

2014

The clinical utility of the Halosperm assay and the development of a simplified method of human semen storage for the testing of sperm DNA fragmentation

Ashleigh Cavell McEvoy
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**THE CLINICAL UTILITY OF THE HALOSPERM ASSAY
AND THE DEVELOPMENT OF A SIMPLIFIED METHOD
OF HUMAN SEMEN STORAGE FOR THE TESTING OF
SPERM DNA FRAGMENTATION**

A thesis submitted in partial fulfilment of the requirements for the
degree of Master of Science (Human Biology)

by

Ashleigh Cavell McEvoy (BSc)

in the School of Medical Sciences

Faculty of Health, Engineering and Science

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April 2014

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Abstract

Male infertility is typically diagnosed upon routine semen analysis following the World Health Organisation's (WHO) semen analysis manual. Recent editions of the manual have essentially changed the diagnosis of a semen sample, prompting debate between experts as to which edition should be followed. Deoxyribonucleic Acid (DNA) integrity analysis is proving to be a useful adjunct to semen analysis as 15% of infertile men have a normal semen analysis but they have an increased DNA fragmentation level (DFL) which has been associated with increased disease incidence in any resultant offspring. However, such tests are not endorsed by the WHO, possibly due to a lack of standardisation in the implementation, analysis and clinical interpretation of methods used to evaluate DNA integrity. Improved efficiency of testing is achieved by batch testing or sending samples to a central laboratory for analysis, requiring an effective storage system. Most current protocols for semen storage and related DNA integrity testing are complex, expensive and require specialised equipment. Nevertheless, since the Halosperm® G2 Kit, requires only standard laboratory equipment, a simple, convenient and stable storage method for the purpose of testing sperm DNA fragmentation would be advantageous. DNA has been successfully extracted from air-dried semen and one particular study has investigated the use of air-dried semen slides as a method of storage prior to DNA fragmentation testing, however, the effects of time and temperature on the integrity of spermatozoa DNA has not been considered.

The first objective of this present study was to investigate the relationship between sperm DNA fragmentation (using the Halosperm® G2 Test Kit) and semen analysis results (measured according to the 4th and 5th Edition WHO semen

analysis manuals) to determine the clinical utility of the Halosperm assay. The second objective was to consider extrinsic effects on the DNA integrity of air-dried semen in order to develop an alternative storage method for semen prior to DNA fragmentation testing using the Halosperm assay.

A retrospective analysis was carried out on 905 consecutive semen samples with 4th and 5th Edition semen analysis and Halosperm result. Pearson correlations, analysis by ANOVA and post-hoc testing by Tukey's HSD were used for statistical analysis. Multiple aliquots of semen samples were prepared to achieve fresh, snap frozen and air-dried samples. Samples were sequentially assessed for sperm DNA fragmentation using the Halosperm® G2 kit (Halotech DNA SL, Spain) and scored against 300 sperm, with fragmentation results $\geq 30\%$ considered positive. Fragmentation levels were compared between the different protocols. Multiple aliquots of semen samples were then air-dried to test the fragmentation levels between different slide types, reconstituting fluids, times and temperatures. Pearson's correlation coefficient and paired t-tests were used for statistical analysis.

In summary, whilst significant associations exist between sperm DNA fragmentation and sexual abstinence, volume of the ejaculate, sperm concentration, normal sperm morphology and sperm motility, the Halosperm assay may provide an explanation for infertility where semen analysis cannot. Furthermore, air-drying semen is a simple and stable storage method, for up to one month at -22 degrees, prior to DNA fragmentation testing with the Halosperm® G2 kit.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

- i. Incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;
- ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
- iii. Contain any defamatory material.
- iv. Contain any data that has not been collected in a manner consistent with ethics approval.

The Ethics Committee may refer any incidents involving requests for ethics approval after data collection to the relevant Faculty for action.

Signed:

Date: 06-06-2014 .

Acknowledgements

Firstly, to the Fertility Society of Australia for the research grant which provided much of the funding to conduct this research.

To everyone who has given me their support, time and encouragement not only with this research project but also throughout my studies, I thank you most sincerely.

To my supervisors: Dr Peter Roberts and Dr Phillip Matson. I have been privileged to learn from you both. Thank you for your unwavering guidance and support and your belief in me. I will be eternally grateful.

To Kailin Yap, you taught me so many of the protocols used in this study without which none of this would have been possible. Your kind nature and crazy humour are wonderful.

To all the laboratory staff at Fertility North: Thank you for helping me collect and store the samples used in this study.

Finally my heartfelt thanks go to my family and friends. To my wonderful husband Adam, you have been an inspiration for me and I thank you for your love, understanding, encouragement and fortitude during the stressful times. To my beautiful daughter Becci, thank you for your love, patience and support my angel - I hope I make you proud. To each and every other member of my family and all my friends, you have each played an important role in this journey, and I thank you.

Table of Contents

Abstract	iii
Declaration.....	v
Acknowledgements.....	vi
Table of Contents	vii
List of Figures	x
List of Tables	xii
Abbreviations.....	xiii
1 Introduction.....	1
2 Literature Review	5
2.1 Human Infertility	5
2.2 Male Infertility	6
2.2.1 Human Semen	6
2.2.2 Mechanisms in Sperm Production and Ejaculation	8
2.2.3 Spermatozoon Anatomy	9
2.2.4 Damage to Sperm Genomic Material	10
2.3 Diagnostic Role of Semen Analysis	13
2.4 Relevance of Sperm DNA Damage	15
2.5 Evaluation of Sperm Nuclear DNA Integrity	15
2.5.1 Sperm Chromatin Structure Assay (SCSA)	16
2.5.2 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)	17
2.5.3 Single Cell Gel Electrophoresis Assay (Comet)	17
2.5.4 Sperm Chromatin Dispersion (SCD)	18
2.6 Clinical Utility of the Halosperm® G2 kit	19
2.7 Storage of Human Semen	21
2.7.1 Alternative Methods of Storage	22
2.8 Reconstitution Media	23
2.8.1 Seminal Plasma	24
2.8.2 Phosphate Buffered Saline and Bovine Serum Albumin	24
3 Summary and Theoretical Framework.....	26
4 Research Questions and Hypotheses	28
4.1 Research Question 1	28
4.1.1 Hypothesis	28
4.2 Research Question 2	28
4.2.1 Hypothesis	28
4.3 Research Question 3	28
4.3.1 Hypothesis	29
4.4 Research question 4	29
4.4.1 Hypothesis	29
5 Materials and Methods	31
5.1 Ethics Approval	31
5.2 Sample Collection	31

5.3	Clinical Utility of the Halosperm Assay	32
5.3.1	Semen Analysis	32
5.3.2	DNA Fragmentation Analysis	35
5.4	Quality Control of DNA Fragmentation Scoring	37
5.5	Processing Protocols	38
5.5.1	Freshly Ejaculated Semen	38
5.5.2	Snap Frozen/Thawed Semen	38
5.5.3	Air-dried Semen	39
5.5.4	Storage of Air-dried Slides	39
5.5.5	Reconstitution of Air-dried Slides	39
5.5.5.1	Seminal Plasma	40
5.5.5.2	Phosphate Buffered Saline (PBS) and Bovine Serum Albumin (BSA) Solution	40
5.6	Factors Affecting DNA Fragmentation Levels of Air-dried Semen	41
5.6.1	Reconstituting fluid	41
5.6.2	Slide Type	41
5.6.3	Semen Resource	43
5.6.3.1	Snap Frozen versus Freshly Ejaculated	43
5.6.3.2	Freshly Ejaculated versus Air-dried	43
5.6.4	Time and Temperature	44
5.6.4.1	Room Temperature: 1 Day versus 7 Days	44
5.6.4.2	4°C: 1 Day versus 7 Days	45
5.6.4.3	4°C: 1 Day versus 30 Days	45
5.6.4.4	-22°C: 1 Day versus 30 Days	46
5.6.4.5	Air-dried Semen Stored over Time at -22°C	47
5.7	Statistical Analysis	48
6	Results.....	49
6.1	Quality Control and Assay Precision	49
6.2	Mean Semen Analysis and DNA Fragmentation Results	54
6.3	Semen Analysis According to Different WHO Semen Analysis Guidelines 4 th and 5 th Editions	55
6.4	Relationship between DNA Fragmentation and Semen Analysis	57
6.5	Effects of Extrinsic Factors on Air-dried Slides	59
6.5.1	Comparing SDF between Different Reconstituting Fluids	59
6.5.2	Comparing SDF Levels between Slide Type	60
6.5.3	Comparing SDF between Processing Protocols	63
6.5.3.1	Snap Frozen Semen to Fresh Semen	63
6.5.3.2	Air-dried Semen to Fresh Semen	65
6.5.4	Comparing SDF between Different Times and Temperatures	67
6.5.4.1	Air-dried Semen Stored at Room Temperature: 1 Day compared to 7 Days	67
6.5.4.2	Air-dried Semen Stored at 4°C: 1 Day compared to 7 Days	68
6.5.4.3	Air-dried Semen Stored at 4°C: 1 Day compared to 30 Days	69
6.5.4.4	Air-dried Semen Stored at -22°C: 1 Day compared to 30 Days	70
6.5.4.5	Air-dried Semen Stored over Time at -22°C	71
7	Discussion	75

7.1	Summary	75
7.2	Clinical Utility of the Halosperm® G2 Kit	75
7.3	Quality Control and Assay Precision	77
7.4	Reconstitution Media	79
7.5	Effect of Slide Type	80
7.6	Snap Frozen Semen Storage	81
7.7	Air-dried Semen Storage	82
8	Limitations and Future Studies	83
9	Conclusion	84
10	References	85
	Appendix I: Participant Information Sheet and Consent Form	105
	Appendix II: SOP Halosperm Testing.....	109

List of Figures

Figure 1: Experimental protocols 30

Figure 2: Section from “Figure 1: Experimental protocols” - Determining the clinical utility of the Halosperm assay..... 32

Figure 3: Criteria to determine unfragmented and fragmented DNA in spermatozoa (Halotech DNA, Spain)..... 36

Figure 4: Section from “Figure 1: Experimental protocols” – Comparing the affects that an alternative reconstituting fluid and slide type has on DNA fragmentation levels of air-dried semen. 41

Figure 5: Section from “Figure 1: Experimental protocols” – Comparing the affect that semen resource in terms of processing has on DNA fragmentation levels of air-dried semen. 43

Figure 6: Section from “Figure 1: Experimental protocols” – Comparing the affect that time and temperature has on DNA fragmentation levels of air-dried semen..... 44

Figure 7: Comparing DNA fragmentation results to determine consistency of results between Halosperm runs..... 49

Figure 8: Bland Altman plot comparing DNA fragmentation obtained from the same semen sample processed and scored in duplicate by the same technician and within the same Halosperm run (n=34). 52

Figure 9: Difference of DNA fragmentation levels between technician 1 and technician 2. 53

Figure 10: Comparing DNA fragmentation levels to determine effects of reconstituting fluids..... 59

Figure 11: Comparing DNA fragmentation levels to determine effects of slide type after 1 day..... 60

Figure 12: Comparing DNA fragmentation levels to determine effects of slide type after 7 days..... 61

Figure 13: Comparing DNA fragmentation levels of snap frozen and fresh semen..... 63

Figure 14: Mean DNA fragmentation differences between snap frozen semen and fresh semen..... 64

Figure 15: Comparing DNA fragmentation levels of air-dried semen and fresh semen..... 65

Figure 16: Mean DNA fragmentation differences between air-dried semen and fresh semen.....	66
Figure 17: DNA fragmentation levels of air-dried semen stored at room temperature for 1 and 7 days.....	67
Figure 18: DNA fragmentation levels of air-dried semen stored at 4°C for 1 and 7 days.	68
Figure 19: DNA fragmentation levels of air-dried semen stored at 4°C for 1 and 30 days.....	69
Figure 20: DNA fragmentation levels of air-dried semen stored at -22°C for 1 and 30 days.	70
Figure 21: DNA fragmentation levels of 'QC1 High' (A) and 'QC2 High' (B) high value quality control samples measured at different time points from air-dried slides stored at -22°C.....	71
Figure 22: DNA fragmentation levels of 'QC1 Low' (A) and 'QC2 Low' (B) low value quality control samples measured at different time points from air-dried slides stored at -22°C.....	73
Figure 23: DNA fragmentation levels of 'QC1 Low' (A) and 'QC2 Low' (B) low value quality control samples measured after 28 days storage to show rapid increase in fragmentation after this time point.	74

List of Tables

Table 1: Cut off values for semen parameters according to WHO 4th Edition and WHO 5th Edition semen analysis criteria.	34
Table 2: Repeated Measures ANOVA showing variance among subjects differs significantly from the variance between measures of the same individuals confirming repeatability of the assay.	51
Table 3: Repeated Measures ANOVA showing variance among subjects differs significantly from the variance between measures of the same individuals confirming repeatability of the assay.	53
Table 4: Summary of semen analysis results and Pearson Correlation to DFL.....	54
Table 5: Matrix table showing semen analysis when scored following WHO 4th Edition and WHO 5th Edition semen analysis criteria. Refer to Table 6 for reference guide to abbreviations.....	55
Table 6: Reference guide to abbreviations used in tables 5, 7 and 8	56
Table 7: Mean DNA fragmentation levels according to semen quality when classified by WHO 4th Edition and WHO 5th Edition semen analysis criteria (n=905).....	57
Table 8: Proportion of abnormal DNA fragmentation levels (>30%) when classified by WHO 4th Edition and WHO 5th Edition semen analysis criteria.	58

Abbreviations

BSA	Bovine Serum Albumin
COMET	Single Cell Gel Electrophoresis Assay
DFI	Deoxyribonucleic acid Fragmentation Index
DFL	Deoxyribonucleic acid Fragmentation Level
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
ECU	Edith Cowan University
FN	Fertility North
g	Gram
HCl	Hydrochloric Acid
KCl	Potassium Chloride
KH ₂ PO ₄	Monopotassium Phosphate
L	Litre
LN ₂	Liquid Nitrogen
m	Milli
N	Normozoospermia
NA	Normoasthenozoospermia
NaCl	Sodium Chloride
Na ₂ HPO ₄	Sodium diphosphate
NT	Normoteratozoospermia
NAT	Normoasthenoteratozoospermia
O	Oligozoospermia
OT	Oligoteratozoospermia
OA	Oligoasthenozoospermia

OAT	Oligoasthenoteratozoospermia
PBS	Phosphate Buffered Saline
rpm	Revolutions per minute
ROS	Reactive Oxygen Species
SCSA	Sperm Chromatin Structure Assay
SCD	Sperm Chromatin Dispersion Assay
SDF	Sperm DNA Fragmentation
SEM	Standard error of the mean
SOP	Standard Operating Procedure
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay
WA	Western Australia
WHO	World Health Organisation
µm	Micrometer
°C	Degrees Celsius

1 Introduction

Infertility, which is defined as the failure to conceive a child subsequent to 12 months of unprotected intercourse between a couple of reproductive age (Singh & Agarwal, 2011), is recognised by the World Health Organisation (WHO) as a public health concern (Boivin, Bunting, Collins, & Nygren, 2007). Of all couples attempting to have children, between 17% and 25% will be diagnosed as infertile with approximately 50% of these cases being attributed to male infertility (Singh & Agarwal, 2011; Venkatesh et al., 2011).

Male infertility has traditionally been diagnosed on the basis of a number of standard parameters including seminal volume, pH, morphology, motility and concentration as recommended by the WHO's laboratory manual which focuses on the examination of human semen (World Health Organisation, 2010), hereafter referred to as the guidelines or manual. Periodically, new manuals are released; the first being published in 1980 with updates being released in 1987, 1992, 1999 and the 5th Edition in 2010 (Esteves, Zini, et al., 2012). The 5th Edition guidelines radically change semen analysis interpretation so that patients diagnosed with an abnormal semen analysis under 4th Edition guidelines may be diagnosed as having a normal semen analysis using 5th Edition criteria. The reference values published prior to the 5th Edition were derived from vague reference populations and consequently lack clarity, resulting in some confusion (Catanzariti, Cantoro, Lacetera, Muzzonigro, & Polito, 2013; Cooper et al., 2010) which has been acknowledged by the WHO itself (Esteves, Zini, et al., 2012; Menkveld, Holleboom, & Rhemrev, 2011). Despite clearly defined reference ranges being included in the 5th Edition, debate has ensued over its legitimacy, with concerns being raised

regarding the basis upon which these reference ranges were built (Eliasson, 2010). Therefore there is no definitive consensus between laboratories on which edition (4th or 5th) should be used (Lu, Zhang, Hu, Huang, & Lu, 2010).

However, regardless of which reference values are used, routine semen analysis does not consider sperm DNA integrity and therefore, whilst it plays an integral role in diagnosing male infertility (De Jonge, 2012), it has its limitations. Research has shown that approximately 15% of infertile men tested have semen with normal semen analysis parameters (Omran, Bakhiet, & Dashti, 2013; Schulte, Ohl, Sigman, & Smith, 2010). As a result, there has been considerable focus on sperm DNA fragmentation and its association with reproductive outcomes. Increased levels of DNA damage have been correlated with infertile men (Chohan, Griffin, Lafromboise, De Jonge, & Carrell, 2006; Schulte et al., 2010; Shamsi, Imam, & Dada, 2011; Tamburrino et al., 2012; Yilmaz et al., 2010; Zini, 2011), severe semen defects (Mangiarini et al., 2013; Sun, Jurisicova, & Casper, 1997; Varshini et al., 2012), poor fertilisation rates (Bakos, Thompson, Feil, & Lane, 2007; Lewis et al., 2013), increased risk of pregnancy loss after assisted reproductive technology (ART) (Lewis et al., 2013; Robinson et al., 2012; Zini, Boman, Belzile, & Ciampi, 2008) and negative results in both ART (Simon et al., 2013) and natural conception (Barratt et al., 2010). Consequently, tests focusing on sperm DNA fragmentation (SDF) measurement have been proposed to be a valuable adjunct to routine semen analysis when attempting to evaluate male infertility (Lewis et al., 2013; Schulte et al., 2010).

Since the first proposal more than thirty years ago that suggested changes in sperm chromatin structure may be associated with reduced fertility rates (Evenson, Darzynkiewicz, & Melamed, 1980), a number of tests to measure sperm DNA integrity have been developed; the sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphates nick-end labelling (TUNEL), single cell gel electrophoresis (Comet) and sperm chromatin dispersion (SCD) assays (Chohan et al., 2006). Although none of these assays have been endorsed by the WHO, it acknowledges the use of several of these for "testing the regularity of sperm chromatin and DNA" (2010, p. 157). Conversely, with such an array of tests available, it has become increasingly evident that the standardisation of tests and uniformity in terms of clinical interpretation are now required (Boe-Hansen, Ersbøll, & Christensen, 2005; Shamsi et al., 2011). Until such time as there is standardisation in the execution, analysis and clinical interpretation of DNA fragmentation assays it is unlikely that DNA fragmentation testing will be formally accepted as a part of routine diagnostic testing in andrology laboratories worldwide (Fernández et al., 2005).

Factors which have likely prohibited DNA integrity tests from being standardised before now include the requirement of specialised equipment to not only carry out such tests (Boe-Hansen et al., 2005) but also to store and transport samples (Aitken, Allan, Irvine, & Macnamee, 1996; Riel, Yamauchi, Huang, Grove, & Ward, 2011; Royster et al., 2000). The optimisation of methods used to assess DNA integrity and the development of a simplified method of semen storage is therefore particularly significant to ART.

Whilst sperm DNA integrity can be assessed in a number of different ways, each has its benefits and limitations. In terms of simplicity, cost effectiveness and availability however, only the commercially available kits based on the sperm chromatin dispersion test appear to fulfil these criteria. Other DNA fragmentation assays require either specialised equipment, are labour intensive and/or are complex (Chohan et al., 2006).

Current methods of semen storage require either liquid nitrogen (LN₂) or dry ice entailing high costs or complicated logistical implications (Aitken et al., 1996; Dondero et al., 2006; Royster et al., 2000) and hence a simplified storage method would be advantageous. In the forensic setting, DNA has been successfully extracted from air-dried saliva (Walsh et al., 1992) and semen (Giusti, Baird, Pasquale, Balazs, & Glassberg, 1986; McNally et al., 1989) and is routinely recovered from penile and vaginal swabs which are air-dried (Martin et al., 2006). Additionally, Yap and Matson (2012) have shown that air-dried semen can be successfully air-dried onto a glass microscope slide, reconstituted with seminal plasma and then used to assess sperm DNA fragmentation levels using the Halosperm[®] G2 Test Kit.

This present study aimed to formally investigate the clinical utility of the Halosperm[®] G2 assay using both 4th and 5th Edition WHO Semen analysis guidelines and to evaluate alternative methods for short-term storage of human semen prior to testing DNA fragmentation with the Halosperm[®] G2 Test Kit. Extrinsic factors such as the storage protocol, nature of reconstituting fluid and stability over time and temperature were also considered.

2 Literature Review

2.1 Human Infertility

The likelihood of a healthy couple of reproductive age having unprotected sexual intercourse and achieving pregnancy for each reproductive cycle is approximately 20 to 25% with increasing probabilities of conception over time: 60% in the first 6 months, 84% within the first 12 months and 92% in the first 48 months (Kamel, 2010). Infertility or subfertility is diagnosed in a couple who have not achieved conception after 12 months of unprotected regular sexual activity where the female partner is under 35 years of age, or after 6 months if she is above 35 years of age (Kamel, 2010), or immediately where an obvious cause is evident (Speroff, Glass, & Kase, 1999). This obvious cause being shared equally between men and women (Evenson, Larson, & Jost, 2002). There are two different types of infertility: Primary infertility which is determined when a couple has not previously had a live birth and secondary infertility when the same couple has had at least one live birth previously (Buy & Ghossain, 2013).

A number of different factors can cause infertility, some of which include: defects relating to genetics, urogenital and reproductive systems, gametogenesis, gamete function, fertilization and embryonic development (Matzuk & Lamb, 2008). Additionally, lifestyle factors have been shown to affect fertility in both men and women (Ferreira et al., 2010). In most world regions, infertility incidence was similar in 2010 to that in 1990 (Mascarenhas et al., 2013), however, reports suggesting an increase in male subfertility are of concern (Sharpe & Irvine, 2004), particularly since there appears to be a common belief that subfertility has changed since the WHO introduced lower reference ranges when analysing human

semen (Barratt, Björndahl, Menkveld, & Mortimer, 2011). Notwithstanding this, dramatic advances have been made in ART with the first baby to be born by in vitro fertilization in 1978, followed by the introduction of intracytoplasmic sperm injection (ICSI) in 1992. ICSI essentially circumvented male factor infertility (Varghese, du Plessis, & Agarwal, 2008). Not surprisingly therefore, assisted reproductive technology (ART) appears to be increasing with 4% more couples per year attempting ART (Lewis et al., 2013).

