

**A STUDY OF FUNCTIONAL MARKERS IN RAW AND
PROCESSED BOVINE SPERM AND THEIR POTENTIAL USES
FOR FERTILITY PREDICTION AND PROCESS REFINEMENT**

A THESIS SUBMITTED IN ACCORDANCE
WITH THE REQUIREMENTS OF THE
UNIVERSITY OF LIVERPOOL
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE FACULTY OF HEALTH AND LIFE SCIENCES

BY

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NOVEMBER, 2012

DECLARATION

I declare that, unless stated otherwise, this thesis is entirely my own work carried out in the laboratories of the Genus Breeding Limited, Freezing Unit, Llanrhydd, Ruthin, North Wales and Centre for Cell Imaging, School of Biological Sciences, University of Liverpool, UK, under the supervision of Dr. R.D. Murray, S.G. Revell, Dr. C.G. Argo and D.G. Spiller between August 2009 and April, 2011. No part of this thesis, in any form, has been submitted to any other University or for any other degree.

Sahib Khan Shahani, November, 2012

Table of Contents		Pages
Chapter	Title page	i
1.	General Introduction and Literature Review	1
	Historical background of artificial insemination	1
	Semen collection, extension and preservation	6
	Morphology and ultra structure of bovine spermatozoa	13
	<i>The head or caput</i>	13
	<i>The flagellum</i>	16
	Transport and maturation of spermatozoa	17
	<i>Epididymal transition</i>	17
	<i>Female genital tract</i>	19
	Sperm capacitation and hyperactivated motility	20
	Energy metabolism related to spermatozoa	23
	<i>Role of mitochondria</i>	23
	<i>Energy production</i>	25
	<i>Energy and sperm motility</i>	27
2.	General Materials and Methods	31
	Preparation of artificial vagina	31
	Teasing bulls and semen collection	32
	Semen handling and assessment	32
	<i>Colour</i>	32
	<i>Semen volume</i>	32
	<i>Sperm motility</i>	32
	<i>Sperm concentration</i>	33
	<i>Sperm morphology</i>	33
	<i>Semen dilution, packing and freezing</i>	33
	<i>Assessment of motility after freeze-thaw process</i>	34
	<i>Bull stud records</i>	34
	Non-return rates in bulls	34
	Preparation of reagents	35
	<i>Nigrosin-eosin stain</i>	35
	<i>Phosphate buffered saline extender</i>	35
	<i>2-dexoxy-D-glucose suspension</i>	35
	<i>Iodoacetamide suspension</i>	35
	<i>Valinomycin</i>	36
	<i>Sodium sulphite</i>	36
	<i>JC-1 fluorescent dye</i>	36
	<i>Propidium iodide fluorescent dye</i>	36
	<i>SYBR-14 fluorescent dye</i>	36
	<i>Hoechst 33258 fluorescent dye</i>	36
3.	Mid-piece length of spermatozoa in different cattle breeds and its relationship to fertility	37
	Introduction	37
	Materials and methods	39

	Data collection and statistical analysis	41
	Results	41
	<i>Differences between ejaculates from the same bull</i>	41
	<i>Differences between breeds</i>	43
	<i>Mid-piece length and 49-day non-return rates</i>	44
	Discussion	45
4.	Validation of a method for estimating bovine sperm mitochondrial membrane potential	48
	Introduction	48
	Materials and methods	51
	<i>Preparation of frozen-thawed and fresh semen</i>	51
	<i>Fluorescent staining</i>	51
	<i>Regional and logical gates</i>	51
	<i>Precision and trueness of regional/logical gates</i>	53
	<i>Application of colour gates</i>	53
	<i>Auto-fluorescence</i>	55
	<i>Incubation times</i>	55
	Statistical analysis	55
	Results	56
	<i>Precision and trueness of regional/logical gates</i>	56
	<i>Application of colour gates</i>	57
	<i>Auto-fluorescence</i>	58
	<i>Comparison of incubation times</i>	60
	Discussion	60
	Conclusions	62
5.	Effects of some respiratory and glycolytic inhibitors on mitochondrial functionality in bovine semen	63
	Introduction	63
	Materials and methods	66
	<i>Preparation of fresh and frozen-thawed semen</i>	66
	<i>Semen concentration related to different reagents</i>	66
	<i>Addition of reagents and control</i>	66
	<i>Fluorescence staining of sperm and flow cytometry</i>	67
	Statistical analysis	68
	Results	68
	<i>Comparison of control with treatment</i>	69
	<i>Comparison between fresh and frozen-thawed semen</i>	72
	Discussion	72
	Conclusions	75
6.	Oxygen metabolism and its relationship to sperm mitochondrial membrane potential	76
	Introduction	76
	Materials and methods	78
	<i>Preparation of raw semen</i>	78
	<i>Preparation of frozen-thawed semen</i>	78

	<i>Measurement of mitochondrial membrane potential</i>	78
	<i>Oxygen consumption in live sperm</i>	79
	<i>Oxygen biosensor plate</i>	79
	<i>Sperm live:dead ratio</i>	79
	<i>Fluorescence intensity collection and normalization and calculation of oxygen concentration and oxygen consumption rate</i>	79
	<i>Amplitude of lateral head displacement</i>	80
	Statistical analysis	81
	Results	81
	<i>Differences in ZO₂ between fresh and frozen-thawed semen</i>	81
	<i>Differences in ZO₂ between ejaculates</i>	81
	<i>Differences between bulls</i>	82
	<i>Oxygen consumption compared to mitochondrial membrane potential</i>	84
	Discussion	86
	Conclusions	87
7.	Effect of glycolytic and respiratory inhibitors on bovine sperm motility	88
	Introduction	88
	Materials and methods	90
	<i>Preparation of semen</i>	90
	<i>Assessment of sperm motility</i>	91
	Data handling and statistical analysis	92
	Results	92
	<i>Relationship between sperm motility parameters</i>	92
	<i>Differences between fresh and frozen-thawed semen</i>	92
	<i>Comparison of sperm motility characteristics between bulls</i>	94
	<i>Comparison of motility characteristics of fresh and frozen-thawed spermatozoa diluted in EYTG and Ruthin extender</i>	94
	<i>Sperm motility in presence of glycolytic and respiratory inhibitors</i>	95
	2-deoxy-D-glucose	95
	Iodoacetamide	96
	Valinomycin	96
	<i>Sperm progressive motility, oxygen consumption and mitochondrial membrane potential compared to bull non-return rate (NRR) and sperm mid-piece (MPL)</i>	98
	<i>Sperm progressive motility compared to oxygen consumption and mitochondrial membrane potential</i>	99
	Discussion	100
	Conclusions	104
8.	General discussion and conclusion	105
	References	113
	Appendices	
	3.1. Representing number of mature bulls from different breeds, their mid-piece length (MPL) measurement and percentage of non-return rates (NRR %).	137

