI thresholds of 0-15% DFI, 16-29% DFI and ≥ 30% DFI are respectively related to high fertility, moderate fertility and very low fertility (Evenson and Jost, 2000).

It has also been reported in men that > 27% DFI is associated with failure to establish a pregnancy after IVF, even though fertilisation and initial embryonic development can occur (Larson *et al.* 2000, 2003; Agarwal and Said, 2003). Preliminary data for recreational (horse) and production (boar, bull) animals would suggest that relationships exist between the SCSA and fertility for males across species (Evenson and Jost, 2000; Boe-Hansen, 2005). The SCSA has recently attracted considerable interest in bulls because of the importance of the bull in reproductive outcomes in both dairy and beef production. The results to date for bulls are equivocal with respect to the relationship between the SCSA and fertility (Boe-Hansen *et al*, 2005; Hallap *et al*, 2005; Madrid-Bury *et al*, 2005). It has been acknowledged that information on the SCSA needs to be obtained for a considerably larger number of bulls before its significance to bull fertility can be assessed. In this regard, the % DFI thresholds in the SCSA that relate to fertility have only been established for men.

The semen of men is in general notoriously unstable compared with that of production animals and the 27-30% DFI threshold may not apply to animals. It will be necessary to conduct extensive mating studies before there can be confidence between DFI thresholds and fertility for production animals. It could be predicted that DFI thresholds as determined by the SCSA might be lower for production animals compared with men. In the absence of relevant data, the decision was made in the current project to use DFI thresholds of < 15% DFI, 15-27% DFI and > 27% DFI as respective indicative thresholds of stable, moderately stable, and unstable chromatin in bulls. If the above argument that DNA in production animals is in general more stable than DNA of men is substantiated, then a 15-27 % DFI could be indicative of unstable chromatin in bulls; however, this is speculation.

The SCSA does not appear to be correlated with conventional measures of semen quality (Spano *et al.* 1998; Evenson *et al.* 1999; Sills *et al.* 2004; Virro *et al.* 2004; Peris *et al.* 2004). It has therefore been concluded that the SCSA is an independent descriptor of semen quality which complements the information derived from conventional semen assessments (Peris *et al.* 2004). To date, the SCSA has been largely overlooked as a possible cause of sub-fertility in herd bulls. However, it may prove to be an important component of 'male factor' infertility that contributes to embryonic mortality and reproductive wastage in cattle.

2.2 Project Objectives

The objectives in this project were:

1. Report on the prevalence of sperm chromatin structure and instability in bulls in northern Australia
2. Report on the preliminary assessment of the inheritance of sperm chromatin structure and instability in bulls in northern Australia
3. Report on whether there are significant reproductive losses associated with sperm chromatin instability in bulls
4. Make recommendations as to the extent and direction of further research in sperm chromatin instability

2.3 Materials and Methods

Animals and Semen Samples

The aim in the project was to obtain as broad a geographical representation as practically possible on the prevalence of sperm chromatin instability in bulls in northern Australia. Accordingly, bulls were evaluated at locations across central and northern Queensland and included a range of genotypes (Table 1). A subset of bulls was sampled on two occasions to obtain preliminary information on the repeatability over time of the SCSA for individual bulls. The latter information was considered to be important in determining whether the SCSA has practical utility as a measure of sperm chromatin status in bulls. The ages of bulls ranged from 1 to 10 years with the majority being 2-year-old bulls that underwent a breeding soundness evaluation (BSE) test before their first mating season. This provided the opportunity to obtain a sample of semen for the SCSA (Table 2).

The BSE test was conducted according to McGowan *et al.* (2002). After the BSE test semen was collected by electroejaculation and underwent standard semen analysis that included volume, colour, density, mass activity and individual motility (Fitzpatrick *et al.* 2002). An aliquot of each semen sample was diluted in glutaraldehyde (1:20) for morphological evaluation and 1 ml of undiluted semen was placed into a 2 ml cryovial (Nunc or Greiner Bio-one, Cellstar) and snap frozen in liquid nitrogen. The latter semen samples were stored in liquid nitrogen or an ultra-cold freezer (-80°C) until required for the SCSA.

Sperm Chromatin Structure Assay

The SCSA was carried out according to the method described by Evenson and Jost (2000) with minor modifications. Individual semen samples (stored in liquid nitrogen or at -80°C) were thawed in a 37°C water bath and then immediately placed on crushed ice. All subsequent steps were carried out at 4°C. A 5μl to10μl aliquot was taken from each semen sample, placed in a 1.5ml centrifuge tube (Quality Scientific Plastics, Australia) and diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA; pH 7.4) to obtain a sperm concentration of 1 to 2 x 10^6 sperm/ml. A 60μl aliquot of diluted semen was then placed in a 1.2 ml microtitre tube (Quality Scientific Plastics, Australia). The sample was admixed with 120μl of a low pH (pH 1.2) detergent solution containing 0.15 M NaCl, 0.08 N HCl and 0.1% Triton X-100. Triton X-100 permeabilises sperm cell membranes thus providing greater accessibility to DNA of the low pH solution and acridine orange (AO) (Larson *et al.* 2000). After 30 sec of acid denaturation, 360μl of staining solution (6μg/ml AO, 0.2 M Na_2HPO_4, 0.1 M citric acid monohydrate, 1 mM disodium EDTA and 0.15 M NaCl, pH 6.0) was added and the sample was allowed to stand in ice slurry for at least 3 min before assessment on a flow cytometer.

Table 1. The location, collection times, number and genotype of bulls used in the project.

	Collection 1	Collection 2	n	Genotype
Allansleigh	Nov 2003		49	31BB, 4BR, 13COMP, 1D
Belah Valley	Mar 2004	July 2004	62	62BB
Belmont	Nov 2003	Nov 2004	37	16BB, 12BR, 9COMP
Berrigurra	Oct 2003	July 2004	29	29COMP
Brian Pastures	Oct 2003	Nov 2004	17	5BR, 12COMP
Commoron Park	Sep 2003		7	7COMP
Homeview	Sep 2003		11	1BB, 9BG, 1DM
Meteor Downs	Oct 2003		114	52BB, 58COMP, 6SE
Rockley	Sep 2003		17	17BB
Stratford	Dec 2003	Aug 2004	55	33BB, 14BBX, 8BG
Swan's Lagoon	Dec 2003	Sep 2004	79	14BB, 65BBX
Thirsty Creek	Sep 2003		10	10BG
Toorak	Oct 2003	Nov 2004	12	3BB, 9BR
Weetalaba	Aug 2003		66	66BB

BB	Brahman	(n= 295)	COMP	Composite	(n = 126)
BBX	Brahman cross	(n = 79)	DM	Droughtmaster	(n = 2)
BG	Brangus	(n = 27)	SE	Senepol	(n = 6)
BR	Belmont Red	(n = 30)			
				TOTAL	565

Table 2. The location and age distribution of bulls used in the project. Information on age was not available for all bulls.

	AGE (years)													
LOCATION	1	1.5	1.75	2	2.5	3	4	5	6	7	8	9	10	TOTAL
Allansleigh				21		6	13	7		1	1			49
Belah Valley						20	14	7	8	5	4	3	1	62
Berrigurra	9			12		8								29
Commoron Park		4		3										7
Meteor Downs				108	3	2	1							114
Stratford				42		6	4	3						55
Swan's Lagoon		30		24		17	8							79
Weetalaba			5	44		3	7					4	2	65
TOTAL	9	34	5	254	3	62	47	17	8	6	5	7	3	460

To determine whether there was existing sperm chromatin instability in the absence of acid denaturation, 60μl of each semen sample was prepared in a similar manner to the SCSA outlined above but without exposure to low pH. Instead, 60μl of diluted semen was admixed with 120μl of detergent solution (0.15 M NaCl, 0.1% Triton X-100; pH 7.0). Exactly 30 sec later 360μl of the AO staining solution was added and the sample allowed to rest in an ice slurry at least 3 min before flow cytometer measurement. To determine the relationship between length of exposure to low pH and apparent sperm chromatin instability a time-course was conducted with exposure times of 0.5, 2.0, 5.0, 10.0, 20.0, 30.0 and 60.0 min. Samples in the project were subsequently assessed at 0.0, 0.5 and 5.0 min.

Chromatin status after the exposure of sperm to SCSA assay was determined using a FACSCalibur Flow Cytometer (Becton Dickson, Australia) equipped with an argon ion laser (488nm) and interfaced with a Macintosh computer. For instrument calibration and set up, a reference semen sample was collected from a bull external to this project and prepared as described by Evenson and Jost (2000). The reference sample was repeated after every 5 test bull samples to ensure instrument settings remained calibrated between samples and sample days. Each sample was assayed twice for statistical purposes at a flow rate of < 300 sperm/sec. A total of 5,000 sperm were evaluated for each replicate sample. The flow cytometer measured the amount of red and green fluorescence emitted from individual sperm resulting in a two-parameter scatter-gram. Off-line analysis of the flow cytometric data was carried out using CellQuest software (Becton Dickson, Australia).

Statistical Analyses

Spearman correlation coefficients were used to determine relationships between the SCSA, bull age, scrotal circumference and standard semen analyses (volume, density, mass activity, motility and morphology). Relationships between genotype, location and testis tone with % DFI were tested by one-way ANOVA.

2.4 Results and Discussion

Two-parameter scatter-grams

Examples of typical two-parameter scatter-grams generated from the flow cytometric evaluation of sperm subjected to the SCSA are shown in Figure 1.

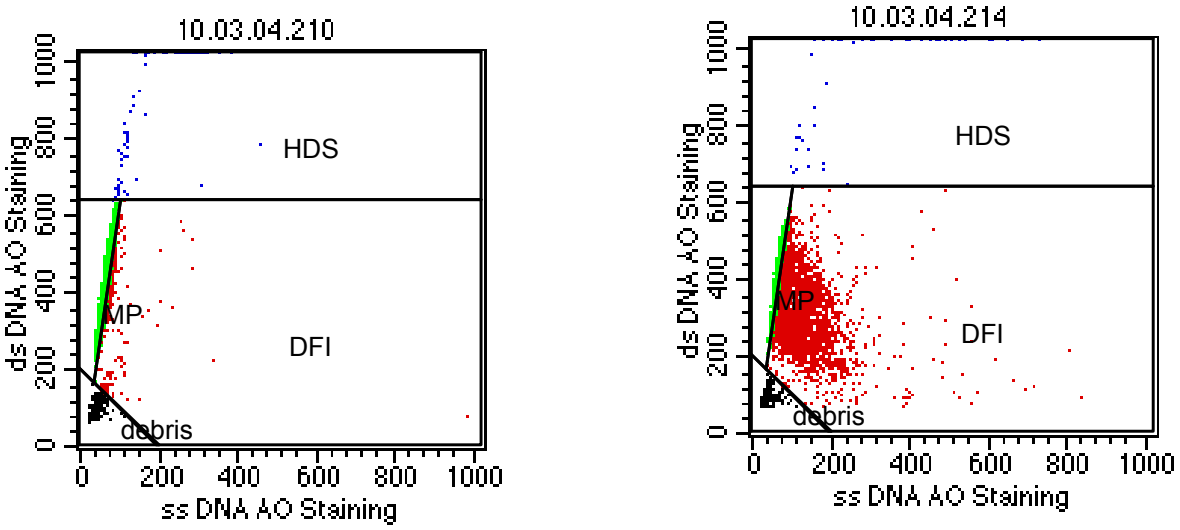

Figure 1. Examples of two-parameter scatter-grams generated from the flow cytometric evaluation of sperm subjected to the SCSA. The sample on the left had a DFI of 8% (red zone) and is representative of stable chromatin whilst the sample on the right had a DFI of 53% and is representative of highly unstable chromatin.

Each point on a scatter-gram represents the co-ordinates of red and green fluorescence for a single sperm cell. Gates and statistical regions were drawn using CellQuest software (Becton Dickson, Australia) and sperm populations were divided into 4 significant statistical regions; the main population of cells (MP), Cells Outside the Main Population ($COMP\alpha_t$) which was subsequently replaced by the DNA Fragmentation Index (DFI) (Evenson *et al.* 2002), cellular debris, and sperm with high DNA stainability (HDS). The main population of sperm in a sample remain as an elliptical coherent population (Evenson and Jost, 2000). DFI represents the proportion of sperm in a sample with abnormal chromatin structure and is considered the most important variable of this assay for fertility assessment (Evenson and Jost, 2000). Cellular debris appears in the bottom left corner of the cytogram and is gated so that DFI is easily distinguished from all other cell types. The final region of interest is HDS, which represents sperm that have a high AO stainability. These sperm are likely to have incorrectly condensed sperm DNA thus allowing greater accessibility of dyes. However it has been shown that many of these points are also cellular debris and this information was not utilised in the present project.

SCSA time-course

Results for the SCSA time-course are shown in Figure 2. There was a progressive increase in the % DFI with increasing exposure to low pH from 0.5 to 60 min with the most dramatic increase occurring between 0.5 and 5.0 min. Based on the results of the time-course study test samples in the project were routinely exposed to the SCSA for 0.0, 0.5 and 5.0 min. A representative example of the latter is shown in Figure 3.

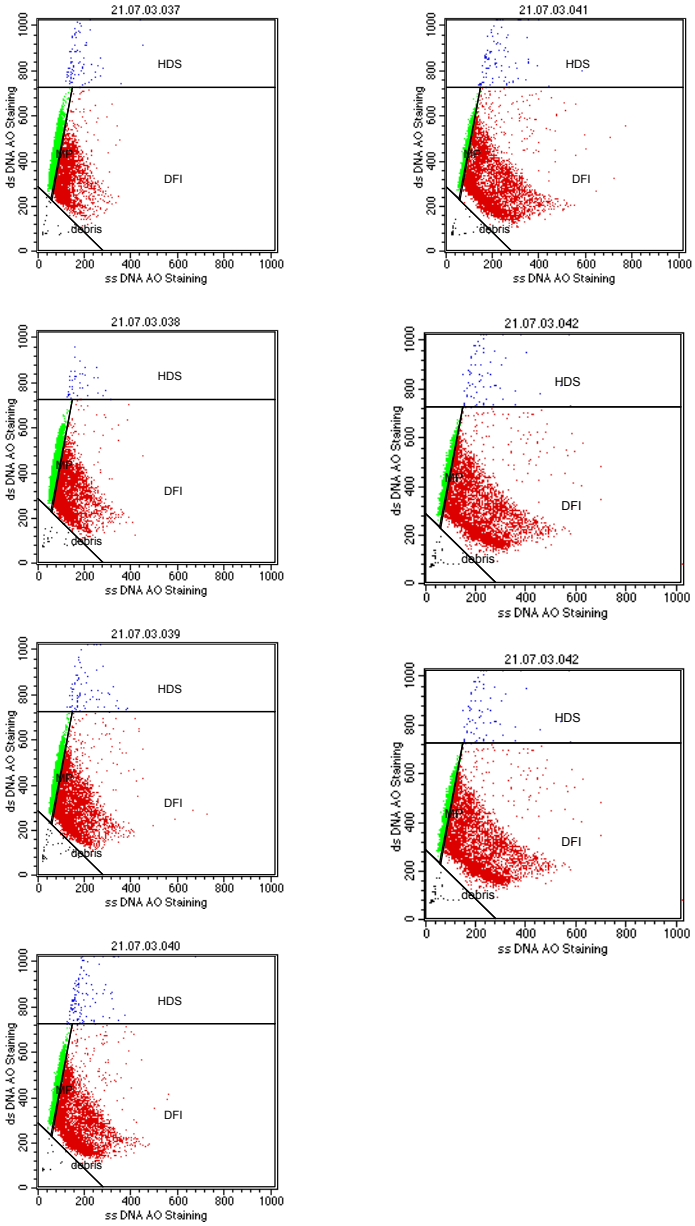

Figure 2. Time-course study of the SCSA assay. Sperm were exposed to low pH for 0.5 (left, top), 2.0 (left, upper middle), 5.0 (left, lower middle), 10.0 (left, bottom), 20.0 (right, top), 30.0 (right, middle) and 60 (right, bottom) min. The respective % DFI progressively increased from 29%, 36%, 42%, 43%, 50%, 52% and 57%.

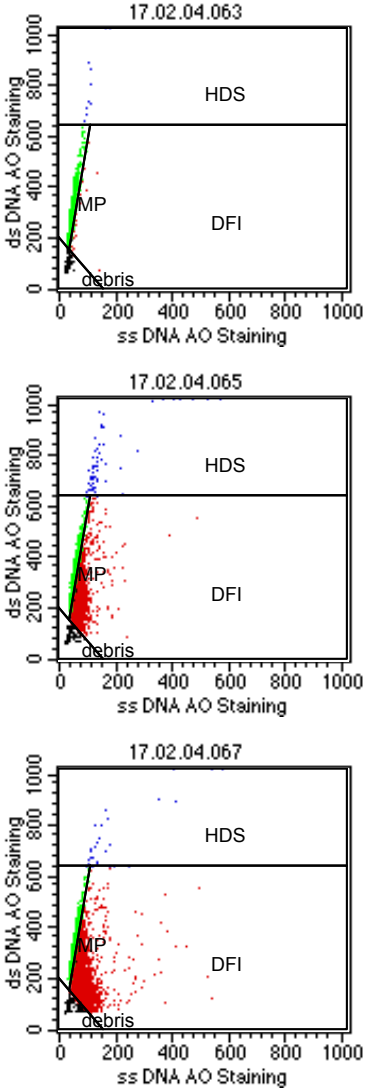

Figure 3. Representative example of the SCSA outcome for sperm of a bull exposed to low pH for 0.0 (top), 0.5 (middle) and 5.0 (bottom) min. At 0.0 min the DFI was 0.5% indicating that semen collection and processing did not induce sperm chromatin instability. At 0.5 min the DFI was 29% suggesting that this bull had sperm chromatin instability. The DFI increased to 37% at 5.0 min SCSA.

Summary data for 565 bulls for the relationship between time of exposure to low pH and apparent chromatin instability are shown in Table 3. The relevant observation to note at this stage of the report is that at 0.0 min SCSA sperm chromatin showed high stability in 561 samples indicating that the procedures used for semen collection and processing did not induce sperm chromatin instability.

Table 3. Values for DNA fragmentation index (DFI) for semen samples from 565 bulls exposed to the SCSA for 0.0, 0.5 and 5.0 min. At 0.0 min SCSA, sperm of 561 bulls had high chromatin stability.

	SCSA (min)		
	0.0	0.5	5.0
DFI (%)	Number of Bulls		
< 15	561	513	432
15 – 27	3	24	68
> 27	1	28	65

Sperm chromatin in bulls in northern Australia

As shown in Table 3, sperm chromatin for 561/565 bulls was found to be stable in the absence of exposure to low pH. Results for 0.0 min SCSA are therefore not reported in further detail.

Results for the SCSA at 0.5 min and 5.0 min are presented in Figure 4 and Figure 5, respectively, and summary data are shown in Table 3. At 0.5 min SCSA, 513 bulls (91%) had < 15% DFI, 24 bulls (4%) had 15-27% DFI, and 28 bulls (5%) had > 27% DFI. At 5.0 min SCSA, 432 bulls (77%) had < 15% DFI, 68 bulls (12%) had 15-27% DFI, and 65 bulls (11%) had > 27% DFI.