2.2 Male Infertility

In couples of reproductive age with fertility related issues, male infertility has been found to be responsible for 50% of cases, whether directly or indirectly (Singh & Agarwal, 2011) with more than half of these cases being idiopathic (Poongothai, Gopenath, & Manonayaki, 2009). Any aspect that disrupts the process of sperm production and/or its quality has the potential to cause male infertility with the major causes including varicocoele, cryptorchidism, testicular failure, genital tract obstruction, ejaculatory dysfunction, gonadotrophin exposure, hormone dysfunction, infections, genetic conditions, cancer and systemic diseases (Esteves, Miyaoka, & Agarwal, 2011).

2.2.1 Human Semen

Human semen is primarily composed of seminal fluid, secreted by a number of different accessory glands and spermatozoa which are produced in the testis (Johnson, 2013). The accessory glands comprise the seminal vesicles, prostate and bulbourethral glands which contribute approximately 60%, 30% and 5% of the volume of seminal fluid respectively (Martini & Nath, 2011). When ejaculated, these components are not entirely mixed and the semen therefore is not a fully

homogenous fluid (Fleming & Cooke, 2009). Within 15 to 60 minutes at room temperature after ejaculation however, semen will liquify, resulting in a more homogenous liquid. An average ejaculate is between 2ml and 5ml and dependent on the concentration of spermatozoa, characteristically has a grey-opalescent appearance (World Health Organisation, 2010). Human semen however is known to vary between ejaculates of different men and can also vary between ejaculates of the same man at different time points (Amann, 2009; World Health Organisation, 2010). From a biological perspective, such variations are attributed to many different factors including the composition of semen, the source of these components and the secretions associated with them (Björndahl, 2011).

Whilst the primary role of the spermatozoon is to deliver paternal genetic material to the oocyte at fertilisation (Ramalho-Santos et al., 2007), the seminal fluid has no direct role in fertilisation, despite being a complex biological fluid composed of inorganic and organic constituents which allow it to fulfill several different functions (Rolland et al., 2012). The seminal fluid instead acts as a transport medium for spermatozoa, is actively involved in the maturation process of the spermatozoa, provides an energy source from fructose and due to its prostaglandin levels, protects the spermatozoa from the acidic nature of vaginal fluids (Fleming & Cooke, 2009; Mann, 1964; Owen & Katz, 2005; Rolland et al., 2012). In addition to these functions, antioxidant enzymes within seminal plasma have been shown to have a protective role against lipid peroxidation with sperm chromatin being stabilised because of its zinc content (Rolland et al., 2012; Tavilani, Goodarzi, Vaisi-raygani, Salimi, & Hassanzadeh, 2008).

2.2.2 Mechanisms in Sperm Production and Ejaculation

Under normal conditions, spermatozoa are produced in the seminiferous tubules within the testes, transforming diploid spermatogonial germ cells into haploid spermatozoa in a complex biological process of cellular transformation referred to as spermatogenesis (Hess & de Franca, 2008). This is a process of precisely timed and extremely organised cycles whereby each stem cell first multiplies by continual mitotic divisions followed by meiosis 1 and 2 in half the cells resulting from these mitotic divisions. The remaining cells are used to renew their own population. Meiosis entails chromosomal duplication, genetic recombination and a reductional division to produce four haploid spermatids in a cycle which lasts around 12 days (Esteves et al., 2011). Spermatids will then differentiate into spermatozoa, via spermiogenesis (Hess & de Franca, 2008). During spermiogenesis, the cell undergoes dramatic changes in terms of structure and function. The majority of the cell's cytoplasm is shed, the nucleus elongates, the acrosome and mid-piece are formed, and the chromatin is condensed (Marcon & Boissonneault, 2004). The highly condensed spermatozoa are then released into the lumen of the seminiferous tubules prior to storage in the epididymis to begin maturation until the time of ejaculation (Hess & de Franca, 2008). The spermatozoa however, will only complete maturation within the female reproductive tract (Esteves et al., 2011). According to Mortimer et al., (2013) this is not an efficient storage process as dependent on the individual, spermatozoa become moribund or die after a relatively short period. As a result, without ejaculation occurring every few days, spermatozoa in an ejaculate may well be compromised (Mortimer et al., 2013). Under sympathetic stimulation, ductal and epididymal muscles contract conducting the spermatozoa to the prostatic urethra.

Fluids secreted by the male reproductive glands (seminal vesicles, prostate and bulbourethral) are combined with the spermatozoa to form semen. The semen is then expelled through the urethra under the influence of periurethral muscle contraction (Esteves et al., 2011). This process, from the beginning of sperm production to ejaculation is around 60 to 70 days (Misell et al., 2006). Male infertility may result from any interference in any step throughout the entire process from sperm production to ejaculation (Esteves, Hamada, Kondray, Pitchika, & Agarwal, 2012).

2.2.3 Spermatozoon Anatomy

Although a deceptively simple cell, the mature spermatozoon is highly specialized (Martini & Nath, 2011) and plays a vital role in the continuance of mammalian species with its primary purpose being to deliver an intact complement of the paternal haploid genome to the oocyte at fertilization (Ramalho-Santos et al., 2007). Unlike somatic cells, mature spermatozoa lack intracellular structures permitting optimal size, mass and motility. Typical spermatozoa are approximately 50µm in length and are organized into three distinctive regions known as the head, middle piece and tail (Martini & Nath, 2011).

The majority of the mature spermatozoon head is taken up by a flattened and elongated nucleus (Martini & Nath, 2011) which contains highly condensed chromatin with deoxyribonucleic acid (DNA) being arranged in a specific manner, quite different to that of somatic cells (Ramalho-Santos et al., 2007; Zini & Libman, 2006). The DNA in spermatozoa is stabilised by protamines which counterbalance the repulsion of phosphodiester bonds in the backbone of DNA allowing the DNA to fold up onto itself and create tightly wound toroids. The protamine toroids are

arranged in linear arrays, further maximising compaction. A small percent of DNA is bound to histones, however, the majority of these are replaced by protamines during the maturation process to provide even tighter compaction (Singh & Agarwal, 2011). As a result of this differentiation during spermatogenesis a predominantly protamine based DNA packaging arrangement is evident, as opposed to the histone packaging configuration in somatic cells (Balhorn, 2007). In the mature spermatozoon, approximately 4% of the DNA is histone bound (Singh & Agarwal, 2011). The paternal genome therefore is somewhat protected during the transportation from male to female reproductive tracts (Schulte et al., 2010) which eases the delivery and transference of genetic information to the oocyte (Miller, Brinkworth, & Iles, 2010). DNA integrity of spermatozoa is however vulnerable to a number of environmental and lifestyle factors which may result in abnormal chromatin structures, prior to being ejaculated from the male (Agarwal & Said, 2003).

The balance of the mature spermatozoon is made up of the middle piece containing condensed mitochondrial rods arranged around axial filaments which provide energy for the tail to “lash” providing mobility to the spermatozoon (Martini & Nath, 2011).

2.2.4 Damage to Sperm Genomic Material

DNA damage in spermatozoa may range from chromosomal aberrations such as deletions and aneuploidies to epigenetic modifications and mutations to single and/or double strand DNA breaks which is commonly referred to as sperm DNA fragmentation (SDF) (Sharma, Said, & Agarwal, 2004). It is SDF which is considered to be the most common of all DNA damage in spermatozoa that results

in abnormal transmission of paternal DNA (Tamburrino et al., 2012). Although much research has focused on SDF in ejaculated spermatozoa (Chohan et al., 2006; Evenson et al., 1999; Sakkas, 1999; Simon et al., 2010; Smit, Romijn, Wildhagen, Weber, & Dohle, 2010; Venkatesh et al., 2011) and various mechanisms have been linked with it, the exact source and nature of such damage remains controversial (Sharma et al., 2004).

A number of mechanisms that have been suggested to explain the incidence of these anomalies in the human ejaculate include damage to chromatin packaging induced during the maturation process (Singh & Agarwal, 2011; Twigg, Fulton, Gomez, Irvine, & Aitken, 1998), oxidative stress and abortive apoptosis (Aitken, Jones, & Robertson, 2012; Sharma et al., 2004; Singh & Agarwal, 2011).

A disparity between antioxidant capacity and the production of reactive oxygen species (ROS) in spermatozoa causes oxidative stress (Agarwal, Saleh, & Bedaiwy, 2003; Paick, 2003) and practically every human ejaculate is tainted with potential sources of ROS which can cause chromosome deletions, chromatin cross-linking and DNA base oxidation (Agarwal & Said, 2005). Additionally, ROS plays an important role in mediating apoptosis which sequentially results in a high incidence of both single and double stranded DNA breaks (Aitken & Baker, 2002). Since spermatozoa have little cytoplasm, there is a lack of cytoplasmic antioxidants and as such the spermatozoon is vulnerable to oxidative stress brought about by ROS. Furthermore, unsaturated fatty acids which attract unwarranted ROS are common in the spermatozoon's plasma membrane. This mechanism brings about oxidative stress resulting in degradation of the cell membrane and therefore

weakening the spermatozoon's defence mechanisms (Singh & Agarwal, 2011). Increased levels of ROS have also been correlated with reduced sperm motility (Agarwal, Ikemoto, & Loughlin, 1994; Armstrong et al., 1999; Lenzi, Lombardo, Gandini, Alfano, & Dondero, 1993), however the exact mechanism of action is not fully understood (Makker, Agarwal, & Sharma, 2009).

Apoptosis is a key mechanism to control the number of proliferating germ cells and to remove old or defective spermatids and spermatozoa via activation of Fas surface proteins and associated ligands (Agarwal & Said, 2003). Inappropriate Fas-ligand production may result in spermatozoa with damaged DNA avoiding apoptosis and being integrated into the gene pool (Singh & Agarwal, 2011).

Owing to the intrinsic nature of chromatin compaction and organisation, strand breaks are a common form of DNA damage in spermatozoa. The tension on the phosphodiester backbone created by the formation of toroids can only be released by single strand breaks. These sporadic breaks allow the DNA to further fold on itself, adding to increased compaction. Where insufficient mechanisms are in place to eliminate such cells, (such as apoptosis) spermatozoa in the ejaculate will have increased levels of DNA fragmentation (Singh & Agarwal, 2011).

Many external environmental factors have also been shown to cause SDF such as environmental toxicants (Evenson & Wixon, 2005), heat stress (Pérez-Crespo, Pintado, & Gutiérrez-Adán, 2008; Singh & Agarwal, 2011) and radiation (Singh & Agarwal, 2011). Recreational drugs, cigarette smoke, certain medications and coffee contain chemicals which can also impact on the DNA in spermatozoa, either

by directly causing strand breaks or via oxidative methods (Singh & Agarwal, 2011).

None the less, fertile men have been shown to have some degree of SDF and infertile men substantially more SDF than their fertile counterparts (Fernández-Gonzalez et al., 2008; Tamburrino et al., 2012; Zini & Libman, 2006). Hence the level of DNA fragmentation should play a role in the clinical management of male infertility (Evenson & Wixon, 2006).

2.3 Diagnostic Role of Semen Analysis

Semen analysis has long been regarded as the diagnostic cornerstone for male factor infertility (De Jonge, 2012) and it is well documented that it should be carried out according to standardised procedures (Barratt et al., 2010; Castilla et al., 2006; Keel, 2002). As such, more recent diagnosis has predominantly been based on routine semen analysis as described by the WHO laboratory manual 4th (1999) and/or 5th (2010) Editions. These guidelines outline standardised parameters to characterise normal spermatozoa (Singh & Agarwal, 2011) and in its most basic part describes the testicular function (De Jonge, 2012) with focus on sperm concentration, motility and morphology (Cooper et al., 2010). Due to the guidelines having radically changed between these two editions, a man presenting with an abnormal semen analysis under 4th Edition guidelines may be considered to have a normal semen analysis when tested using 5th Edition guidelines.

There are differing opinions on the value of the 5th Edition (Barratt et al., 2011; Eliasson, 2010; Jequier, 2010; Lewis et al., 2013) and consequently not all laboratories have formally adopted the latest guidelines (Lu et al., 2010). One

particular concern over the WHO 5th Edition reference values is due to the reference population, despite this being significantly better than previous editions (Lewis et al., 2013; Penn et al., 2011). It is worth noting, that the European Society of Human Reproduction and Embryology (ESHRE) basic semen analysis course material is produced by the ESHRE and Nordic Association for Andrology (NAFA), the standard of which is grounded in protocols defined by the WHO and *Practical Laboratory Andrology* (Barratt et al., 2011; Mortimer, 1994). Previously ESHRE and NAFA standards have been updated in response to new findings, however recently they have specifically deviated from the WHO 5th Edition because they suggest that some factors are scientifically and clinically inappropriate (Barratt et al., 2011).

According to WHO guidelines a semen analysis performed on an infertile man may reveal a number of abnormal conditions ranging from oligozoospermia (low concentration), asthenozoospermia (reduced motility), teratozoospermia (poor morphology) or an amalgamation of each of these such as oligoasthenoteratozoospermia (low concentration with reduced motility and poor morphology) (Poongothai et al., 2009). Conversely, standard semen parameters can reveal a high degree of variability (Zini, 2011) and have been considered as only a poor predictor of fertilising potential and a meagre predictor of reproductive outcomes (Guzick et al., 2001). Furthermore, some infertile men have been shown to have a normal semen analysis (Avendaño et al., 2009; French, Sabanegh Jr., Goldfarb, & Desai, 2010).

In an attempt to improve male infertility diagnosis, sperm DNA fragmentation has become one of the most researched sperm factors in the last decade (Agarwal &

Said, 2011) and is now regarded by some as a more promising test (Barratt et al., 2010; Evenson, Kasperson, & Wixon, 2007; Lewis et al., 2013).

2.4 Relevance of Sperm DNA Damage

Whilst studies have shown that spermatozoa with compromised DNA are associated with reduced fertilisation rates (Ji et al., 2012), longer times to conceive and early pregnancy loss (Simon et al., 2013), advances in ART have allowed spermatozoa with an altered genome to fertilise oocytes *in vitro* (Lewis et al., 2013). Although oocytes can repair a certain amount of sperm DNA damage (Menezo, Dale, & Cohen, 2010), residual DNA damage from the male gamete can still be translated into chromosomal aberrations and gene mutations after fertilisation, amplifying the possibility of genetic diseases, cancer and developmental defects in the offspring (Aitken, Baker, & Sawyer, 2003). Furthermore, children conceived by ICSI have a higher occurrence of disease than those spontaneously conceived (Basatemur & Sutcliffe, 2008; Katari et al., 2009) with literature showing a link between the higher occurrence and the father's sperm (Aitken, De Iuliis, & McLachlan, 2009). Therefore, quantifying SDF levels not only provides a guide for ART treatment (Sharma et al., 2004) but also protects the integrity of future generations.

2.5 Evaluation of Sperm Nuclear DNA Integrity

A number of different methods to assess sperm DNA damage have been devised, however, the SCSA, TUNEL, and Comet are recognised as the most commonly used (Sharma et al., 2004; Venkatesh et al., 2011). The SCD assay although being a relatively new technique (Chohan et al., 2006) appears to have gained popularity due to its simplicity, cost effectiveness (Absalan et al., 2012), reproducibility and

the fact that its results are highly correlated with the SCSA (Fernández et al., 2005). Despite technical differences between each of these assays, they all fundamentally detect damaged DNA in spermatozoa (Dugum, Sandlow, & Brannigan, 2011). It is important however to consider not only the strengths and weaknesses of each assay, but also how well they correlate with each other when considering how DNA fragmentation testing can be standardised.

2.5.1 Sperm Chromatin Structure Assay (SCSA)

SCSA is based on the principle that sperm DNA is susceptible to low pH. Spermatozoa are therefore subjected to an acid treatment to denature the DNA and then stained using a metachromatic dye named acridine orange (Evenson et al., 1980). Using flow cytometry, fluorescence signals are then measured whereby denatured single stranded DNA will emit a red signal whilst intact and double stranded DNA breaks will emit a green signal. Quantifying the metachromatic shift from green to red fluorescence provides the extent of DNA denaturation (Agarwal & Said, 2003) which is commonly referred to as the DNA fragmentation index (DFI); the higher the DFI value, the higher the percent of cells with DNA damage (Evenson et al., 2002). Semen samples with a DFI of >30% using this method are associated with a reduced level of pregnancy success (Evenson & Wixon, 2006). Whilst the SCSA has been clinically validated for statistical robustness (Shamsi et al., 2011), according to some experts in the field, the equipment requirements of a flow cytometer and its associated software and technical expertise are its foremost limitations (Fernández et al., 2005; Shamsi et al., 2011) and which for some andrology laboratories remains unattainable due to cost factors (Chohan et al., 2006).

2.5.2 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

The TUNEL assay is based on the principle of attaching a labelled nucleotide, deoxyuridine triphosphate (dUTP) to the 3' OH end of single and double stranded DNA breaks by means of the template-independent enzyme terminal deoxynucleotidyl transferase (TdT). The incorporated dUTP is labelled with a fluorescent tag which produces a signal allowing the breaks to be measured by flow cytometry, fluorescent microscopy or bright field microscopy (Gorczyca, Gong, & Darzynkiewicz, 1993; Shamsi et al., 2011). The strength of the signal illustrates the number of strand breaks; spermatozoon heads with highly fragmented DNA will emit a bright fluorescent light whilst sperm with normal chromatin integrity produce only background fluorescence (Hekmatdoost, Lakpour, & Sadeghi, 2009).

The most recognised limitation to this assay is that the TdT is unlikely to access all 3'OH ends due to the highly compacted nature of sperm chromatin (Mitchell, De Luliis, & Aitken, 2011) and because there is no lysis of the spermatozoon membrane, the chromatin remains highly compacted (Tesarik, Mendoza-Tesarik, & Mendoza, 2006). Zini and Sigman (2009) advocate that whilst fluorescent microscopy or flow cytometry are not necessary, a consequence of having variable assay protocols is that thresholds are not standardised.

2.5.3 Single Cell Gel Electrophoresis Assay (Comet)

The Comet assay involves staining spermatozoa with a fluorescent DNA-binding dye (Fluorochrome), suspending these in an agarose gel and then subjecting them

to electrophoresis. The fragmented DNA will migrate during electrophoresis, with shorter fragments or low-molecular weight DNA moving further through the gel toward the positive anode than high-molecular weight intact DNA. Sperm with fragmented DNA will therefore display a comet tail characteristic (Klaude, 1996). Using imaging software, the comet tail is measured by length and fluorescence intensity to determine the level of DNA fragmentation (Schulte et al., 2010). Whilst the main advantage of the Comet assay is that it is a direct method that quantifies both single and double stranded breaks (Agarwal & Said, 2003), according to Shamsi et al., (2011), the labour intensity of this assay is a major limitation.

2.5.4 Sperm Chromatin Dispersion (SCD)

The SCD assay determines the vulnerability of sperm DNA to acid denaturation and follows the principle that induced condensation is directly related to sperm DNA fragmentation (Muriel et al., 2006). Sperm are suspended in an agarose micro gel before being placed on an agarose pre-treated slide which renders the sperm in a “suspension-like environment” on the slide (Fernández et al., 2005, p. 834). An acid solution is applied to the agarose/sperm matrix to denature the DNA followed by treatment with a lysis buffer to lyse cell membranes and remove nuclear proteins. As a result a halo of dispersed DNA loops surround the central core which can be viewed under bright field microscopy after staining the slides with Wright’s stain. Sperm with intact DNA will show as having large halos whilst those with elevated DNA fragmentation will generate no halo or at the most a very small halo (Fernández et al., 2005).

The most commonly cited advantages of the Halosperm® G2 assay (which is modelled on the SCD assay) include the relatively short and simple protocol, the requisite of only standard laboratory equipment and its cost effectiveness (Fernández et al., 2005; Shamsi et al., 2011). The only limitation identified by Chohan et al., with this assay, is inter-observer subjectivity (Chohan et al., 2006).

Chohan and colleagues (2006) have shown a significant correlation ($r > 0.866$ and $P < 0.001$) for sperm DNA fragmentation on the same samples between SCSA, TUNEL and SCD. In contrast however Sharma and colleagues (2004) claim a significant correlation between the SCSA, Comet and TUNEL assays but do not provide any statistical evidence, nor do they comment on the SCD assay.

2.6 Clinical Utility of the Halosperm® G2 kit

The ultimate sperm function test or series of tests should determine the reason for male factor infertility, predict the pregnancy rate and guide the clinician towards the therapeutic route which would alleviate the dysfunction (Franken & Oehninger, 2012). Whilst a standard semen analysis may assist a clinician in selecting the treatment approach for some infertile couples, repeatedly normal results in other couples may go unexplained, providing the clinician with little direction (Saleh et al., 2002). A number of studies have been undertaken to assess the relationship of specific semen analysis factors and DNA fragmentation such as motility (Peluso et al., 2013), morphology (Franken & Oehninger, 2012; Mangiarini et al., 2013), motility and morphology (Avendaño et al., 2009; Avendaño & Oehninger, 2011; Irvine et al., 2000; Lopes, Sun, Jurisicova, Meriano, & Casper, 1998; Tandara et al., 2013) with none having considered all semen analysis factors

in the same study (volume, concentration, motility and morphology) and several having used alternative DNA fragmentation assays to the Halosperm assay (Avendaño et al., 2009; Avendaño & Oehninger, 2011; Lopes et al., 1998; Mangiarini et al., 2013; Saleh et al., 2002). Furthermore, no study has considered the association of DFL (DNA fragmentation level) to semen quality as classified by both the WHO 4th and 5th Edition semen analysis guidelines. Catanzariti and colleagues (2013) have assessed the difference in semen quality (considering count, motility and morphology) using both these editions, however they did not compare this to DNA fragmentation levels. Due to the debate surrounding the WHO 4th and 5th Editions and the fact that the WHO laboratory manual for the examination and processing of human semen has long been regarded as the 'gold standard' (Walczak-Jedrzejowska et al., 2013) plus the WHO 4th Edition guidelines are predominantly followed in the laboratory where this research was carried out, the inclusion of both editions are warranted when comparing DFLs to WHO reference ranges. This comparison will provide some insight into the clinical utility of the Halosperm assay.

One particular study that has assessed concentration, motility and morphology in relationship to DNA fragmentation levels used the SCSA method and only considered the WHO 4th Edition guidelines when determining the normality of semen analysis parameters (Saleh et al., 2002). The results of this study indicated that poor semen quality (based on 4th Edition guidelines) was associated with increased DFL. Using the TUNEL assay, Avendaño and colleagues (2009) reported that morphologically normal and highly motile spermatozoa (as categorised by the WHO 4th Edition guidelines) may have DNA fragmentation and infertile men with

severe teratozoospermia had significantly higher DNA fragmentation levels. Based on WHO 5th Edition guidelines, using the TUNEL assay, Mangiarini et al., (2013) also concluded that teratozoospermic samples had a significantly higher incidence of DNA fragmentation than normozoospermic samples. Using the Halosperm assay, Peluso and colleagues (Peluso et al., 2013) showed a significant correlation between percentage spermatozoa motility (based on 4th Edition guidelines) and the DFL, as did Tandara et al., (2013). Interestingly, Catanzariti et al., (2013) concluded that the 5th Edition guidelines did not change the final verdict of semen analysis from the 4th Edition guidelines since concordance was very high when all three parameters (concentration, motility and morphology) were considered together and when each parameter was analysed on its' own.