4.1. Fresh spermatozoa washed in Ruthin extender and stained with JC-1, and PI.	138
4.2. Frozen-thawed spermatozoa washed in Ruthin extender and stained with JC-1, and PI.	138
4.3. Fresh unwashed spermatozoa in Ruthin extender and stained with JC-1, and PI.	138
4.4. Frozen-thawed unwashed spermatozoa in Ruthin extender and stained with JC-1, and PI.	139
4.5. Fresh unwashed spermatozoa in PBS extender and stained with JC-1, and PI.	139
4.6. Frozen-thawed unwashed spermatozoa in PBS extender and stained with JC-1, and PI.	139
4.7. Fresh spermatozoa washed in Ruthin extender unstained with JC-1, and PI.	140
4.8. Frozen-thawed spermatozoa washed in Ruthin extender unstained with JC-1, and PI.	140
5.1. Schematic presentation of how different modes of actions of 2-Deoxy-D-glucose and iodoacetamide affect glycolytic pathways.	141
7.1. Differences in motility parameters between the bulls and fresh and frozen-thawed semen.	142
7.2 (a & b). Motility characteristics of fresh sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors and their comparison with control (CON).	143
7.2 (a & b). Motility characteristics of frozen-thawed sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors and their comparison with control (CON).	144

Publications

Shahani, S.K., Revell, S.G., Argo, C.G. and Murray, R.D. (2010). Mid-piece length of spermatozoa in different cattle breeds and its relationship to fertility. *Pakistan Journal of Biological Sciences*, **13**: 802-808.

Shahani, S.K., Revell, S.G., Argo, C.G. and Murray, R.D. (2012). Effects of some respiratory and glycolytic inhibitors on mitochondrial functionality in bovine semen. *The Open Andrology Journal*, **4**:12-20.

Oral presentation

Shahani, S.K., Revell, S.G., Argo, C.G. and Murray, R.D. (2010). Bovine spermatozoon mid-piece length in different breeds and its relationship to fertility. *Annual Conference of Association of Veterinary Teachers and Research Workers*, York, pp, 44.

Shahani, S.K., Revell, S.G., Argo, C.G. and Murray, R.D. (2011). Assessment of sperm motility and its relationship to bull fertility. *Annual meeting of British Andrology Society*, Birmingham.

Poster presentation

Shahani, S.K., Revell, S.G., Argo, C.G. and Murray, R.D. (2011). Sperm oxygen metabolism and its relationship to mitochondrial membrane potential. *Annual Conference of Society of Reproduction and Fertility*, Brighton. pp,64.

List of Tables	Pages
3.1 Mean mid-piece length (SD) of 200 spermatozoa examined from five ejaculates, each obtained from either juvenile and mature Holstein or five other breeds of bull.	43
4.1 Logical gates set on quadrant 1-4.	54
4.2 Outcome of populations at altered logical gates set on quadrant one to four.	58
4.3 Mean low and high mitochondrial membrane potential ($\Delta\Psi_m$) (\pm SD) and high:low $\Delta\Psi_m$ ratio of fresh and frozen-thawed semen after 40 and 80 minutes incubation time.	60
5.1 Comparison of high:low $\Delta\Psi_m$ ratio between fresh (FR) and freeze-thaw (FT) sperm.	72
6.1 Comparison of live sperm (%) and mean ZO_2 between fresh (FR) and frozen-thawed (FT) sperm.	81
6.2 Comparison of mean ZO_2 between the first (E1) and second (E2) ejaculates of fresh and frozen-thawed sperm.	82
6.3 Comparison of ZO_2 , high:low $\Delta\Psi_m$ ratio and sperm amplitude of lateral head displacement (ALH) between first and second ejaculates (mean \pm SD).	82
7.1 Sperm motility characteristics and their description.	91
7.2 Correlation between sperm motility parameters in 16 frozen-thawed spermatozoa samples obtained from four bulls.	93
7.3 Differences in mean motility parameters (SE of mean) between four bulls and their fresh and frozen-thawed semen.	93
7.4 Effect of egg-yolk/tris/glycerol (EYTG) and Ruthin (RUTH) extenders on mean sperm motility parameters (SE of mean) for fresh and frozen-thawed bull semen.	95
7.5 The relationship of CASA assessed sperm motility parameters, oxygen consumption (ZO_2) and high:low $\Delta\Psi_m$ ratio of frozen-thawed semen with 49 day bull non-return rates (NRR) and sperm mid-piece length (MPL), using Pearson correlation test.	99
7.6 The relationship between sperm progressive motility and ZO_2 and high:low $\Delta\Psi_m$ ratio of fresh (FR) and frozen-thawed (FT) spermatozoa using Pearson correlation test.	99

List of Figures	Pages
1.1 Trends in number of cows bred by artificial insemination in England and Wales, 1944/45-1957/58.	5
1.2 Trends in number of cows bred by artificial insemination in England and Wales, 1975/76-1987/88.	5
1.3 Diagrammatic representation of a bovine spermatozoa.	13
1.4 Outline of the glycolytic pathway.	26
1.5 Electron transport chain (ETC) of the inner mitochondrial membrane involved in oxidative phosphorylation, including ETC complexes (I, II, III, IV and V), electron carriers ubiquinone reductase (QH ₂) and cytochrome c (cyt c). FMN = Flavin mononucleotide; Fe = Ferrum (Iron); Pi = Inorganic phosphate.	27
2.1 Parts of artificial vagina used for bull semen collection. A= AV body; B= inner liner; C= receiving cone.	31
3.1 Microscope (a) and digital camera (b) used to measure sperm mid-piece length.	40
3.2 Spermatozoa with both straight and bent mid-pieces that were measured either as a single straight line or the sum of several measurements.	41
3.3 Variation in sperm mid-piece length of 40 sperm in each of five ejaculates for each bull within breeds: a) juvenile (●) and mature (■) Holsteins, b) Belgian Blue (●) and Friesian (■), and c) Aberdeen Angus (●) and Limousin (■).	42
3.4 Variation in sperm mid-piece length between breeds: Aberdeen Angus (AA), Belgian Blue (BB), Charolais (CH), Friesian (FR), Holsteins (HO) and Limousin (LN).	43
3.5 Sperm mid-piece length compared to 49 day non-return rate (NRR) between dairy and beef breeds (n=6), using Pearson correlation test. AA= Aberdeen Angus; HO= Holstein; BB= Belgian Blue; CH= Charolais; FR= Friesian; LN= Limousin.	44
3.6 Sperm mid-piece length compared to 49 day non-return rate (NRR) of bulls from the dairy and beef breeds (n=29), using Pearson correlation test. AA= Aberdeen Angus; AH= Holstein; BB= Belgian Blue; CH= Charolais; FR= Friesian; LN= Limousin.	45
4.1 Arrangement of regional (RN) gates on different fluorescence histograms and logical (LG) gates on quadrants (Q).	53
4.2 Spermatozoa labelled with PI presented a single population on logical gate G5.	56
4.3 Spermatozoa labelled with JC-1 and PI presented unified population on logical gate G10.	56
4.4 Sperm labelled with JC-1, SYBR 14 and PI presented unified population on logical gate G10.	57
4.5 Grown populations on quadrants after application of colour gating.	57
4.6 Fresh spermatozoa washed in PBS extender and labelled with JC-1, and PI.	59
4.7 Frozen-thawed spermatozoa washed in PBS extender and labelled with JC-1, and PI.	59
4.8 Fresh spermatozoa washed in PBS extender unlabelled with JC-1, and PI.	59
4.9 Frozen-thawed spermatozoa washed in PBS extender unlabelled with JC-1, and PI.	59