The majority of bulls (76%) had < 15% DFI irrespective of whether sperm were subjected to 0.5 min or 5.0 min SCSA. This finding could be interpreted to suggest that most bulls in northern Australia have relatively stable chromatin. Only 28 bulls (5%) had > 27% DFI at 0.5 min SCSA which is the protocol that is used routinely in humans to test for sperm chromatin instability. As noted above, > 27% DFI (at 0.5 min SCSA) is the threshold that in humans is associated with the failure to maintain a pregnancy. Whether this is also the case for bulls at 0.5 min SCSA remains to be determined. At 5.0 min SCSA, 65 bulls (11%) had > 27% DFI but the significance of this to fertility is likewise unknown. There was no apparent relationship between the SCSA and genotype (Table 4).

Table 4. Relationships between parameters of the SCSA and genotype. Genotypes are defined in Table 1.

SCSA	DFI	BB (295)	BBX (79)	BG (27)	BR (30)	COMP (126)	DM (2)	SE (6)
0	<15%	293	79	27	30	124	2	6
	15-27%	2	0	0	0	1	0	0
	>27%	0	0	0	0	1	0	0
0.5 min	<15%	268	75	25	29	110	1	5
	15-27%	11	3	1	1	8	0	0
	>27%	16	1	1	0	8	1	1
5.0 min	<15%	220	68	19	28	91	1	5
	15-27%	36	5	5	1	21	0	0
	>27%	39	3	3	1	14	1	1

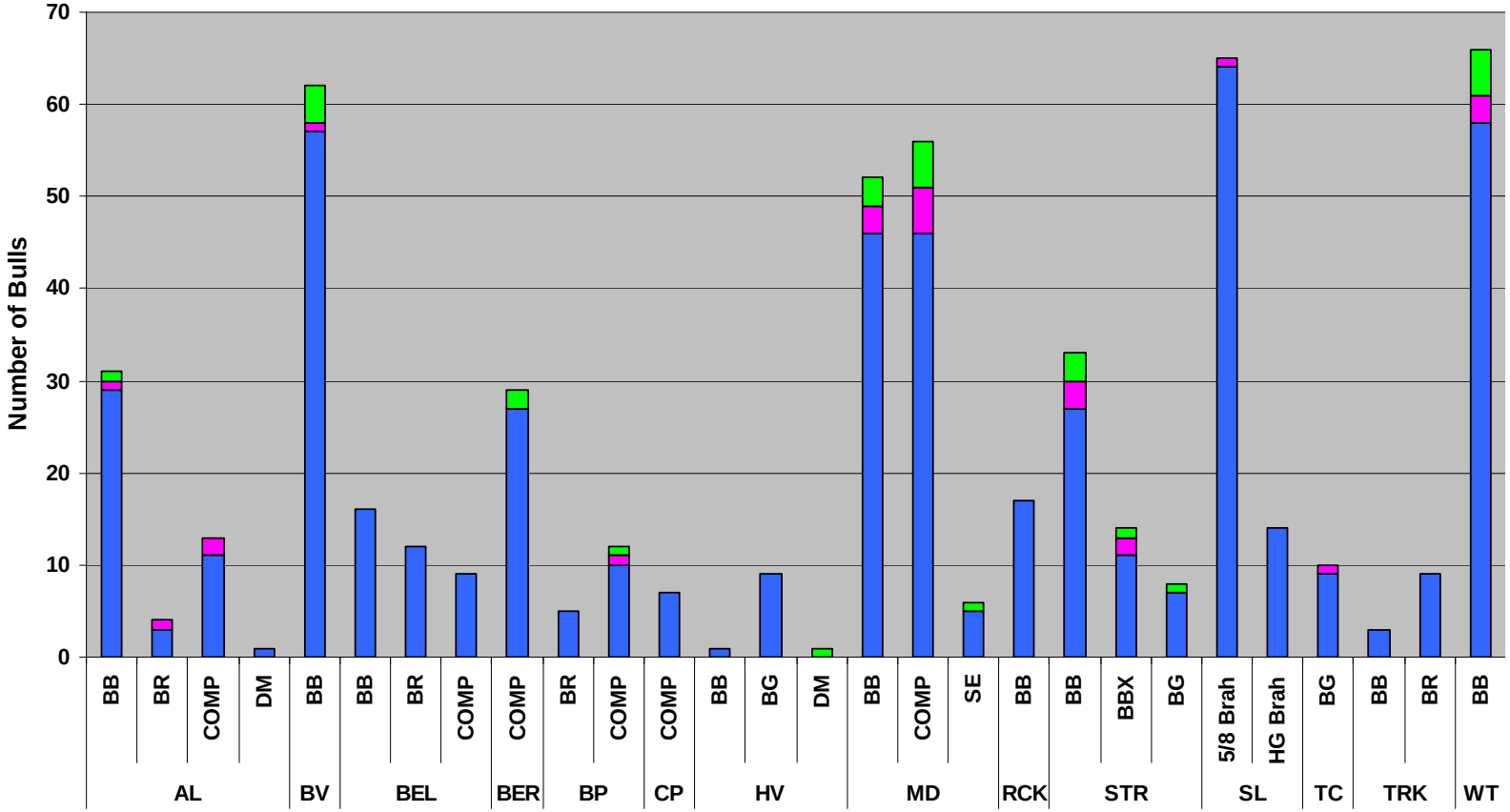

Figure 4. Summary data for the 0.5 min SCSA for 565 bulls in northern Australia. The distribution of bulls in the 3 categories of DNA fragmentation index (DFI) is shown as < 15% DFI (blue), 15-27% DFI (pink) and > 27% DFI (green). Genotype identification is given in Table 1. Locations were as follows: AL, Allansleigh; BV, Belah Valley; BEL, Belmont; BER, Berrigurra; BP, Brian Pastures; CP, Commoron Park; HV, Homeview; MD, Meteor Downs; RCK, Rockley; STR, Stratford; SL Swan's Lagoon; TC, Thirsty Creek; TRK, Toorak; WT, Weetalaba.

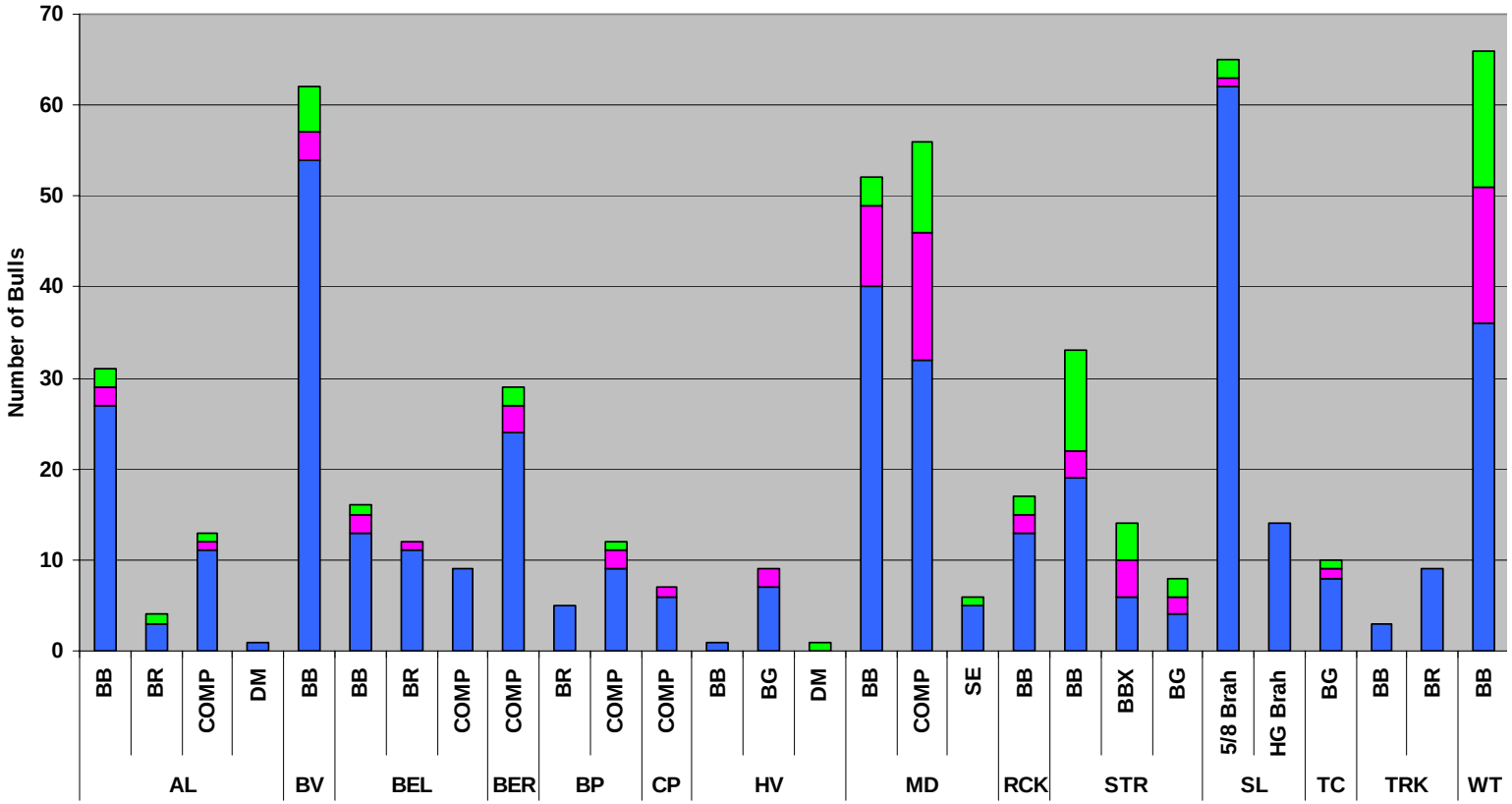

Figure 5. Summary data for the 5.0 min SCSA for 565 bulls in northern Australia. The distribution of bulls in the 3 categories of DNA fragmentation index (DFI) is shown as < 15% DFI (blue), 15-27% DFI (pink) and > 27% DFI (green). Genotype identification is given in Table 1. Locations were as follows: AL, Allansleigh; BV, Belah Valley; BEL, Belmont; BER, Berrigurra; BP, Brian Pastures; CP, Commoron Park; HV, Homeview; MD, Meteor Downs; RCK, Rockley; STR, Stratford; SL Swan's Lagoon; TC, Thirsty Creek; TRK, Toorak; WT, Weetalaba.

Changes in DFI for individual bulls

Results for changes in DFI for individual bulls at 0.5 min and 5.0 min SCSA are shown in Table 5. For 58 bulls, the DFI increased from < 15% DFI (0.5 min SCSA) to 15-27% DFI (5.0 min SCSA) with increased exposure of sperm to low pH and for 23 bulls the DFI increased from < 15% DFI (0.5 min SCSA) to > 27% DFI (5.0 min SCSA) with increased exposure to low pH. Similarly, 15 bulls that had 15-27% DFI at 0.5 min SCSA had > 27% DFI at 5.0 min SCSA. These findings suggested that sperm chromatin instability in bulls becomes increasingly more apparent with increased exposure to low pH. It should be noted that there was not a consistent and progressive increase in DFI with increasing exposure to low pH for all bulls. For example, some bulls (n = 23) increased from < 15% DFI at 0.5 min SCSA to > 27% DFI at 5.0 min SCSA. Further studies are required to determine the relationship of this orchestration of apparent sperm instability to fertility for individual bulls.

Table 5. Changes in the DNA fragmentation index (DFI) for individual bulls at 0.5 min SCSA and 5.0 min SCSA.

	% DFI (5.0 min SCSA)			
	<15	15-27	>27	TOTAL
% DFI (0.5 min SCSA)	Number of Bulls			
<15	432	58	23	513
15-27	0	9	15	24
>27	0	1	27	28
TOTAL	432	68	65	565

Repeatability of the SCSA

The repeatability of the SCSA was examined in 149 bulls and the results for 0.5 min SCSA and 5.0 min SCSA are shown in Table 6 and Table 7, respectively (see also Table 1). At 0.5 min SCSA, 136/140 bulls had < 15% DFI at both collections whilst of 4 bulls with < 15% DFI at Collection 1, 2 had 15-27% DFI and 2 had > 27% DFI at Collection 2. At 5.0 min SCSA, 94/127 bulls had < 15% DFI at both collections whilst of 33 bulls with < 15% DFI at Collection 1, 20 had 15-27% DFI and 13 had > 27% DFI at Collection 2. Therefore, at 0.5 min SCSA 3% of bulls showed a shift in DFI from < 15% to higher DFI's whilst at 5.0 min SCSA 26% of bulls showed a shift in DFI from < 15% to higher DFI's. This finding could be interpreted to suggest that the 0.5 min SCSA provides a more repeatable measure of sperm chromatin status in bulls than the 5.0 min SCSA.

Table 6. Results for the 0.5 min SCSA for 149 bulls at two collection times. Details of the collection times are provided in Table 1.

Collection 1	Collection 2			
	< 15% DFI	15-27% DFI	> 27% DFI	TOTAL
< 15% DFI	136	2	2	140
15-27% DFI	3	0	0	3
> 27 % DFI	2	0	4	6
TOTAL	141	2	6	149

Table 7. Results for the 5.0 min SCSA for 149 bulls at two collection times. Details of the collection times are provided in Table 1.

Collection 1	Collection 2			
	< 15% DFI	15-27% DFI	> 27% DFI	TOTAL
< 15% DFI	94	20	13	127
15-27% DFI	8	1	3	12
> 27 % DFI	4	2	4	10
TOTAL	106	23	20	149

Results for repeatability of the SCSA at different locations are shown in Figure 6. There were no changes (P > 0.05) in the proportions of bulls in the different DNA fractionation index categories (< 15%, 15-27% and > 27% DFI) between the 1st and 2nd semen collections for any of the properties. This is likely explained, at least in part, by virtue of the fact that the vast majority of bulls on all properties had a repeatable DFI measure of < 15%.

The SCSA was repeated on 4 occasions for three properties and the findings are shown in Figure 7. Similar to the finding when the SCSA was repeated on 2 occasions, there was no change (P > 0.05) in the proportions of bulls in the different DNA fractionation index categories between the 4 collection times for the three properties. The majority of bulls at the three properties had a repeatable DFI measure of < 15%.

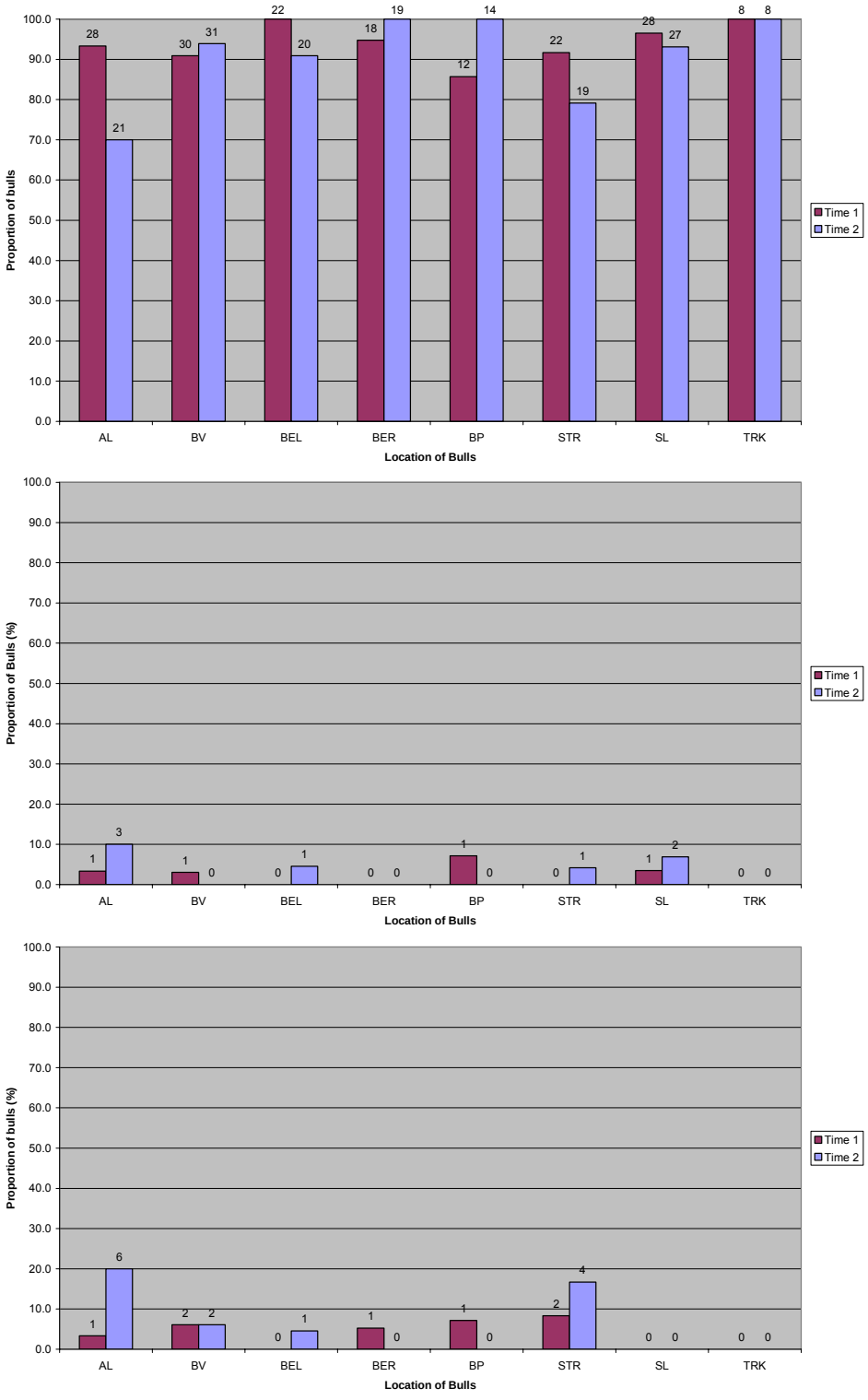

Figure 6. Repeatability of the SCSA. The SCSA was repeated on 2 occasions for 8 properties. The properties are identified in the legend to Figure 4 above. Results are for DFI < 15% (top panel), DFI 15-27% (middle panel) and DFI > 27% (bottom panel). Numbers above columns are number of bulls.