2.7 Storage of Human Semen

Although the protocols of semen storage in LN₂ have advanced substantially since Jahnke's first report in 1938 of successful long term storage of human spermatozoa (Macpherson, 1960), the use of LN₂ remains a costly process which entails complicated logistical and technical implications (Aitken et al., 1996; Dondero et al., 2006; Royster et al., 2000). Whilst LN₂ may be mandatory in cases where spermatozoa must be preserved for subsequent insemination, e.g. donor sperm insemination, prior to cancer therapy and where the male partner spends long periods of time away from his female partner (Boe-Hansen et al., 2005), alternative methods of storage are possible where only the DNA must be preserved (Imoedemhe, Holiva, & Adam, 2004).

2.7.1 Alternative Methods of Storage

A study conducted by Imoedemhe and colleagues (2003) demonstrated that it is possible to achieve successful fertilization of human oocytes via intra-cytoplasmic sperm injection with sperm that had been air-dried and stored for four weeks at 21°C or 8°C. Following these observations, they continued their research to assess the long term storage of air-dried sperm to determine its effects on the morphology of the spermatozoa and reported no evidence of deterioration in air-dried spermatozoa which was stored at 0°C for ten months and at 8°C for six months before being resuspended in Earle's medium (Imoedemhe et al., 2004). Whilst this study has provided valuable information concerning air-dried semen samples, the DNA integrity following these storage conditions was not considered. Furthermore, the study size was a limiting factor as only ten semen samples were analysed (Imoedemhe et al., 2004).

In the forensic setting, DNA has been successfully extracted from air-dried semen (Giusti et al., 1986; Martin et al., 2006; McNally et al., 1989) and is often recovered from penile and vaginal swabs which are typically exposed to air (Martin et al., 2006). Additionally, Robbins and colleagues (1993) have effectively performed fluorescence in situ hybridisation on air-dried semen microscope slides. Such evidence suggests that sperm DNA remains unchanged when semen is air-dried.

A preliminary study by Yap and Matson (2012) ascertained that human semen can be air-dried on flat microscope slides for short term storage prior to sperm DNA fragmentation testing using the Halosperm G2 kit. The research compared DNA fragmentation results in snap frozen/thawed semen samples in LN₂ with air-dried semen samples from the same men and reported a significant correlation ($r=0.982$,

p=0.000). Moreover, they advocated that further analysis was required, including the effects of time and temperature on air-dried semen and the use of alternative reconstituting fluids to further refine this technique. Consequently, this present study aimed to formally evaluate air-dried semen (with particular consideration of the storage protocol, nature of reconstituting fluid, slide type and stability over time and temperature) as an alternative storage method for semen prior to testing DNA fragmentation levels with the Halosperm G2 kit.

Whilst Yap and Matson did not report any difficulties associated with using flat microscope slides (2012), the preliminary stages of this study revealed that where semen was air-dried on flat slides, in some samples the semen pool became flaky around the edges. Since concave slides have been used in other settings for long term culture (Thomas et al., 2001), it was hypothesised that concave slides would be beneficial for this study, and may prevent the semen from thinning at the edges, therefore avoiding potential flaking of the semen pool. Furthermore the semen pool would be confined by the well of the concave slide ensuring a consistently sized pool across all slides.

2.8 Reconstitution Media

In order to liquefy a dried semen sample, a reconstitution medium is required so that the spermatozoa are resuspended and can be removed from a variety of sources (Giusti et al., 1986). Both phosphate buffered saline (PBS) and seminal plasma are regularly used in andrology laboratories and have each been used in previous studies involving air-dried semen (Martin et al., 2006; Yap & Matson, 2012).

2.8.1 Seminal Plasma

After centrifuging a semen sample and removing the pellet, seminal plasma is obtained. This has previously been used as a reconstituting medium prior to testing the DNA integrity of air-dried semen (Yap & Matson, 2012; Yap et al., 2012). Prior to DNA integrity testing of the air-dried semen samples, each air-dried semen slide was gently mixed with 40 µl of seminal plasma (which had previously been centrifuged and stored at 4°C) to resuspend the dried spot and then placed in a 0.5ml conical tube (Eppendorf, Germany). Whilst seminal plasma was shown to be an effective reconstituting medium (Yap & Matson, 2012), the nature of its composition and variability (Milardi et al., 2012) prompted this writer to consider using phosphate buffered saline as a reconstituting medium. Furthermore, in addition to organ specific proteins, the origin and function of other proteins found in seminal plasma remain poorly understood (Milardi et al., 2012). Since the whole semen sample was air-dried, the use of seminal plasma as a reconstituting fluid would mean the reconstituted air-dried samples would contain double strength seminal plasma.

2.8.2 Phosphate Buffered Saline and Bovine Serum Albumin

Forensic laboratories are commonly required to recover spermatozoa from vaginal swabs or items of clothing to determine if sexual intercourse has taken place in an alleged sexual assault case. Furthermore, to determine the identity of the alleged attacker, the extraction of spermatozoa must not affect the subsequent DNA profiling (Martin et al., 2006). A number of different methods are used to recover spermatozoa prior to applying these to a microscope slide for assessment, however PBS has been shown to be an effective reconstituting fluid for this purpose (Giusti et al., 1986; Martin et al., 2006). PBS is a non-toxic, neutral

solution readily used in most biological laboratories which allows cells to remain in their current state. The protocol used by Giusti and colleagues (Giusti et al., 1986) required that fabric containing dried semen was scrubbed with 30ml of PBS (136mM sodium chloride, 8mM sodium phosphate, dibasic, 17mM sodium phosphate, monobasic, pH7.0). After 24 hours of mild agitation in 100ml of PBS (containing 2% Sodium lauryl sarcosinate (Sarkosyl®) at 4°C, the solution was pressed through nylon mesh and centrifuged for 10 minutes at 3600 rpm at 4°C. After resuspending the pellet in 1ml of PBS, the sperm count of each sample was conducted before isolating DNA. The successful extraction of DNA from dried semen samples on fabric using PBS as a reconstituting medium has prompted the hypothesis that PBS may be a potential medium for reconstituting air-dried semen on microscope slides. Since Sarkosyl® is an ionic detergent which disrupts biological membranes (Garvin, Bottinelli, Gola, Conti, & Soldati, 2009), allowing for DNA extraction (Griffin, 2013), the addition of this solution was foreseen to be detrimental prior to DNA fragmentation testing as the Halosperm assay has its own lysing solution.

Bovine serum albumin (BSA) is used with many laboratory techniques due to its stability, lack of intrusion within biological reactions (Farell & Alexandre, 2012) and the fact that it prevents adhesion to glass surfaces (Harrison, Dott, & Foster, 1982; Namasivayam, Robin, Bharani, Vigneshwaraprakash, & Vivek, 2013). BSA has also been shown to have a favourable effect when amplifying ancient DNA and that which has been found in fresh and marine water as well as in faeces (Tarrand, Krieg, & Döbereiner, 1978; Woide, Zink, & Thalhammer, 2010). For these reasons,

it was postulated that the addition of BSA to PBS, as opposed to Sarkosyl®, would be advantageous.

3 Summary and Theoretical Framework

The methods used to assess human semen as described in the WHO 4th (1999) or 5th (2010) Edition manuals are widely used in fertility laboratories as a standard reference to begin evaluating male factor infertility (Sánchez, Wistuba, & Mallidis, 2013). Since consensus over which edition should be used has not been established as yet, some laboratories are using the 4th Edition, whilst others refer to the 5th Edition (Eliasson, 2010; Esteves, Zini, et al., 2012). Despite the exclusion of DNA integrity testing in WHO recommendations to evaluate male factor infertility (World Health Organisation, 2010) many studies have reported that DNA integrity testing is a valuable diagnostic tool for assessing male infertility (Lewis et al., 2013) and as such numerous laboratories have included sperm DNA fragmentation analysis as an adjunct to the traditional semen analysis. Few studies have considered the clinical significance of semen analysis and its relationship to DNA fragmentation levels, whilst none have measured this against both the 4th and 5th Edition WHO semen analysis guidelines.

Given the increasing focus on male factor infertility and the role that DNA fragmentation plays, it is important to develop strategies that provide a storage medium that allows for the analysis of DNA integrity in semen samples to be standardised, more efficient and provide a potential platform for both internal and external quality assurance. Whilst other studies have evaluated the use of air-dried semen, only one study (Yap & Matson, 2012) has considered this as a method

of semen storage prior to DNA fragmentation testing, however, the extrinsic factors associated with such storage methods were not analysed. Consequently, this research project evaluated the relationship of semen analysis as measured using the WHO 4th and 5th Edition semen analysis manuals to DNA fragmentation levels and then considered the extrinsic factors associated with air-dried semen.

4 Research Questions and Hypotheses

4.1 Research Question 1

What are the relationships between semen quality according to the WHO 4th and 5th Editions and sperm DNA fragmentation levels as detected with the Halosperm G2[®] kit?

4.1.1 Hypothesis

It was expected that the proportion of semen samples which score above the recommended DNA fragmentation level will shift, dependent on whether they are compared with the WHO 4th or 5th Edition semen quality parameters.

4.2 Research Question 2

Can a solution of phosphate buffered saline and bovine serum albumin be used as a reconstituting fluid instead of seminal plasma without any detrimental effect on the DNA integrity of air-dried semen?

4.2.1 Hypothesis

A simple medium (e.g. phosphate buffered saline and bovine serum albumin) is equally as effective as seminal plasma to reconstitute air-dried semen.

4.3 Research Question 3

What (if any) is the difference in DNA fragmentation levels between snap frozen and fresh samples when performing the Halosperm[®] G2 test?

4.3.1 Hypothesis

Current storage protocols prior to DNA fragmentation testing using the Halosperm® G2 test include snap freezing in liquid nitrogen. It was expected that there would be no difference in DNA fragmentation levels when comparing snap frozen and fresh samples.

4.4 Research question 4

How stable are air-dried slides over time and at different temperatures prior to DNA fragmentation analysis using the Halosperm® G2 Test Kit?

4.4.1 Hypothesis

After consideration of the literature, it was envisaged that air-dried slides would be stable at 4°C.

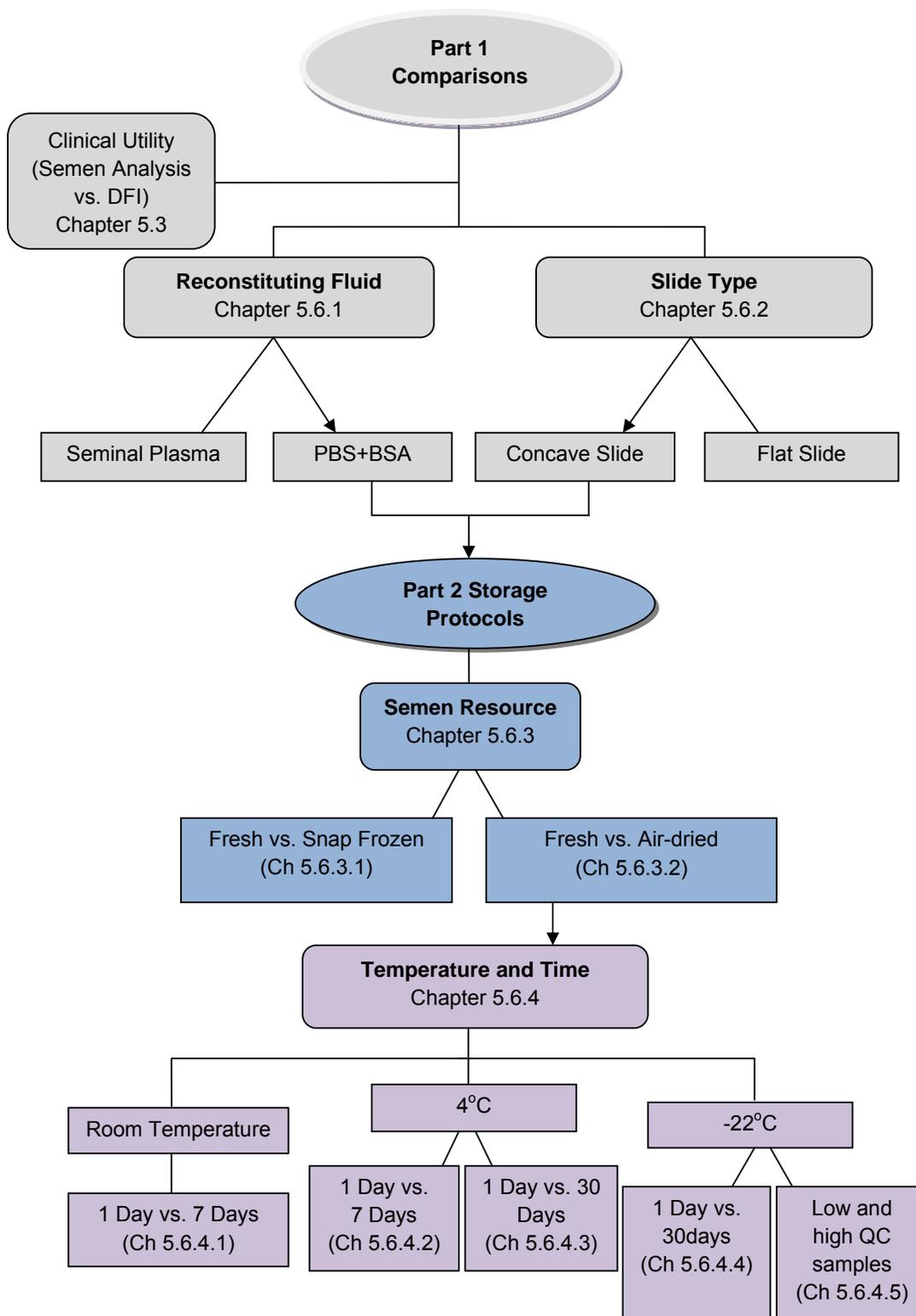


Figure 1: Experimental protocols

5 Materials and Methods

Figure 1 shows a diagrammatic representation of the experimental protocols used in this study. Part 1 illustrates the protocols used to establish the materials and methods to be used in part 2 of the project. In summary a semen analysis was conducted on all samples and the DNA fragmentation level established for each of these. Seminal plasma and a solution of PBS together with BSA (PBS+BSA) were the two different types of reconstituting fluids that were compared. The type of slide to be used for part 2 of the project was determined by comparing DNA fragmentation levels on flat and concave slides. PBS+BSA (as the reconstituting fluid) and concave slides (as the slide type) were used in part 2 of the project where DNA fragmentation levels were compared using different testing protocols, followed by a range of temperatures over different periods of time.

5.1 Ethics Approval

Ethics approval was granted by Joondalup Health Campus Human Ethics Committee under reference number 1222 and the Human Research Ethics Committee of Edith Cowan University under reference number 9147.

5.2 Sample Collection

The semen that was analysed for this research project was collected from patients attending Fertility North, Suite 213, Specialist Medical Centre, Joondalup Health Campus. The participants in the study were men aged between 18 and 64 years undergoing semen analysis and DNA fragmentation testing as part of their fertility investigations at Fertility North. All participants included in this study were provided with an information letter explaining the details of the study and signed a

consent form (Appendix I). Semen samples were collected via masturbation in a private room adjacent to the laboratory after sexual abstinence for a period of between two and five days. Each sample was collected in a clean 60ml wide-mouthed universal container and then immediately labelled with the man’s name, laboratory identification number, date and time of collection before storage at 37°C in an incubator (Mettmert GmbH, Germany) for a maximum of one hour to allow liquefaction. From eight hundred and sixty three men, nine hundred and five semen samples were analysed. Full details of the semen analysis results are shown in chapter 6.2, Table 2.

5.3 Clinical Utility of the Halosperm Assay

5.3.1 Semen Analysis

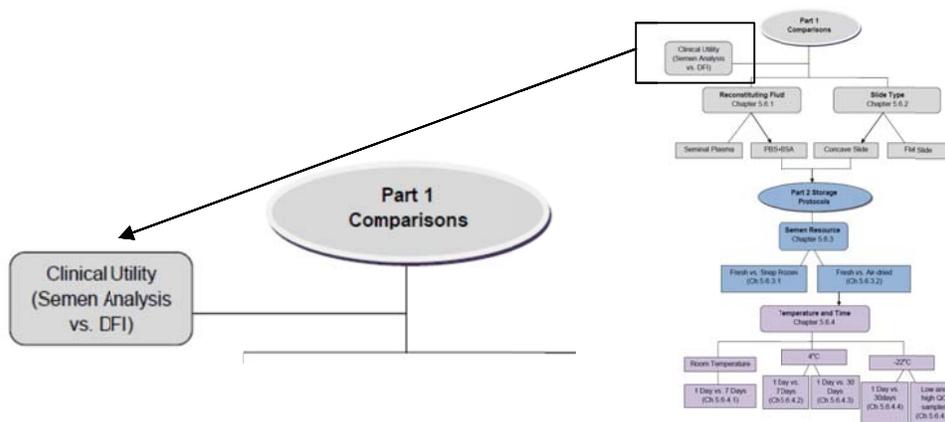


Figure 2: Section from “Figure 1: Experimental protocols” - Determining the clinical utility of the Halosperm assay.

To determine the clinical utility of the Halosperm assay (Figure 2), first a semen analysis was performed according to WHO guidelines (World Health Organisation, 1999, 2010). In summary, semen volume was measured by aspirating the whole sample into a 5 or 10ml graduated pipette. To measure the pH level of the sample, a drop of semen was placed and spread evenly onto pH test paper. After <30seconds, the colour of the impregnated zone was compared to the calibration

strip. For sperm motility assessment, a wet mount approximately 20µm deep was examined with a phase contrast microscope after the sample had stopped drifting. The motility of all spermatozoa within the field of a defined area were assessed and scored as progressive, non-progressively motile or non-motile. Spermatozoa were considered progressively motile where they actively moved, regardless of their speed in either a linear or large circular pattern. All other patterns of motility, where there was no progression, were considered non-progressively motile. The absence of any movement rendered the spermatozoa non-motile. Sperm numbers for each ejaculate were determined using a Neubauer Haemocytometer with a single count of 200 spermatozoa per replicate. This varies from the WHO 5th Edition guidelines which suggest that several counts of 200 spermatozoa will reduce sampling error (World Health Organisation, 2010). A single count was used as this follows the Fertility North laboratory protocol which is predominantly based on the WHO 4th Edition guidelines. To identify the percentage of morphologically normal spermatozoa in an ejaculate a drop of semen was placed onto a labelled microscope slide and then smeared by dragging a 22 x 22mm coverslip over the drop before it was allowed to dry. The slide was then stained using "Diff Quick" solutions (Bacto Laboratories Pty Ltd, Mt Pritchard, NSW 2170) as per the manufacturer's instructions and 200 consecutive spermatozoa counted. A morphologically normal spermatozoon has an oval head with 40% to 70% being covered by the acrosome. No abnormalities should be seen in the neck, midpiece or tail (World Health Organisation, 1999).

Semen parameters were then classified as normal or abnormal using the WHO laboratory manuals for examining human sperm 4th and 5th Editions as shown in Table 1.

Table 1: Cut off values for semen parameters according to WHO 4th Edition and WHO 5th Edition semen analysis criteria.

(Adapted from Esteves and Agarwal, 2011)

Semen Parameters	WHO 4 th Edition	WHO 5 th Edition ¹
Volume (mL)	2	1.5
Sperm Concentration (10 ⁶ /mL)	20	15
Motility	50% (a + b)	32% (a + b)
Morphology (% normal)	14	4

Semen samples that scored below the recommended levels for each of the following parameters were classified as follows:

1. Sperm concentration: Oligozoospermia (O)
2. Sperm concentration and morphology: Oligoteratozoospermia (OT)
3. Sperm concentration and progressive motility: Oligoasthenozoospermia (OA)
4. Sperm concentration, progressive motility and morphology: Oligoasthenoteratozoospermia (OAT)

Semen samples that scored above or equal to the sperm concentration recommended values, but below the recommended levels for each of the following parameters were classified as follows:

1. Morphology: Normoteratozoospermia (NT)
2. Progressive motility: Normoasthenozoospermia (NA)

3. Progressive motility and morphology: Normoasthenoteratozoospermia (NAT)

Semen samples that scored above or equal to all parameters were classified as Normozoospermia (N).

5.3.2 DNA Fragmentation Analysis

The second part of determining the clinical utility of the Halosperm assay (Figure 2), required snap frozen samples (refer chapter 5.5.2) to be analysed and their DNA fragmentation results compared with the semen analysis. Analysis was carried out using the reagents supplied in the Halosperm® G2 Test Kit (Halotech DNA SL, Spain) plus additional reagents which included ethanol (95%), microscope slides and cover slips, distilled water and a water bath (37°C). The protocol for the Halosperm® G2 Assay was used according to Appendix II. The low-melting-point agarose gel supplied with the kit was placed in a water bath at 90°-100°C for 5 minutes to melt before 50µL was transferred into an Eppendorf tube at 37°C for 5 minutes to permit temperature equilibration. 25µL of the semen sample was then added to the 50µL of liquefied and temperature equalised agarose gel and mixed until fully incorporated. 8µL of the semen-agarose mix was pipetted onto a Halosperm® pre-treated slide, covered with a 22mm by 22mm coverslip and placed on a pre-cooled metal tray inside a refrigerator at 4°C for 5 minutes to enable the spermatozoa to become embedded in the agarose gel. The slide was then placed in a horizontal position on top of a petri dish inside a glass container before the coverslip was gently removed and an acid denaturation solution (solution 1) applied for 7 minutes and then drained. This was followed by

20 minutes of incubation in a lytic solution (solution 2) before draining. Following this, the slide was washed in distilled water for 5 minutes and drained. This was followed by dehydration in 70% and 100% ethanol for 2 minutes each with drainage of the ethanol between each step. Subsequently the slide was air-dried and covered with Eosin solution (solution 3) for 7 minutes, drained and then Azur B solution (solution 4) added for a further 7 minutes before final draining.

DNA fragmentation levels were determined by viewing each slide under bright field microscopy using the 40x objective lens. A total of 300 sperm were counted on each slide, with sperm classified as having either fragmented DNA or unfragmented DNA. Criteria to determine fragmented and non-fragmented DNA was followed in accord with the classification set out and provided by Halotech DNA, Spain (shown in Appendix II). In essence and as shown in Figure 3, spermatozoa with either a large or medium sized halo depict unfragmented DNA. Spermatozoa with either a small halo or without a halo and those with a weak or irregular stained core depict fragmented DNA. The percentages of sperm with fragmented DNA were calculated by dividing the number of fragmented sperm by the total number of sperm counted.

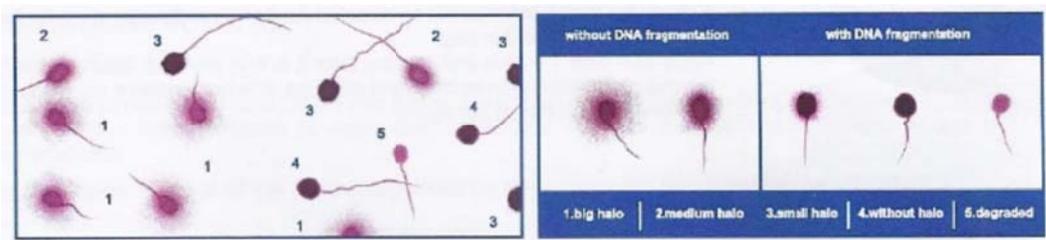


Figure 3: Criteria to determine unfragmented and fragmented DNA in spermatozoa (Halotech DNA, Spain).