5.1	Flow chart for JC-I protocol. The acronyms CON, VAL, DOG and IAM refer to treatments control, valinomycin, 2-deoxy-D-glucose and iodoacetamide.	67
5.2	Dot plots resulting from flow cytometric analyses of spermatozoa stained with JC-1. Dot plot of sperm population with highly functional mitochondria displayed oval outline (A, C) and elongated in less functional mitochondria (B, D). A) population in presence of glycolytic substrate. B) population treated with IAM in presence of respiratory substrate. C) population treated with DOG in presence of respiratory substrate. D) population treated with VAL in presence of glycolytic substrate.	69
5.3 (a&b)	Comparison of low and high mitochondrial membrane potential ($l\Delta\Psi_m$ and $h\Delta\Psi_m$) and high:low $\Delta\Psi_m$ ratio between controls and glycolytic and respiratory inhibitors for 12 semen samples.	70
5.4 (a&b)	Comparison of low and high mitochondrial membrane potential ($l\Delta\Psi_m$ and $h\Delta\Psi_m$) and high:low $\Delta\Psi_m$ ratio between controls and glycolytic and respiratory inhibitors for 12 semen samples.	71
6.1	Confocal images of OBS wells a- blank well, the oxygen-sensitive fluorescent, compound tris (4,7-diphenyl-1,10 phenanthroline) ruthenium (II) chloride is embedded b- fluorescence intensity after addition of sodium sulphite in well, c-f- fluorescence intensity after addition of sperm at 0, 15, 30 and 45 min.	80
6.2 (a&b)	Comparison of mean ATP (μl ATP synthesised by 10^8 sperm/hr) between bulls of fresh and frozen-thawed spermatozoa.	83
6.3 (a&b)	Comparison of mean ATP (μl ATP from 10^8 sperm/hr) between bulls of fresh and frozen-thawed spermatozoa.	84
6.4	Correlation between ZO_2 and high:low $\Delta\Psi_m$ ratio of fresh spermatozoa from six bulls.	85
6.5	Correlation between ZO_2 and high:low $\Delta\Psi_m$ ratio of frozen-thawed spermatozoa from six bulls.	85
7.1 (a&b)	Motility characteristics of fresh sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors compared with control, shown as variance (\pm) from the '0' mean control value.	97
7.2 (a&b)	Motility characteristics of frozen-thawed sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors compared with control, shown as variance (\pm) from the '0' mean control value.	98

A study of functional markers in raw and processed bovine sperm and their potential uses for fertility prediction and process refinement

Abstract

The extensive assessment of bull's reproductive potential prior to breeding is highly important and includes examination of general physical soundness, external and internal genitalia and semen quality. Breeding success depends on the efficient use of bulls with high breeding value but simultaneously semen quality imposes restrictions on the use of these bulls in AI. Several techniques have been devised to assess quality of either fresh or frozen-thawed semen. Among a variety of traditional parameters sperm concentration, sperm raw and post-thaw motility and sperm morphology are commonly used for routine semen assessment in the laboratory. In this study, we investigated differences in sperm metabolic activity relative to their motility that may reflect better the fertility of bulls from their non-return rates (NRRs).

To investigate the relationship between mid-piece length and fertility of bovine spermatozoa, sperm biometry was performed on ejaculates obtained from 34 bulls representing six breeds: Holstein (yearlings and mature), Friesian, Belgian Blue, Aberdeen Angus, Charolais and Limousin. Significant differences ($P < 0.01$) between ejaculates were found in 9/34 bulls, as well as differences ($P < 0.001$) between individual bulls within the same breed. The average mid-piece length for Aberdeen Angus was $13.35\mu\text{m}$, for Belgian Blues and Limousin around $13.8\mu\text{m}$, and for Charolais $13.68\mu\text{m}$: for dairy breeds (Holstein and Friesian) it was about $13.4\mu\text{m}$. The mean value of mid-piece length for breed was compared with their 49 day non-return rate; a negative correlation was found in the dairy breeds, while in bulls from beef breeds this correlation was positive but very low: the small numbers of bulls involved prevented meaningful statistical relationships being established.

To differentiate live and dead sperm and non-sperm-specific particles, a flow cytometry method was developed by labelling sperm with JC-1 and propidium iodide (PI) dyes and to determine maximum mitochondrial membrane potential ($\Delta\Psi\text{m}$) at minimum incubation. This method entailed setting regional and logical gates to exclude dead sperm and other non-cellular components from live sperm present within an ejaculate. It was confirmed that spermatozoa of both fresh and frozen-thawed semen exhibited maximum high:low $\Delta\Psi\text{m}$ ratio after 40 min incubation. Flow cytometric dot plots of analyses of fresh and frozen-thawed spermatozoa incubated with JC-1 could identify a unified sperm population of membrane-intact cells, each population characterised by both low and high $\Delta\Psi\text{m}$ but to varying degrees suggesting that this flow cytometric method simplifies the determination of mitochondrial membrane potential using JC-1. This method serves two purposes: using this method, one could be able to evaluate sperm $\Delta\Psi\text{m}$ as well as the proportion of live:dead.

Changes in mitochondrial structure and integrity appear to be an important component associated with sperm motility and reduced fertility. The $\Delta\Psi_m$ was assessed using JC-1 and PI in the presence of glycolytic and respiratory inhibitors. Mean high $\Delta\Psi_m$ was significantly greater for control compared to the treatments in fresh and frozen-thawed semen. In samples treated with valinomycin (VAL) and iodoacetamide (IAM) $\Delta\Psi_m$ was lowered significantly. The proportion of sperm with a high ratio of high:low $\Delta\Psi_m$ was higher in control and 2-deoxy-D-glucose (DOG) treated samples representing more active mitochondria: in samples treated with VAL and IAM the ratio was reduced, representing loss in activity of mitochondria. Cryopreservation significantly decreases high:low $\Delta\Psi_m$ ratio in control suggesting that lower mitochondrial activity may be associated with oxidative stress produced by reduced antioxidant levels due to the freeze/thaw cycle.