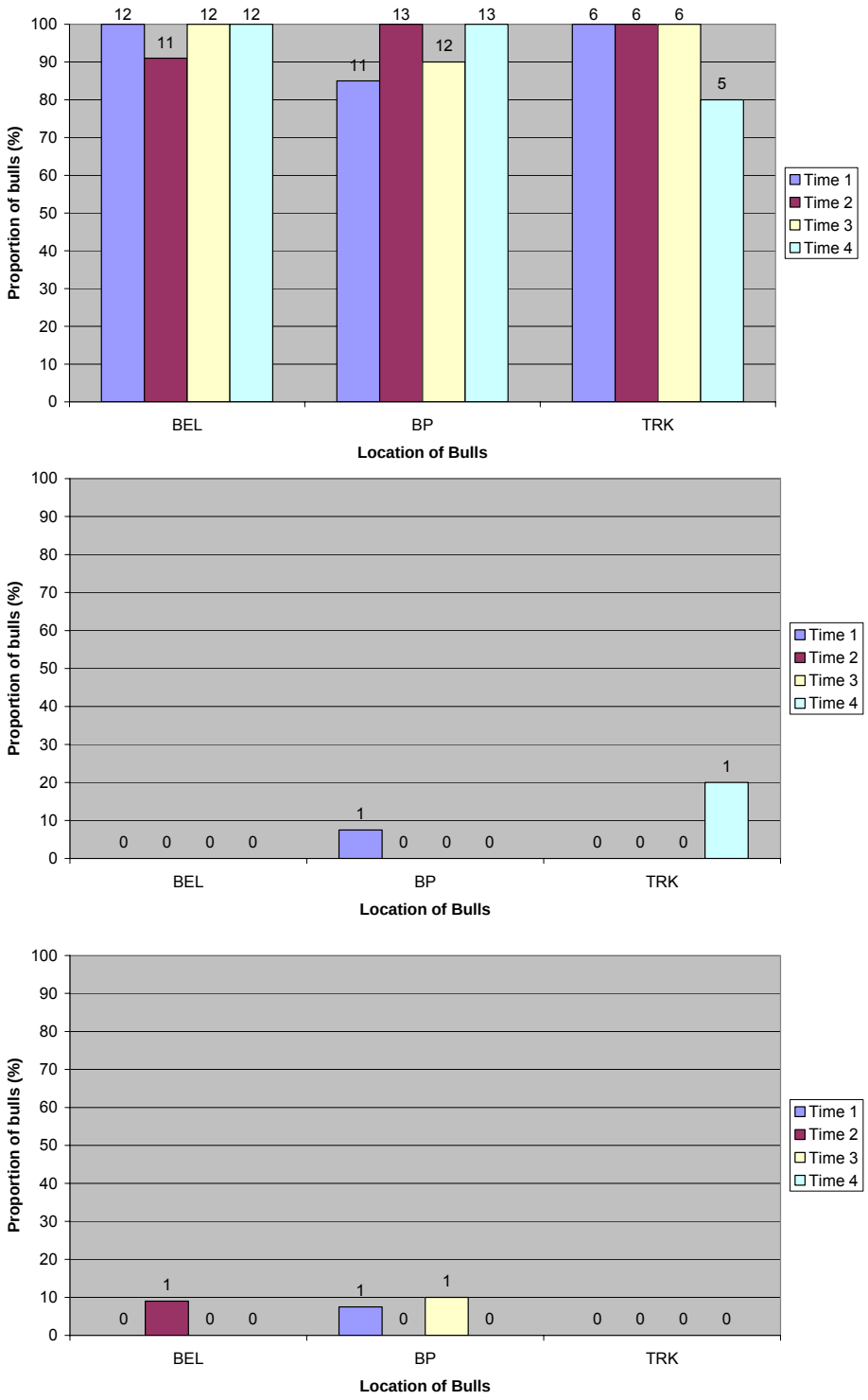

Figure 7. Repeatability of the SCSA. The SCSA was repeated on 4 occasions for 3 properties. The properties are identified in the legend to Figure 4 above. Results are for DFI < 15% (top panel), DFI 15-27% (middle panel) and DFI > 27% (bottom panel). Numbers above columns are number of bulls.

Correlations between the SCSA, age, scrotal size and standard semen parameters

Results for correlations (Peason *r* value) between the SCSA, age, scrotal size and standard measures of semen quality are shown in Table 8. Correlations between the SCSA and measures of semen quality were mostly negative for both the 0.5 min SCSA and 5.0 min SCSA. The outstanding exception was a positive correlation ($P < 0.01$) between head abnormalities and the SCSA for both the 0.5 min SCSA (0.41) and 5.0 min SCSA (0.47).

Table 8. Correlations (Pearson *r* value) between the SCSA, age, scrotal circumference (SC) and standard measures of semen quality.

SCSA	Age (y)	SC (cm)	Standard semen quality measures				
			Volume (ml)	Density (1-5)	Mass activity (1-5)	Motility (%)	Normal (%)
0.5 min SCSA	-0.14 [b]	-0.06 [n]	-0.13 [b]	-0.29 [a]	-0.41 [a]	-0.38 [a]	-0.42 [a]
5.0 min SCSA	-0.05 [n]	-0.10 [b]	-0.16 [b]	-0.20 [a]	-0.38 [a]	-0.37 [a]	-0.45 [a]

[a] $P < 0.01$
[b] $P < 0.05$
[n] non significant

Relationship of the SCSA to location, genotype and testicular tone

A strong relationship existed between the SCSA and location (Table 9). This information was derived primarily from Brahman which was the predominant genotype in the project. A preliminary conclusion could be that location had a greater effect on the SCSA than did differences between genotypes at the one location. However, it should be noted that some genotypes were represented by only a relatively small number of bulls. There were no apparent relationships between the SCSA and genotype (see Table 4 also) or testicular tone.

Table 9. Relationships between the SCSA, location, genotype and testicular tone.

	0.5 min SCSA	5.0 min SCSA
	P-value	P-value
Location	< 0.01	< 0.01
Genotype	0.17	0.07
Testicular Tone	0.19	0.14

SCSA and fertility

Results for relationships between the SCSA outcome and the ability of bulls to sire calves are shown in Table 10 and Table 11. Bulls represented in Table 10 all had a DFI of < 15% and they all sired calves in multiple-sire herds. The results in Table 10 are only for bull progeny. Notwithstanding, all bulls with a DFI ranging from 0.8% DFI to 8.6% DFI sired calves.

Table 10. Relationship between SCSA outcome and progeny for bulls in northern Australia. The results are for bulls monitored in Beef CRC II in multiple-sire herds in which the progeny are determined using DNA fingerprinting. The data are only for bull progeny as these are retained for ongoing assessment of reproductive development and reproductive function in successive generations. The DNA Fractionation Index (DFI) was assessed using the 0.5 min SCSA. BB, Brahman; BR, Belmont Red; COMP, Composite.

Sire ID	Sire Genotype	Sire DFI (%)	Number of Bull Progeny
00-029	BR	1.8	31
00-083	BR	0.9	7
00-106	BB	3.7	11
00-130	BR	0.6	11
00-132	COMP	3.5	12
00-142	COMP	3.9	13
00-150	BR	2.0	16
00-175	BR	1.2	12
00-179	BR	3.9	2
00-209	BR	8.6	7
00-212	BB	0.8	8
00-217	BR	2.2	13
00-237	BB	4.8	20
00-264	BR	1.3	26
00-266	BB	2.8	3
00-322	BR	0.8	23
00-379	BB	1.8	6

At one property, 4 bulls were identified that had a relatively high DFI in the SCSA. The high DFI was repeated on 2 occasions for 2 bulls and on 3 occasions for 1 bull (Table 11). These bulls were at a breeding property and part of a single-sire genetic evaluation program. They had been mated as single sires on a seasonal basis for 1 to 6 years. Bulls with a repeatable relatively high DFI all had sired calves (Table 11).

Table 11. Mating outcomes for bulls with a relatively high DNA fragmentation index (DFI). Bulls were mated to groups of around 40-70 heifers and cows as part of a sequential mating strategy where individual bulls are allowed to mate for 21 days. Each mating period is separated by 10 days and the full mating program is typically carried out from October to February in central Queensland. The capacity of individual bulls to sire calves is influenced by the mating period in the Oct-Feb program when they are introduced to a group of females and the pregnancy status of that group of females at the time of introduction. The DFI was assessed using the 0.5 min SCSA and all bulls were Brahman.

Bull	DNA Fragmentation Index (%)			Total number of calves	Years mated	Average calves per mating program
	Sampling Period					
	1	2	3			
97-7507	38		35	161	6	27
98-8196	28			64	4	16
00-9157	58	51	51	20	3	7
02-0979	56	32		6	1 *	6

* Bull was mated to yearling heifers that had experienced a relatively dry period before mating

Genetic background and sperm chromatin structure

Initial results for sperm chromatin structure amongst half-sib bulls are shown in Table 12. Consistent with other findings, the majority of half-sib bulls had a DFI < 15% (82/87, 94%; comprised of smaller half-sib groups) in the SCSA and there was only the odd animal with a DFI 15-27% (4/87, 5%) and DFI > 27% (1/87, 1%).

For a small number of bulls the DFI was measured for sires and their progeny. All sires (n = 6) had a relatively low DFI (0.8 to 3.1%) as did 41/45 (91%) of the total group of progeny. Three progeny (7%), each from a separate sire, had a DFI 15-27% and one progeny (2%) had a DFI > 27%. Although this data set is small it might be interpreted to suggest that environment is an important factor that determines sperm chromatin structure.

Table 12. DNA fragmentation index (DFI) amongst half-sib bulls. Also shown are results for DNA fragmentation index (DFI) scores of bulls and their progeny (shaded area). Results were obtained using the 0.5 min sperm chromatin structure assay (SCSA). BB, Brahman, BBX, Brahman cross; BR, Belmont Red.

			DNA Fragmentation Index of Progeny			
		Sire DFI	<15%	15-27%	>27%	Total
Sire ID	Genotype	(%)	Number of Progeny			
931084	BBX		4			4
941296	BBX		12			12
970026	BBX		7	1		8
970078	BBX		2			2
980678	BBX		5			5
BEL079/96	BB		2			2
CBV96-7085	BB		3			3
RJB5/139	BB		2			2
TTS983511	BB		2			2
Y7X539	BB		2			2
BELMONT 000175	BR	1.2	7	1		8
BELMONT 000209	BR	3.1	4	1		5
BELMONT 000237	BB	2.0	13			13
BELMONT 000266	BB	2.2	2			2
BELMONT 000322	BR	0.8	13	1		14
BELMONT 000379	BB	1.8	2		1	3

SCSA and DNA methylation

In men, a DFI > 27% in the SCSA is typically associated with fertilisation and initial embryonic development but a high likelihood of early pregnancy failure. It has been suggested that the latter might be related to abnormal temporal expression of developmental genes of paternal origin. A possible cause of abnormal gene expression is a change in the epigenetic status of the genome. A mechanism for epigenetic regulation of gene expression involves methylation at CpG islands on DNA which can occur in promoter regions and/or the gene itself. A methylated gene or a gene regulatory element prevents expression of that gene. The inherited methylation profile of sperm is established during early spermatogenesis but methylation status can also be influenced by the environment. A preliminary investigation of the relationship of sperm chromatin structure to DNA methylation in bulls was undertaken in this project. Methylation status was compared for 4 bulls (Bulls 2, 3, 4 and 5 in Figures 8 and 9) with a low DFI and 8 bulls that had recorded a relatively high DFI in the SCSA. The results in Figure 8 are for DNA digested with the Hpa*II* enzyme that cleaves DNA at methylation sites. The findings would suggest that Bulls 4-5 had a similar methylation status (bulls grouped together) which differed to that of bulls with a relatively high DFI. If bulls with a high DFI do indeed have an altered methylation status then the changes in methylation related to chromatin

instability may not occur at the same sites on the genome as these bulls did not group together (Figure 8). Three of 4 bulls with a low DFI remained grouped when methylation was assessed for genomic (undigested) DNA (Figure 9) suggesting that the apparent differences in DNA methylation status between bulls with a low and relatively high DFI were not explained by nucleotide polymorphisms. Based on these preliminary findings, the relationships between sperm chromatin stability and methylation warrant further investigation.

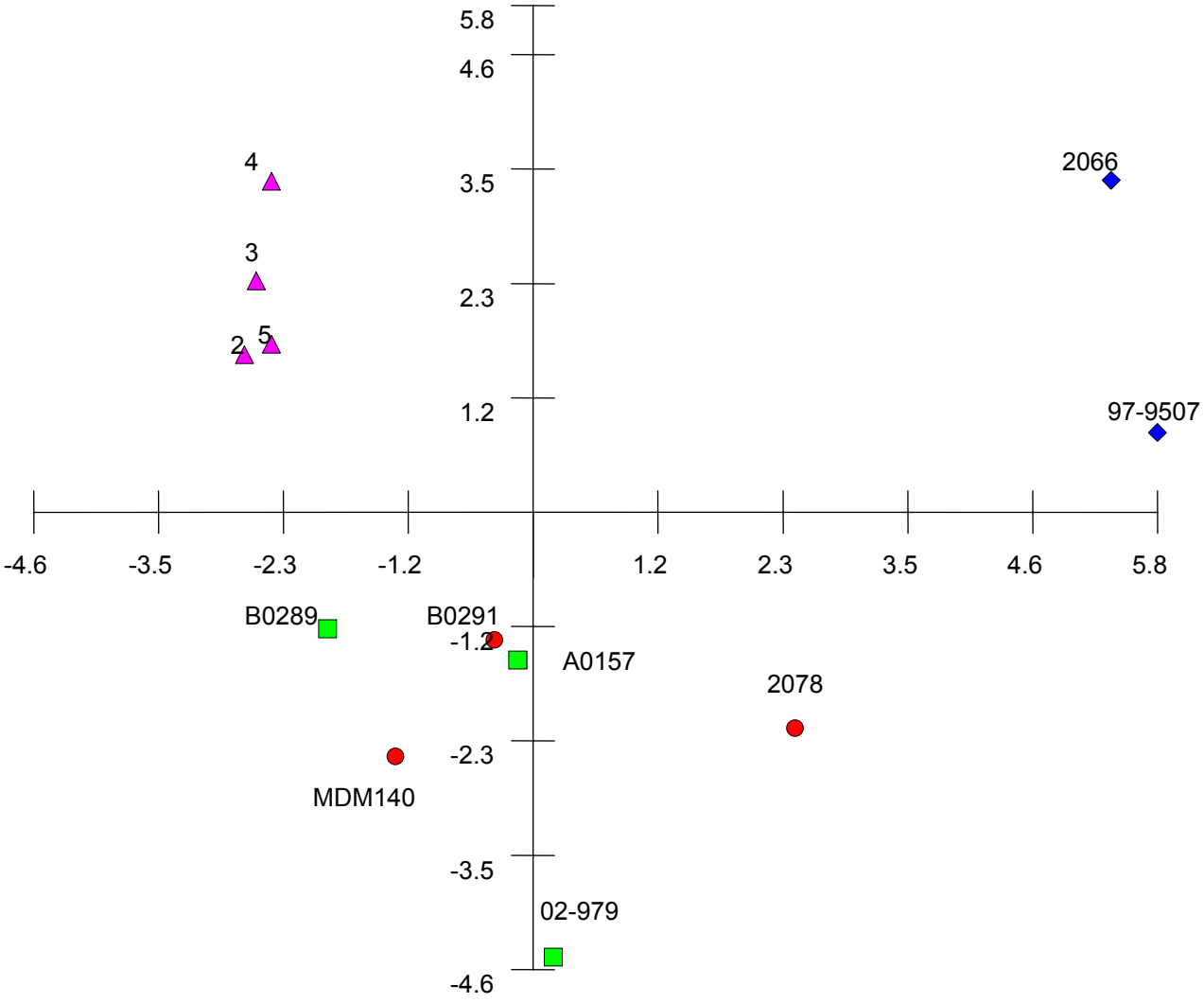

Figure 8. DNA methylation scattergram for bulls with a low DFI (Bulls 2, 3, 4, 5) and bulls with a relatively high DFI. The results are for Hpa*II* digested DNA.

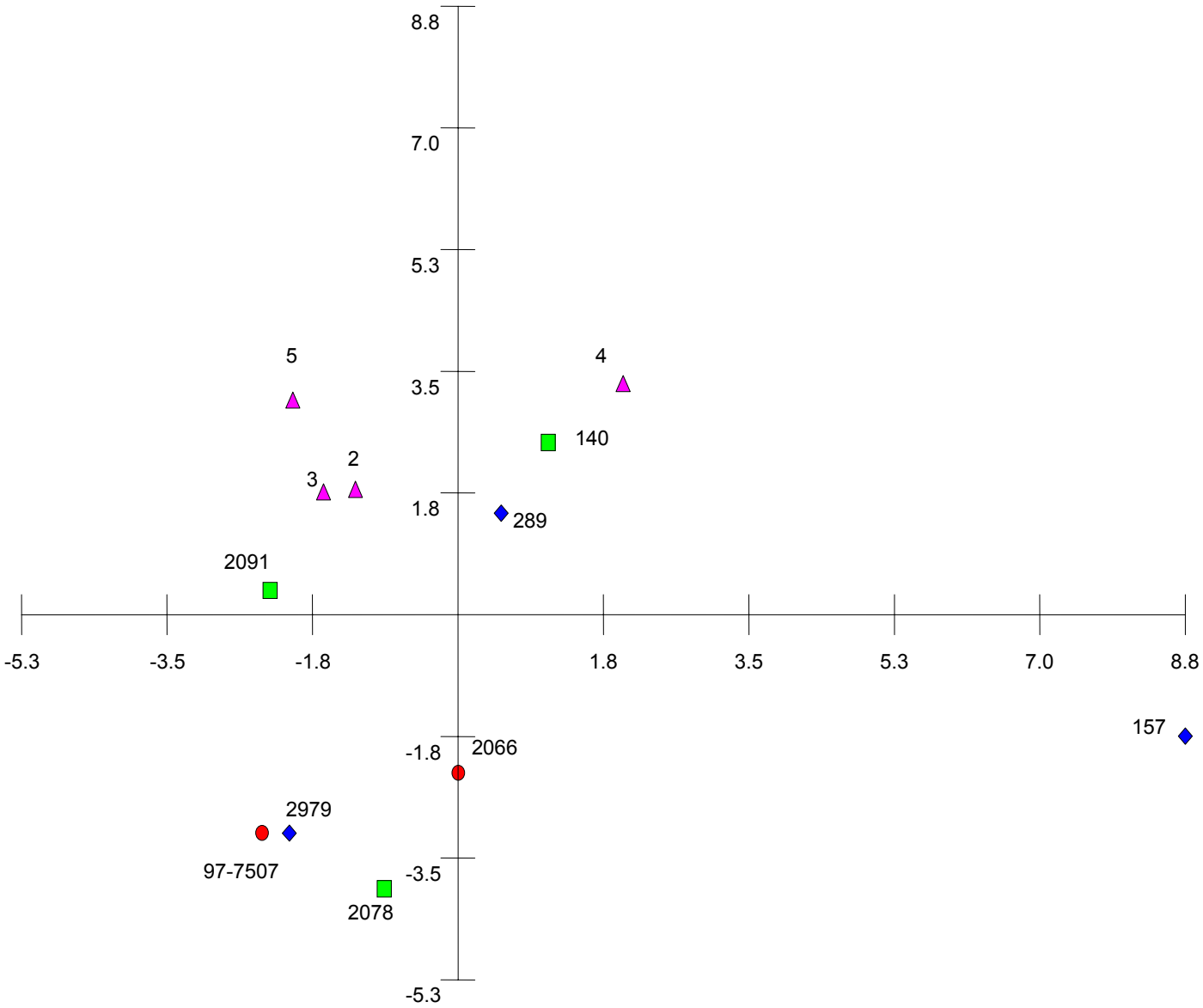

Figure 9. DNA methylation scattergram for bulls with a low DFI (Bulls 2, 3, 4, 5) and bulls with a relatively high DFI. The results are for non-digested genomic DNA.