5.4 Quality Control of DNA Fragmentation Scoring

To assess the degree of variability in DNA fragmentation levels in the same sample when run in different batches using the Halosperm kit, low and high DNA fragmentation level quality control samples were assessed. Two batches, each of 34 positive and 34 negative samples were processed with the Halosperm assay over 68 different batch assays.

Positive quality control samples were made from pooled semen which was placed in a water bath at 90° to 100°C for 2 hours. Negative quality control samples were made from pooled semen known to have DNA fragmentation levels below 30%. This semen was aspirated into orange (positive control) and green (negative control) labelled 0.5ml IMV AI straws (Genetics Australia, Bacchus Marsh, Australia) before heat sealing.

The straws were then placed in a freezer (-22 °C) for 30 minutes, then into a mixture of LN₂ vapour and air in the neck of a LN₂ container for 10–15 minutes. The frozen straws were then placed in plastic storage tubes and inserted into larger storage goblets in a LN₂ dewar. Prior to DNA fragmentation testing, the straws were removed from the LN₂ and placed on a workbench in a polystyrene tray to thaw at room temperature for between 30 and 60 minutes. Whilst the actual temperature of the laboratory was not monitored for this purpose, the expected temperature would have been approximately 25°C as this is the Fertility North laboratory protocol.

To consider if there was any variability when the same sample was tested in the same Halosperm run, 34 samples were tested twice in the same Halosperm run

and the DNA fragmentation levels compared by the same technician using a blind counting technique.

In evaluating the level of inter operator scoring, 164 Halosperm slides were analysed by 2 different operators using a blind count. The variability of DNA fragmentation levels reported from each technician was compared.

5.5 Processing Protocols

5.5.1 Freshly Ejaculated Semen

After liquefaction, freshly ejaculated semen samples (in the original container) were placed on bench coat for a maximum of 35 minutes before running the Halosperm assay. This time frame allowed for a portion of the same semen sample to either be snap frozen and thawed (refer 5.5.2 below) or air-dried and reconstituted (refer 5.5.3, 5.5.5 and 5.5.5.2 below) so that the same sample could be tested in the same Halosperm run.

5.5.2 Snap Frozen/Thawed Semen

1.5ml of liquefied semen was placed into a 1.8ml Cryotube™ vial which was then immediately plunged into LN₂ for snap freezing and stored until further processing. Prior to further processing, the Cryotube™ vial was removed from the LN₂ and placed on a work bench in a polystyrene tray to thaw at room temperature for between 30 to 60 minutes. The lid of the Cryotube™ vial was loosened to allow trapped LN₂ to escape to prevent explosion and then tightened until thawed.

5.5.3 Air-dried Semen

Air dried slides were made by dispensing 50µl of thawed and/or fresh liquefied semen onto a microscope slide and immediately placed on a 37°C warming stage (Minitüb GmbH, Germany) for between 25 and 30 minutes. After 25 minutes, the semen pool was assessed and where the centre of the semen pool was translucent, the slide remained on the warming stage for a further 5 minutes. This allowed for the semen pool to be completely dry before the slide was removed from the warming stage. The air-dried slide was placed on bench coat for 2 minutes to allow the slide to return to room temperature, before being placed in a sealed plastic container for storage or further processing.

5.5.4 Storage of Air-dried Slides

The air-dried slides that were stored at room temperature were placed in an enclosed wooden slide box in Fertility North's Andrology laboratory until further processing. Those air-dried slides that were stored at approximately 4°C or -22°C were placed in a sealed plastic container before being placed in a fridge or freezer respectively (Fisher and Paykel™) at Fertility North's Andrology laboratory until further processing. The temperatures of both the fridge and freezer are tested daily and no temperature fluctuations were found during the project, however as both appliances are of the frost free variety, it is possible that there may have been some fluctuation in temperature between the daily recordings.

5.5.5 Reconstitution of Air-dried Slides

Immediately prior to assessing the sperm DNA fragmentation of an air-dried semen sample, 50µL of a specific reconstituting fluid was applied to the dried

semen. This was gently mixed until the dried semen was free from the glass slide and the solution appeared homogenous. The solution of semen and reconstituting fluid was then placed into a 0.5ml conical tube (Eppendorf, Germany) for further analysis.

5.5.5.1 Seminal Plasma

Seminal plasma was obtained by centrifuging the semen sample at 16,000 rpm for 15 minutes followed by removal of the seminal supernatant without disturbing the pellet. An additional centrifugation of 16,000 rpm for a further 10 minutes preceded the final removal of seminal plasma (again without disturbing the pellet) before its placement in a Cryotube™ vial and storage at 4°C.

5.5.5.2 Phosphate Buffered Saline (PBS) and Bovine Serum Albumin (BSA) Solution

The preparation of phosphate buffered saline is described by Sambrook, Fritsch and Maniatis (1989) and is briefly described as follows:

The following was added to 800ml of distilled water:

8g of NaCl

0.2g of KCl

1.44g of Na₂HPO₄

0.24g of KH₂PO₄

Using HCl, adjust the pH to 7.4

Distilled water was added to bring the total volume to 1 litre.

This solution was then dispensed into 10ml conical tubes and 0.3g of BSA added before storage. Following the addition of BSA, the PBS+BSA solution was stored at 4°C for a maximum of 10 days.

5.6 Factors Affecting DNA Fragmentation Levels of Air-dried Semen

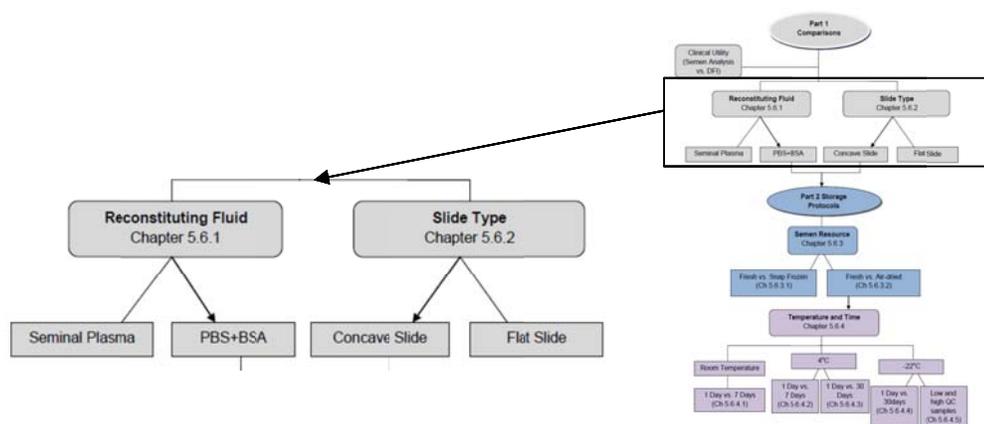


Figure 4: Section from “Figure 1: Experimental protocols” – Comparing the affects that an alternative reconstituting fluid and slide type has on DNA fragmentation levels of air-dried semen.

5.6.1 Reconstituting fluid

To determine if the reconstituting fluid had any effect on the DNA fragmentation levels of air-dried semen (Figure 4), from each of 15 samples, 2 aliquots of semen (total 30 aliquots) were prepared using the methods as described in chapter 5.5.2. Air-dried slides (total 30 slides) were made according to the protocol as described in chapter 5.5.3 using thawed semen. Each slide was stored for one day at room temperature in the same manner as in chapter 5.5.4. The following day one slide from each sample was reconstituted with seminal plasma as outlined in chapter 5.5.5.1 and the second slide from each sample was reconstituted with PBS+BSA as described in chapter 5.5.5.2. All samples were processed in the same Halosperm assay and DNA fragmentation levels for each reconstituting solution from the same sample were compared.

5.6.2 Slide Type

To determine if the slide type had any effect on the DNA fragmentation levels of air-dried semen (Figure 4), from each of 8 samples, 2 air-dried slides (one a flat

glass microscope slide and the other a glass microscope slide with a concave middle section) were prepared as described in chapter 5.5.2, using thawed semen. A total of 16 slides were stored for one day at 4°C using the methods outlined in chapter 5.5.4 and then reconstituted with the PBS+BSA solution using the method stated in chapter 5.5.5.2. DNA fragmentation levels for each slide type from the same sample were compared using the Halosperm assay as discussed in chapter 5.4.

To assess the effect of slide type, when stored at 4°C for 7 days, 10 samples were used to make up 2 air-dried slides using the different slide types (a total of 20 slides). Again slides were reconstituted with PBS+BSA before running the Halosperm assay. DNA fragmentation levels for each slide type from the same sample were then compared.

5.6.3 Semen Resource

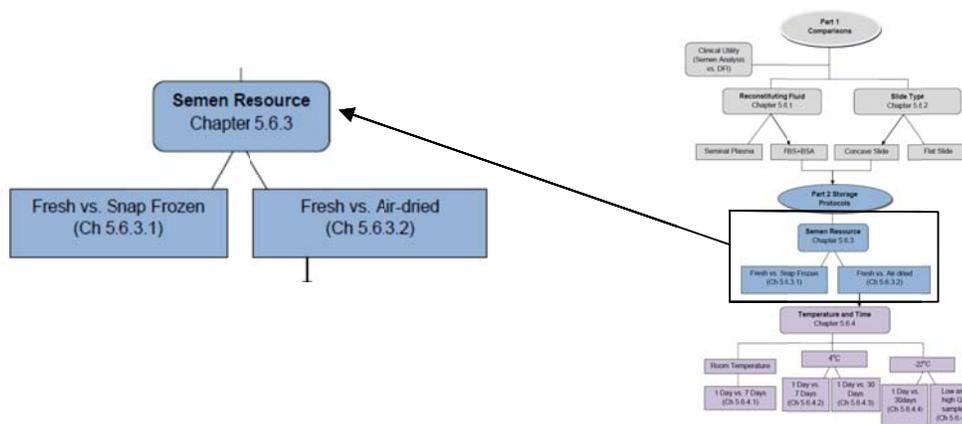


Figure 5: Section from “Figure 1: Experimental protocols” – Comparing the affect that semen resource in terms of processing has on DNA fragmentation levels of air-dried semen.

5.6.3.1 Snap Frozen versus Freshly Ejaculated

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 5), firstly the DNA fragmentation levels from snap frozen samples were compared with those of freshly ejaculated samples. From each of 12 freshly ejaculated samples, 1 aliquot of semen (total 12 aliquots) was snap frozen as described in chapter 5.5.2 and immediately thawed. DNA fragmentation levels were then assessed using the protocol as described in chapter 5.4 and compared between the freshly ejaculated samples (as described in chapter 5.5.1) and snap frozen samples in the same Halosperm run.

5.6.3.2 Freshly Ejaculated versus Air-dried

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 5), secondly the DNA fragmentation levels from freshly ejaculated samples were compared with those of air-dried samples. From 13 freshly ejaculated samples, 1 air-dried slide (total 13 slides) was made using the protocol as described in chapter 5.5.3 and then immediately reconstituted with PBS+BSA (as per chapter 5.5.5.2). DNA fragmentation levels were then assessed

using the protocol as described in chapter 5.4 and compared between the freshly ejaculated samples (as described in chapter 5.5.1) and reconstituted samples in the same Halosperm run.

5.6.4 Time and Temperature

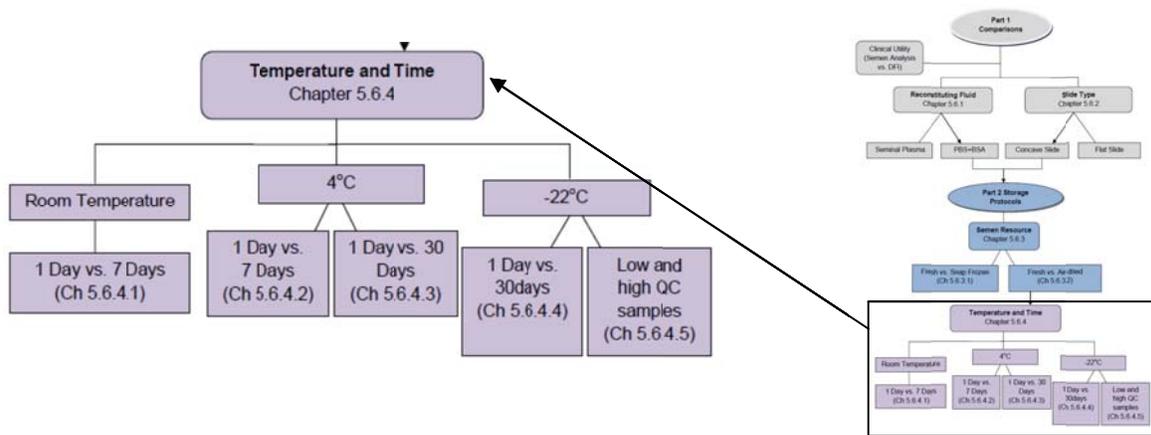


Figure 6: Section from “Figure 1: Experimental protocols” – Comparing the affect that time and temperature has on DNA fragmentation levels of air-dried semen.

5.6.4.1 Room Temperature: 1 Day versus 7 Days

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 6), firstly the DNA fragmentation levels from snap frozen and air-dried samples stored at room temperature (approximately 25°C) for 1 day were compared with those processed in the same manner but stored for 7 days. From each of 11 freshly ejaculated samples, 2 aliquots of semen (total 22 aliquots) were prepared using the protocol as described in chapter 5.5.2. From each aliquot, one air-dried slide was made using the protocol as described in chapter 5.5.3. One slide from each semen sample was stored for one day and the other stored for 7 days at room temperature using the protocol from chapter 5.5.4. On the 8th day each of the air-dried slides was reconstituted using the protocol as

described in chapter 5.5.5.2 and DNA fragmentation levels measured using the Halosperm assay. The DNA fragmentation levels for air-dried slides stored for 1 day were compared against the same sample whose air-dried slide was stored for 7 days.

5.6.4.2 4°C: 1 Day versus 7 Days

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 6), the DNA fragmentation levels from snap frozen and air-dried samples stored at 4°C for 1 day were compared with those processed in the same manner but stored for 7 days. From each of 15 samples, 2 aliquots of semen (total 30 aliquots) were prepared using the protocol as described in 5.5.2. From each aliquot, one air-dried slide was made using the protocol as described in chapter 5.5.3. One slide from each semen sample was stored for one day and the other stored for 7 days at 4°C using the protocol as described in chapter 5.5.4. On the 8th day each of the air-dried slides was reconstituted using the protocol as described in chapter 5.5.5.2 and DNA fragmentation levels measured using the Halosperm assay. The DNA fragmentation levels for air-dried slides stored for 1 day were compared against the same sample whose air-dried slide was stored for 7 days.

5.6.4.3 4°C: 1 Day versus 30 Days

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 6), the DNA fragmentation levels from snap frozen and air-dried samples stored at 4°C for 1 day were compared with those processed in the same manner but stored for 30 days. From each of 10 samples, 2

aliquots of semen (total 20 aliquots) were prepared using the protocol as described in chapter 5.5.2. From each aliquot, one air-dried slide was made using the protocol as described in chapter 5.5.3. One slide from each semen sample was stored for one day and the other stored for 30 days at 4°C in the same manner as in chapter 5.5.4. On the 31st day each of the air-dried slides was reconstituted as described in chapter 5.5.5.2 and DNA fragmentation levels measured using the Halosperm assay. The DNA fragmentation levels for air-dried slides stored for 1 day were compared against the same sample whose air-dried slide was stored for 30 days.

5.6.4.4 -22°C: 1 Day versus 30 Days

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 6), the DNA fragmentation levels from snap frozen and air-dried samples stored at -22°C for 1 day were compared with those processed in the same manner but stored for 30 days. From each of 11 samples, 2 aliquots of semen (total 22 aliquots) were prepared using the protocol as described in chapter 5.5.2. From each aliquot, one air-dried slide was made using the protocol as described in chapter 5.5.3. One slide from each semen sample was stored for one day and the other stored for 30 days at -22°C as described in chapter 5.5.4. On the 31st day each of the air-dried slides was reconstituted in the same manner as in chapter 5.5.5.2 and DNA fragmentation levels measured using the Halosperm assay. The DNA fragmentation levels for air-dried slides stored for 1 day were compared against the same sample whose air-dried slide was stored for 30 days.

5.6.4.5 Air-dried Semen Stored over Time at -22°C

To determine if the processing protocols had any effect on the DNA fragmentation levels of air-dried semen (Figure 6), the DNA fragmentation from snap frozen and air-dried samples stored at -22°C were measured over a period of time. From a single semen sample known to have a low DFI, eight air-dried slides were prepared from a frozen-thawed sample (as described in chapter 5.5.2, followed by the air-drying protocol described in chapter 5.5.3). Each slide was individually placed into a sealed plastic container and stored at -22 °C. These samples were used as negative quality controls during routine clinical Halosperm tests. On the day of a clinical Halosperm run, one slide was removed from the freezer, reconstituted as described in chapter 5.5.5.2 and included in the Halosperm assay as a negative quality control sample. All eight samples were reconstituted and used as negative quality control samples in consecutive clinical analyses. From a different semen sample, also known to have a low DFI, six air-dried slides were prepared and used as negative quality control samples in the following consecutive clinical analysis. Protocols as discussed in chapters 5.5.2, 5.5.3 and 5.5.5.2 were again followed.

From a single semen sample known to have a high DFI, eight air-dried slides were prepared from a frozen-thawed sample (as described in chapter 5.5.2, followed by air-drying protocol described in chapter 5.5.3). Each slide was individually placed into a sealed plastic container and stored at -22 °C. These samples were used as positive quality controls during routine clinical Halosperm tests. On the day of a clinical Halosperm run, one slide was removed from the freezer, reconstituted as described in chapter 5.5.5.2 and included in the Halosperm assay as a positive quality control sample. All eight samples were reconstituted and used as positive quality control samples in consecutive clinical analyses. From a different semen

sample, also known to have a high DFI, six air-dried slides were prepared and used as positive quality control samples in the following consecutive clinical analysis. Protocols as discussed in chapters 5.5.2, 5.5.3 and 5.5.5.2 were again followed.

5.7 Statistical Analysis

To examine the relationship between sperm DNA fragmentation and semen analysis results, Pearson correlations and analysis by ANOVA were performed using StatistiXL (Nedlands, Western Australia), with post-hoc testing by Tukey's HSD. Proportions were compared by Chi-squared test and $P < 0.05$ was set as the minimum level of significance. To measure the variation of results within each batch and confirm the quality assurance of scoring between batches, the coefficient of variation was calculated (standard deviation \times 100/mean) and a precision profile built. The within run and between operator variability in scoring was analysed using repeated measures analysis of variation (ANOVA), difference plots and Student's t-tests. Similarly, the DNA fragmentation levels for each of the above processing protocols (chapter 5.6) were statistically analysed using Student's t-tests to compare the different experimental regimes. Confidence intervals were set at 0.05 and significance levels are reported as $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$. All statistical analyses were performed using the Microsoft Excel and StatistiXL (Nedlands, Western Australia) statistical packages.

6 Results

6.1 Quality Control and Assay Precision

The mean DNA fragmentation levels \pm the standard error of the mean (SEM) of the first batch of low and high controls were reported as $10.5 \pm 0.55\%$ and $81.3 \pm 2.01\%$ respectively. The fragmentation levels of the second batch of controls were $20.7 \pm 0.82\%$ and $56.6 \pm 1.80\%$ for the low and high controls respectively (Figure 7).

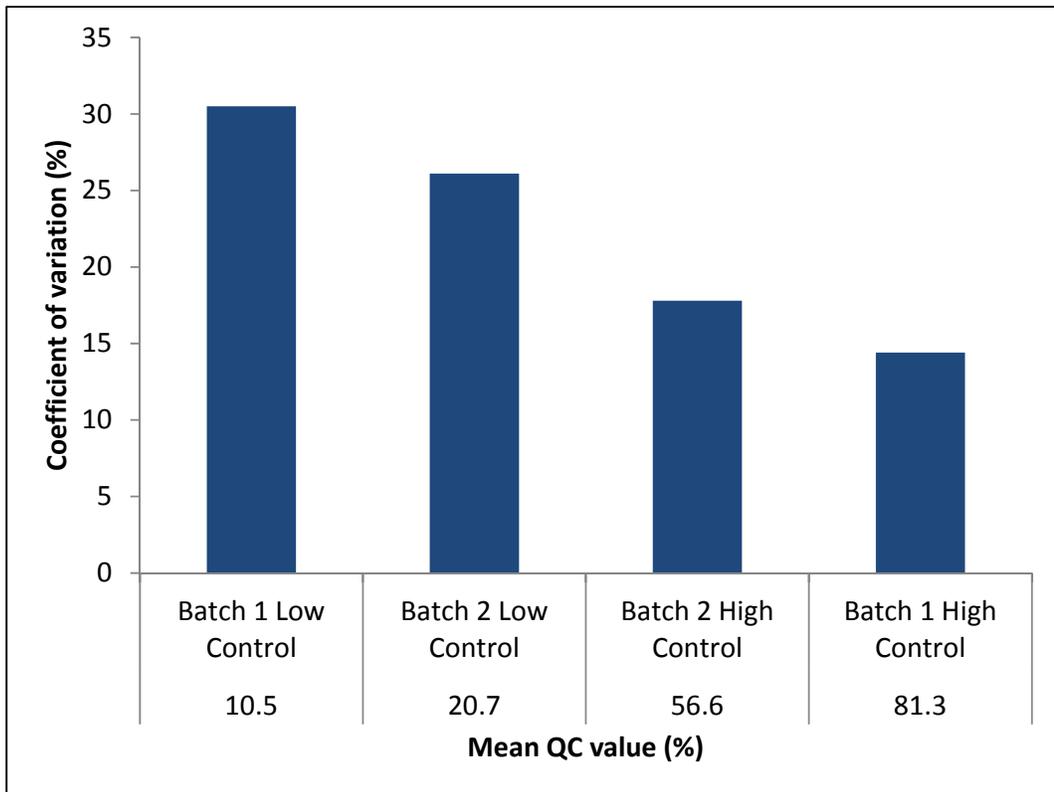


Figure 7: Comparing DNA fragmentation results to determine consistency of results between Halosperm runs.

Precision profile of 2 batches of controls each with one low and one high known DNA fragmentation level. Values for each batch are the mean per group (n=34).

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Using a repeated measures ANOVA, the variance of DNA fragmentation levels between subjects is significantly different, but no significant variance is seen between different measurements of the same individual (Table 2). The mean DNA fragmentation percentage \pm SEM for two replicates of the same semen sample was 27.81 ± 2.67 and 26.79 ± 2.78 for replicates 1 and 2 respectively. Student's paired t-test between the two replicates was $t=1.84$; $p=0.08$. The Bland-Altman plot (Figure 8) shows the DNA fragmentation difference between the two replicates.