The relationship between ZO_2 (μl oxygen consumed / 10^8 spermatozoa/hr) and mitochondrial function was assessed in fresh and frozen-thawed semen. Sperm oxygen consumption was greater in fresh compared to frozen-thawed semen. Insignificant positive correlations existed between ZO_2 and ratio of high:low $\Delta\Psi_m$ in fresh ($r=0.82$) and frozen-thawed ($r= 0.49$) semen suggesting that the $\Delta\Psi_m$ measured in this way by flow cytometry can be used as an indicator of ZO_2 .

Finally, the metabolic pathways by which spermatozoa produce energy to support their motility were investigated in fresh and frozen-thawed semen diluted in media containing glycolytic and respiratory inhibitors. Total and progressive motilities were not significantly different in sperm incubated with DOG and VAL but decreased significantly with IAM compared to control. This indicates that sperm can maintain a similar degree of motility when generating their energy exclusively from either glycolysis or mitochondrial activity. IAM significantly lowered sperm motility as well as mitochondrial activity (as described above) and was found to be an inhibitor of both glycolysis and respiration possibly linked with either modification of mitochondrial cysteine and/or glutathione levels. Sperm are considered in a state of hyperactivation/capacitation when their amplitude of lateral head displacement (ALH) increases and path straightness (STR) and linearity (LIN) decrease. In the present study higher ALH and lower STR and LIN were observed when spermatozoa were dependent on mitochondrial energy (DOG), whereas these estimates were reversed when they were on glycolytic energy (VAL) indicating that sperm hyperactivation and capacitation are associated with mitochondrial function. There was a positive correlation of sperm progressive motility, ZO_2 and high:low $\Delta\Psi_m$ ratio with bull NNRs suggesting that these sperm characteristics may be useful for predicting bull fertility. Furthermore sperm mid-piece length was significantly correlated with sperm average curvilinear velocity and amplitude of lateral head displacement. Since the mitochondria are localized on the sperm mid-piece, it is likely that its energy may contribute in high sperm velocity and also hyperactivation that helps sperm disengagement from oviduct epithelium and positioning at the site of fertilization.

Acknowledgements

First I offer my humblest and profound gratitude to 'Almighty Allah', 'Who bestowed me the potential and ability in conducting research and knowledge of the subject.

I am grateful to a number of people for contributing to this work. Amongst them I would like to acknowledge my supervisor Richard Murray and Stuart Revell for their continuous guidance, constructive criticism and support throughout the project. Also thanks for my co-supervisor Caroline Argo especially for her support in fundamental biochemistry during the early days of study, Peter Cripps for his aid in statistics awareness and Dave Spiller for his help in laboratory experiments based at Centre for Cell Imaging, School of Biological Sciences, University of Liverpool.

I would also like to thank Secretary, Government of Sindh, Livestock and Fisheries Department, and Dr. Abdul Qadir Junejo, Director Animal Breeding Sindh for their encouragement, administrative and financial support under the development project 'Development of Milk and Meat Production in Sindh.

Many thanks to the people at Genus Freezing Unit Ruthin, for their technical assistance in the semen laboratory, especially Eleanor for her valuable support on flow cytometer, Sian and Matt for their encouragement and guidance in semen handling, processing, and freezing and also keeping me supplied with semen.

I am also grateful to Professor Stuart Carter, allowing me to work in the Veterinary Science Building Liverpool during the last stage of my thesis writing. Many thanks also go to Chris Broadband for her administrative support at Leahurst, and Patricia, for her friendly attitude and providing articles and books.

I would personally grateful to all my colleagues, well wisher, relatives and friends, especially my father and mother in-law, brothers and sisters whose constant blessing and encouragement have helped me to complete this project.

Finally, I want to express my warmest gratitude to my boundless and loving family, my wife Shama and my little princes Suhayb and Hariz for their tolerance, patience, blessing and encouragement, to whom I dedicate the thesis.

Chapter 1

General Introduction and Literature Review

Artificial Insemination (AI) was the first significant biotechnology applied to improve reproduction and genetics of farm animals. It has had an enormous impact worldwide in many species, particularly in dairy cattle (Foote, 2002) where the speed of genetic improvement, a lowering of the financial costs associated with cattle breeding, the potential to control herd fertility, and reduction in the incidence of bovine venereal diseases have been greatest.

Historical background of Artificial Insemination

Historical accounts from the Middle East, dated around 1322 A.D, described an Arab chieftain who wanted to mate his prize mare to an outstanding stallion owned by an enemy. He introduced a wand of cotton into the mare's reproductive tract, then used it to stimulate sexually the stallion that caused him to ejaculate. The semen was collected, placed in the mare's vagina, and this resulted in a successful pregnancy (Foote, 2005).

It was not until the late 17th century that Leeuwenhoek and his assistant, Hamm from Holland, first observed human sperm within an ejaculate which they called "animalcules". Leeuwenhoek did not receive a formal education so he did not study Latin, which was the scientific language of the day. However, he was a clever, capable man who ground lenses so precisely that individual sperm could be visualized; indeed, one such lens still exists today with a magnification of x270. Another century passed before Spallanzani performed the first successful insemination of a bitch in Italy in 1784: she whelped three pups 62 days later. He originally trained as a priest, but had a great interest in natural history which he pursued with vigour. He was Professor of Natural History in Pavia at the age of 25, collecting, analyzing, and classifying a large array of butterflies, shells, and other marine animals and mammals (Foote, 2002).

In 1785 the Scottish surgeon, John Hunter, described the first use of artificial insemination in humans: he obtained an ejaculate from a patient suffering from hypospadias and placed it with a warmed syringe into the wife's vagina resulting in the birth of a healthy child (Herman, 1981).

In 1897, Heape reported that AI had been used to breed rabbits, dogs, and horses. He was an outstanding reproductive biologist, establishing many of the principles that, nowadays, relate to the seasonality of reproduction. He described the recovery of pre-implantation embryos after flushing a rabbit's oviduct and transferring them into a recipient doe which supported their normal development subsequently (Heape, 1891). Cambridge became a world centre for studying reproductive physiology, attracting such eminent scientists as Marshall, Hammond, Walton, and students such as M. C. Chang (Foote, 2002).

Pioneering efforts to establish AI as a practical procedure continued in Russia under the direction of Ivanov who developed artificial insemination in farm animals, dogs, foxes, rabbits, and poultry with varying degrees of success. The technique resulted in higher pregnancy rates compared to natural service (Ivanoff, 1922). In 1909, he established a laboratory to train veterinary personnel to collect semen from stallions with either superior performance or excellent conformational traits that enhanced their breeding potential through AI. Ivanov extended the technique for cattle and sheep with such good results that, by 1938, 1.2 million cows and 15 million sheep had been inseminated in the USSR. (Foote, 2002). Much of this pioneering AI work in Russia was continued subsequently by Milovanov who established major centres for breeding sheep and cattle (Milovanov, 1964).