2.5 Summary and Conclusions

The contribution of sperm chromatin status to male fertility generally, and reproductive wastage more specifically, has largely been overlooked in production animals. The likely reasons for this are that proper assessment of chromatin status requires the use of a relatively expensive flow cytometer and personnel trained in the principles of flow cytometry. However, the recent emergence of compelling information from assisted reproduction in humans of a relationship between sperm chromatin stability and the establishment and maintenance of pregnancy has led to considerable interest in production and recreational animals. Evidence for the latter is the series of articles that have appeared in the past two years that describe studies on sperm chromatin in bulls (Boe-Hansen *et al.* 2005; Hallap *et al*. 2005), boars (Boe-Hansen *et al*. 2005), rams (Peris *et al*. 2004) and stallions (Love *et al*. 2005), plus reviews in this area (Gillan *et al*. 2005). The findings in the current project are therefore timely and represent a unique data set globally which makes an important contribution to the field.

As noted earlier in this report, the relationships between sperm chromatin stability categories (< 15% DFI (stable chromatin), 15-27% DFI (moderately stable chromatin) and > 27% DFI (unstable chromatin)) and fertility have yet to be established for bulls. In the interim, a DNA fragmentation index (DFI) of > 27% has been adopted as the threshold for significant sperm chromatin instability. The apparent DFI can be altered depending on the length of time that sperm are exposed to a low pH environment and the sperm chromatin structure assay (SCSA) parameters that accurately reflect chromatin status in bulls remain to be determined. The standard SCSA uses 0.5 min exposure to low pH and the decision was made in the current project to use 0.0 min, 0.5 min and 5.0 min. When chromatin status was determined on two occasions it was found that there was greater repeatability in the SCSA outcome with 0.5 min SCSA than with 5.0 min SCSA. This may indicate that the 0.5 min SCSA provides a more reliable and consistent measure of chromatin status in bulls. The majority of results are therefore reported for the 0.5 min SCSA.

The categories < 15% DFI (stable chromatin), 15-27% DFI (moderately stable chromatin) and > 27% (unstable chromatin) were used in the current project. Of 565 bulls tested, 432 (76%) had < 15 % DFI at 0.5 min SCSA. It was concluded from this finding that the majority of bulls in northern Australia tend to have relatively stable sperm chromatin. It needs to be stressed, however, that the % DFI thresholds were based on data in men and it was noted in Section 2.1 that men have notoriously unstable sperm DNA compared with other species. When bulls were evaluated on two occasions, 136/149 (91%) bulls had a repeatable < 15% DFI (0.5 min SCSA). For bulls evaluated on four occasions, 17/21 (81%) had a repeatable < 15% DFI (0.5 SCSA). The findings from the repeat sampling suggested that sperm chromatin status remains consistent over time for the majority of bulls, and below < 15% DFI.

Using the standard 0.5 min SCSA, 28 bulls (5%) had > 27% DFI. This could be interpreted to suggest that sperm chromatin instability is not a major factor in bull fertility and reproductive wastage in northern Australia. Also, a relatively small number of bulls that recorded a repeatedly high DFI (> 27%) in the project had sired

calves in a single-sire mating program over the preceding 1-6 years. This finding would suggest that a high DFI in bulls does not impede fertility. However, there are several factors to consider. Firstly, there was a time lag between when bulls were mated and when sperm chromatin was assessed. If chromatin stability has a major environmental component then it is possible that chromatin status for individual bulls may change over time, although this would not be supported by the repeat data discussed above. Notwithstanding, elevated scrotal temperature induces chromatin instability in bulls but the period required to reinstate chromatin status has not been accurately established. Secondly, a high DFI in men does not induce absolute infertility. Rather, men with a high DFI (> 27%) are able to achieve fertilisation that is followed by embryonic development. However, there is often failure to either establish or maintain a pregnancy. A similar scenario may also occur in cattle whereby the most common outcome for bulls with chromatin instability is fertilisation and early embryonic development, but pregnancy failure. A proportion of matings by these bulls would however result in the maintenance of a pregnancy. Heifers and cows that had pregnancy failure would need to re-cycle and re-mate and the overall outcome would be a delay in herd cumulative pregnancies and subsequent extended calving period. It was the latter occurrence in industry that led to the proposition that sperm chromatin instability might be a contributing factor in delayed conception and extended calving periods, even when the majority of heifers and cows are undergoing regular oestrous cycles at the start of mating. Whilst further research is required to clarify the relationships between the DNA fragmentation index category (< 15%, 15-27% or > 27% DFI) and fertility in bulls, the relatively small number of bulls with > 27% DFI (around 5%), together with mating data for bulls with > 27% DFI, would tend to lead to the conclusion that sperm chromatin instability in bulls is not a major cause of reproductive wastage in northern Australia. Seventeen bulls with a DFI ranging from 0.8 to 8.6% all sired calves.

A preliminary investigation was undertaken on the genetic component of chromatin stability in bulls. The majority of half-sib bulls examined (82/87, 94%; comprised of smaller half-sib groups) had < 15% DFI and the odd progeny, amongst different sires, had a 15-27% DFI (4/87, 5%) or > 27% DFI (1/87, 1%). The DFI was not available for the preceding sires. Six sires had a DFI ranging from 0.8 to 3.1% and of their progeny 41/45 (91%) had < 15% DFI, 3/45 (7%; different sires) had 15-27% DFI and 1/45 (2%) had > 27% DFI. The findings would suggest that DFI is heritable but information on sire-progeny relationships for sires with 15-27% DFI and > 27% DFI is required before the heritability of sperm chromatin structure can be confirmed.

The DNA methylation status of sperm chromatin was compared for a small number of bulls with low (< 15%) and high (> 27%) DFI. The reason for examining DNA methylation was that studies in men had shown that sperm from individuals with > 27% DFI fertilised oocytes and there was embryonic development, but most often this was followed by early pregnancy failure. The suggestion had been made that the failure to establish a pregnancy was due, at least in part, to inappropriate expression of developmentally related genes of paternal origin. DNA methylation is associated with epigenetic regulation of gene expression and it is possible that chromatin instability is liked to changes in DNA methylation which, in turn, results in altered patterns of gene expression. These relationships are hypothetical and remain to be tested. In the present project it was found that bulls with < 15% DFI

were grouped together on the DNA methylation scattergram whilst bulls with > 27% DFI mapped individually. This finding could be interpreted to suggest that bulls with > 27% DFI have an altered DNA methylation status which is different for different bulls with > 27% DFI. However, this remains to be substantiated.

A notable finding was the apparent relationship between location and prevalence of sperm chromatin instability. The environment and possibly management might therefore be important factors in sperm chromatin status in bulls. This observation warrants further investigation as it may provide at least a partial explanation for location-related low fertility and reproductive wastage in northern Australian beef herds. It is recognised for example that heat stress can induce transient chromatin instability in bulls. It is not known whether longer-term chromatin instability occurs in a proportion of bulls exposed to an environmental effect that influences sperm chromatin.

In conclusion, the current project represents the first attempt to determine the prevalence of sperm chromatin instability amongst bulls in northern Australia. Based on the standard criteria of > 27% DFI (0.5 min SCSA) then 5% of 565 bulls tested would be classified as having unstable sperm chromatin. Whether a > 27% DFI impacts on reproductive wastage in cattle remains equivocal since a small sample of bulls with > 27% DFI sired calves. However, the latter observation had limitations as chromatin status was not determined close to the periods of mating. Seventeen bulls with 0.8 to 8.6% DFI all sired calves. The prevalence of sperm chromatin instability was higher at some locations and appeared not to be related to genotype. The latter observation is preliminary as Brahman bulls were the predominant genotype (295/565) in the current project. An attempt was made to determine the genetic component of sperm chromatin instability in bulls. The vast majority of half-sib bulls had a < 15% DFI (80-90% of bulls; comprised of smaller half-sib groups) and there was only the occasional bull with 15-27% DFI (~ 8%) and rarely > 27% DFI (1-2%). A group of six sires with 0.8 to 3.1% DFI had progeny predominantly with < 15% DFI (91% of progeny). Based on the 3 categories of sperm chromatin status adopted in the project, < 15% DFI (stable), 15-27% DFI (moderately stable) and > 27% (unstable), it would appear that the majority of bulls in northern Australia have stable sperm chromatin and no data showed that sperm chromatin instability contributed to reproductive wastage, although the latter data are limited.

2.6 Recommendations

Before making specific recommendations it is reiterated that the % DFI thresholds used in the present project were adopted from studies in men which had shown that % DFI thresholds of < 15%, 15-27% and > 27% were respectively related to stable chromatin (high fertility), moderately stable chromatin (moderate fertility) and unstable chromatin (very low fertility). It had also been reported in men that > 27% DFI was associated with failure to establish a pregnancy after IVF, even though fertilisation and initial embryonic development typically occurred. Men characteristically have poor semen quality compared with other species so it is possible that the % DFI threshold-fertility relationships in men do not apply directly in other species. For instance, a % DFI 15-27% in bulls might be equivalent, in relative terms, to a % DFI > 27% in men. Extensive fertility trials would be required to

establish SCSA-fertility relationships in bulls. This investment might be questioned based on the low numbers of bulls with % DFI > 15% in the present project. Sperm morphology has been shown to account for 35-57% of the variation between bulls in calf siring ability, but there is still little understanding of why a small proportion of bulls (typically < 15%) sire a relatively large number of calves (around 30%) and many bulls (> 50%) only sire around 10% of calves in extensive, multiple-sire herds in northern Australia. Based on the importance of bulls to herd reproductive outcomes and genetic improvement the search for predictors of bull siring ability remains important. Notwithstanding the preliminary nature of sperm chromatin studies in bulls a set of recommendations follows.

1. The project has identified a relatively small number of bulls with apparent sperm chromatin instability (> 27% DFI) that was repeatable. It is recommended that the fertility of these bulls, as determined by the rate of cumulative pregnancies (and monitoring of embryonic development) in single-sire mating groups, should be investigated. The SCSA should be conducted immediately before and after the mating period. An alternate approach is to monitor the fertility of bulls before and after experimental induction of sperm chromatin instability. The latter can be done in bulls by transient scrotal insulation and exposure of the testes to elevated temperature.

2. The finding of an apparent location-related effect on sperm chromatin warrants further investigation as environment and management could be important determinants of sperm chromatin status in bulls.

3. Information on the repeatability of chromatin status is important if the SCSA is to be considered as a meaningful and practical parameter in semen assessment. In the present project SCSA repeatability was determined on 2 occasions for 149 bulls and on 4 occasions for 21 bulls. The collaborators in the current project will continue to monitor selected groups of bulls for chromatin status over time and MLA may wish to participate in this monitoring.

4. Preliminary data were obtained in the current project on the genetic component of sperm chromatin status. A feature of these data was that the vast majority of half-sib bulls, and sires and their progeny, had a DFI of < 15% (stable chromatin category). This led to the preliminary conclusion that chromatin status in bulls has a genetic component. This conclusion must however be confirmed with increased numbers of half-sib bulls, and sires and their progeny, in the categories 15-27% DFI and > 27% DFI. This information will emerge from the ongoing monitoring noted in Recommendation 3.

2.7 Publications and communication arising from the project

D'Occhio MJ (2003) Sperm chromatin instability in bulls. Annual Meeting of the Australian Society for Embryo Transfer 15-17 August, Gold Coast, Queensland.

Hengstberger K, Johnston SD, Sester D, Tutt D, Holroyd RG, Fordyce G and D'Occhio MJ (2004) Incidence of sperm chromatin instability amongst bulls in a tropical environment: A preliminary investigation. 15th International Congress on Animal Reproduction, 8-12 August, Porto Seguro, Brazil. Volume 1, 275 (abstract).

D'Occhio MJ (2004) Sperm chromatin instability in bulls: An update I. Annual Meeting of the Australian Society for Embryo Transfer, 20-22 August, Dubbo, NSW.

D'Occhio MJ (2004) The decline in sperm production. ABC Radio National. Multi-participant feature on the decline in sperm production in males.

Hengsberger K, Sester D, Tutt, D, Holroyd RG, Fordyce G, Johnston S and D'Occhio MJ (2005) Prevalence of sperm chromatin instability amongst bulls in a subtropical environment. Submitted to Animal Reproduction Science.

Hengsberger K, Sester D, Tutt, D, Holroyd RG, Fordyce G, Johnston S and D'Occhio MJ (2005) Prevalence of sperm chromatin instability amongst bulls in a subtropical environment. A preliminary investigation. Proceedings of the Annual Scientific Meeting of the Society for Reproductive Biology, 4-7 September, Perth, Western Australia (abstract).

D'Occhio MJ (2005) Sperm chromatin instability in bulls: An update II. Annual Meeting of the Australian Society for Embryo Transfer, 18-21 August, Darwin, NT.

2.8 Project Objectives

1. *Report on the prevalence of sperm chromatin structure and instability in bulls in northern Australia*

Sperm chromatin status has been determined for 565 bulls representing 7 genotypes at a total of 14 locations in central and northern Queensland. The prevalence of apparent sperm chromatin instability has been described. Additionally, sperm chromatin status was recorded on 2 occasions for 149 bulls and 4 occasions for 21 bulls.

2. *Report on the preliminary assessment of the inheritance of sperm chromatin structure and instability in bulls in northern Australia*

The bulls utilised in the current project represented:

(a) Bulls on properties owned by individuals or pastoral companies that underwent standard breeding soundness evaluation (BSE) in conjunction with routine selection of mating bulls that were utilised in the 2004/2005 mating season; this was the first time that the bulls were assessed for sperm chromatin and the SCSA had not been performed on their sires; there were only limited numbers of bulls in half-sib clusters

(b) Bulls on breeding properties that likewise were monitored for the first time and included small half-sib clusters that had not been precisely genotyped

(c) Beef CRC II bulls at research stations that had been identified as half-sibs, and as sire-progeny groups, using DNA fingerprinting

For bulls in Group (c) above, the vast majority of half-sib bulls had a DFI < 15% (82/87, 94%; comprising smaller half-sib groups) in the SCSA and there was only the odd animal, across half-sib groups, with a DFI 15-27% (4/87, 5%) and DFI > 27% (1/87, 1%). Based on these data it could be suggested that there is a genetic component to sperm chromatin status in bulls. However, greater numbers of half-sibs in the 15-27% DFI and > 27% DFI categories will need to be evaluated. Sire-

progeny relationships were determined for 6 sires and a total of 45 progeny. All sires had a relatively low DFI (0.8 to 3.1%) as did 41/45 (91%) of the total group of progeny. Three progeny (7%), each from a separate sire, had a DFI 15-27% and one progeny (2%) had a DFI > 27%. Similar to the results above for half-sibs, the interpretation of these data is limited by the preponderance of bulls in the < 15% DFI category. An alternate view is that the majority of bulls have stable sperm chromatin (< 15% DFI) which is a heritable trait. The limitations in the data do not allow the calculation of an actual heritability value.

It should be noted that a relatively large number of bulls utilised in the current project were undergoing monitoring in Beef CRC II (R Holroyd, G Fordyce) which will continue for a proportion of their progeny in Beef CRC III. Also, the research in the project is the topic of a PhD thesis being undertaken by Ms Kirstin Hengstberger. Hence, additional genetic and phenotypic data will be obtained over the next 12-18 months that will contribute to the determination of the heritability of sperm chromatin structure in bulls. The current project had a 12-month duration which was inadequate time to obtain sufficient data, particularly for sire-progeny phenotypic relationships, to draw major conclusions on the genetics of sperm chromatin structure. The progeny of bulls involved in the initial screen of sperm chromatin structure are now starting to undergo phenotypic assessment as well as assignment to sires using DNA fingerprinting. In due course the information will be communicated to MLA as part of the ongoing research dialogue, and also to industry.

3. *Report on whether there are significant reproductive losses associated with sperm chromatin instability in bulls*

Preliminary information on the relationship between sperm chromatin structure and fertility was obtained in the current project. A group of 17 bulls with DFI ranging from 0.8 to 8.6% (i.e. all with stable chromatin < 15% DFI) each sired calves. Another group of 4 bulls that recorded a relatively high DFI ranging from 28% to 58% also sired calves as shown in the table below that is reproduced from the Results section of this report. For 3 of 4 bulls the high DFI was consistent on 2 occasions (2 bulls) and 3 occasions (1 bull). The repeatability of the DFI measures would suggest that apparent chromatin instability was a feature of these bulls, although this assertion remains to be confirmed. Notwithstanding the latter, the fact that bulls with a high DFI had previously sired calves tends to question whether there are any relationships between sperm chromatin structure and fertility in bulls. Based on these findings it could be concluded that sperm chromatin instability might not be expected to contribute significantly to reproductive wastage in cattle. Fertility data are not available for bulls with moderately stable sperm chromatin (15-27% DFI).

Bull	DNA Fragmentation Index (%)			Total number of calves	Years mated	Average calves per mating program
	Sampling Period					
	1	2	3			
97-7507	38		35	161	6	27
98-8196	28			64	4	16
00-9157	58	51	51	20	3	7
02-0979	56	32		6	1 *	6

* Bull was mated to yearling heifers that had experienced a relatively dry period before mating

It has been previously noted in this report that the environment can influence sperm chromatin structure and stability in bulls. The fertility data for bulls with > 27% DFI was obtained retrospectively and semen was not collected immediately before and after mating. Hence, it remains possible that sperm chromatin status could have changed over time in these bulls. But, the latter is not supported by the repeatedly high DFI on 2 occasions in 2 bulls and 3 occasions in 1 bull in the above table.

The findings in the current project highlight the need to conduct controlled studies in order to establish clear relationships between sperm chromatin structure, fertility and reproductive wastage. These studies could include the screening of bulls for sperm chromatin status and immediate mating of bulls with extremes in DFI, or the induction of sperm chromatin instability (e.g. scrotal insulation and exposing testes to elevated temperature) followed by mating. These types of studies were beyond the scope of the current project.

4. Make recommendations as to the extent and direction of further research in sperm chromatin instability

Recommendations on the extent and direction of further research in sperm chromatin instability have been made in this report (see Section 2.6 and Section 2.8 (3)).

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3.0 Appendix 1: Literature Review

Mammalian Sperm Chromatin Structure and Function and its Relationship to Male Fertility and Embryonic Survival

Table of Contents

1.0 Abstract

Embryonic mortality in mammals is generally considered to result from 'female factor' infertility. There is growing evidence to suggest, however, that the status of sperm chromatin (DNA) at the time of fertilisation can also influence embryonic survival. During the final stages of sperm production (spermiogenesis) a number of unique biochemical, morphological and physiological processes take place that are associated with changes in the structure of sperm chromatin. In early spermatogenesis sperm DNA is bound to histone necleoproteins and structured into nucleosome core particles similar to that of all other somatic cells of the body. However, as spermiogenesis proceeds the histones of the nucleosome are replaced by transition proteins, which are subsequently replaced by protamines. At the completion of spermiogenesis the chromatin of mature sperm has a toroidal structure that is very compact and resistant to damage. This is necessary for protection of sperm DNA during transit through the epididymis and female reproductive tract. Disruption to chromatin remodelling processes, at the molecular level, during spermiogenesis can result in poorly packaged chromatin that is susceptible to denaturation. This has been shown in several mammalian species, including humans, to be related to male infertility. Several techniques have been developed to assess the chromatin status in sperm but the most sensitive technique to date is the sperm chromatin structure assay (SCSA), which is a flow cytometric assay that uses the metachromatic properties of acridine orange to test the susceptibility of sperm DNA to acid-induced denaturation *in situ.* A relationship has been found, primarily in humans, between SCSA parameters and the probability of continued embryonic development and the establishment of pregnancy after fertilisation. However, the SCSA technique has yet to be applied on a regular basis in human fertility clinics and animal breeding centres, where conventional WHO semen analysis is still the most common method of semen evaluation. The contribution of sperm chromatin instability to reproductive wastage in both natural mating and assisted reproduction in humans and domestic and production animals deserves further consideration as it may prove invaluable as a means for decreasing the incidence of embryonic mortality.