Table 2: Repeated Measures ANOVA showing variance among subjects differs significantly from the variance between measures of the same individuals confirming repeatability of the assay.

ANOVA							
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>	
Same sample	16535.68	33	501.0812	96.00666	8.4E-25	1.787822	
Between samples	17.60529	1	17.60529	3.373157	0.075289	4.139252	
Error	172.2347	33	5.219234				
Total	16725.52	67					

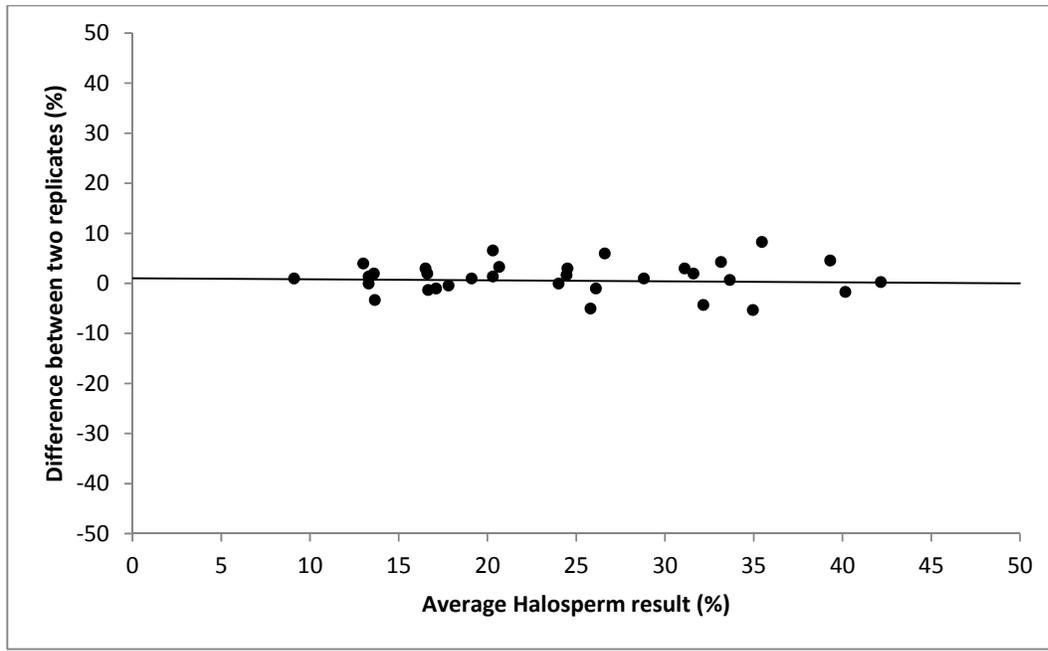


Figure 8: Bland Altman plot comparing DNA fragmentation obtained from the same semen sample processed and scored in duplicate by the same technician and within the same Halosperm run (n=34).

Replicate 1 and replicate 2 from the same semen sample were scored by the same technician and the mean DNA fragmentation difference was compared. The x axis represents the DNA fragmentation level obtained from replicate 1 and the y axis represents the percentage difference in the two replicates. Each point represents a sample analysed by both replicas.

Using a repeated measures ANOVA, the variance of DNA fragmentation levels between subjects is significantly different, but no significant variance is seen between different measurements of the same individual (Table 3). The mean DNA fragmentation percentage \pm SEM for technician 1 was 22.86 ± 1.28 and for technician 2 the percentage was 22.65 ± 1.37 . Student's paired t-test between the two replicates was $t=0.37$; $p=0.71$. The Bland-Altman plot (Figure 9) shows the DNA fragmentation difference between technician 1 and technician 2.

Table 3: Repeated Measures ANOVA showing variance among subjects differs significantly from the variance between measures of the same individuals confirming repeatability of the assay.

ANOVA							
Source of Variation	SS	df	MS	F	P-value	F crit	
Same sample	90134.26	163	552.9709	20.60958	1.42E-63	1.294886	
Between samples	3.734756	1	3.734756	0.139197	0.709566	3.899142	
Error	4373.415	163	26.83077				
Total	94511.41	327					

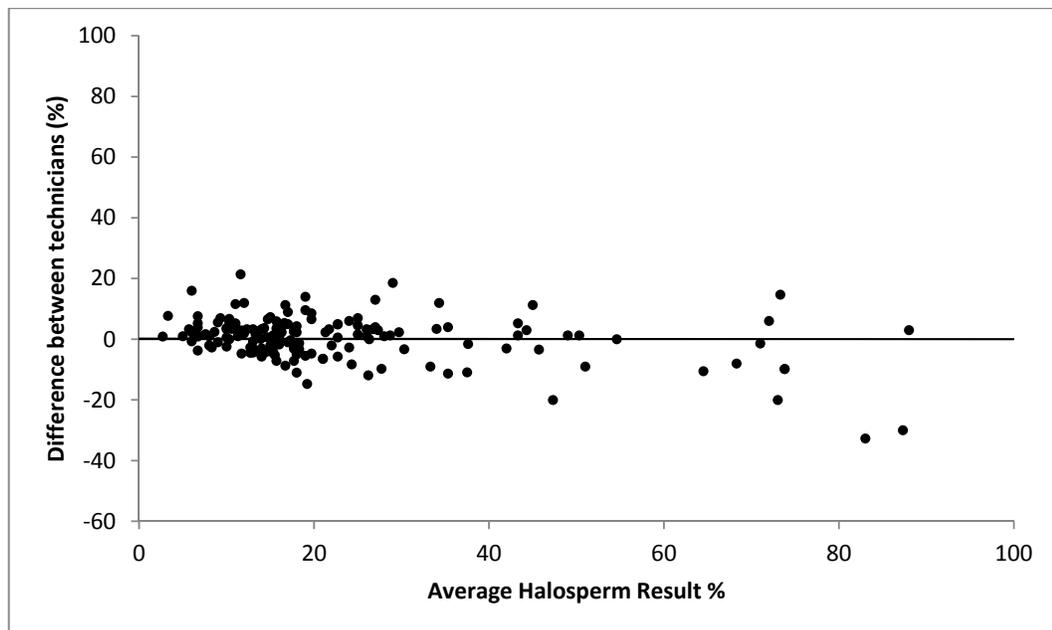


Figure 9: Difference of DNA fragmentation levels between technician 1 and technician 2.

The same semen samples were scored on different occasions by 2 different technicians (n=164)

6.2 Mean Semen Analysis and DNA Fragmentation Results

The total number of semen samples obtained for semen analysis and Halosperm testing was 905, from a total of 863 men. A summary of the semen analysis results and their association to the mean DNA fragmentation result are shown in Table 4.

Table 4: Summary of semen analysis results and Pearson Correlation to DFL.
Values are for the 905 semen samples from 863 men. *Significant association.

Variable	Value	P	Pearson's r
Number of men	863		
Number of semen samples	905		
DNA fragmentation	23.0±0.6		
Abstinence (days±SEM)	4.1±0.2	0.001*	0.11
Volume (ml±SEM)	3.5±0.1	0.159	0.05
Sperm concentration (x10 ⁶ /ml±SEM)	68.6±2.2	0.000*	-0.17
Sperm motility (%±SEM)	59.0±0.6	0.000*	-0.30
Sperm morphology (%±SEM)	14.0±0.2	0.000*	-0.24

6.3 Semen Analysis According to Different WHO Semen Analysis Guidelines 4th and 5th Editions

Of 905 samples, the number of samples that were classified as normozoospermic according to the 4th Edition was 385 whilst 697 were classified as normozoospermic according to the 5th Edition (Table 5). Of the semen samples that were classified as normozoospermic in the 5th Edition, 34 were classified as normoasthenozoospermic, 190 were classified as normoteratozoospermic and 61 as normoasthenoteratozoospermic in the 4th Edition. Of the 31 samples that were classified by the 4th Edition as oligozoospermic, 9 were classified as normozoospermic by the 5th Edition. From 10 samples classified as oligoasthenozoospermic according to the 4th Edition, 7 were classified differently

Table 5: Matrix table showing semen analysis when scored following WHO 4th Edition and WHO 5th Edition semen analysis criteria. Refer to Table 6 for reference guide to abbreviations.

		WHO 5th Edition								TOTAL
		N	NA	NT	NAT	O	OA	OT	OAT	
WHO 4th Edition	N	385	0	0	0	0	0	0	0	385
	NA	34	11	0	0	0	0	0	0	45
	NT	190	2	11	0	0	0	0	0	203
	NAT	61	13	7	4	0	0	0	0	85
	O	9	0	0	0	22	0	0	0	31
	OA	1	1	0	0	5	3	0	0	10
	OT	10	0	2	0	53	0	12	0	77
	OAT	7	5	1	0	19	18	6	13	69
	TOTAL	697	32	21	4	99	21	18	13	905

according to the 5th Edition; 1 sample as normozoospermic, 1 as normoasthenozoospermic and 5 samples as oligozoospermic. Of 85 samples classified as normoasthenoteratozoospermic by the 4th Edition, 81 were classified

differently by the 5th Edition: 61 samples as normozoospermic, 13 as normoaesthenozoospermic and 7 as normoteratozoospermic. Similarly, 12 of 77 samples were classified as oligoteratozoospermic by both editions, whilst 53 samples were graded as oligozoospermic, 2 as normoteratozoospermic and 10 as normozoospermic by the 5th Edition. From 69 samples that were classified as ologoasthenoteratozoospermic by the 4th Edition, 13 were classed the same by the 5th Edition whilst the balance was graded in 3 other categories: 10 as normozoospermic, 2 as normoteratozoospermic and 53 as oligozoospermic.

Table 6: Reference guide to abbreviations used in tables 5, 7 and 8

Abbreviation	Nomenclature	Deviation from reference limits
N	Normozoospermia	All semen analysis criteria are within reference limits
NA	Normoaesthenozoospermia	Progressive motility is outside of reference limits
NT	Normoteratozoospermia	Morphology is outside of reference limits
NAT	Normoaesthenoteratozoospermia	Progressive motility and morphology are outside of reference limits
O	Oligozoospermia	Sperm concentration is outside of reference limits
OA	Oligoaesthenozoospermia	Sperm concentration and progressive motility are outside of reference limits
OT	Oligoteratozoospermia	Sperm concentration and morphology are outside of reference limits
OAT	Oligoaesthenoteratozoospermia	Sperm concentration, progressive motility and morphology are outside of reference limits

6.4 Relationship between DNA Fragmentation and Semen Analysis

Table 7 shows the number of semen samples classified by semen quality according to WHO semen analysis recommendations 4th and 5th Editions and the mean DNA Fragmentation levels (as detected with the Halosperm® G2 Test Kit) of each.

Table 7: Mean DNA fragmentation levels according to semen quality when classified by WHO 4th Edition and WHO 5th Edition semen analysis criteria (n=905).

Values with the same superscript, within the same manual edition are significantly different from each other (p<0.05). NS = Not significant. Refer to Table 6 for reference guide to abbreviations.

Semen Quality	WHO 4 th Edition		WHO 5 th Edition		Significance between 4 th & 5 th Editions p
	n	SDF (%)	n	SDF (%)	
O	31	23.3±2.8 ^h	99	27.0±1.7 ^{l, o, p}	NS
OT	77	27.6±2.0 ^{e, i}	18	31.0±4.4	NS
OA	10	28.1±6.4	21	33.4±4.3 ^m	NS
OAT	69	37.0±2.6 ^{d, g, h, i}	13	46.6±5.8 ^{n, p}	NS
N	385	17.5±0.6 ^{a, b, c, d, e, j}	697	20.3±0.6 ^{j, k, l, m, n}	0.001
NT	203	21.8±1.2 ^{c, f, g}	21	29.9±4.9	NS
NA	45	28.0±3.2 ^a	32	39.4±4.6 ^{k, o}	NS
NAT	85	30.7±2.1 ^{b, f}	4	36.1±9.6	NS

Following the 4th Edition semen analysis criteria, OAT samples had significantly higher DNA fragmentation compared to oligozoospermic samples (p<0.01). Similarly, when samples were classified using the 5th Edition criteria, OAT samples had significantly higher DNA fragmentation compared to oligozoospermic samples (p<0.001). Equally when samples were classified using the 4th Edition criteria with a concentration ≥ 20M/ml but with reduced motility, DNA fragmentation levels were significantly higher than compared to normozoospermic samples (p<0.001). Again DNA fragmentation levels were significantly higher in samples with reduced

motility alone when compared with normozoospermic samples ($p < 0.0001$) classified under the WHO 5th Edition guidelines.

Table 8 shows the proportion of semen samples classified by semen quality according to WHO semen analysis recommendations 4th and 5th Editions which gave an abnormal sperm DNA fragmentation result as detected with the Halosperm ® G2 Test Kit.

Table 8: Proportion of abnormal DNA fragmentation levels (>30%) when classified by WHO 4th Edition and WHO 5th Edition semen analysis criteria.

Refer to Table 6 for reference guide to abbreviations.

Semen quality	Abnormal SDF / Total (%)	
	WHO 4 th Edition	WHO 5 th Edition
O	6/31 (19.4%)	31/99 (31.3%)
OT	22/77 (28.6%)	7/18 (38.9%)
OA	4/10 (40.0%)	10/21 (47.6%)
OAT	37/69 (53.6%)	10/13 (76.9%)
N	45/385 (11.7%)	112/697 (16.0%)
NT	34/203 (16.8%)	9/21 (42.9%)
NA	13/45 (28.9%)	17/32 (53.1%)
NAT	38/85 (44.7%)	3/4 (75.0%)

The greatest percentage of samples with abnormal DFLs (>30%) were recorded where each of sperm concentration, morphology and progressive motility were outside of WHO reference limits for WHO 4th and 5th Editions respectively. The lowest percentage of samples with abnormal DFLs were recorded where none of these semen analysis parameters (concentration, morphology and progressive

motility) were outside the WHO reference limits for WHO 4th and 5th Editions respectively.

6.5 Effects of Extrinsic Factors on Air-dried Slides

6.5.1 Comparing SDF between Different Reconstituting Fluids

The mean DNA fragmentation percentage \pm SEM for slides reconstituted with PBS+BSA solution was 19.74 ± 3.90 and for those reconstituted with seminal plasma the percentage was 18.54 ± 3.26 . Student's paired t-test between the two replicates was $t=1.11$; $p=0.29$. Figure 10 shows the DNA fragmentation percentage recorded for each sample after the airdried sample was reconstituted with either seminal plasma or PBS+BSA. The mean difference for the two reconstituting fluids was $1.21 \pm 1.13\%$.

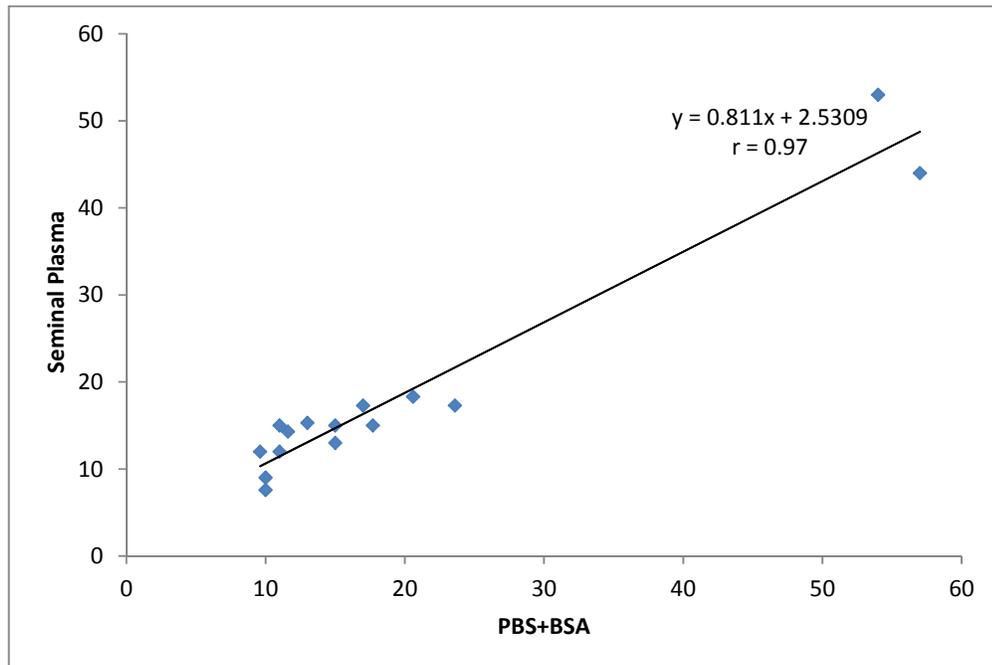


Figure 10: Comparing DNA fragmentation levels to determine effects of reconstituting fluids.

Results are from the same original semen samples ($n=15$) after being air-dried and reconstituted with either seminal plasma or PBS+BSA and being processed in the same Halosperm run.

6.5.2 Comparing SDF Levels between Slide Type

The mean DNA fragmentation levels \pm SEM of the semen air-dried on flat slides and stored at 4°C for 1 day, was $13.98 \pm 1.55\%$ and $14.39 \pm 1.94\%$ for those air-dried on concave slides and stored in the same manner for the same amount of time. Student's paired t-test between the two slide types was $t = -0.55$; $p = 0.60$. The mean difference per sample was $0.41 \pm 0.75\%$. Figure 11 shows the DNA fragmentation percentage recorded for each sample, after being stored on either a flat or concave slide for 1 day.

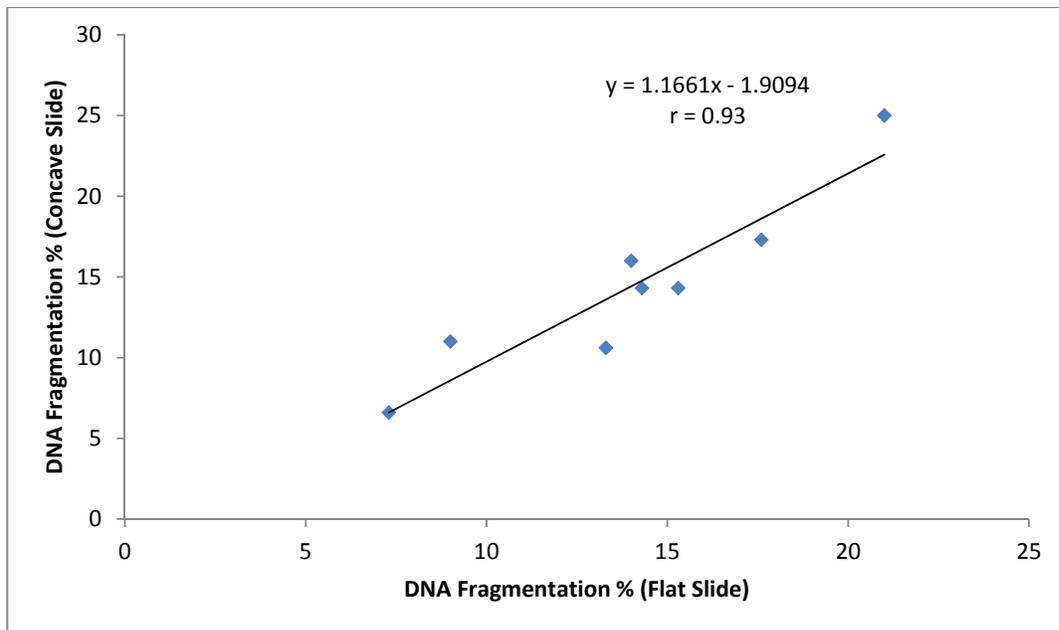


Figure 11: Comparing DNA fragmentation levels to determine effects of slide type after 1 day.

Results are from the same original semen samples ($n=8$) air-dried and stored on either a concave or flat slide and processed in the same Halosperm run.

The mean DNA fragmentation levels \pm SEM of the semen air-dried flat slides and stored at 4°C for 7 days was $16.26 \pm 1.74\%$ and $17.34 \pm 2.02\%$ for those air-dried on concave slides. Student's paired t-test between the two slide types was $t = -1.56$; $p = 0.15$. The mean difference per sample was $1.08 \pm 0.69\%$. Figure 12 shows the DNA fragmentation percentage recorded for each sample after being stored on either a flat or concave slide for 7 days.

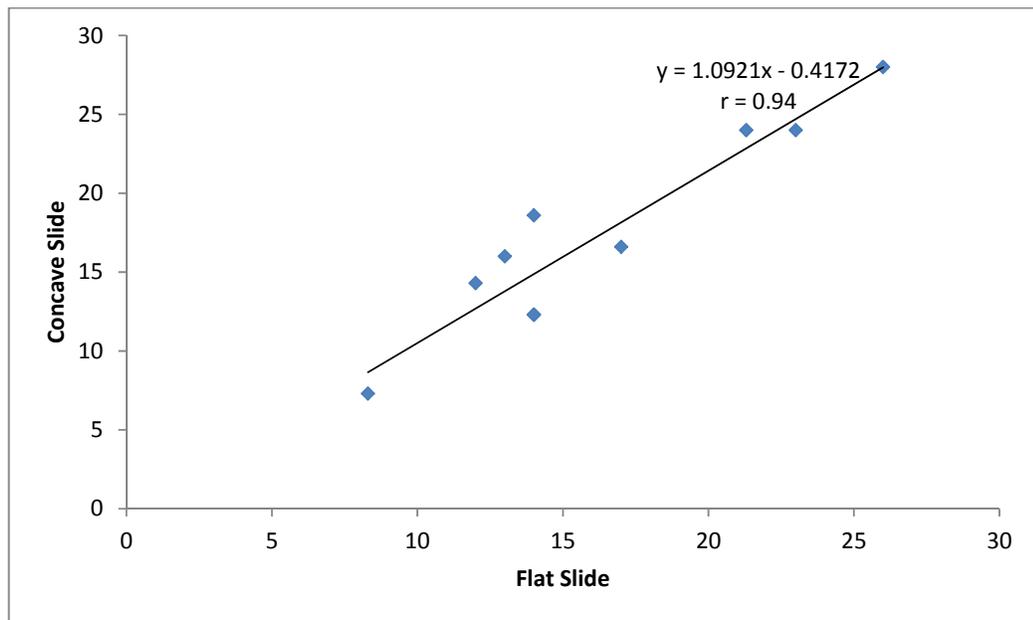


Figure 12: Comparing DNA fragmentation levels to determine effects of slide type after 7 days.

Results are from the same original semen samples ($n = 10$) air-dried and stored on either a concave or flat slide and processed in the same Halosperm run.

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6.5.3 Comparing SDF between Processing Protocols

6.5.3.1 Snap Frozen Semen to Fresh Semen

The average DNA fragmentation percentage \pm SEM in snap frozen samples was 21.83 ± 3.16 and those obtained from freshly ejaculated samples was 16.33 ± 2.69 . Student's paired t-test between the snap frozen and fresh semen was significant ($t=5.00$; $p=0.0004$). Figure 13 shows the DNA fragmentation percentage recorded from the same semen samples that were tested using either fresh semen or following snap freezing. The Bland-Altman plot (Figure 14) shows the mean DNA fragmentation difference between the two protocols.