In the United States during 1906, Lewis of the Oklahoma Agriculture Experiment Station described the use of an 'impregnator' for carrying out artificial insemination in mares. He said, "the use of the impregnator or other artificial means of introducing the semen in horse breeding has passed beyond the experimental stage" (Herman, 1981). In 1907, a white face calf was born into the breeding herd of R.L. Hughey, Alva, Oklahoma, after its dam had been inseminated artificially. He collected semen from the vagina of another cow in season that had just been bred naturally, then placed it in a gelatin capsule which was then inserted into that particular

cow that was in season also. Later he used the capsule method to breed eight hundred mares using semen from a single stallion (Herman, 1981).

The Danish “invented” the AI straw for storing semen. A Dr. Sorensen kept natural oat straws on his desk. At a birthday party given for his daughter, he saw children drinking punch with cellophane straws, and suddenly realised that he had found a method for storing semen (Sorensen, 1940). Over twenty years later, French worker Cassou produced straws commercially (Cassou, 1964): they were plastic containers 113 mm long, varying in diameter from 2-4.2 mm with a capacity of 0.25-1.2 ml.

The first AI Society in the United States was formed in 1937 in New Jersey and within six years had grown to 99 members. Originally, a Bull Association was formed in 1906 in Michigan whose membership had grown steadily to over four hundred active associations in 1936. There were four or more units in each association that comprised four herd owners who shared the use of a registered bull, kept at on member’s farm. These bulls were relocated every two years to avoid inbreeding within former units. While this program helped to provide better genetic potential, it had the disadvantage of farmers having to lead cows some distance for mating. A remarkable feature was the rate at which the number of dairy cows bred by AI had overtaken those bred by Bull Association members using natural service; the average number of cows bred using AI being ten-fold greater compared to those bred naturally. This change resulted in the number of Bull Associations declining steadily to be replaced by AI Societies (Edwards and Ritchie, 1944). Since AI was first introduced, the US domestic market for semen has risen to 15.5 million units annually in 2002. Improved efficiency of reproduction has increased profit potential for the US dairy producer, allowing the industry to meet higher consumer demand for a safe, wholesome, and inexpensive food supply (DeJarnette *et al.*, 2004). As the AI industry in the United States evolved from regional to national to international businesses, the industry simultaneously went through various consolidations, acquisitions, and mergers. In 1981, eleven AI companies produced 90% of bovine semen required: today, that same proportion of semen is produced from only five cattle breeding companies- the co-operatives Select Sires, Genex Co-operative, and Accelerated

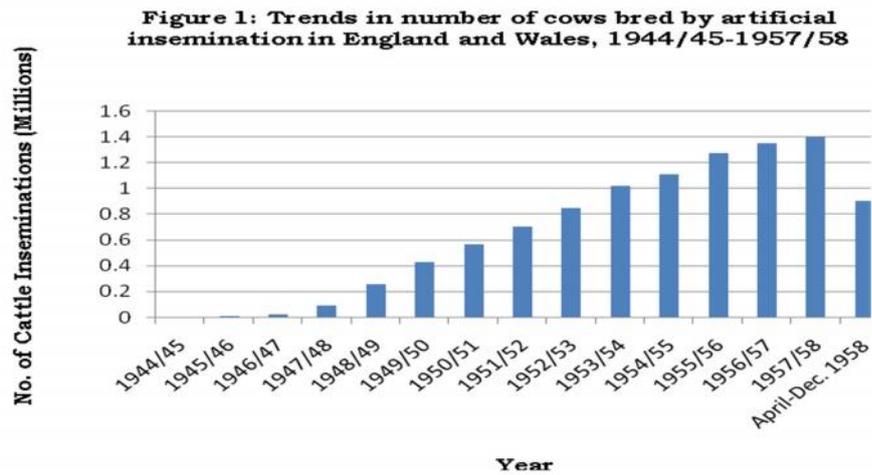
genetics, one private company Alta Genetics, and one public trading company ABS Global (Genus plc) (Funk, 2006).

Edward Sorensen and others, working in the Royal Agriculture and Veterinary College, Copenhagen, organized the first co-operative artificial breeding association in Denmark in 1936: in that year 1,070 cows were inseminated. The number of services required per pregnancy was 1.7, slightly better than using natural service. They developed the recto-vaginal technique for AI in cattle, which made more efficient use of semen and improved fertility by about 10%. Ten years later, Denmark had 100 co-operative cattle breeding associations that delivered 500,000 inseminations per annum (Herman, 1981). Their cattle-breeding programme grew steadily and by 1960 almost all their cattle were bred by AI.

AI was first considered for the cattle breeding industry in the United Kingdom during 1942 (Anon, 1959). Two years later, a plan was formulated to expand the AI service throughout England and Wales by creating regional AI centres, except for farms serviced already by the pioneering AI centre in Cambridge, Warwickshire, Somerset and Devon. Using a crude cattle population map, territorial areas containing approximately 60,000 cows were selected as being suitable for this service supplied by one centre. Each centre contained about 30 dairy bulls, based on the assumption that around 50% of dairy cows in an area would be bred using AI. Furthermore, it was estimated that about 1000 cows could be inseminated using semen collected from just one bull annually. Thus the Milk Marketing Board (MMB) A.I Service came into being. Initially the Ministry of Agriculture supported cattle breeding centres, but after 1951 they were able to deliver a commercial AI service through their own resources. Most of the AI centres were supervised by a local committee, which met every three months to receive veterinary, management and livestock reports. Once a year, the Chairmen of all the centres met the MMB to discuss the year's results and to consider future plans. In 1952, there were some 40 main centres and 100 sub-centres in operation with an enrolled membership of more than 100,000 farmers (Anon, 1959).

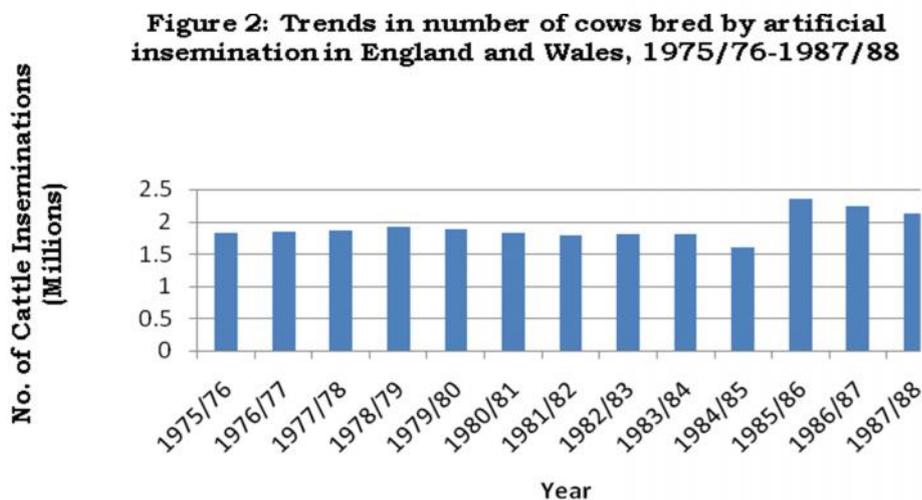
Dairy, dual-purpose, and beef bulls were kept at each Centre. The dairy and dual-purpose breeds were British Friesian, Ayrshire, Jersey, Dairy

Shorthorn, Red Poll and Welsh Black: Aberdeen Angus, Devon, and Hereford represented the beef breeds. During the first 14 years of the MMB existence service, ten million cattle were inseminated using MMB bulls and three million inseminations were sold from bulls kept at non-Board AI centres (see Figure 1.1). By 1957-58, 60% of all cattle bred in England and Wales were served using artificial insemination.