2.0 Introduction

Male fertility can be defined as the success of sperm to fertilise oocytes and the resulting zygotes to continue on through embryonic development until birth (Eid *et al.* 1994). Sperm dysfunction is one of the most common single causes of male infertility, yet our knowledge of the cellular and biochemical basis for this is relatively poor (Esterhuizen *et al.* 2000). For a number of years the role of a spermatozoon in fertilisation was perceived as being limited to penetration of the oocyte in order to deliver its DNA. However, it is now apparent that there are many other significant attributes inherent within the spermatozoon that play a distinct role in post-fertilisation processes and in the production of fertile, healthy offspring (De Jonge, 2000).

Mammalian spermatogenesis is a complex and unique process that involves several physiological, biochemical and morphological changes which results in the transformation of round diploid spermatogonia into fully differentiated haploid spermatozoa with a condensed nucleus, acrosome and flagellum. Disruption to these processes can have a significant effect on the short- and longer-term fertilising potential of a spermatozoon and its ability to sustain a viable pregnancy.

Spermatogenesis can be divided into two distinct stages; spermatocytogenesis and spermiogenesis. Spermatocytogenesis consists of the mitotic divisions involving proliferation and maintenance of spermatogonia (Senger, 1999). Spermatogonia then undergo meiosis to form primary spermatocytes and then secondary spermatocytes, which differentiate into spermatids (Figure 1).

Spermiogenesis occurs during the final stage of spermatogenesis and involves the transformation of early round spermatids into fully differentiated mature spermatids, or spermatozoa. The tissue remodelling that occurs during spermiogenesis is unique in that it is one of the only mammalian systems that produces a cell type in which the nucleus is transcriptionally inactive and the majority of the cytoplasm is lost and shed in the form of a residual body (Sakkas *et al.* 2002).

Spermatids can be divided into 3 distinct categories during mammalian spermiogenesis, according to appearance; (1) early spermatids with round nuclei, (2) intermediate spermatids with elongating nuclei and (3) mature spermatids with condensed nuclei (Figure 2) (Dadoune, 2003).

In mammals, during mid-spermiogenesis the shape and size of the nucleus and the compaction state of the chromatin change dramatically as the nucleosomal histones of the meiotic germ cells are replaced by spermatid specific nucleoproteins (Dadoune, 2003). These proteins are thought to be responsible for the highly compact nature of sperm DNA. Thus they are of great interest, as it has been demonstrated that abnormal expression of spermatid nucleoproteins can alter the packaging of sperm chromatin. The integrity of sperm chromatin has been shown to be an important factor in male fertility, where alterations to the condensation of chromatin can reduce the ability of a spermatozoon to sustain embryo development.

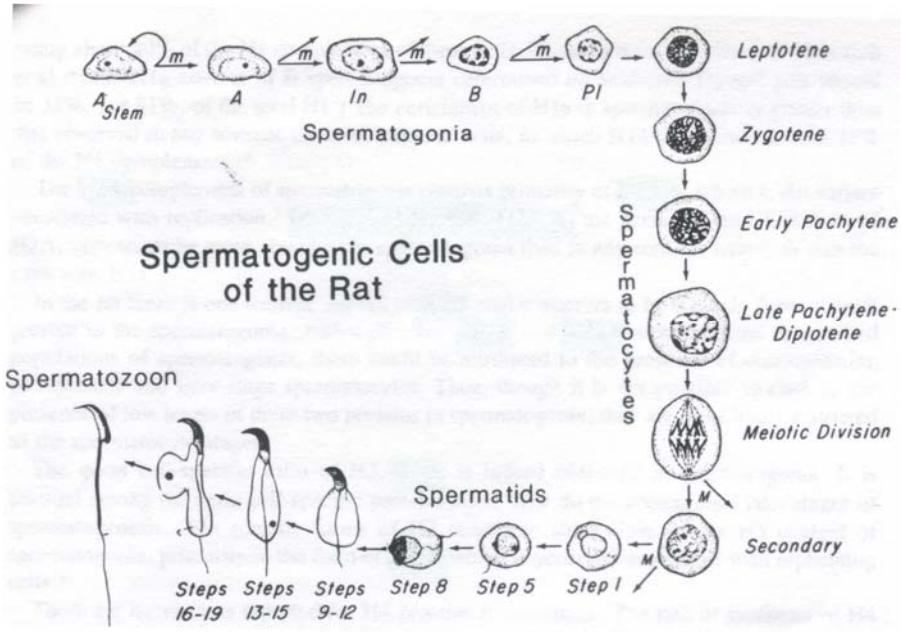

Figure 1. Sequence of spermatogenesis in the rat, showing changes in morphology (Meistrich, 1989).

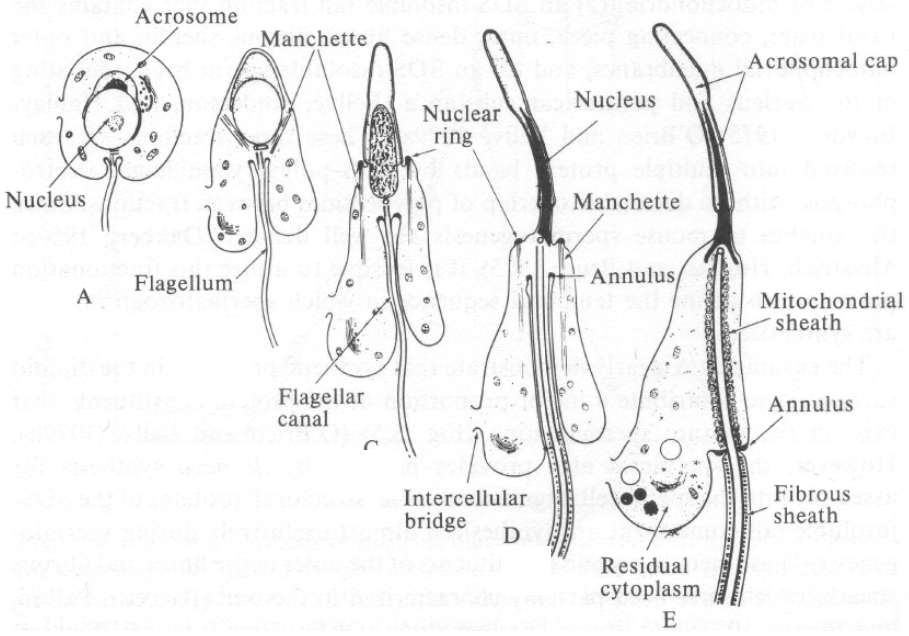

Figure 2. Diagrammatic representation of typical mammalian spermiogenesis depicting the major morphological events that are associated with assembly of the spermatozoon (Bellvé, 1979).

The aim of the current review is to present a comprehensive description of the molecular processes that are involved in mammalian sperm cell differentiation, the influence of these processes on the paternal contribution to embryo survival, and outline the current diagnostic tools used to identify sperm chromatin defects.

3.0 Sperm chromatin structure

The chromatin packaging of sperm cells is strikingly different from that of somatic cells (Ward, 1993). Sperm chromatin is a highly organised, compact structure that consists of DNA complexed with several different unique nucleoproteins (Evenson *et al.* 2002). Sperm cell chromatin is maintained in a somatic cell-like state through the last meiotic division of spermatocytes leading to round spermatids with histone-complexed DNA (Evenson, 1999a) organised into nucleosome core particles (Figures 3 and 4).

A model describing the structure of a nucleosome was first described by Kornberg (1974) where each nucleosome core particle consisted of DNA wrapped around an octamer of core histones. Each nucleosome core particle is connected by linker DNA, to give somatic chromatin an appearance similar to that of beads on a string (Wolffe, 1998)(Figures 3 and 4). In most mammals electron microscope examination of dispersed spermatid chromatin has shown that the nucleosomal organisation of chromatin found in early spermatids disappears and is replaced by smooth fibrils (Dadoune, 2003). Aggregation of these fibrils gives rise to larger fibres that coalesce into a dense homogenous mass (Loir *et al.* 1985). This homogenous mass can largely be attributed to the interaction of sperm DNA with protamines.

Concomitant with visible changes in chromatin organisation, histones are removed from the DNA of spermatocytes and early spermatids and replaced by transition proteins (TP). Transition proteins are then replaced by protamines (P) that are responsible for the final condensation and stabilisation of sperm chromatin (Meistrich, 1989; Barratt, 1995; Kistler *et al.* 1996; Steger, 1999; Kierszenbaum, 2001; Zhao *et al.* 2001). Sperm DNA interacts with protamines in a unique fashion that involves the coiling of sperm DNA into toroidal subunits, also known as the doughnut loop, containing ~50kb of DNA (Figures 3 and 4). At the completion of spermiogenesis it has been estimated one sperm cell contains as many as 50,000 of these toroidal structures packed inside the nucleus (Balhorn *et al.* 1999).

Other well-documented nuclear events associated with the modification of sperm chromatin structure include an increase in histone acetylation (Meistrich *et al.* 1992), an increase in ubiquitin system activity (Baarends *et al.* 2000) and a change in DNA topology resulting from the elimination of negative supercoiling (Ward and Coffey, 1990), which is discussed in detail in later in this review.

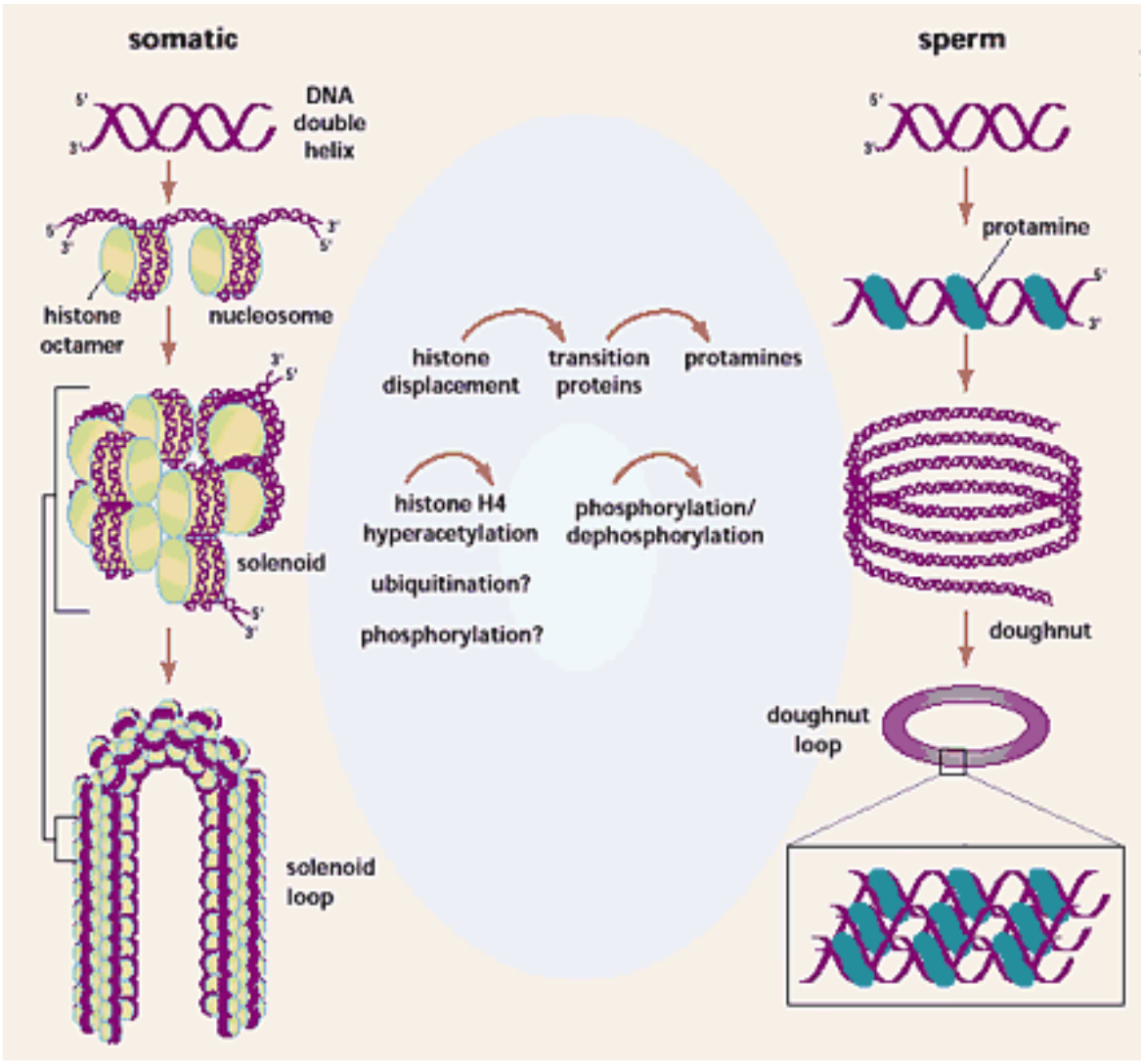

Figure 3. Changes to the structure of sperm chromatin during mammalian spermatogenesis. In somatic cells DNA is wound around histone octamers twice to form a nucleosomes, which are then coiled into solenoids. Solenoids are attached at intervals to the nuclear matrix and form DNA loop domains. In the sperm nucleus transition proteins replace the histones and are in turn replaced by protamines. Protamines bind in the major groove of DNA and coil the DNA into a doughnut shape (toroid). Displacement of the histones is facilitated by post-translational modifications of the proteins in the form of histone acetylation, ubiquitination and phosphorylation. Phosphorylation and dephosphorylation of the transition proteins is also important and is thought to facilitate their displacement before protamines bind to DNA.

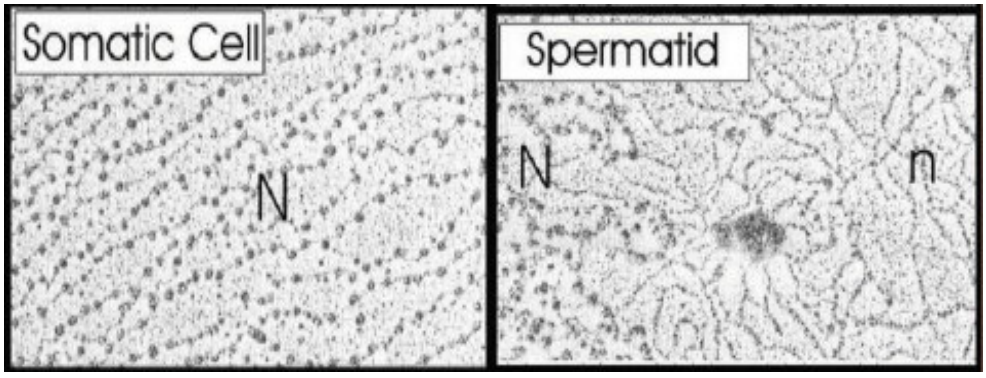

Figure 4. Comparison between somatic chromatin and sperm chromatin structure. The sperm chromatin image is taken during the transition from histone-complexed DNA to protamine-complexed DNA. Somatic chromatin (N) is distinctly arranged into nucleosomal structures that look like beads on a string, whereas sperm chromatin is in a finer, more fibrillar form (n) (O'day, 2002).

Mammalian sperm DNA is said to be the most tightly packed eukaryotic DNA (Barratt, 1995) and this is predominantly due to the unique biochemical changes, induced by spermatid nucleoproteins, that occur during spermiogenesis. The highly compact chromatin structure of spermatozoa is important, biologically, as it allows for protection of the DNA during epididymal transit and transit through the female reproductive tract to the site of fertilisation. Sperm chromatin integrity is also extremely important in the transfer of genetic information to the oocyte and in the early stages of embryo development.

4.0 Spermatid nucleoproteins

The mechanism of chromatin transition from a nucleohistone type to a nucleoprotamine fibre is still one of the most poorly understood events during spermiogenesis (Kistler *et al.* 1996). However, it is known that the chromatin structure of the differentiating sperm cell is characterised by several successive changes in nucleoprotein composition (Table 1) (Meistrich, 1989; Hecht, 1989).

Table 1. Nucleoproteins present in mammalian spermatogenic cells (Meistrich, 1989)

	Nucleoproteins
Spermatogonia	H1c, H1a, H2A.1, H2A.2, H2A.X, H2B, TH2B, TH2A, TH3, H3.2, HMG1, HMG2
Spermatocytes	H1t, TH2A, TH2B, H2A.1, H2A.2, H2A.Z, H4, HMG2
Early Round Spermatids	H1t, TH2A, TH2B, H2A.1, H2A.2, H2A.Z, H4, HMG1, HMG2
Elongating and Condensing Spermatids	TP1, TP2, TP4**,
Condensed Spermatids	TP3**, P1, P2***

H – Histone

TH – Testis-specific Histone

HMG – High Mobility Group Protein

TP – Transition Protein

P - Protamine

**only present in boar, bull, man, mouse, ram and rat sperm cells

***only present in hamster, human, stallion, chinchilla, mouse, monkey, baboon and macaque sperm cells

Early spermatids remain round, in a decondensed state and possess the same histone and high mobility group (HMG) proteins as late primary spermatocytes, but none of the transition proteins (TPs) or protamines (Meistrich, 1989) (Figure 5). Transition protein 2 (TP2) is the first nucleoprotein that replaces histones from the spermatid nucleus and its appearance is associated with the onset of nuclear elongation (Kistler *et al.* 1996). Transition protein 1 (TP1) generally appears 24h later and this offset order of appearance suggests differing functions for the two transition proteins (Kistler *et al.* 1996).

The pattern of protamine binding is roughly identical in all the mammals studied (Dadoune, 2003). Immunocytochemical studies have revealed a detectable amount of protamine is present in elongated spermatids at step 12 in the mouse (Biggiogera *et al.* 1992), step 10 in the rabbit (Courtens *et al.* 1995), step 12 in the rat (Kistler *et al.* 1996), step 10 in the boar (Courtens *et al.* 1988) and steps 4-5 in man (Prigent *et al.* 1996).

5.0 Histones

Histones represent approximately 20% of the human sperm basic nuclear proteins (Dadoune, 2003). In most mammals histones can be divided into three categories (Meistrich, 1989)(Table 2). The somatic histones are those that are found in somatic

cells and in most spermatogenic cells of the testis. 'Germ cell enriched' variants have been labelled as such because although present in somatic cells, they are prolific in sperm cells of the testis (Meistrich, 1989) and the third group, the testis-specific histones, are only found in sperm cells and testicular cells.