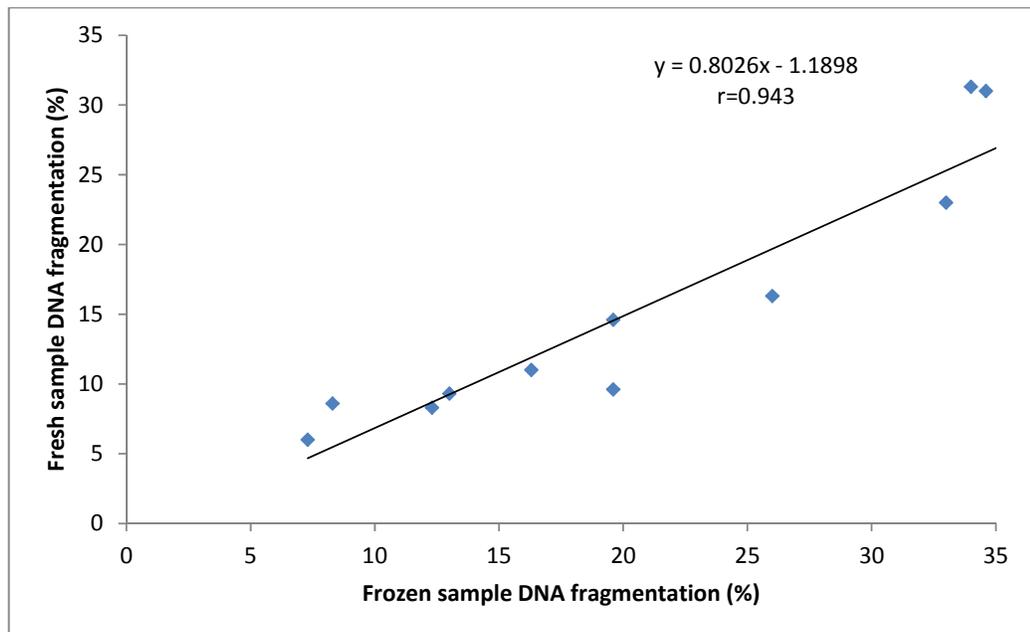


Figure 13: Comparing DNA fragmentation levels of snap frozen and fresh semen.

Both protocols originate from the same semen sample ($n=12$) and were processed in the same Halosperm run.

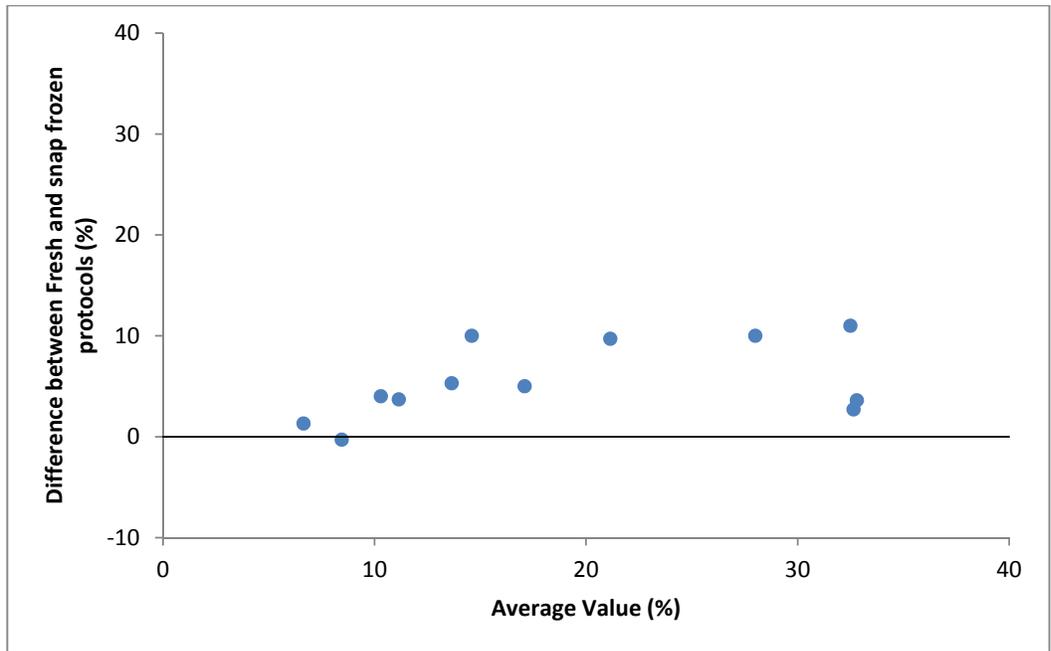


Figure 14: Mean DNA fragmentation differences between snap frozen semen and fresh semen.

Both protocols originate from the same semen sample (n=12) and were processed in the same Halosperm run.

6.5.3.2 Air-dried Semen to Fresh Semen

The average DNA fragmentation percentage \pm SEM in air-dried semen samples was 20.17 ± 4.54 and those obtained from freshly ejaculated samples was 19.09 ± 4.77 . Student's paired t-test between the air-dried and fresh semen was $t=1.65$; $p=0.12$. Figure 15 shows the DNA fragmentation percentage recorded from the same semen samples that were tested using fresh semen or following air-drying. Considering the Bland-Altman plot (Figure 16) the mean difference per sample was $1.08 \pm 0.65\%$.

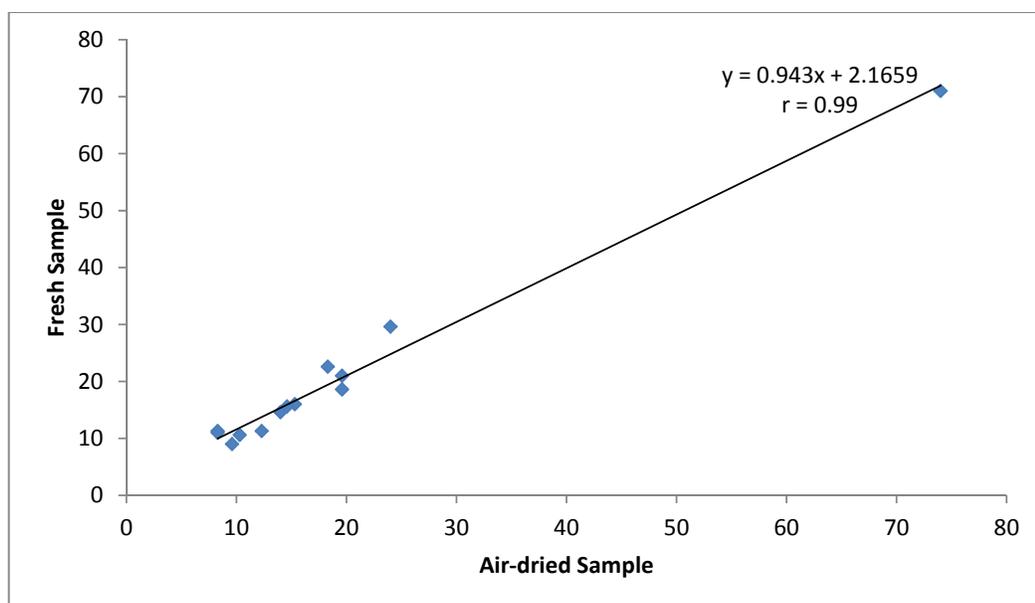


Figure 15: Comparing DNA fragmentation levels of air-dried semen and fresh semen.

Both protocols originate from the same semen sample ($n=13$) and were processed in the same Halosperm run.

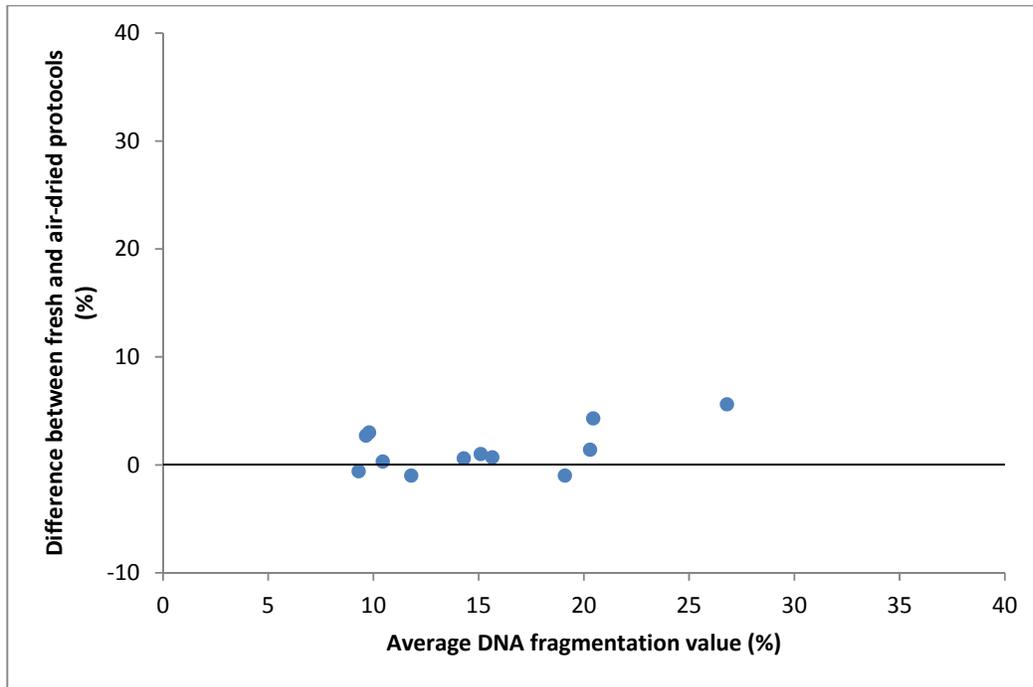


Figure 16: Mean DNA fragmentation differences between air-dried semen and fresh semen.

Both protocols originate from the same semen sample (n=13) and were processed in the same Halosperm run.

6.5.4 Comparing SDF between Different Times and Temperatures

6.5.4.1 Air-dried Semen Stored at Room Temperature: 1 Day compared to 7 Days

The mean DNA fragmentation levels \pm SEM of the semen air-dried and stored at room temperature for 1 day, was $23.95 \pm 4.40\%$ and $70.25 \pm 8.58\%$ for those air-dried and stored in the same manner for 7 days. Student's paired t-test between 1 day and 7 days was $t = -5.08$; $p = 0.0005$. The mean difference per sample was $46.29 \pm 9.12\%$. Figure 17 shows the DNA fragmentation percentage recorded from the same semen samples that were air-dried and stored at room temperature for either 1 day or 7 days.

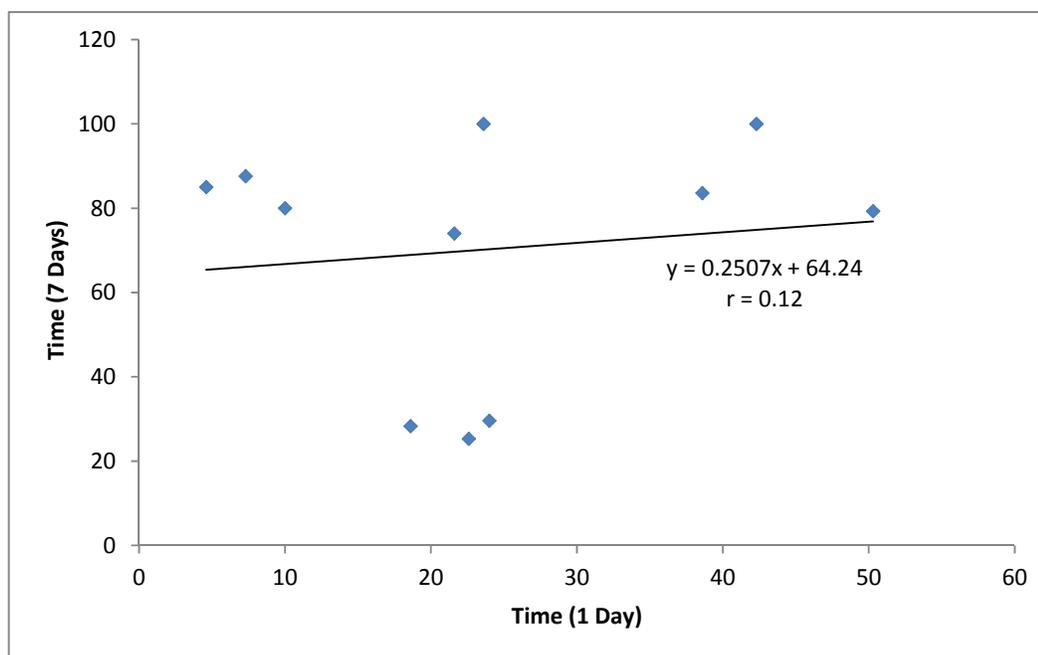


Figure 17: DNA fragmentation levels of air-dried semen stored at room temperature for 1 and 7 days.

Both protocols originate from the same semen sample ($n = 11$) and were processed in the same Halosperm run.

6.5.4.2 Air-dried Semen Stored at 4°C: 1 Day compared to 7 Days

The mean DNA fragmentation levels \pm SEM of the semen air-dried and stored at 4°C for 1 day, was $23.79 \pm 4.75\%$ and $24.62 \pm 4.79\%$ for those air-dried and stored in the same manner for 7 days. Student's paired t-test between 1 day and 7 days was $t = -1.01$; $p = 0.33$. The mean difference per sample was $0.83 \pm 0.82\%$. Figure 18 shows the DNA fragmentation percentage recorded from the same semen samples that were air-dried and stored at 4°C for either 1 day or 7 days.

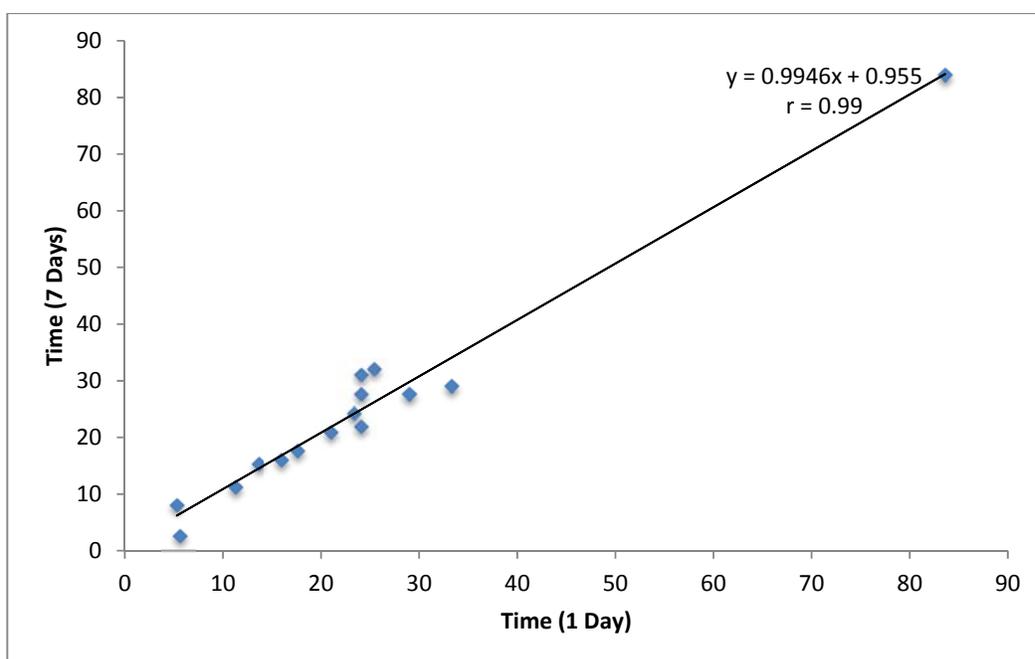


Figure 18: DNA fragmentation levels of air-dried semen stored at 4°C for 1 and 7 days.

Both protocols originate from the same semen sample ($n = 15$) and were processed in the same Halosperm run.

6.5.4.3 Air-dried Semen Stored at 4°C: 1 Day compared to 30 Days

The mean DNA fragmentation levels \pm SEM of the semen air-dried and stored at 4°C for 1 day, was $11.37 \pm 1.66\%$ and $30.96 \pm 5.32\%$ for those air-dried and stored in the same manner for 30 days. Student's paired t-test between 1 day and 30 days was significant ($t = -3.42$; $p = 0.007$). The mean difference per sample was $19.59 \pm 5.72\%$. Figure 19 shows the DNA fragmentation percentage recorded from the same semen samples that were air-dried and stored at 4°C for either 1 day or 30 days.

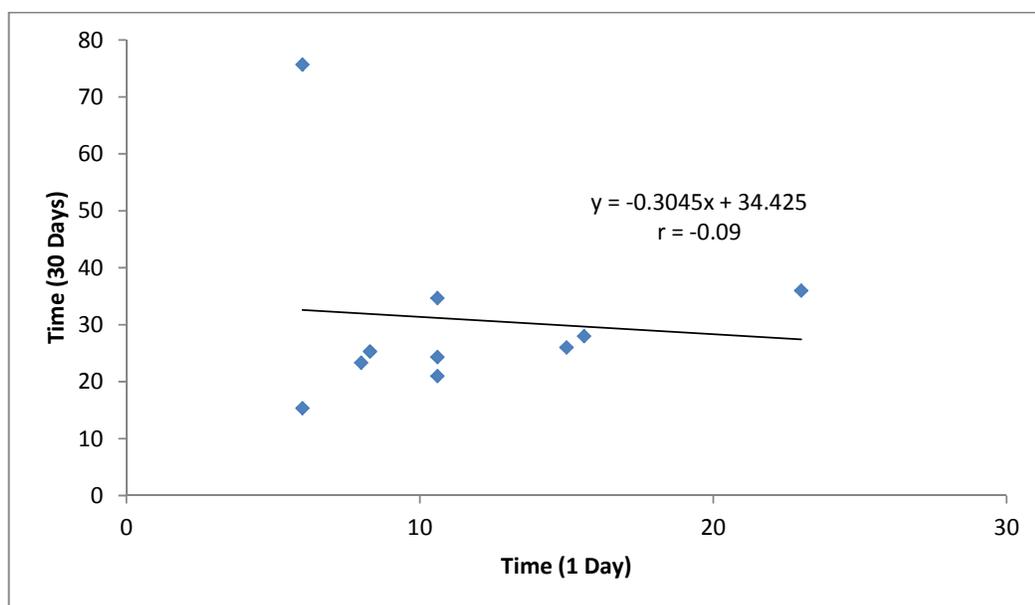


Figure 19: DNA fragmentation levels of air-dried semen stored at 4°C for 1 and 30 days.

Both protocols originate from the same semen sample ($n = 10$) and were processed in the same Halosperm run.

6.5.4.4 Air-dried Semen Stored at -22°C: 1 Day compared to 30 Days

The mean DNA fragmentation levels \pm SEM of the semen air-dried and stored at -22°C for 1 day was $22.81 \pm 4.20\%$ and $23.22 \pm 3.84\%$ for those air-dried and stored in the same manner for 30 days. Student's paired t-test between 1 day and 30 days was $t=0.75$; $p=0.47$. The mean difference per sample was $0.68 \pm 0.53\%$. Figure 20 shows the DNA fragmentation percentage recorded from the same semen samples that were air-dried and stored at -22°C for either 1 day or 30 days.

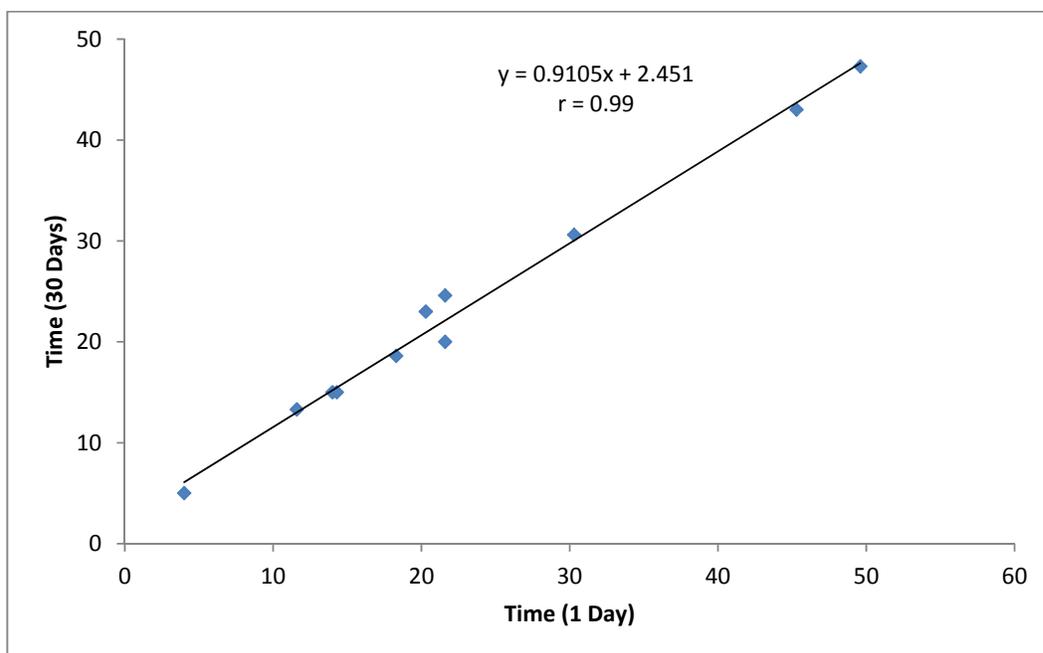


Figure 20: DNA fragmentation levels of air-dried semen stored at -22°C for 1 and 30 days.

Both protocols originate from the same semen sample ($n=11$) and were processed in the same Halosperm run.

6.5.4.5 Air-dried Semen Stored over Time at -22°C

Figure 21 shows results from the first and second batch of high value DNA fragmentation quality control samples, 'QC1 High' and 'QC2 High' respectively.