(Anon, 1959)

Since 1975, the number of semen doses sold for AI by the MMB and other private AI centres has been reasonably static at around 1.8 million. The sharp reduction in AI demand in 1984/85 was due to the imposition of milk quotas in April 1984, consequently increased numbers of dairy farmers leaving the industry: within five years, this trend had slowed down so that the demand for AI rose to over two million doses each year (see Figure 1.2).



During this same period, the demand for semen collected from Friesian and Canadian Holstein bulls has risen from 58% to 64%. There has been a trend for dairy farmers to serve their cows with beef semen, reflecting the increasing demand for beef crossbred calves for rearing to slaughter weight. Until 1980/81 the dominant beef breed was the Hereford: nowadays, the demand is Limousine, Charolais Belgian Blue semen. Today, the Belgian Blue is popular as a terminal sire because of its relatively short gestation length, low feed conversion ratio and high percentage of lean meat (Anon, 1988) and also the more extreme beef breed is needed to compensate for increasing dairy type of Holstein cows.

Semen collection, extension and preservation

Over the last sixty years methods have been developed to meet the demand of an expanding cattle industry worldwide. The idea of the artificial vagina originated in Italy when an artificial vagina (AV) was used to obtain semen from a dog (Amantea, 1914). Later the Russians designed an artificial vagina suitable for collecting from a bull: the first attempts allowed the bull to mate naturally with a cow, and its semen was removed from the cow's vagina with either a spoon or ladle, by aspiration, or using a sponge (Lambert and McKenzie, 1940). The semen collected by this method was always contaminated by bacteria and the risk of venereal disease transmission was considerable. Later, a specially designed rubber bag was placed in the vagina, so that the bull ejaculated into it: whilst this eliminated some of the problems of collecting semen directly from a cow's vagina, the semen was difficult to handle (Komarov and Nagaev, 1932; Miller and Evans, 1934).

The first AV for bulls was designed in Russia (Sorensen, 1938). It was made of a rigid rubber cylinder 24 inches long with an internal diameter of 2.2 inches. Inside the cylinder was placed a thin-walled rubber tube whose ends were turned back over the outer cylinder, forming a watertight jacket. A graduated glass receptacle of small volume was fitted to the inner rubber tube at one end of the AV. Through a screw-plug hole in the outer rubber, hot water was introduced between inner cylinder and inner rubber tube to bring 37-39°C measured by a thermometer placed within the inner tube.

Several modifications of this early Russian model were made. In the English model, the inner receptacle tube had a larger capacity to accommodate any increase in air pressure, built up when the bull ejaculates. The Danish model had a much shorter cylinder, and the semen receptacle was attached by means of a rubber funnel. The disadvantage with both these models was that the semen collecting vessel was exposed to light and ambient temperature, and there was danger of cold shock to spermatozoa if collected in cold weather (Sorensen, 1938). To overcome this, MacMillan *et al.*, (1966) designed an AV in which the collection receptacle was placed inside the inner liner thus protecting it from the surrounding air temperature. This modification preserved the motility of spermatozoa much better, especially for collections made in adverse weather conditions. In the United States today, the AVs used are based on the Danish model, having a cylinder approximately 16 inches long with an internal diameter of 2.25 inches: smaller AVs are used for bulls of lesser stature so that their ejaculate is deposited as near the collecting vessel as possible.

Case (1925) described how he collected semen from a bull by massaging its seminal vesicles and ampullae *per rectum*; semen was ejaculated within two minutes. Semen collection by this method is used today in bulls that, for some reason, are unable to mount or whose libido is poor. Semen collected by this method may be contaminated with urine, epithelial cells, debris, or have too high a proportion of seminal vesicle secretion that imbalances the different components of a healthy ejaculate.

The first attempts at inducing ejaculation by electrical means was made by Batteli (1922), who stimulated the base of the brain of a mature male guinea pig with an electrical current of 30 volts. Gunn (1936) pioneered the development of electro-ejaculation in farm animals with his work on rams by stimulating their spinal cord between the fourth lumbar and first sacral vertebrae. He placed one electrode in the rectum the other on the back muscle, and passed a similar voltage delivered at 5 - 10 second intervals. Ejaculation occurred and the semen was collected in a glass tube.

In 1948, the French investigators Ortavant and Thibault obtained semen by introducing a multi-ringed, bipolar electrode into the rectum and applied up to 30 volts a.c (Laplaud *et al.*, 1948). Dziuk *et al.*, (1954) used this

bipolar electrode technique to bulls using a 1.5 x 22-inch probe, having at one end six metal rings spaced 1.75 inches apart; the rings formed positive and negative alternative electrodes. The probe was held in contact with the floor of the rectum, the voltage was increased gradually from zero to 10-15 volts in two volt increments at 5-10 second intervals, returning to zero after each increase. They found that semen collected in this way had a larger volume and lower sperm concentration compared to an ejaculate collected by AV.

Marden (1954) modified the design, to apply a sine-wave pulse at a frequency of 20-30 cycles per second, gradually increasing the intensity of the signal over a 3-8sec period. As the stimulation progressed, the intensity of signal was increased gradually from 0-5.5 volts with a current of 900 milliamperes. No harmful effects were recorded in two bulls who were collected from by this method over a continuous 12-month period. Today, a variety of manufactured probes are available, from them the Lane Pulsator IV (Lane Manufacturing, Denver, CO, USA) being available worldwide. The maximum voltage of this relatively new and popular model is 16 V, with a maximum current of <900mA. Ejaculation in bulls occurs usually with voltage impulses between 8-10 V (Palmer *et al.*, 2005).

The concentration of spermatozoa in healthy, freshly ejaculated bull semen ranged from 200 - 2200 x 10⁶ per ml. It has been estimated that a mature bull can produce over 40 billion sperm per week, or more than two trillion sperm per year. Normally, 15 million spermatozoa in extended semen are delivered in an A.I straw. Ultimately a bull kept on a continuous semen collection schedule, can produce over 100,000 straws of semen per year (Funk, 2006). Clearly there is a balance between providing a volume in an A.I straw that contains sufficient spermatozoa that allows optimal fertility to be expressed in the cow without unduly wasting sperm. There are two major functions of semen extenders: to preserve the integrity and fertilisation potential of spermatozoa and increase the volume within which the correct number of viable male gametes can access the uterine isthmus and the ovum (Salisbury *et al.*, 1978a).