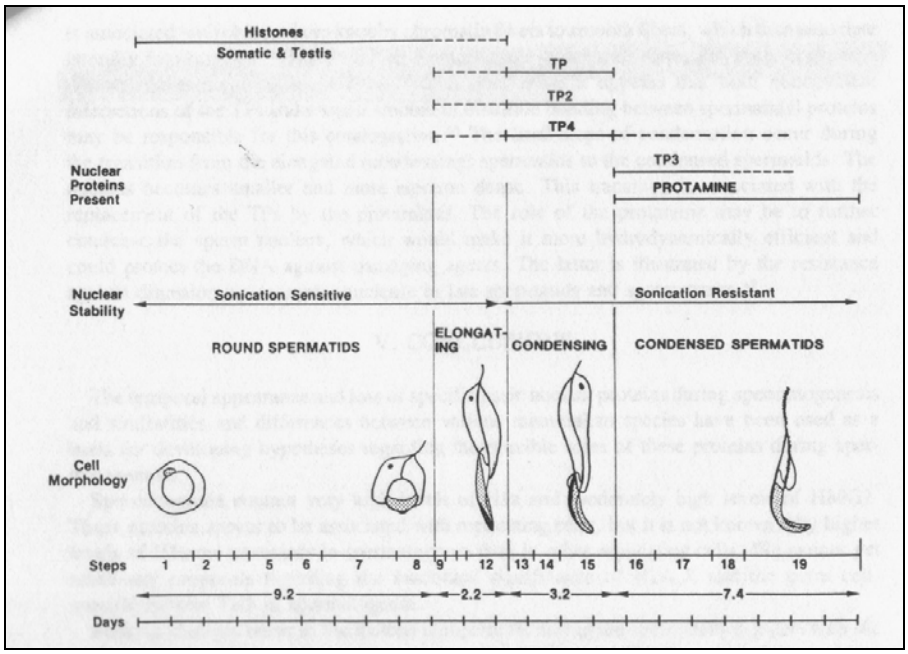

Figure 5. Stages of spermiogenesis and expression of spermatid nucleoproteins in the rat (Meistrich, 1989).

Table 2. Histone variants present in mammalian spermatogenic cells (Meistrich, 1989; Courtens et al. 1995)

Histone	**Somatic Type**	**Germcell enriched**	**Testis-Specific**
H1	H1b, H1c, H1d, H1e, H1°	H1a	H1t
H2A	H2A.1, H2A.2, H2A.Z	H2A.X	TH2A
H2B	H2B.1	-	TH2B
H3	H3.1, H3.2, H3.3	-	TH3
H4	No variants	-	-

Major attention has been focussed on histone acetylation and ubiquitination, which are thought to facilitate chromatin transformation from the nucleosome structure to the nucleoprotamine structure during spermatid differentiation (Dadoune, 2003). Specifically, the lysine-rich amino-terminal tail of H4 becomes hyperacetylated, which reduces its affinity for DNA (McCarrey, 1998). This is proposed to reduce the rigidity of DNA for possible interactions with the more basic spermatidal proteins, resulting in the replacement of histones by TP's (Meistrich *et al.* 1992).

In humans and some other mammals it has been shown that there is some persistence of histones in mature ejaculated spermatozoa. It has been assumed that histone retention is an abnormal characteristic, leading to poor packaging of the sperm chromatin and instability of the genome (Evenson, 1999b) as retention of histones is related to a lack in P2 processing. However, it has also been shown that retained histones are not localised in one region of the nucleus, rather evidence shows that they are strategically placed within the chromatin and perhaps mark sets of genes that will be preferentially activated during early embryo development (Evenson, 1999b).

6.0 Transition proteins

Transition proteins (TP's)are the major basic proteins of spermatids during the period of transition from histone-associated to protamine-associated DNA (Meistrich, 1989). There is still a great deal to be learnt about the specific functions of TP's, however, it has been demonstrated that 3 important events occur during the TP phase of spermiogenesis: (1) transformation of nucleosomal type chromatin into a smooth chromatin fibre, (2) initiation of chromatin condensation and (3) cessation of transcription (Kundu and Rao, 1996). It is assumed therefore that TP's are involved in one or more of these events.

To date, TP1 and TP2 are the best characterised, despite the presence of at least 4 TP's in rat (Kistler *et al.* 1975), mouse (Kleene *et al.* 1988), boar and bull (Kremling *et al.* 1989), ram (Chirat *et al.* 1991) and human (Luerssen *et al.* 1988) spermatozoa.

Transition protein 1 is a 54-amino-acid protein rich in arginine, lysine, histidine and serine (Heidaran *et al.* 1989; Dadoune, 1995; Dadoune, 2003), with cysteine also present in the TP1 of bull, boar and ram spermatozoa (Dadoune, 2003). Basic residues are distributed at regular intervals along TP1 where it is thought they bind to DNA by aligning themselves lengthwise along the major groove. In contrast, TP2 is a much larger (117-138 residues according to species) zinc metalloprotein rich in serine, proline and cysteine (Dadoune, 2003).

Transition protein 1 and TP2 seem ubiquitous among mammals (Alfonso and Kistler, 1993) whilst TP3 and TP4 have only been characterised in some species. Transition protein 3 (76-103 residues according to species) has only been isolated and characterised in the rat, ram and boar (Dadoune, 2003). The amino acid sequence of boar TP3 is characterised by the presence of two tryptophan residues in the N-terminal part of the molecule and by a C-terminal region showing a high degree of homology with boar TP1 (Wouters-Tyrou *et al.* 1998). Rat TP3 has been characterised as being the primary translational product of rat P2 gene (Wouters-Tyrou *et al.* 1998).

The transition protein TP4 has been characterised in rat and boar late spermatid nuclei and in the boar it is a basic protein of 138 residues and a highly basic amino-terminal half and a less basic carboxy-terminal half (Wouters-Tyrou *et al.* 1998). Akama *et al.* (1995) demonstrated that boar TP4 has different domain structures to that of TP1-3 and has a higher affinity for double-stranded DNA (ds-DNA). In the rat, TP4 is a unique protein immunologically distinct from TP1, TP2, TP3, P1 and histones and shares no sequence homologies with any known protein (Unni and Meistrich, 1992).

According to their stage-specific expression, TP's are thought to be actively involved in the process of nuclear condensation that transforms the somatic chromatin into the metabolically quiescent fibrillar chromatin of the mature spermatozoon (Lévesque *et al.* 1998). Transition protein 1 has DNA destabilising properties and relaxes DNA, reducing the interaction of DNA with the nucleosome core (Dadoune, 2003). This has led to the proposal that TP1 is actively involved in the displacement of histones. In addition to assisting in the condensation of chromatin it is thought that TP1 facilitates DNA strand break repair, by neutralising the phosphodiester backbone of DNA and bringing nick-ends into close proximity, allowing a still unknown ligase to repair the break (Caron *et al.* 2001).

In contrast, TP2 exhibits better DNA-stabilising and condensing properties than TP1 and compacts the DNA within the nucleosome cores (Levesque *et al.* 1998; Kundu and Rao, 1996). Transition protein 2 has therefore been implicated in the initiation of chromatin condensation prior to the expression of protamines. Being a zinc metalloprotein TP2 has been shown to condense DNA in a zinc-dependent manner, showing a distinct preference for GC-rich sequences in DNA (Kundu and Rao, 1996). One of the well defined and well characterised GC-rich sequences in the eukaryotic genome is CpG islands which are associated with 5' or 3' domains (gene promoter domains) of all known housekeeping genes and a set of tissue specific genes (Antequera and Bird, 1993).

Kundu and Rao (1996) examined the interaction of TP2 with an oligonucleotide containing a human CpG island sequence and found that experimental methylation of the oligonucleotide completely inhibited the formation of the TP2-CpG island complex. On the basis of this information it can be suggested that the methylation status of CpG islands in sperm DNA plays a significant role in the amount of TP2 that can bind to DNA and thus deserves further scrutiny as such a relationship may be implicated in the correct condensation of sperm chromatin.

Another important factor essential for the correct binding of proteins to DNA is the phosphorylation-dephosphorylation cycle. Protein phosphorylation plays an important role in several intracellular processes, including modulation of function of DNA binding proteins and transcription factors (Strahl and Allis, 2000). Several studies have shown that protamines undergo a phosphorylation-dephosphorylation cycle, *in vivo* to facilitate chromatin condensation and it has recently been proposed that TP2 completes a similar pattern of phosphorylation-dephosphorylation *in vivo* (Meetei *et al.* 2002).

Meetei *et al.* (2002) proposed that immediately after synthesis TP2 becomes phosphorylated, which temporarily inhibits the condensation property of the basic C-terminal domain. This allows the TP2 molecule to diffuse along the chromatin to

facilitate the binding of zinc-finger modules onto CpG island sequences. Subsequent dephosphorylation of TP2 triggers the initiation of chromatin condensation (Figure 6) (Meetei *et al.* 2002).

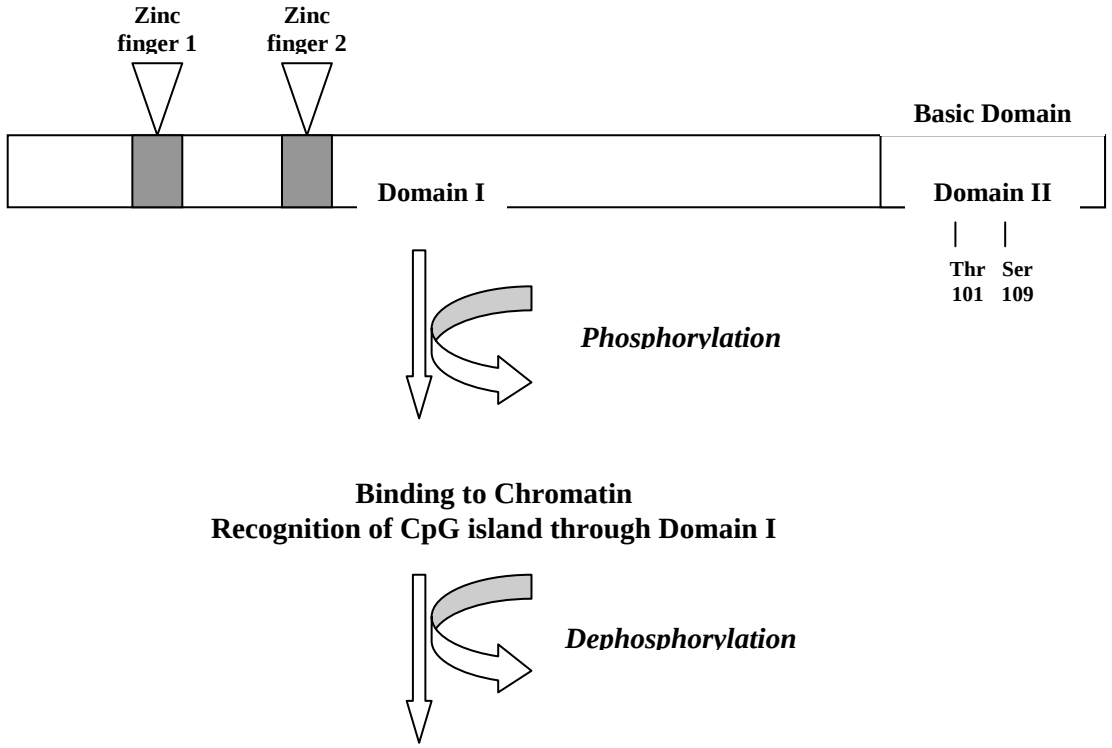

Figure 6. Model depicting the probable sequence of events during TP2-mediated condensation. Reproduced from Meetei et al. (2002).

Levesque *et al.* (1998) observed that both recombinant TP1 and TP2 are phosphorylated *in vitro* by protein kinase C (PKC) and that protein kinase A (PKA) strongly phosphorylated TP1 but had little effect on TP2. In contrast, Green *et al*. (1994) found that TP1 is not phosphorylated *in vivo* and Meetei *et al.* (2002) found that TP2 phosphorylation is mediated by PKA, not PKC. Despite these contradictions, it is clear that the role of the phosphorylation-dephosphorylation cycle is important in the sequence of events taking place in chromatin remodelling. The relationship between TP2 binding and phosphorylation status needs to be further investigated as current information suggests that phosphorylation of TP2 molecules ensures optimal binding of TP2 to CpG island sequences in sperm DNA and disruption to the phosphorylation-dephosphorylation cycle could have a significant impact on the condensing properties of TP2.

7.0 Protamines

Biochemical and primary sequence analyses of isolated sperm nuclear proteins have demonstrated that two different types of arginine and cysteine-rich protamine molecules package DNA in the sperm of eutherian mammals (Corzett *et al.* 1999). These

protamines, which are low in molecular mass and highly basic, appear in the last stages of sperm maturation and are responsible for the final condensation of sperm chromatin.

Protamine 1 (P1) is an arginine- and cysteine-rich polypeptide of about 50 residues (Dadoune, 2003). The central arginine-rich region of P1 is presumed to bind to DNA and the cysteine residues allow P1 to bind to zinc (Dadoune, 2003). All eutherian mammals contain the P2 gene, however, only some actually express the P2 protein (Lewis *et al.* 2003) (Table 3). For example, in the boar and bull the absence of the P2 protein has been attributed to a mutation within the P2 gene (Maier *et al.* 1990) and in the rat, the lack of P2 protein has been shown to be the result of suppression at both the transcriptional and translational level (Hecht, 1989).

Unlike P1, P2 is synthesised as a precursor and modified after binding into DNA (Evenson *et al.* 2002). Protamine 2 is histidine-rich and has approximately 54-63 amino-acid residues according to species. The genes encoding P1, P2 and TP2 are grouped together in a small region of the same chromosome in most mammals and are clustered in a large methylated domain in round spermatids (Choi *et al.* 1997).

Initially it was assumed that in mammalian sperm containing both protamine variants P2 performs a function essential for male fertility, as in some cases of human male infertility P2 is completely absent (de Yebra *et al.* 1993). However, recent studies indicate that both protamine variants are essential and that haploin sufficiency, caused by a mutation in one allele of P1 or P2, prevents the production of structurally and functionally intact sperm in mice (Cho *et al.* 2001).

As briefly mentioned previously, the interaction of DNA with protamines forms toroid-shaped structures, also called DNA loop doughnuts. Each doughnut represents one DNA loop domain attached to the sperm nuclear matrix where protamine-bound DNA is coiled, with a very slight bend in the protamine-DNA complex, into concentric circles (Dadoune, 1995). These concentric circles collapse into a toroid or DNA loop doughnut and are tightly packed together by Van der Waal's forces (Dadoune, 1995).

In a model proposed by Balhorn *et al.* (1984), bull sperm protamines interact with DNA lengthwise and the central arginine-rich segment of P1 lies within the minor groove of the DNA double helix, cross-linking and neutralising the phosphodiester backbone. The carboxy- and amino-terminal residues of P1 then participate in the formation of inter- and intra-protamine bridges and hydrophobic disulfide bonds, where the protamine-DNA complex of one DNA strand fits into the major groove of a parallel strand so that the DNA strands are packaged side by side in a linear array within the nucleus (Figure 7a) (Balhorn *et al.* 1984; Dadoune, 1995).

This model however does not take into consideration the interaction between DNA and both protamine variants. Therefore, another model has been proposed that involves both P1 and P2, where the DNA structure is an extensive assembly of roughly parallel DNA molecules in a hexagonal array, interconnected by globular protamine molecules placed between them (Raukas and Mikelsaar, 1999) (Figure 7b).

Table 3. Protamine expression in the spermatozoa of different mammalian species

Protamines	**Species**	**Reference**
P1	Rat	Evenson *et al.* 2002; Dadoune, 2003
	Ram	Sautiére *et al.* 1984; Evenson *et al.* 2002
	Bull	Corzett *et al.* 2002; Dadoune, 2003
	Boar	Corzett *et al.* 2002; Dadoune, 2003
	Rabbit	Calvin, 1976
	Guinea pig	Lee and Cho, 1999; Evenson *et al.* 2002
	Dog	Dadoune, 2003
	Black monkey	Lee and Cho, 1999
	Domestic cat	Corzett *et al.* 2002
P1 and P2	Hamster	
	Human	Corzett *et al.* 2002; Evenson *et al.* 2002
	Stallion	Corzett *et al.* 2002; Evenson *et al.* 2002
	Chinchilla	Corzett *et al.* 2002
	Mouse	Corzett *et al.* 2002
	Australian native mouse	Corzett *et al.* 2002
		Corzett *et al.* 2002
	Rhesus monkey	Corzett *et al.* 2002
	Baboon	Corzett *et al.* 2002
	Pigtail macaque	Corzett *et al.* 2002
	Crab-eating macaque	Corzett *et al.* 2002

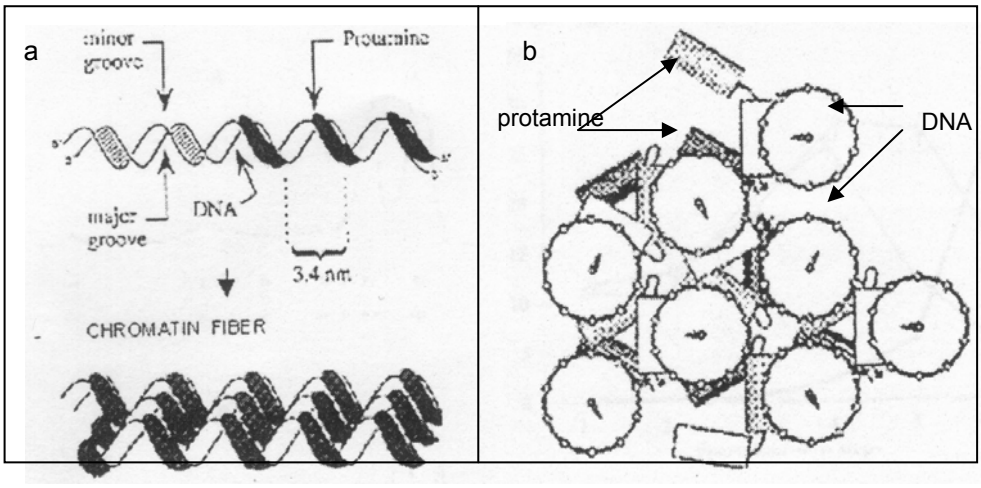

Figure 7. Diagrams illustrating how protamines bind to DNA (a) Balhorn's model where protamins package DNA into linear, side-by-side arrays (b) Raukas' and Mikelsaar's model where protamine molecules are located in channels where large grooves of the double helices are juxtaposed (Dadoune, 2003).

Two distinct stages of chromatin remodelling have been described involving the protamines (Love and Kenney, 1999; Dadoune, 2003). Stage 1 involves the phosphorylation and dephosphorylation of serine and threonine residues of protamines (Dadoune, 2003) and the second stage involves stabilisation of DNA initiated by the formation of inter- and intra-molecular disulfide bonds between cysteine residues of protamine molecules (Love and Kenney, 1999).

Protamine phosphorylation is a rapid process that facilitates correct binding to DNA, while dephosphorylation is associated with an increase in sperm chromatin condensation (Dadoune, 2003). Phosphorylated forms of protamines have been shown to persist in the ejaculated sperm from the bull, boar, human and stallion and this has been proposed to result from an incomplete dephosphorylation process (Marushige and Marushige, 1978). This incomplete dephosphorylation is accompanied, at least in man and mouse, with the persistence of intermediary maturation forms of the protamine precursor (Wouters-Tyrou *et al.* 1998), which results in a less stable sperm chromatin structure.