DNA fragmentation levels for 'QC1 High' were recorded at 67% after 2 days in

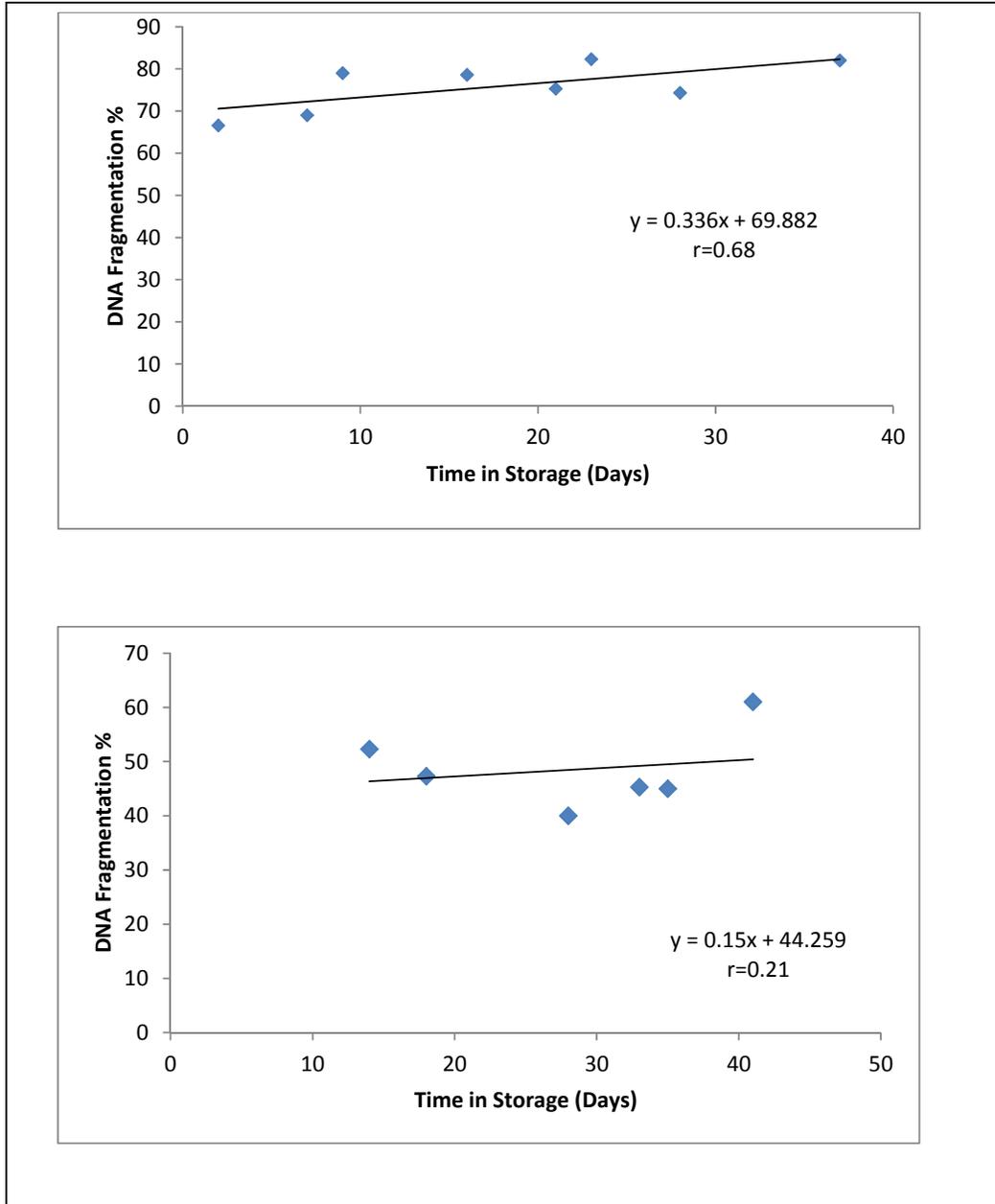


Figure 21: DNA fragmentation levels of 'QC1 High' (A) and 'QC2 High' (B) high value quality control samples measured at different time points from air-dried slides stored at -22°C.

'QC1 High' and 'QC2 High' originate from different semen samples. All 'QC1 High' samples were stored on the same day, as were all 'QC2 High' samples.

storage, 69% after 7 days in storage, 79% after 16 days in storage, 74% after 28 days in storage and 82% after 37 days in storage. The results from 'QC2 High' for similar time frames as 'QC1 High' were 52% after 14 days in storage, 40% after 28 days in storage and 45% after 35 days in storage. For 'QC1 High' the mean DFL \pm SEM for samples stored up to 28 days was 75.01 ± 2.12 compared to 78.15 ± 3.85 for samples stored between 28 and 41 days, resulting in a 4% increase between the two time frames. The mean DFL \pm SEM for 'QC2 High' was 46.53 ± 3.57 for samples stored up to 28 days and 47.83 ± 4.56 for samples stored between 28 and 41 days, resulting in a 3% increase between the two time frames.

Figure 22 shows results from the first batch (A) and second batch (B) of low value DNA fragmentation quality control samples, 'QC1 Low' and 'QC2 Low' respectively.

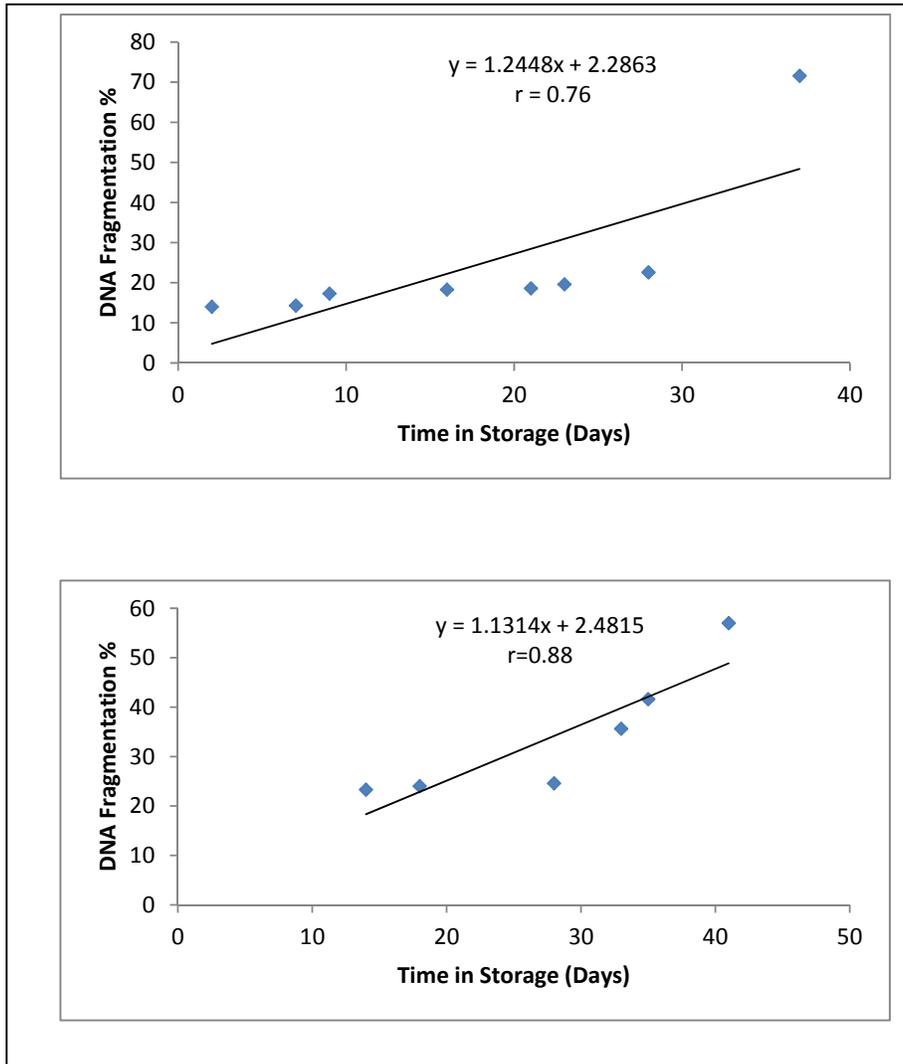


Figure 22: DNA fragmentation levels of 'QC1 Low' (A) and 'QC2 Low' (B) low value quality control samples measured at different time points from air-dried slides stored at -22°C.

'QC1 Low' and 'QC2 Low' originate from different semen samples. All 'QC1 Low' samples were stored on the same day, as were all 'QC2 Low' samples.

DNA fragmentation levels for 'QC1 Low' were recorded at 14% after 2 days in storage, 18% after 16 days in storage, 23% after 28 days in storage and 72% after 37 days in storage. The results from 'QC2 Low' for similar time frames as 'QC1 Low' were 23% after 14 days in storage, 25% after 28 days in storage and 42% after 35 days in storage. For 'QC1 Low' the mean DFL \pm SEM for samples stored up

to 28 days was 17.81 ± 1.14 compared to 47.10 ± 24.50 for samples stored between 28 and 41 days, resulting in a 164% increase between the two time frames. The mean DFL \pm SEM for 'QC2 Low' was 23.97 ± 0.38 for samples stored up to 28 days and 39.70 ± 6.76 for samples stored between 28 and 41 days, resulting in a 66% increase between the two time frames. Figure 23 shows the rapid increase in DNA fragmentation after 28 days storage.

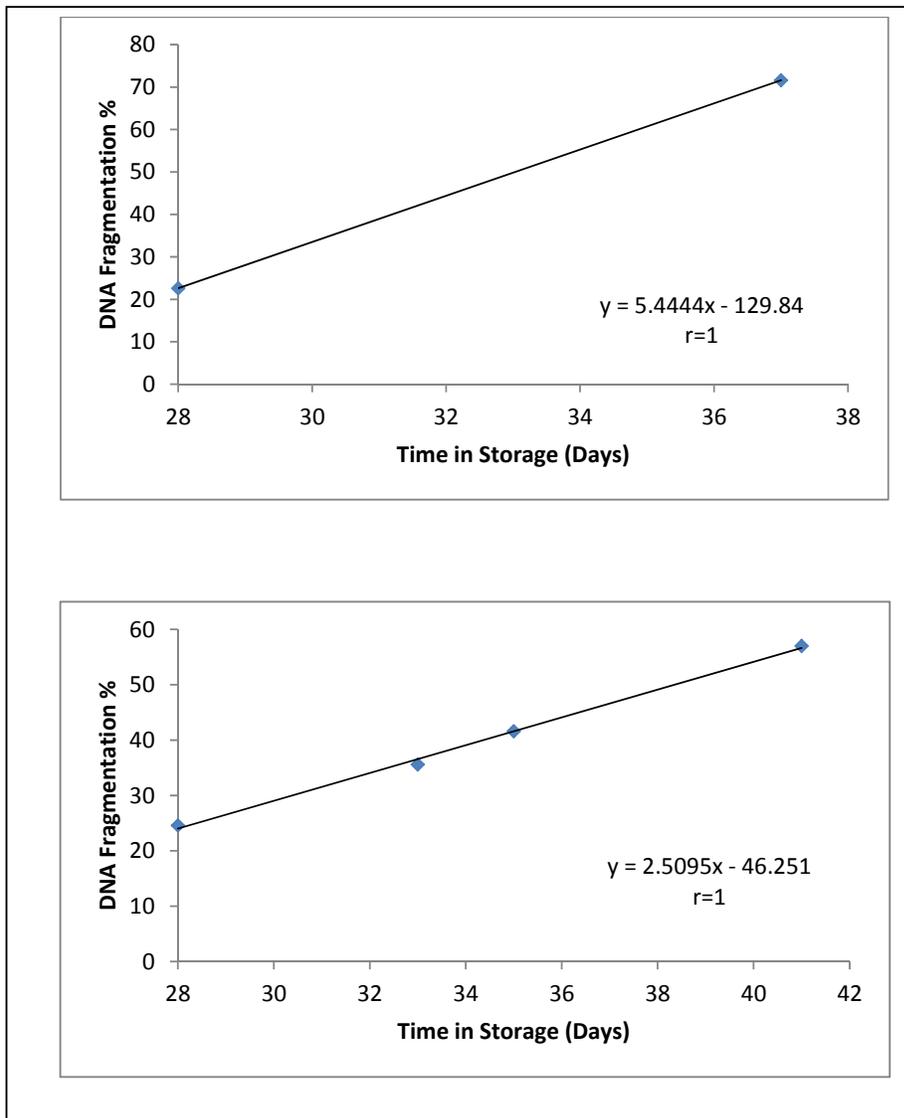


Figure 23: DNA fragmentation levels of 'QC1 Low' (A) and 'QC2 Low' (B) low value quality control samples measured after 28 days storage to show rapid increase in fragmentation after this time point.

7 Discussion

7.1 Summary

There were two major purposes of this study. The first was to consider the clinical utility of the Halosperm assay by investigating the relationship between DNA integrity and semen analysis. The main finding was that DNA fragmentation is more prevalent in abnormal semen samples when considering both WHO 4th and 5th Edition semen analysis criteria. Conversely, elevated DNA fragmentation levels have been found in normal semen samples using the same criteria. From this it can be concluded that the Halosperm test provides additional information over and above semen analysis and therefore is a valuable adjunct to semen analysis in an andrology laboratory.

The second purpose of this study was to develop an alternative method to store sperm, over a period of time, prior to DNA integrity testing using the Halosperm assay. This was achieved by air-drying whole semen samples onto microscope slides and storing the slides at -22°C for one month whilst maintaining the DNA integrity of that sample.

7.2 Clinical Utility of the Halosperm® G2 Kit

The first research question asked what relationships exist between semen quality (according to both the WHO 4th and 5th Edition semen analysis guidelines) and DNA fragmentation levels as detected by the Halosperm® G2 Kit. The results from this study indicate that DNA fragmentation levels, defined by the Halosperm assay, are significantly higher in samples categorised as abnormal than those samples which were categorised as having a normal semen analysis, regardless of which guidelines were used. Semen samples categorised as OAT by both WHO editions

have the highest DNA damage load compared to those of oligozoospermic, asthenozoospermic and teratozoospermic samples. Whilst the compounding effects of impaired parameters (concentration, morphology and motility) may increase fragmentation levels, 11.7% and 16.0% of samples classified as normozoospermic in WHO 4th and 5th Edition guidelines respectively had elevated DFLs. Furthermore, there was a statistically significant difference in DNA fragmentation levels for these samples. There was however, no statistical difference between the WHO 4th and 5th Edition guidelines for DNA fragmentation levels of specifically categorised semen defects. All samples scored using 5th Edition criteria, with some abnormality in terms of motility and/or morphology showed a mean abnormal DNA fragmentation result. In contrast, using 4th Edition criteria, only samples with both abnormal motility and morphology show a mean abnormal DNA fragmentation result. Whilst no studies have considered all of these same parameters, the general finding that there is a relationship between DFL and sperm motility and morphology is in agreement with other reports (Irvine et al., 2000; Lopes et al., 1998; Mangiarini et al., 2013; Peluso et al., 2013; Velez de la Calle et al., 2008).

Interestingly the mean DNA fragmentation level for samples with only abnormal concentration levels were within normal limits when using both 4th and 5th Edition semen analysis criteria. Irvine et al., (2000) also concluded that DNA fragmentation levels were within normal limits when sperm concentration was below normal limits, when they followed the WHO 3rd Edition semen analysis criteria. Since the guidelines of the WHO 3rd and 4th Editions provide the same sperm concentration criteria (Esteves et al., 2011) the use of a different manual

was not considered relevant. They did however use the Comet assay to interpret DNA fragmentation levels and as such a direct comparison between this study and that of Irvine and colleagues should be considered cautiously.

The actual proportion of normozoospermic samples with abnormal DNA fragmentation levels (>30%) were 11.7% and 16% following 4th and 5th Edition guidelines respectively, which concurs with others' findings (Omran et al., 2013; Schulte et al., 2010) that 15% of infertile men have semen within normal parameters. This demonstrates that the Halosperm assay can explain infertility where semen analysis cannot.

The proportion of abnormal semen samples with high DNA fragmentation levels is higher when semen analysis is measured using the 5th Edition compared to semen analysed using 4th Edition criteria. Consequently, there is considerable risk that DNA damaged spermatozoa could be used in ART. In considering this, it is proposed that the inclusion of the Halosperm assay, particularly where laboratories are using 5th Edition guidelines, is necessary when diagnosing infertility and planning treatment options.

7.3 Quality Control and Assay Precision

As with all diagnostic tests, corroboration between technicians' testing procedures and interpretation of results within andrology laboratories is an important factor to be considered (Matson, 1997). In order to establish the potential fertility status of a semen sample accurately, the method of analysis must have specific, precise and objective parameters that allow for a correlation of the data to be recognised and validated. Furthermore, analysing the internal sources of potential

error such as results produced by the same technician and those between technicians, are paramount to confirm the validity of any study (Franken & Oehninger, 2012).

In this present study it was apparent that there was some variability between different Halosperm runs using the same samples. The greatest variability was evident at the lower values which is similar to that seen with semen analysis (Leushuis et al., 2010).

Intra assay variability appears to be different dependent on which DNA fragmentation assay is being used, with previous studies reporting this to be either small but significantly different with the TUNEL assay (Sharma et al., 2010) or small and not significantly different using a modified Comet assay (Hughes, Lewis, McKelvey-Martin, & Thompson, 1997) and no difference using the SCSA method (Erenpreiss et al., 2006). It is not surprising therefore that the results of this present study show some variability. As the mechanisms behind such variations remain unclear, it is difficult to speculate why intra assay variability exists, particularly when the same semen sample is used. Data from Cortes and colleagues (2010) has shown that DNA fragmentation levels in semen, when measured with the SCD test are not consistent within a male over different time frames and that such variation is similar in scale to that which can be seen between individuals. It seemed obvious therefore, that where different protocols were being compared, it was necessary that each protocol be tested and run in the same Halosperm batch assay.

Having considered between run variability, within-run variability was assessed. In this study no significant variation was seen in DFI (DNA fragmentation Index) when the same semen sample was tested in duplicate within the same batch assay. This is correlates with other reports that have tested more than one aliquot of the same semen sample in the same batch assay (Shamsi et al., 2010; Sharma et al., 2010) although again different DNA fragmentation assays were used. Following this, inter-observer variability (results from one technician compared to those from another technician for the same samples) was found to be very similar and as such the quality assurance of this present study was confirmed.

7.4 Reconstitution Media

In the present study it was clear that both seminal plasma and phosphate buffered saline plus bovine serum albumin solution (PBS+BSA) were effective methods of reconstituting air-dried semen. The primary aim of comparing the reconstitution fluids was to establish if a readily available solution could be used without affecting DNA fragmentation levels of air-dried semen. Bovine serum albumin is a very stable serum which not only stabilizes proteins whilst in solution but also prevents non-specific binding to laboratory equipment. The serum also acts as an extracellular antioxidant which is useful for the elimination of free radicals which could potentially cause DNA damage (Namasivayam et al., 2013). In the forensic setting PBS is commonly combined with saliva and semen stains prior to DNA extraction (Alvarez, Juusola, & Ballantyne, 2004) and therefore it was expected that the PBS+BSA solution would have no detrimental effect on DNA integrity of air-dried semen.

Recent research assessed the stability of DNA integrity after air-drying semen using seminal plasma as the reconstituting fluid (Yap & Matson, 2012), however these authors did not consider the effects of PBS+BSA solution. In the present study, it was found that reconstituting air-dried semen with PBS+BSA had several advantages over seminal plasma: Firstly, the use of seminal plasma required a sufficient ejaculate volume to be able to air-dry samples and centrifuge the balance to enable the separation of seminal plasma from sperm whereas PBS+BSA is readily available. Secondly, the air-dried semen became homogenous quickly and easily, without the need for prolonged mixing when the PBS+BSA solution was used as the reconstituting fluid, compared to seminal plasma which required extensive mixing. Thirdly, the time to separate spermatozoa from seminal plasma and confirm that no spermatozoa remain was disruptive to the andrology laboratory with regards to centrifuge availability, as was technician time. This was not a problem with PBS+BSA. Finally, the antibacterial properties of seminal plasma diminish greatly over time resulting in a short shelf life (Edström et al., 2008) whereas PBS+BSA can be stored in an airtight container for several months at 4°C.

7.5 Effect of Slide Type

The present study has demonstrated no significant difference in DNA integrity when semen was air-dried on flat or concave microscope slides. There was however a marked difference in the ease of reconstituting the air-dried semen in the confines of the concave as opposed to the flat surface. Furthermore collection of the 25µl was substantially easier from the concave slide with air bubbles being fairly common when collecting resuspended semen off flat microscope slides. This

was particularly evident if the angle of the slide was not sufficient to pool the semen without it running off the slide. On flat slides, the edges of the air-dried semen pool on occasions became flaky whereas the air-dried semen pool on concave slides remained consistently uniform. It is possible that flakes breaking away from the original pool could potentially contaminate other samples and is therefore considered to be a distinct disadvantage.

7.6 Snap Frozen Semen Storage

The DNA fragmentation levels of the snap frozen versus fresh semen sample experiment indicate that snap freezing induces DNA fragmentation, with the results showing a significant decline in DNA integrity following snap freezing of the same fresh sample. This is in accordance with results from Jackson et al., (2010) who reported a significant decrease in DNA integrity using the SCD assay after snap freezing. In contrast, Wakefield and colleagues (2010) reported that snap freezing had no effect on the result of DNA fragmentation when using the SCSA assay. This difference may be accounted for by the different methods of thawing. Wakefield et al., (2010) thawed the semen rapidly in a water bath at 37°C for 30 seconds, whilst this present study thawed the samples at room temperature for 30 to 60 minutes (refer chapter 5.5.2). Jackson and colleagues did not however disclose their thawing method. The slow thaw option was used in this present study for two reasons: the first from a safety perspective as there was less risk of the nunc cryovial exploding and secondly to prevent DNA damage caused by the heat of the water bath. The method of DNA fragmentation analysis used by Wakefield et al., is unlikely to have had an effect on the differing fragmentation

levels since SCSA and SCD produce statistically similar results (Chohan et al., 2006; Ebner et al., 2011; Velez de la Calle et al., 2008).

7.7 Air-dried Semen Storage

In the present study no significant variation between DNA fragmentation levels of fresh semen and air-dried semen were observed. This study has also demonstrated that air-dried semen can be stored for up to one week at 4°C and up to one month at -22°C with only slight (non-significant) changes to the DNA integrity. The presence of some DNA degradation within the air-dried semen sample is consistent with previous research in the forensic setting using saliva. Such research has shown that DNA can be successfully extracted and analysed from fresh saliva that was stored for one week at 4°C and -20°C and from saliva stored at -20°C for 20 days (Walsh et al., 1992).

Whilst there was no significant change in DFL when air-dried semen slides were stored at -22°C for 30 days, there was a significant decline when samples were stored for this time period at 4°C. Furthermore the DFLs, of the two low value quality control samples post 28 days storage, increased substantially over those recorded up to 28 days of storage. This data suggests that the DNA in air-dried samples does degrade; the pace of which being dependent on the temperature used for storage. In this present study DNA degradation is far slower at -22°C than at 4°C showing that the colder temperature is more effective at slowing down DNA degradation. It is therefore recommended that whilst air-dried semen samples can be stored at -22°C for up to one month, this method of storage is not suitable for

longer term storage and may therefore not suffice for use in internal and external quality assurance programs.

The high value control samples produced erratic changes which may be explained by the degree of sperm DNA integrity having an influence on the stability of the air-dried samples and/or being vulnerable to the freeze thawing process. Since only two samples were available for analysis however, caution should be exercised in this regard. These results indicate that frozen-thawed samples with excessively high DFLs are not stable when air-dried and stored at -22°C.

8 Limitations and Future Studies

One particular limitation of this study was the need to snap freeze all semen samples in order to run DNA fragmentation analysis on different storage time protocols within the same batch assay. Spermatozoa from infertile men are more susceptible to the freeze thawing process with DNA fragmentation levels increasing significantly more than in their fertile counterparts. Whilst this is unlikely to have affected the difference in fragmentation levels between protocols, the writer is aware that snap freezing does induce some DNA damage and may have exacerbated the level of DNA degradation.

Another limitation of this study was with the test involving the high and low quality control samples to measure changes in DNA fragmentation over time. This test was not purposefully designed but high and low quality control samples were included in routine clinical analysis where space within the assay run was available. There was therefore no way of ensuring consistent gaps in time between

analyses as the timing of these tests were dictated by when the routine analysis was conducted and where space within the assay run was available. Consequently the sample size was small, limiting the statistical power of these results.