Salt or sugar solutions were used first to dilute semen to extend ejaculate volume for immediate use. When the suitability of egg yolk was recognised,

a “preserving” extender was developed: it lowered the advantages of reducing the metabolic activity of spermatozoa and prolonging their fertility by allowing them to be stored at around 5°C when combined with phosphate buffer (Philips, 1939). Using 100 millilitres of ordinary egg yolk, the pH of this buffered medium was 6.75. Later Knoop (1941) added small amounts of gelatine to extend the storage period. The gelatine held the spermatozoa inactive, assisted in maintaining them in suspension, and supplied extra nutrients sufficient to maintain 50% of sperm motility for around 12 days when kept at 4-6°C. Later, Knoop and Kraus (1944) produced a diluent containing 1.0-% glycine or 1% l-proline to replace the gelatine and increased sperm survival rates from 50% to 86%. At around the same time Salisbury *et al.*, (1941) buffered the egg yolk with sodium citrate. Being a chelating agent, it caused a noticeable dispersion of the fat globules within the suspension that produced better visibility of individual spermatozoa within the medium. Further addition of glucose and dibasic sodium phosphate, or dibasic-sodium-phosphate-glucose and adenylic acid extended sperm motility (Lardy and Philips, 1942).

The yolk-based buffer was displaced when the Cornell University Extender (CUE) was developed, using a bicarbonate-citric acid buffer system (Foote *et al.*, 1960) which resulted in 77% pregnancy rates achieved within AI in cattle (Foote and Gray, 1960).

A major indication for using AI in dairy cattle was to control venereal diseases. In 1917, Ivanov added ethyl alcohol, atoxyl and salvarsan to contaminated semen to limit possible spread of infection from bull to cows. Knodt and Salisbury (1946) first studied the feasibility of using bacteriostatic drugs such as sulfanilamide to control bacterial growth in semen. Later, Almquist *et al.*, (1949) showed that both penicillin and streptomycin inhibited the growth of several Gram-positive and Gram-negative bacteria that survived in yolk-citrate diluted bull semen, without affecting its viability. When studying the biology of *Trichomonas fetus* infection in cattle, Williams and Plastridge (1946) used penicillin and streptomycin to eliminate other bacterial contaminants that allowed *T. fetus* to be studied alone together with *Pseudomonas aeruginosa*. Keeping bulls in studs away from cows and never used for natural service also has a key

role in breaking the cycle of these disease; antibiotics were only a precautionary back-up.

Freezing and thawing is injurious to spermatozoa with the percentage of fully functional sperm with intact cellular and organelle membranes being decreased significantly (Holt, 1997). This is due to formation of intracellular ice crystals, osmotic stress, lipid phase transitions in the plasma membrane, oxidative damage, premature aging, and capacitation-like changes (Watson, 2000). Thawing increases sperm membrane permeability that causes ionic influx within the plasma membrane and damages it by creating lipid phase transitions (Holt and North, 1984). Phospholipids, glycolipids and sterols are the major lipids found in the mammalian spermatozoa. Unusual distribution of phospholipid and glycolipid can be induced as a result of temperature-related phase transitions from the gel to liquid-crystalline state and *vice versa* (Holt and North, 1985, Parks and Lynch, 1992). Spermatozoa with equal proportion of cholesterol and phospholipid are less susceptible to cold shock. Cholesterol is the major sterol present in the sperm that modulate membrane fluidity and stability by interacting with membrane phospholipid. Therefore any change in the membrane lipid composition can be resulting in membrane fluidity or disruption (Holt and North, 1985, Parks and Lynch, 1992).

Premature capacitation-like changes such as spontaneous acrosome reaction and poor motility are associated with the freeze-thaw cycle. Any increase in sperm membrane permeability after cooling results in the uptake of calcium, leading to capacitative changes and fusion events between the plasma membrane and underlying outer acrosomal membrane. Therefore capacitation like changes after freeze-thawing may limit the sperm to live for short period and this short period of capacitation may be crucial to encourage *in vitro* fertilization, (Watson, 1995a; 1995b; 2000). DNA damage, lipid peroxidation and impaired motility in sperm are also the factors induced by cryopreservation. Alteration in these biochemical and physical characteristics are provoked mainly by reactive oxygen species (ROS). Superoxide dismutase (SOD) activity, a scavenger of the superoxide radical, is reduced in freeze-thawed spermatozoa: therefore, a significant decrease in SOD activity will increase ROS (Bilodeau *et al.*, 2000). Higher concentrations of ROS destroys poly-unsaturated fatty acids present in cell

membrane leading to a cascade characteristic of lipid peroxidation (Agarwal *et al.*, 2008) that decreases sperm motility through a rapid loss of intracellular ATP. Sperm DNA integrity is reduced significantly by oxidative stress/ROS resulting from freeze-thawing, through either breaks in single and double-stranded DNA, DNA base modification, production of base-free sites, deletion, frame shifts, or DNA cross links and chromosomal rearrangement (Kemal *et al.*, 2000).

Mitochondrial activity is reduced significantly following the freeze-thaw cycle, caused by increased extracellular extender osmolarity and decreased antioxidant activity (Bilodeau *et al.*, 2000; Guthrie *et al.*, 2008).

A major change in storing semen occurred in the 1950s, away from solid carbon dioxide in alcohol storage at -79°C to liquid nitrogen at -196°C . Bull spermatozoa remains fertile when stored at -79°C for 8 years and at -196°C for up to 12 years (Salisbury *et al.*, 1978b) or almost indefinitely (Foote, 2002). Bull semen stored in liquid nitrogen has been used successfully for more than 40 years and is still used currently.

In the conventional freeze/thaw process, spermatozoa are diluted at $30 - 39^{\circ}\text{C}$, close to body temperature then cooled to 5°C at which point the cryoprotectant glycerol is added (Polge *et al.*, 1949). This protects the plasma membrane which is the primary site of injury for cryopreserved sperm during the freeze/thaw process (Hammerstedt *et al.*, 1990; Parks and Graham, 1992). As sperm are frozen, ice crystals form in the extracellular medium that increases the osmolarity of any unfrozen water. Intracellular water diffuses out of the sperm in response to this increase in osmotic gradient, thus dehydrating the cell and injuring the plasma membrane. At thawing, the phenomenon is repeated in reverse as the extracellular ice crystals melt and water diffuses into the sperm. Ultrastructural deformation of the plasma membrane occurs as a consequence of osmotic stress and drastic changes in cell volume during the freeze/thaw process: the inclusion of a cryoprotectant, commonly glycerol for mammalian sperm, is essential for sperm survival. The efficacy of glycerol is related to the increase in osmolarity of the medium, which retards ice formation (Hammerstedt *et al.*, 1990). This effect is only transitory and the ensuing rapid change in cell volume does not appear to be detrimental to sperm

function (Liu and Foote 1998). Also glycerol acts directly on the sperm plasma membrane reducing some of the phase transitions and increasing membrane fluidity during cooling (Noiles *et al.*, 1995). The effect of glycerol is species specific: beside the positive effects on bull sperm, it has also negative effects on the spermatozoa of pig and mouse. It causes severe acrosomal damage and reduces fertility in pigs at 3% concentration whereas at the concentration of 1.75% damages mouse spermatozoa (Holt, 2000).