The stability of chromatin is determined by the number of disulfide cross-links formed between thiol groups of adjacent protamine chains (Dadoune, 2003). The efficiency of the formation of intermolecular disulfide bonds depends on the optimum positioning and binding of the protamines to DNA, which is dependent on the phosphorylation/dephosphorylation process (Marushige and Marushige, 1978; Balhorn *et al.* 1984). Evaluation of the number of disulfide bonds in protamines from mature spermatids and epididymal sperm has shown that the stabilisation of chromatin starts in the testis and develops with passage from the cauda to the caput epididymis (Dadoune, 2003). Numerous studies have demonstrated that the cysteine sulfhydryls in testicular sperm are progressively oxidised to disulfides as the sperm descend through the epididymis (Balhorn *et al.* 1991). The levels of disulfide bonds in ejaculated

spermatozoa may be critical in the decondensation process immediately following fertilisation as the decondensation process is dependent upon the reduction of disulfide bonds (Love and Kenney, 1999) and the timing of decondensation is dependent on the number of disulfide bonds within the sperm nucleus (Perreault *et al.* 1987).

8.0 Intrinsic regulation of the expression of nucleoproteins during mammalian spermatogenesis

The sequential expression of sperm nucleoproteins is dependent on transcriptional and translational regulation activities during spermatogenesis. Genes expressed during spermatogenesis encode proteins necessary both for general activities and for processes specific to germ cells (Eddy, 1998). The intrinsic regulation of gene expression in spermatogenesis occurs at three levels: transcription, translation and post-translation (Eddy, 1998). Numerous studies have shown that the genes for the transition protein and protamine families are transcribed early in round spermatids (Hecht 1990; Morales *et al.* 1991; Eddy *et al.* 1993; Dadoune, 1995; Kleene, 1996). The messenger ribonucleic acids (mRNAs) for these proteins are stored as ribonucleoprotein (RNP) particles in a repressed state within the cell cytoplasm for several days before being actively translated in elongating and condensed spermatids (Eddy, 1998).

The synthesis and storage of mRNAs prior to their translation is a necessity during spermatogenesis as global transcription ceases once histones are replaced by TP's (Braun, 1998). This has significant consequences for gene expression throughout spermiogenesis as it means all gene expression events following the displacement of histones rely on post-transcriptional processes. Thus, mRNA storage and translational activation play prominent roles in the expression of many spermatid and spermatozoan proteins that are synthesised in the later stages of germ cell maturation (Sassone-Corsi, 1998).

There are two categories of transcriptional regulation: methylation and trans-acting factors that bind to the TATA-box, the CRE-box or other specific DNA sequences in the promoter regions of nucleoproteins (Steger, 1999). In mammalian DNA only cytosines present in 5'-CpG-3' dinucleotides are found in a methylated state (McCarrey, 1998). It is generally thought that the methylation of the 5'-site of cytosine in DNA CpG islands leads to a reduction or arrest of gene transcription (Bird *et al.* 1985; Grosveld *et al.* 1987), whereas demethylation has been associated with an increase in gene activity (Steger, 1999). However, for genes specifically expressed in sperm cells the relationship between DNA methylation and gene activity is not as clear (Dadoune, 2003).

For some sperm specific genes an increase in methylation leads to gene transcription. The P1, P2 and TP2 genes located on mouse chromosome 16 are fully methylated when actively transcribed (Choi *et al.* 1997) In contrast, the TP1 gene shows demethylation in the 5'region associated with gene activity (Trasler *et al.* 1990). Due to the fact that sperm-specific methylation patterns appear to be strikingly different and more complex than that of somatic cells it is clear that more research needs to be conducted in order to determine the precise role that DNA methylation has in the transcriptional regulation of sperm-specific genes.

The TATA-box is known to play an important role in the correct initiation of transcription though the binding of transcription factors to the promoter region (Dynan and Tjian, 1985). The TATA-box is present in all protamine genes described so far (Oliva and Dixon, 1990) and in the mouse TP1 gene (Heidaran *et al.* 1989). Both RNA polymerase II components and TATA-binding protein (TBP) have been found to accumulate in rodent round spermatids, while the amount of total RNA increases in the testis (Schmidt and Schibler, 1997).

Transcriptional regulation via the adenyl-cyclase signalling pathway is mediated by cAMP-response element (CRE) nuclear factors, cAMP-response element binding (CREB) protein and cAMP-response element modulator (CREM) protein (Steger, 1999). Within the nucleus, the PKA-catalytic subunits phosphorylate and thereby activate CREB or CREM through phosphorylation of a serine residue at position 133 (CREB) or position 117 (CREM) (Steger, 1999). Activated CREB or CREM can then bind to CRE, which then turns on its target gene (Delmas *et al.* 1993; Tamai *et al.* 1997).

The presence of TP and protamine transcripts long before their corresponding proteins are synthesised argues for control mechanisms of translation (Dadoune, 2003). Translational repression, after transcription, is essential for spermatid differentiation as premature translation of proteins can lead to an arrest in differentiation and cause dominant male sterility (Braun, 1998). In round spermatids the vast majority of mRNA's are translationally repressed by long poly-A tails and are sequestered in cytoplasmic RNP particles for up to a week (Steger, 1999). Translation subsequently takes place after mRNA's undergo a partial poly-A tail shortening by deadenylation (Steger, 1999).

The series of nucleoprotein exchanges during spermiogenesis provides an excellent model for the regulation of sequential gene expression at both the transcriptional and translational level (Steger, 1999). Although the mechanisms regulating expression of basic nucleoproteins during spermiogenesis are still poorly understood, current data suggests that a disruption to gene regulatory mechanisms influence nucleoprotein binding and subsequently may affect the condensation and stability of sperm DNA.

9.0 Detection of sperm chromatin abnormalities

Evenson *et al.* (2002) have stated that for a test to be useful it must have a threshold above and below one that will provide discriminatory and predictive capabilities, with little overlap between fertile and infertile patients. Although a number of methods involving a variety of dyes and fluorchromes have been used to evaluate the chromatin integrity of mammalian sperm, the majority of these are labour intensive and lack statistical power. This stems in part from the use of light microscopy, where the number of sperm analysed is typically only 100–200, thus the coefficient of variation, repeatability and statistical soundness of these measures are relatively poor (Evenson *et al.* 2002).

10.0 Microscopic methods for assessing sperm chromatin and DNA integrity

Microscopic evaluation of sperm chromatin integrity can incorporate the use of electron microscopy (EM), transmission electron microscopy (TEM), light microscopy (LM) and/or fluorescence microscopy (FM). Typically LM and FM are teamed with the use of a variety of dyes and fluorochromes that specifically bind to components of chromatin

and reflect abnormalities in chromatin packaging or condensation.

Examination of human sperm by EM has revealed a wide spectrum of head malformations generally affecting the nuclear shape of sperm and often concomitant with incomplete condensation of chromatin (Dadoune, 1995). Transmission electron microscope image cytometry, which is more informative than conventional quantitative EM, has also revealed significant differences between fertile and infertile men form parameters of nuclear shape and chromatin texture (Dadoune, 1995). Commonly vacuoles, inclusions and disturbances of the nuclear condensation process are observed within the chromatin (Dadoune, 1995) (Figure 8). Evidence of this is demonstrated in a study by Parkinson (2000) where extensive nuclear vacuolation was found in the sperm of a single Angus bull, which was also, as a consequence of this vacuolation, sterile (Figure 8).

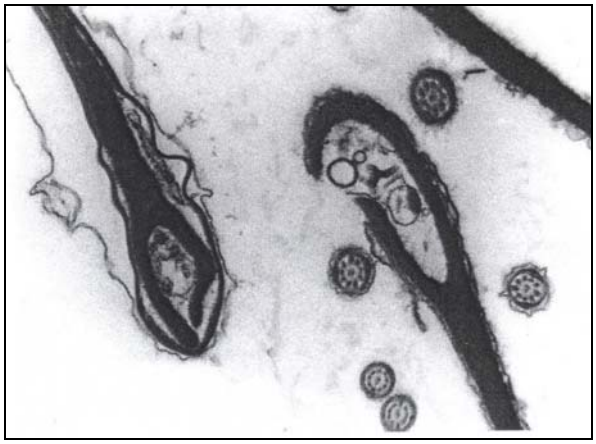

Figure 8. Nuclear vacuoles in the post-acrosomal part of the nucleus of two spermatozoa. These images are a longitudinal section of sperm heads (Parkinson, 2000).

The most commonly used dyes and fluorochromes for detection of chromatin abnormalities include; Feulgen, aniline blue, acridine orange (AO) and chromomycin A_3 (CMA_3) (Esterhuizen *et al.* 2000; Evenson *et al.* 2002). The binding capacity of these dyes is believed to reflect abnormalities in the packaging of chromatin, such as DNA strand breaks and nicks and abnormal nucleoprotein expression.

Chromomycin A_3 is a fluorochrome specific for G-C-rich sequences of DNA and has been used for indirectly visualising protamine-deficient, nicked and partially denatured DNA (Dadoune, 1995). CMA_3 is believed to compete with protamines for association with DNA and is therefore related to the degree of protamination of mature spermatids (Bianchi *et al.* 1993).

Esterhuizen *et al.* (2000) classified patients according to CMA_3 staining, using cut-off values of 44.5% and 60% stainability, and found that implantation and clinical pregnancy rates of the ICSI and IVF groups with CMA_3 percentages of > 60% were significantly lower than those with percentages of 44.5%. However, Esterhuizen *et al.* (2000) also stated that results should be interpreted with caution, as each laboratory

should determine its own cut-off values for CMA_3 staining. Therefore, CMA_3 staining may be a useful method for screening patients for abnormal spermiogenesis that has resulted in intrinsic chromatin abnormalities, but its use in the IVF laboratory remains limited due to the absence of a predictive threshold for fertility (Evenson *et al.* 2002).

Aniline blue selectively stains lysine-rich nucleoproteins (histones and transition proteins) and appears to be a good tool to evaluate chromatin condensation anomalies on sperm smears (Dadoune, 1995). It has been proposed that aniline blue staining is not related to IVF success solely on the basis of chromatin condensation assessment, but that the relationship to IVF success may be attributed to the ability of aniline blue to identify abnormal epididymal maturation and associated sperm function abnormalities.

Problems with interpretation of fluorescent microscopic samples are exacerbated by indistinct colour, rapidly fading fluorescence and heterogenous slide staining which results in artefacts and false-positive or false-negative results (Evenson *et al.* 2002).

11.0 Assays used for the analysis of sperm DNA (chromatin) integrity

Assays using fluorochromes with more specific and complex DNA (chromatin) interactions have been developed to identify more subtle defects in DNA and chromatin integrity. These include single cell gel electrophoresis assay (COMET), terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) assay, *in situ* nick translation (NT), acridine orange test (AOT) and sperm chromatin structure assay (SCSA) (Evenson *et al.* 2002).

COMET assay

In the COMET assay sperm are suspended in a thin agarose gel on a microscope slide, lysed, electrophoresed and stained with a fluorescent DNA-binding dye (Evenson *et al.* 2002). Sperm with increased levels of DNA strand breaks have increased comet tail fluorescent intensity (Hughes *et al.* 1996) and comet tail length (Singh and Stephens, 1998).

The alkaline COMET assay (pH >10) denatures sperm DNA and identifies both single- and double-stranded DNA breaks. In contrast, the neutral COMET assay is more sensitive to double DNA-strand breaks and therefore better able to identify DNA damage related to fertility because the conditions of the assay (pH 9) do not denature DNA (Evenson *et al.* 2002).

To date, clinically useful thresholds have not been established for either alkaline or neutral COMET assay, but it has been demonstrated that the COMET assay is a more sensitive measure of DNA damage than NT (Irvine *et al.* 2000).

TUNEL assay

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single- and double-stranded DNA breaks, in a reaction catalysed by the template-independent enzyme, terminal deoxynucleotidyl transferase (TdT) (Evenson *et al.* 2002). Deoxyuridine triphosphate is labelled so that DNA strand breaks can be quantified using flow cytometry, fluorescence microscopy or light microscopy.

Flow cytometric TUNEL provides clinically significant results and the freezing of neat or extended semen samples does affect the results obtained (Sailer *et al.* 1995). The use of the flow cytometry TUNEL assay is still limited and is not likely to be used extensively in IVF laboratories, as a standardised assay has not yet been validated to make the assay statistically robust.

The microscopic TUNEL assay is labour intensive and possesses all the major weaknesses associated with other microscopic chromatin analysis techniques. However some laboratories have modified the technique to include a peroxidase enzyme labelling system that catalytically generates an intense signal from chromogenic substrates, thus eliminating the problems associated with fluorescence fading and allows technicians more time to analyse a greater number of cells (Host *et al.* 1999a, b; 2000a, b).

In situ NT assay

The *in situ* NT assay quantifies the level of single stranded DNA breaks in a reaction catalysed by DNA polymerase I (Dnase I) (Evenson *et al.* 2002). Biotinylated dUTP is added to the Dnase I treated sperm and the fluorescein-isothiocyanate (FITC) fluorochrome is used to detect the incorporation of biotinylated dUTP at single stranded DNA breaks (Acevedo, 2001).

Currently NT thresholds for embryo viability have not been established, which severely limits the clinical usefulness of this assay (Evenson *et al.* 2002).

Acridine Orange Test

This is a simplified microscopic method of the SCSA that does not require expensive flow cytometry equipment and an SCSA-trained technician (Evenson *et al.* 2002). Both the SCSA and the AOT measure the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ*, by quantifying the metachromatic shift of AO fluorescence from green (normal double-stranded DNA) to red (denatured single-stranded DNA) (Evenson *et al.* 1980; Evenson *et al.* 2002).

Any predictive value by AOT for fertility is still controversial however as AOT relies on visual interpretation of fluorescing sperm and debris that fall into a broad range of colours under microscopic examination (Duran *et al.* 1998). The AOT, being a microscopic test, possesses the same weaknesses as other microscopic tests which renders the AOT of very limited value for critical diagnosis and prognosis of a semen sample (Evenson *et al.* 2002).

Sperm Chromatin Structure Assay

During the SCSA, the extent of DNA denaturation following heat or acid treatment is determined by measuring the metachromatic shift from green fluorescence (AO intercalated into normal double-stranded nucleic acid) to red fluorescence (AO associated with single-stranded DNA) and is based on the principle that abnormal sperm chromatin has a greater susceptibility to physical induction of DNA denaturation *in situ* (Evenson, 1990).

In contrast to light microscopic methods the SCSA is the most objective and statistically sound measure of all tests in the infertility clinic for evaluating sperm chromatin status (Evenson *et al.* 2002). When compared to other sperm DNA integrity assays including TUNEL, COMET and NT, the SCSA proved to be the most practical assay for assessing the DNA integrity of a semen sample (Evenson and Jost, 2000).

The SCSA detects DNA defects (within hours) at the molecular level that may only be observed by light microscopy 1-3 weeks later as metaphase chromosome breaks or discontinuation of embryonic development (Evenson and Jost, 2000).

Evenson *et al.* (2002) outlined the following advantages that the SCSA has over other assays for sperm chromatin:

1. Aliquots of the same unprocessed semen samples used for ART can be easily snap frozen in liquid nitrogen or an ultra cold freezer (-80^0C), stored and sent for later analysis to a laboratory proficient in SCSA. Importantly, this provides a snapshot of the same semen sample used or, for prognostic purposes, a semen analysis performed within a few days prior to attempting fertilisation by IVF or *in vivo* methods.
2. The unprocessed semen is thawed, diluted to a concentration of 1 to 2 x 10^6 sperm/ml, treated to a low pH buffer to potentially denature the sperm DNA, stained with AO and measured by flow cytometry. The unique metachromatic and equilibrium staining properties of AO provide a very sensitive measurement and resolution between double- and single-stranded DNA by a respective shift from green to red fluorescence.
3. Five thousand to 10,000 sperm are measured by flow cytometry in less than 5min, providing (1) objective, equipment-defined criteria rather than biased human visual measures, (2) a higher level of repeatability (0.98-0.99) than any other currently measured semen parameter and (3) data that are related to 'male factor' infertility with reasonable sensitivity, high precision and a statistical threshold for fertility prognosis.

Perhaps the greatest impact of the SCSA technique will be for the assessment of animal and human sub-infertility (Evenson, 1990). From studies in men, bull, stallion and boar semen samples, there is strong evidence that with the current protocol, COMPα_t (the percentage of sperm with denatured DNA) thresholds of 0-15%, 16-29% and ≥ 30% relate to high fertility, moderate fertility and very low fertility, respectively (Evenson and Jost, 2000). The ≥ 30% threshold has been labelled the 'tip of the iceberg' effect; that is, chromatin damage identified in 30% or more of the sperm indicates an abnormality in the entire population causing infertility (Evenson *et al.* 2002).

The current techniques used to measure sperm DNA integrity cannot be performed in such a way as to identify sperm that can be subsequently used for IVF or ICSI due to irreversible sperm damage from fixation, denaturation and DNA staining (Zini *et al.* 2001). However these techniques, especially the SCSA, using the established thresholds, allow technicians in the likelihood of a donor establishing a pregnancy, assuming all other semen parameters are normal.

12.0 Correlation between conventional fertility assessment and SCSA parameters

Clinical evaluation of male infertility is usually confined to World Health Organisation (WHO) recommended parameters, including measures of volume, total sperm count and concentration, morphology, motility and seminal factors such as pH, number of leukocytes and anti-sperm antibodies (Evenson *et al.* 2002). These parameters are generally limited in their diagnostic and prognostic value due to the large variation in results between different technicians. Intensive efforts are being made to objectify conventional semen analysis and the development of computer assisted sperm assessment (CASA) is one example (Mortimer, 2000). However, even with the use of CASA these parameters still provide little prognostic and diagnostic value to the IVF setting because whilst some tests of semen quality correlate with fertility, none has been satisfactory as a predictor of superior fertility (Johnson, 1997). Several tests of sperm function have been developed to try and improve this situation including assessment of acrosome reaction, heparin binding to sperm, sperm-zona binding assays and sperm penetration assays.

All human and most animal studies to date have indicated that SCSA data are poorly correlated with conventional semen parameters (Evenson *et al.* 1999). The percentage of sperm with DNA fragmentation is negatively correlated with fertilisation rate, which is likely to be of importance to IVF and other assisted reproduction procedures as it is assumed that morphologically sound, motile sperm are in fact normal (Lopes *et al.* 1998).

However, it has been reported that there is a strong association between the presence of nuclear DNA damage in mature sperm of men and low ranking in conventional semen parameters (Sun *et al.* 1997; Lopes *et al.* 1998; Irvine *et al.* 2000). This is supported by the studies of Larson *et al.* (2000) which demonstrated that ICSI leads to pregnancy in patients with poor sperm morphology and motility because although the prognoses of these patients was poor, based on conventional semen parameters, SCSA parameters of the unprocessed semen were below the threshold for DNA damage, indicating that the chromatin integrity of the sperm population was adequate to support a viable pregnancy.