Future studies using fresh (as opposed to frozen-thawed) semen prior to air-drying are now required. This will be further improved by a purposely constructed study to measure change in DNA fragmentation over time using consecutive daily analysis on a larger sample size to determine at what time frame significant changes in the DNA integrity are observed when semen has been air-dried and stored at -22°C.

9 Conclusion

In conclusion, the Halosperm assay has been shown to be clinically useful in the diagnosis of male infertility, particularly idiopathic infertility. The test is useful, in that it is reproducible; each time a sample is tested in the same batch assay, the results are similar and it provides additional information over and above standard semen analysis. Additionally, this study has shown that semen can be air-dried on concave microscope slides and stored for up to one month at -22°C, before being reconstituted with a readily available reconstituting fluid without any significant DNA degradation when tested with the Halosperm assay. More importantly, air-dried slides provide statistically closer DNA fragmentation results to those of fresh samples, than to those of frozen samples. Not only is this a simple method to store semen prior to DNA integrity testing but the results are more accurate than when current methods of storage are used.

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Appendix I: Participant Information Sheet and Consent Form



RESEARCH PROJECT INFORMATION

This research project is being conducted by Ashleigh McEvoy as part of the requirements of a Masters Degree (MSc) at Edith Cowan University.

The title for this student research project is:

The development of a simplified method of human semen storage for the testing of sperm DNA fragmentation

Background

1. Increased levels of sperm DNA fragmentation are associated with reduced male fertility.
2. Sperm DNA fragmentation is assessed routinely at Fertility North with a commercially-available method, the Halosperm kit.
3. Tests are run twice per week, with samples being stored in liquid nitrogen ready for batch testing.
4. Quality control samples are kept in liquid nitrogen for inclusion each time the test is run.
5. Liquid nitrogen is not an ideal method of storage as it is dangerous to use, and quality control samples take up a large amount of storage space.

Aim of the project

- a) The present study will investigate the possibility of using air-dried slides to store semen samples prior to testing.

- b) The results of the air-dried samples will be compared with the same sample stored in liquid nitrogen in the usual way.
- c) Only once we are confident that we get the same results will we be able to introduce air-drying in to routine practice.

How can you help?

Once your semen sample has been analysed in the normal way, we would like to use the remainder of the sample in the project rather than it being discarded.

How will the sample be used?

Your semen sample will have some stored in liquid nitrogen, and some air-dried on a microscope slide. Both will then be tested in the Halosperm test. Your sperm from both the liquid nitrogen and the air-dried slide will be discarded once the investigation is complete. It will NOT be used for insemination or to achieve a pregnancy.

Will I get to see the results?

If you agree for your sample to be used then we shall send you a copy of the final report of the project.

Will the study help me?

Not directly as the results will not affect your treatment or management. However, it will hopefully enable us to improve techniques in the future, which will be of benefit to everyone seeking treatment.

Who will see the results of my sample?

The sample will be coded so that your identity will only be known by the staff at Fertility North. Please note that no other samples from you will be used.

What if I do not want my sample to be used?

You are free to refuse for your sample to be used. This will not prejudice your treatment in any way.

Who should I ask if there are any questions?

Dr Phillip Matson (Scientific Director) and Dr Vince Chapple (Medical Director) are the senior people involved in the project. These, or any of the laboratory staff at Fertility North, will be happy to answer any questions or queries. If you require assistance, call Dr Matson on 9301 1075.

FNC 22. CONSENT FOR THE USE OF SEMEN IN THE RESEARCH PROJECT

ENTITLED:

The development of a simplified method of human semen storage for the testing of sperm DNA fragmentation.

Name:

Address:

DOB:

I agree for my semen sample produced on *(date)*..... to be used in this project.

I also understand that:

- a) Refusal would not have prejudiced my treatment in any way.
- b) My sperm will NOT be used to achieve a pregnancy, but will be discarded upon completion of the investigation.
- c) I will be sent a copy of the final report of the study so that I may see how the semen samples were used, and the conclusions made.
- d) I am free to ask questions of the investigators during the study.
- e) I will be given a copy of this consent form.

SIGNATURES

Volunteer: date

Witness: date

(Must be FN member of staff)

The ethical aspects of this study have been approved by the Joondalup Health Campus Human Research Ethics Committee. If you have any complaints or reservations about any ethical aspect of your participation in this research, you may contact the Committee through the Executive Office – phone 9400 9404. Any complaint you make will be treated in confidence and investigated, and you will be informed of the outcome

Appendix II: SOP Halosperm Testing

Purpose: To describe in detail the procedure for the assessment of sperm DNA fragmentation in humans using the Halosperm G2 Test Kit.

Associated Documents:

LB-F-398:02	Halosperm Worksheet
LB-S-493	Semen Analysis

Definitions:

Responsibilities:

Role *or* Responsible for:
Department

Andrology • Ensuring that this procedure is adhered to in relation to DNA fragmentation testing

1 Introduction

- 1.1 The SCD assay determines the vulnerability of sperm DNA to acid denaturation and follows the principle that induced condensation is directly related to sperm DNA fragmentation (Muriel et al., 2006).
- 1.2 Sperm are suspended in an agarose micro gel before being placed on an agarose pre-treated slide which renders the sperm in a “suspension-like environment” on the slide (Fernández et al., 2005, p. 834).
- 1.3 An acid solution is applied to the agarose/sperm matrix to denature the DNA followed by treatment with a lysis buffer to lyse cell membranes and remove nuclear proteins.
- 1.4 As a result a halo of dispersed DNA loops surrounds the central core which can be viewed under bright field microscopy after staining the slides with the Wright’s stain.

- 1.5 Sperm with intact DNA will show as having large halos whilst those with elevated DNA fragmentation will generate no halo or at the most a very small halo (Fernández et al., 2005).

2 Pre-analytic Process

2.1 Semen Collection and Handling

- 2.1.1 Refer to LB-S-493 for the protocol for semen analysis.
- 2.1.2 Semen samples will be collected via masturbation into a 60ml wide-mouthed universal container and then immediately stored at 37°C in an incubator (Mettler GmbH, Germany) for a maximum of one hour (from production until snap freezing), to liquefy.
- 2.1.3 **BIOHAZARD:** All products or objects that come in contact with human or animal body fluids should be handled, before and after cleaning, as if capable of transmitting infectious diseases. Wear facial protection, gloves and protective clothing

2.2 Semen Storage and Stability

- 2.2.1 Keep tubes covered at all times.
- 2.2.2 Do not use samples that have been stored at room temperature for more than one hour.

2.3 Reagents Storage and Stability

The Halosperm® G2 test kit must be stored at 4°C and used before the specified expiry date, or destroyed in the Biohazard bin.

3 Analytical Process

3.1 Halosperm® G2 Assay

3.1.1 Equipment Setup

1. Check room temperature is 22°C.
2. At the workspace, half fill the water bath with tap water.

- 3.** As the current temperature probe is not waterproof, ensure the probe is sealed in plastic and weighted down with a brass key to allow conduction of heat from the water to the probe.
- 4.** Check the temperature reading device is connected to the probe and is visible.
- 5.** Place the lid on the water bath.
- 6.** Plug the water bath into a power outlet and switch on.
- 7.** The water bath should reach 37°C (+/-1°C) before it is used for the Halosperm® G2 assay.
- 8.** Remove the Halosperm® G2 test kit from the refrigerator and place this on the workspace adjacent to the water bath. The kit should be at room temperature before use.
- 9.** Remove the required number of slides from the test kit and place these on the bench in single layers to allow the glass to reach the room temperature of 22°C.
- 10.** Remove from under the bench the blue bag comprising of: 4 glass containers, each with a lid and housing one petri dish, one pipette, one diamond tip pen and one stop watch.
- 11.** Place the required number of glass containers and their lids on a free surface at the workspace.
- 12.** Two pipettes (sizes 20 - 200µL and 2 - 20µL) and stop watch should be placed in close proximity to the glass containers.
- 13.** Other items that should be placed on the free work surface include: relevant pipette tips (sizes 20 - 200µL and 2 - 20µL) , 1.5ml eppendorf tubes, 22 x 22mm glass cover slips, a beaker containing 2 glass disposable pipettes, 70% ethanol, 95% ethanol and a beaker of distilled water, polystyrene tray.
- 14.** If the test is being carried out in the biochemistry laboratory, the metal plates should be transferred from the Andrology fridge to the Biochemistry fridge, both of which are set at 4°C.

3.1.2 Specimen Setup

- 1.** Where semen samples to be tested have been previously frozen in Nunc tubes, the frozen samples should be removed from canister 9 in the liquid

nitrogen tank B and placed in a polystyrene tray at the workspace to thaw, (approximately one hour).

2. The required samples should be cross checked with the register, ensuring that the required samples are removed from the tank.
3. Once the Nunc tube has warmed slightly the lid should be slightly loosened so that any trapped liquid nitrogen is released preventing pressure build up and possible explosion.
4. The Nunc tubes should be arranged in the polystyrene tray so that the patient sample that corresponds to the worksheet at # 1 is placed to the most extreme left of the tray, several rows down from the top. The patient sample that corresponds to the worksheet at # 2 should be placed in the space immediately to the right of the previous sample in the same row. This is done for all patients so that the patient samples are in the same order as the corresponding worksheet number.
5. Positive and negative controls are frozen in straws. One positive and one negative control must be tested each time a batch of Halosperm® G2 assays are processed.
6. The control straws must be removed from, Canister 2 in the liquid nitrogen tank B and placed on a sheet of paper towel to thaw at the workspace.

3.1.3 Paperwork Setup

1. A new worksheet should be set up for every batch of Halosperm® G2 assays carried out.
2. Detail of the Halosperm® G2 kit batch number and expiry date are to be recorded on the worksheet.
3. Detail of the semen samples must be copied from the Andrology Halosperm register including patient's first name in small letters and surname name in capital letters, the patient ID# and the date that the sample was produced.
4. At item '7' on the worksheet, record 'Negative control'.
5. At item '8' on the worksheet, record 'Positive control'.
6. Further detail concerning the patient, such as sample number, days of abstinence, volume of sample, concentration, progressive motility and

morphology is obtained from the Powerterm database. This detail can be completed during incubation periods.

3.1.4 Labelling

1. One eppendorf tube should be labelled with the corresponding number as reflected on the worksheet for each patient, plus one tube labelled '7' and one labelled '8' for the negative and positive controls respectively.
2. Additionally, one eppendorf tube to be labelled 'Pos' and one eppendorf tube to be labelled 'Neg'
3. One eppendorf tube to be labelled 'Neg' should be placed in the polystyrene tray to collect the positive semen sample from the straw.
4. One eppendorf tube to be labelled 'Pos' should be placed in the polystyrene tray to collect the negative semen sample from the straw.
5. Cut one end of the positive control sample straw and place the cut end into the eppendorf tube housed in the polystyrene tray labelled 'Neg'. Make a hole at the other end and allow the semen to flow into the eppendorf tube.
6. Clean the scissors by rinsing with water.
7. Cut one end of the negative control sample straw and place the cut end into the eppendorf tube housed in the polystyrene tray labelled 'Pos'. Make a hole at the other end and allow the semen to flow into the eppendorf tube.
8. Clean the scissors by rinsing with water.
9. Dispose of the straws into Biohazard bin.
10. Label each glass slide at the clear end with the diamond tip pen from 1 to 4 together with the day, month and year (dd/mm/yyyy). Ensure the slide is the right side up and the well areas are not touched. Refer Appendix 2
11. Set pipette to 50 μ L

3.1.5 Preparation of Slides

1. Fill a beaker with 800ml of boiling water and stand in blue bag.
Note: Where boiling water is collected from a distant site (i.e. kitchen) the beaker must be carried in the insulated blue bag as a precaution safety measure.

2. Place the Agarose gel tube from the Halosperm® G2 kit into a blue float (also supplied with the kit) with the lid end being exposed enough to hold.
3. Set the stopwatch for 5 minutes.
4. The agarose gel tube in the floating device is then placed in the beaker of boiling water for 5 minutes to liquefy the agarose.
5. Press 'start' on the stopwatch
6. Check the water bath has reached 37°C,
7. Place one of each labelled eppendorf tube into a floating device and then place this into the water bath.
8. Eppendorf tubes numbered '1' and '2' should be placed in the same floating device, '3' and '4' in the same floating device, '5' and '6' in the same floating device and '7' and '8' in the same floating device. At least two thirds of the tube should be immersed in water.
9. After 5 minutes, remove agarose from the boiling water and place in the polystyrene tray.
10. Transfer 50µL of liquefied agarose into each eppendorf tube floating in the water bath. The same pipette tip can be used for transfer of agarose into all eppendorf tubes.
11. Set the timer for 5 minutes and press start. The agarose must be cooled to 37°C otherwise the heat will disrupt the DNA integrity.
12. Remove and dispose of the pipette tip into the biohazard bin.
13. Reset the pipette to 25µL.
14. After this 5 minute timing session, mix the semen sample by pumping the pipette approximately 15 times.
15. Transfer 25µL of semen from patient recorded on the worksheet to the correspondingly labelled eppendorf tube.
Note: This is a high risk step as the eppendorf tubes are only labelled with numbers. Semen from the patient recorded on the worksheet at #1 must be transferred to the eppendorf tube in the water bath labelled '1'.
16. Gently mix the semen and agarose suspension by pumping the pipette approximately 15 times. Ensure no bubbles are created.
17. If the semen sample is very viscous, visually check that the semen is now fully suspended in the liquefied agarose.
18. Discard the pipette tip into Biohazard bin.

19. This is repeated for each patient, and control, cross checking detail to worksheet numbers and eppendorf numbers. The patient sample should be placed into the eppendorf tube that corresponds to their worksheet number as follows:

Semen from the patient recorded on the worksheet at #2 must be transferred to the eppendorf tube in the water bath labelled '2'.

Semen from the patient recorded on the worksheet at #3 must be transferred to the eppendorf tube in the water bath labelled '3'.

Semen from the patient recorded on the worksheet at #4 must be transferred to the eppendorf tube in the water bath labelled '4'.

Semen from the patient recorded on the worksheet at #5 must be transferred to the eppendorf tube in the water bath labelled '5'.

Semen from the patient recorded on the worksheet at #6 must be transferred to the eppendorf tube in the water bath labelled '6'.

Semen from the negative control (eppendorf tube labelled 'Neg'), recorded on the worksheet at #7 must be transferred to the eppendorf tube in the water bath labelled '7'.

Semen from the positive control, (eppendorf tube labelled 'Pos') recorded on the worksheet at #8 must be transferred to the eppendorf tube in the water bath labelled '8'.

20. A new pipette tip must be used for each semen sample.

21. Set the pipette to 8 μ L.

22. Place the glass slides in order from 1 to 4 on the bench, in preparation of placing the cell suspension on the slides.

23. Place 8 μ L of cell suspension from eppendorf tube labelled '1' onto slide labelled '1' in the centre of the well area labelled 'S' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.

24. Place 8 μ L of cell suspension from eppendorf tube labelled '2' onto slide labelled '1' in the centre of the well area labelled 'C' and place a cover slip over this immediately.

Discard the pipette tip into biohazard bin and collect a new tip.

25. Place 8 μ L of cell suspension from eppendorf tube labelled '3' onto slide labelled '2' in the centre of the well area labelled 'S' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.

- 26.** Place 8 μ L of cell suspension from eppendorf tube labelled '4' onto slide labelled '2' in the centre of the well area labelled 'C' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.
- 27.** Place 8 μ L of cell suspension from eppendorf tube labelled '5' onto slide labelled '3' in the centre of the well area labelled 'S' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.
- 28.** Place 8 μ L of cell suspension from eppendorf tube labelled '6' onto slide labelled '3' in the centre of the well area labelled 'C' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.
- 29.** Place 8 μ L of cell suspension from eppendorf tube labelled '7' onto slide labelled '4' in the centre of the well area labelled 'S' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.
- 30.** Place 8 μ L of cell suspension from eppendorf tube labelled '8' onto slide labelled '4' in the centre of the well area labelled 'C' and place a cover slip over this immediately. Discard the pipette tip into biohazard bin and collect a new tip.
- 31.** Remove the metal plates from the fridge and place each slide onto a metal plate. Two slides and be placed onto one metal plate. The slide must have full contact with the metal plate and not be placed over the holes in the plate.
- 32.** Return the metal plates to the same fridge.
- 33.** Set the stop watch for 5 minutes and press start. Incubating in the fridge at 4°C for 5 minutes will solidify the agarose.
- 34.** After 5 minutes, remove the slides from the fridge and gently remove the cover slips by sliding the cover slip to the nearest long edge of the slide to avoid contamination.
- 35.** Place the slide in the horizontal position on the petri dish inside the glass container.
- 36.** Place the lid on the container immediately

37. Dispose of the cover slips into the Biohazard bin.

3.1.6 Sample Processing

1. Set the stopwatch to 7 minutes.
2. Add 4 drops of solution 1 (checking the solution number on the bottle before use) from the Halosperm® G2 kit to each well to full cover the solidified suspension.
3. Press *'start'* on the stop watch when the first half of the slides have been covered with solution 1.
4. Immediately place the lids on each glass container when application of solution 1 is complete for all slides.
5. Place the metal trays back in the Andrology fridge.
6. After 7 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
7. Set the stop watch for 20minutes.
8. Add 5 drops of solution 2 (checking the solution number on the bottle before use) from the Halosperm® G2 kit to each well to fully immerse the solidified suspension.
9. Press *'start'* on the stop watch when the first half of the slides have been covered with solution 2.
10. Immediately place the lids on each glass container when application of solution 2 is complete for all slides.
11. After 20 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
12. Set the stop watch for 5minutes.
13. Using a glass, disposable pipette, wash each slide with an abundant amount of distilled water.
14. Press *'start'* on the stop watch when the first half of the slides have been covered with distilled water.
15. Immediately place the lids on each glass container when application of the distilled water is complete for all slides.

- 16.** After 5 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
- 17.** Set the stop watch for 2minutes.
- 18.** Using the same pipette as before, dehydrate the solidified suspension by flooding with 70% ethanol for 2 minutes
- 19.** Press 'start' on the stop watch when the first half of the slides have been covered with 70% ethanol.
- 20.** Immediately place the lids on each glass container when application of the 70% ethanol is complete for all slides.
Note: This must be done quickly to prevent the ethanol from evaporating.
- 21.** After 2 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
- 22.** Set the stop watch for 2minutes.
- 23.** Using a new glass disposable pipette , dehydrate the solidified suspension by flooding with 95% ethanol for 2 minutes
- 24.** Press 'start' on the stop watch when the first half of the slides have been covered with 95% ethanol.
- 25.** Immediately place the lids on each glass container when application of the 95% ethanol is complete for all slides.
Note: This must be done quickly to prevent the ethanol from evaporating.
- 26.** After 2 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
- 27.** Wipe any excess liquid with a paper towel from the long edge of each slide, being careful not to touch the wells.
- 28.** Stand the slides along their long edge against a glass container which is resting on a paper towel. Leave these to air dry (approximately 10-15 minutes or until the slide is visibly dry).
- 29.** Place the lids back on the glass containers.

3.1.7 Staining

- 1.** Place each slide in the horizontal position on the petri dish inside the glass container.
- 2.** Set the stop watch for 7minutes.
- 3.** Add 5 drops of solution 3 from the Halosperm® G2 kit (checking the solution number on the bottle before use) to each well to fully immerse the solidified suspension.
- 4.** Press '*start*' on the stop watch when the first half of the slides have been covered with solution 3.
- 5.** Immediately place the lids on each glass container when application of solution 3 is complete for all slides.
- 6.** After 7 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish and then returning the slide to the horizontal position on the petri dish inside the glass container.
- 7.** Set the stop watch for 7minutes.
- 8.** Add 5 drops of solution 4 from the Halosperm® G2 kit (checking the solution number on the bottle before use) to each well to fully immerse the solidified suspension.
- 9.** Press '*start*' on the stop watch when the first half of the slides have been covered with solution 4.
- 10.** Immediately place the lids on each glass container when application of solution 4 is complete for all slides.
- 11.** After 7 minutes, remove the glass lids and drain each slide by tapping the long edge of the slide against the petri dish.
- 12.** Wipe any excess liquid with a paper towel from the long edge of each slide, being careful not to touch the wells.
- 13.** Stand the slides along their long edge against a glass container which is resting on a paper towel. Leave these to air dry (approximately 10-15 minutes or until the slide is visibly dry).
- 14.** Place the lids back on the glass containers.

3.1.8 Clean up

The clean-up process can be carried out as each stage is completed, however the eppendorf tubes from the water bath must not be discarded until visualization of

the samples is complete and it has been confirmed that the assay has been successful.

3.1.9 Visualization

With a bright field microscope and using an imaginary grid starting at the west most point of the well, count individual sperm as fragmented or non-fragmented (according to Appendix 2) moving the stage in the forward position until the edge of the well is reached. Move the stage to the left and continue counting whilst moving the stage in the backward position until the edge of the well is reached. Move the stage further to the left and continue counting whilst moving the stage in the forward position until the edge of the well is reached. Continue until a total of 300 sperms are counted.

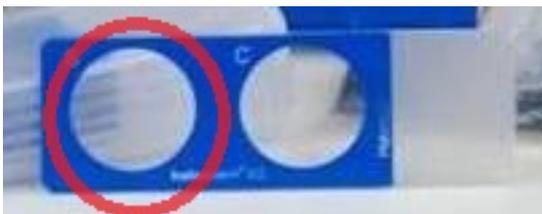
4 References

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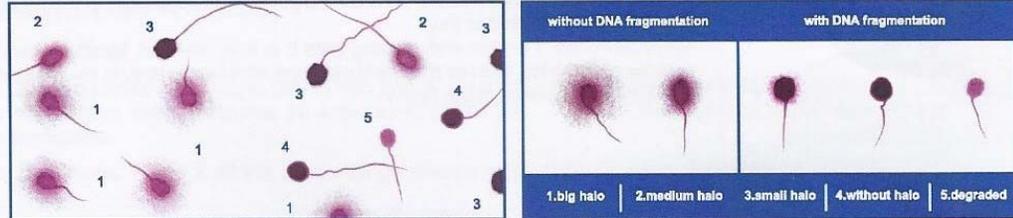
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5 Appendices

Appendix 1 – Photograph of Halosperm® Slide with one well identified



Appendix 2 – Sperm DNA Fragmentation Classification



Sperm classification

Score a minimum of 300 sperm per sample following the criteria:

Sperm with fragmented DNA:

- sperm with small halo: the halo width is similar or smaller than $\frac{1}{3}$ of the minor diameter of the core.
- sperm without halo.
- sperm without halo and degraded: no halo is observed and a irregularly or weakly stained core is present.

Sperm without fragmented DNA:

- sperm with big halo: the halo width is similar or higher than the minor diameter of the core.
- sperm with medium-sized halo: the halo size is between those with large and with very small halo.

“Others”: cell nuclei, which do not correspond to sperm. One of the morphological characteristics, which distinguish them, is the absence of tail. These cells must not be included in the estimation of the frequency of sperm with fragmented DNA.