Computer-interfaced flow cytometry provides a powerful advantage over light microscopy techniques in terms of speed, ability to measure multiple parameters per sperm, objectivity, lack of bias in sample collection, and thousands of cells are evaluated for each sample providing the SCSA with a very high degree of statistical power (Evenson *et al.* 1999). However, favourable SCSA outcomes are not directly predictive of good fertility potential (Evenson *et al.* 1999). Analysis of any single sperm viability parameter does not give a high predictive value of fertility and even sperm with apparently sound chromatin structure, but with other abnormalities and lacking adequate motility, will still be infertile (Evenson *et al.* 2002). Therefore, several attributes of sperm quality including both conventional semen parameters and SCSA data should be combined to determine the quality of sperm from a donor and its fertilising potential.

Relationship between sperm chromatin structure and fertility

It is generally recognised that the survival of cells is dependent on the integrity of their DNA (Ahmadi and Ng, 1999). There is a large body of evidence which suggests that a proportion of ejaculated sperm in most mammals possess a wide range of abnormalities including altered sperm morphology, poor motility and compromised DNA integrity, and that these anomalies can be the cause of male infertility.

Factors that influence sperm chromatin structure

During spermatogenesis, the developing sperm cell is sensitive to external stresses (Ahmadi and Ng, 1999). Stressors may directly cause sperm DNA damage or may generate disturbances in the spermatogenic process. Some external factors including chemicals, radiation and toxicants, type of semen extender used, storage time, heat stress, season and illness are known to cause significant changes to the structure of sperm chromatin (Karabinus *et al.* 1990; Sailer *et al.* 1996; Bochenek *et al.* 2001; Love *et al.* 2002).

Spermatozoa at different stages of development are affected differently by environmental and physiological stresses and commonly chromatin stability appears dependent upon the presumed stage of sperm maturation at the time of stress (Karabinus *et al.* 1997). For example, round spermatids are particularly susceptible to damage by increased temperature, disease and exposure to chemicals, radiation and toxins.

Heat stress/hyperthermia

Sensitivity of mammalian germ cells to environmental heat has been well documented with effects of hyperthermia being recorded for a variety of species including mice (Sailer *et al.* 1997), rats (Chowdhury and Steinberger, 1964), bulls (Vogler *et al.* 1991), rams (Mieusset *et al.* 1991), stallions (Love and Kenney, 1999) and humans (Levine *et al.* 1990). The human testis is more likely to be exposed to high temperature levels than any other species, due to disease (e.g. pyrexia) and/or recreational activities (e.g. hot tubs) (Love and Kenney, 1999).

It has been demonstrated that during the warmer seasons of the year there are increased levels of morphologically abnormal sperm and reduced sperm motility and viability in bulls (Rhynes and Ewing, 1973). Increased environmental temperature can produce abnormalities in the condensation of sperm chromatin. Karabinus *et al.* (1997) demonstrated that the chromatin stability of bull testicular sperm is reduced by heat stress, as evidenced by significantly elevated overall values for the SCSA 12 or more days later. The time delay in appearance of sperm chromatin defects can be attributed to the stage of development and the location of sperm during the period of exposure to thermal stress. The study by Karabinus *et al.* (1997) also revealed that heat stress adversely affected the chromatin of epididymal sperm (sperm collected ≤ 9 days post-stress) but not to the degree exhibited by testicular sperm. The progressive stabilisation of sperm chromatin by disulfide bond formation and the condensation of chromatin that normally occur during epididymal transit would imply greater chromatin resistance to denaturation (Karabinus *et al.* 1997).

Sailer *et al.* (1997) exposed the scrotum of mice elevated temperature (38°C, 40°C and 42°C) for 60 minutes and noted moderate changes to chromatin structure at 40°C and at 42°C virtually all stages of spermatogenesis displayed severe chromatin abnormalities, as determined by the SCSA.

Chemicals, radiation and toxins

A causal relationship between cigarette smoking and impaired reproductive function is highly suspected due to the fact that smokers inhale a host of toxins such as nicotine, carbon monoxide, cadmium, and other mutagenic compounds (Zavos *et al.* 1998). It has been shown that paternal smoking increases sperm DNA damage and this has been suggested to be the cause of the increased incidence of childhood cancer (Sun *et al.* 1997; Potts *et al.* 1999).

An increase in sperm DNA damage in infertile smokers may be caused, at least in part, by the increased levels of seminal oxidative stress (Saleh *et al.* 2002). This is supported by recent reports showing that oxidative stress not only results in damage to the sperm plasma membrane but also to the sperm nuclear DNA by causing high frequencies of single- and double-strand DNA breaks (Twigg *et al.* 1998; Aitken, 1999).

Disease

Evidence has accumulated through routine semen analysis to suggest that *Ureaplasma urealyticum* infection can cause embryo loss without necessarily affecting sperm quality (Reichart *et al.* 2000). Sperm cells isolated from human semen infected with *U. urealyticum* showed no significant difference in motility to that of uninfected semen. However infected semen did exhibit a high percent of denatured DNA as determined by the SCSA (60.9% ± 9.1%) (Reichart *et al.* 2000). This suggests that *U. urealyticum* has a direct effect on sperm chromatin. Interestingly, Reichart *et al.* (2000) reported that after treatment with doxycyclin there was a significant improvement in SCSA results. Evenson *et al.* (1991) demonstrated similar results in patients suffering from prostatitis. These patients had increased nuclear DNA damage in ejaculated sperm cells which was improved by appropriate antibiotic treatment.

Studies by Evenson *et al.* (2002) demonstrated that a fertile man with an influenza-induced fever (39.9°C) expressed an unprocessed P2 precursor in sperm, coincident with an increased histone:protamine ratio, decreased free sulfhydryl groups and increased DNA stainability. This may have been the result of a disturbance to TP expression as incomplete processing of P2 is also evident in TP1-null and TP2-null mice.

Molecular and biochemical disturbances

Stress can cause sperm chromatin abnormalities by inducing chromatin structural problems such as apoptosis or necrosis (Darzynkiewicz *et al.* 1997). The most characteristic feature of apoptosis is DNA double-strand breaks (Gorczyca *et al.* 1993). Apoptosis prevents over proliferation of early germ cells and occurs normally and continuously throughout life (Sakkas *et al.* 1999). Studies on germ cell apoptosis indicate that in sub-fertile men ejaculated spermatozoa with apoptotic markers are escaping programmed cell death resulting in abnormal sperm function (Sakkas *et al.* 1999).

Vinatier *et al.* (1996) proposed that even highly condensed mature spermatozoa may be able to respond to apoptotic triggers by dissociating suicidal machinery from inhibitors and beginning the cascade of molecular events that result in apoptotic DNA degradation. In contrast however it has been proposed that the presence of DNA damage is not directly linked to an apoptotic process occurring in sperm as DNA strand breaks and apoptotic markers did not exist together in the same mature sperm (Sakkas *et al.* 1999). Due to this controversy it is inappropriate to assume that strand breaks are synonymous with apoptotic degeneration (Evenson *et al.* 2002).

It is known that DNA fragmentation in somatic cells can be caused by reactive oxygen species (ROS) and synonymously several pathological processes such as varicocele and infection with pyospermia, as well as repeated centrifugation during the swim-up technique used in IVF, may increase the level of ROS in semen (Lopes *et al.* 1998). In fertile men, the timing and amount of ROS generation is controlled by antioxidants, but high levels of ROS that lead to pathogenicity can result when sperm and contaminating leukocytes produce them in excess of the antioxidant capabilities of the male reproductive tract or seminal plasma (Aitken *et al.* 1992). Reactive oxygen species affect sperm function at both physiologic and pathologic level (Evenson *et al.* 2002). At physiological levels ROS modulate gene and protein activities that are vital to sperm proliferation, differentiation and function, but at the pathological level ROS are implicated in 3 sources of abnormal chromatin structure that include abnormal topo II activity, apoptosis and necrosis.

Structural problems may also be the result of a disturbance in DNA-nicking and ligating activities. DNA-nicks are essential for relief of torsional stress associated with the removal of nucleosomes in elongating spermatids. These nicks are not deleterious normally as they are ligated by topoisomerase II (topo II) prior to completion of spermiogenesis in normal sperm (Evenson *et al.* 2002). However, if topo II ligation activity is disturbed or blocked then nicks may not be repaired resulting in infertility (Evenson *et al.* 2002).

Silvestroni *et al.* (1976) were the first to report that abnormal nucleoprotein expression may be associated with human infertility. It is believed that chromatin packaging anomalies in human sperm arise because of defects in the sperm nuclear condensation mechanisms such as faulty protamine binding during spermatogenesis (Balhorn *et al.* 1988; Belokopytova *et al.* 1993; Manicardi *et al.* 1995). This suggestion is supported by the studies of Foresta *et al.* (1992) which demonstrated increased aniline blue staining of sperm collected from infertile patients, indicating the persistence of histones.

Several other studies have revealed that some infertile men have diminished levels of P2 in ejaculated sperm which has been attributed to the persistence of histones or incomplete processing of P2 precursors (de Yebra *et al.* 1998). This is likely to be the result of a disruption during early to mid-spermiogenesis as it has been found that in TnP-null mice the processing of P2 is disrupted (Meistrich *et al.* 2003). Cauda epididymal sperm from wild-type mice showed complete maturation of P2, with no detectable precursors, but in TnP1-null and TnP2-null mice 58% and 46%, respectively, of the P2 was in the intermediate partially processed form (Meistrich *et al.* 2003).

The role of abnormal P2 expression in infertility is substantiated by the importance of P2 in the formation of intermolecular and intramolecular disulfide bonds that are necessary for chromatin stabilisation (Evenson *et al.* 1980; Balhorn *et al.* 1984; Dadoune, 1995). The importance of spermatid nucleoproteins for chromatin condensation indicates that further studies need to be conducted to determine the relationship between abnormal nucleoprotein expression and environmental and physiological stresses, such as heat stress, toxins, chemicals, periods off illness and disease. It is important to determine the presence of such a relationship as exposure to such stresses may have both short-term and long-term effects on sperm chromatin structure may as a result decrease the chance of successful mating outcomes.

13.0 Relationship of sperm chromatin structure to fertilisation and embryo survival

The integrity of sperm DNA is important for the success of natural or assisted fertilisation as well as normal continued development of the embryo (Morris *et al.* 2002). It is generally considered that the only truly essential component in sperm for embryogenesis is the DNA itself and that all other sperm components evolved solely as the means of transporting the paternal DNA to the oocyte (Ward *et al.* 2000). However, studies by Ward *et al.* (2000) have demonstrated that the sperm nucleus requires other factors for embryogenesis in addition to the genetic information encoded within the DNA. Variation in the highly defined nuclear architecture of sperm chromatin may influence the initiation and regulation of paternal gene expression in early embryonic development (Haaf and Ward, 1995). This has been demonstrated in rodents (Yu *et al.* 2000; Zhao *et al.* 2001), humans (De Yebra *et al.* 1998) and cattle (Evenson, 1999a), where it has been found that sperm with sperm chromatin damage show abnormal chromatin de-condensation patterns and/or take longer to initiate pronucleus formation after fertilisation.

Studies by Ward *et al.* (2000) involving several nuclear destabilising agents indicated that even very subtle modification of sperm nuclear proteins prevented sperm from achieving fertilisation leading to embryonic development. Dithiothreitol (DTT) in the presence of an ionic detergent (ATAB) prevented sperm nuclei from being able to participate in embryogenesis, suggesting that a modification of a protein component(s) was the reason for failure of zygote formation (Ward *et al.* 2000).

It is well known that sperm heads isolated from tails are not capable of entering an oocyte but if such sperm are physically injected into the oocyte (e.g. intra-cytoplasmic sperm injection, ICSI) they are capable of fertilisation that is followed by embryonic development. The ability of ICSI to bypass oocyte-associated boundaries has facilitated the successful use of semen from males with severe oligozoospermia, teratozoospermia, azoospermia and immotile sperm cells (Engel *et al.* 1996). However, some studies have reported that impaired sperm quality, characteristic of ICSI patients, leads to a lower percentage of embryos that form blastocysts (Shoukir *et al.* 1998), poor blastocyst quality (Janny and Ménézo, 1994) and high rates of pregnancy failure (Sanchez *et al.* 1996).

It has been found that ejaculated sperm with compromised DNA integrity, regardless of the degree of DNA damage, have the ability to fertilise an oocyte at the same rate as normal sperm (Ahmadi and Ng, 1999). However, the degree of DNA damage does

significantly affect embryonic development, which has led to the proposal that although the oocyte has the capacity to repair some pre-existing DNA damage in fertilising sperm, if the damage is above a certain level then the oocytes repair capabilities may be inadequate, leading to a low rate of continued embryo development and high early pregnancy failure (Ahmadi and Ng, 1999).

Larson *et al.* (2000) found that no patients achieved pregnancy following IVF or ICSI if > 27% of sperm in a sample showed susceptibility to DNA denaturation by the SCSA. They also found that patients with morphologically abnormal sperm could achieve pregnancy following ICSI, as the SCSA thresholds for sperm chromatin abnormalities were 0-15%, indicating that the sperm DNA was capable of sustaining normal embryonic development.

In another study the SCSA data on 16 boars significantly correlated with farrowing rate and litter size, strongly suggesting the presence of an un-compensable nuclear factor resulting in embryo loss (Didion *et al.* 1999). Similar results have been found in studies on mice where no significant difference in the fertilisation capability of TP1-null (Yu *et al.* 2000) and TP2-null mice (Zhao *et al.* 2001) was found, compared to that of wild-type mice, but there was a significant decrease in the number of litters and the average litter size produced byTP1-null mice.

14.0 Sperm chromatin structure and fertility in bulls

Reproductive wastage, which is predominantly caused by fertilisation failure and early embryonic mortality, is a major cause of production loss in both dairy and beef cattle (Sreenan and Diskin, 1986). This wastage has generally been assumed to be due to 'female factor' infertility. However, it is now thought that 'male factor' infertility may contribute to a higher percent of reproductive wastage than originally thought.

Semen quality and its relationship to fertility are of major importance in the animal production industries (Ballachey *et al.* 1987). Cattle AI centres and industry acquire large numbers of young bulls each year that undergo breeding soundness evaluation (BSE). Despite the evidence that suggests the SCSA is an important parameter for predicting potential fertility, semen evaluation is typically confined to conventional semen parameters, as defined by the WHO, including morphology, concentration, motility and viability.

The failure to incorporate the SCSA technique at an industry-wide level can be attributed to two factors. First, there is a limited understanding of research that has clearly implicated DNA damage in otherwise normal appearing sperm as a major problem for achieving successful pregnancy (Evenson *et al.* 2002) and second the flow cytometry used in the SCSA is relatively expensive.

However, the SCSA should be considered for fertility assessment in bulls as studies have shown strong negative correlations between the susceptibility of sperm nuclear DNA to denaturation, as measured by the SCSA, and measures of bull fertility (Evenson, 1999a). One study showed that SCSA-defined chromatin damage in bovine spermatozoa negatively affects early embryo formation from what appear to be normally fertilised eggs (Ellington *et al.* unpublished).

Other studies have demonstrated that zygotes fertilised by bulls of high fertility (as determined by the non-return rate) are more likely to develop to the morula/blastocyst stage, start DNA synthesis (S phase) earlier, stay in S phase longer and have a shorter gap 2 (G2) phase and mitotic phase than those fertilised by low fertility bulls (Eid *et al.* 1994; Ostermeier *et al.* 2001; Ward *et al.* 2001). S-phase is the period of time during the cell cycle when DNA synthesis and replication occurs and is separated from mitosis by two gap phases (G1 and G2) (Figure 9). It was speculated that the differences observed between bulls of high fertility and low fertility in the zygotic cell cycle were due to varying levels of damaged DNA, un-replicated DNA or both (Eid *et al.* 1994).

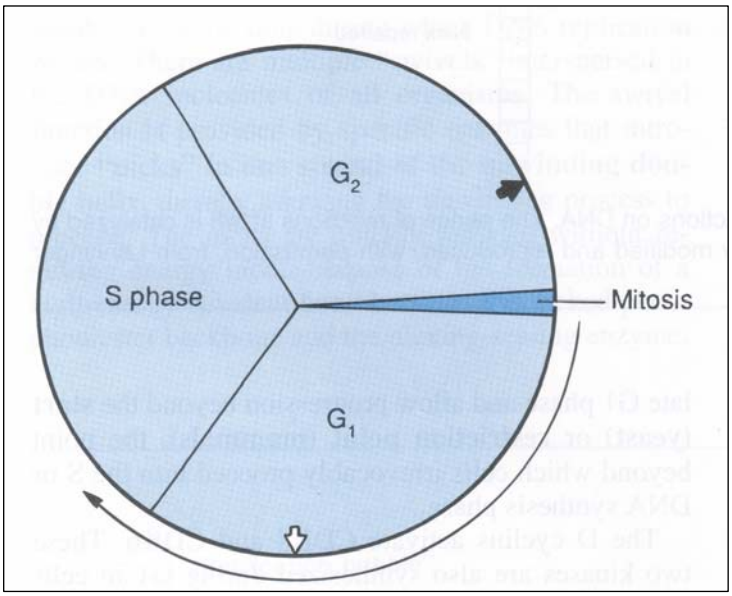

Figure 9. The mammalian cell cycle. The DNA synthetic phase (S phase) is separated from mitosis by gap 1 (G1) and gap 2 (G2). The arrow outside the circle indicates the direction of the progression of the cell cycle (Granner, 1996).

The above speculation is not unsubstantiated since a block or delay in G1 or G2 of the cell cycle has been associated with damaged DNA in somatic cells (Weinert and Hartwell, 1988, 1990; Shiestl et al. 1989 and Brown et al. 1991). It is also interesting to note that *in vivo* artificial insemination with semen from these bulls resulted in differences in conception rates despite the fact that sperm numbers inseminated were more than adequate for fertility (Eid et al. 1994). Nevertheless, the relationship between the zygotic cell cycle and sperm DNA abnormalities is yet to be fully established and despite the extensive literature on the link between sperm DNA damage and male infertility, there remains a great deal to be learnt about the relationship between sperm chromatin defects and early embryo loss in cattle.

15.0 Conclusions

The processes involved in the condensation of sperm chromatin in mammals are complex and unique. It is generally recognised that the greatest potential for abnormal sperm DNA and nucleoprotein interactions occurs during spermiogenesis when histones are replaced by TP's and then protamines (within the testis) and during the transit of sperm through the epididymis when protamines form intra- and inter-molecular disulfide bonds. It has also been demonstrated that disturbances to transcription and translation processes (i.e. phosphorylation-dephosphorylation, methylation) during spermiogenesis can affect the binding properties of nucleoproteins resulting in alterations to the packaging of chromatin in mature sperm.

There is evidence that sperm with normal conventional semen parameters, as outline by the WHO, can still have compromised DNA questioning the viability of using only WHO parameters to assess sperm to determine potential fertility. The SCSA is a technique that has proven, on a number of occasions, to be a statistically robust, repeatable and reliable test for the assessment of sperm chromatin integrity. It has been demonstrated that sperm samples showing ≥ 30% DNA damage, as determined by the SCSA, have a very low probability of sustaining viable embryo development. The SCSA can also be used to provide an indication of whether the sequential changes in binding of nucleoproteins to DNA during spermatogenesis have been successfully completed, by measuring how susceptible sperm DNA is to denaturation *in situ.* The contribution of sperm chromatin instability to reproductive wastage, both with natural and assisted reproduction, should be further investigated as it could prove valuable at an industry-wide level as a means of decreasing the incidence of embryonic mortality.

16.0 References

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