Recently Bailey *et al.*, (2003) studied the use of cyclodextrin to reinforce the plasma membrane structure. Cyclodextrin are cyclic oligomers of glucose that form water-soluble complexes with other organic molecules that may not be water-soluble. They suggested that methyl- β -cyclodextrin could be used to deliver cholesterol to the plasma membrane of ram sperm during cryopreservation, thereby stabilising it and making the sperm more resistant to cryo-capacitation. Indeed, post-thaw sperm quality was improved when a standard egg yolk-glycerol extender was supplemented with cholesterol-saturated methyl- β -cyclodextrin.

Morphology and ultra structure of bovine spermatozoa

The spermatozoon is a highly specialized cell whose function is to transport the male haploid genome, via the female genital tract, and deliver it to the oocyte for fertilization.

The head or caput:

In mature spermatozoa the head or caput is oval with approximately dimensions 8-10 x 4-5 x 0.3-0.5 μ m. The entire spermatozoon is enclosed in a plasma membrane. Within this, the acrosome membranes, inner and outer, surround the acrosome matrix over the anterior part of the head, terminating caudally at the equatorial region (Fig 1.3). This cap-like structure covers about 60% of the anterior portion of the nucleus situated within the caput (Salisbury *et al.*, 1978c).

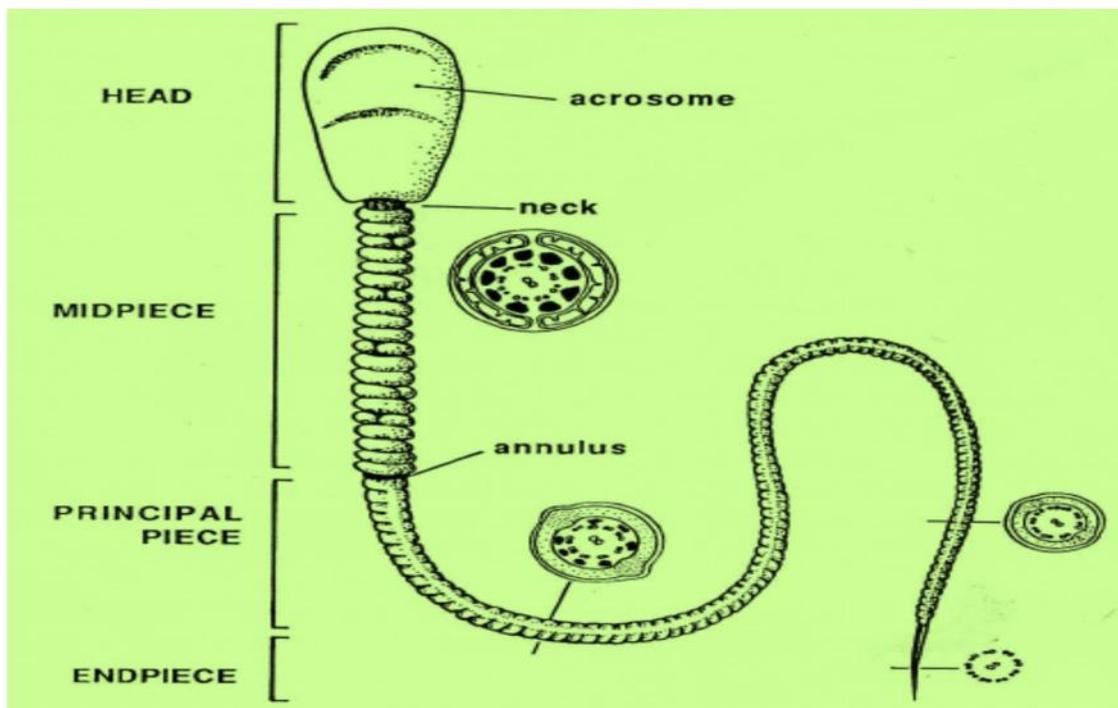


Figure 1.3: Diagrammatic representation of a bovine spermatozoon (Barth and Oko, 1989)

Covering the entire surface of the spermatozoa, from the anterior aspect of the head to the end piece, a continuous plasma membrane modulates interactions between the spermatozoon and its surrounding environment. It is organized into a series of restricted domains that contain unique lipids and proteins which, interact to regulate aspects of sperm physiology and function. In the head region, proteins such as actin and actin-binding

proteins (Howes *et al.*, 2001), cholesterol (de Lamirande *et al.*, 1997) and sphingolipids (Vadnais *et al.*, 2007) are associated with cell-cell recognition and membrane fusion with the ovum during fertilization. In the mid-piece the plasma membrane contains proteins essential for synthesis of mitochondrial ATP and modulating microtubule sliding. As spermatozoa migrate down the epididymis, significant changes occur in this membrane's composition that allows sperm to be free-moving within the female reproductive tract (Millette, 1999).

The acrosomal membrane consists of an outer membrane that lies just below the plasma membrane and an inner portion that overlies the nucleus (Cardullo and Florman, 1993). These membranes run parallel throughout most of their relationship: between them is a narrow cavity containing a complex array of acrosomal proteins and enzymes that are involved in fertilization. For example, the proteins acrosin/acrosomase (Srivastava *et al.*, 1965; Stambaugh and Buckley, 1969) and hyaluronidase (Rowlands, 1944; Austin, 1948) are unique in that they are not found in somatic cell lysosomes. Acrosin is a trypsin-like serine protease: hyaluronidase is a sperm-specific iso-enzyme, distinct from lysosomal hyaluronidase, tightly bound to the interior aspect of the inner acrosomal membrane. Hydrolytic (Dott and Dingle, 1968; Allison and Hartree, 1970) and other enzymes are found in the mammalian acrosome, such as β -*N*-acetylglucosaminidase, acid phosphatase, arylamidase, arylsulfatase A, aspartylamidase, caplain II, a cathepsin D-like protease, collagenase-like proteins, various esterases, β -glucuronidase, neurominidases and phospholipases especially phospholipase A: in most instances, the physiological roles of these molecules are unknown (Millette, 1999). Penetration of the envelope of the ovum by spermatozoa is attributed to a mechanical property of the acrosome, but the localization of these enzymes are also involved in the process.

In human spermatozoa, the acrosome is relatively small and does not extend anteriorly much beyond the leading edge of the nucleus, but in most other mammalian species there is a conspicuous thickening of the acrosomal cap that extends well beyond the anterior border of the nucleus (Fawcett, 1975): this region is called the apical segment or ridge. During fertilisation, in response to contact with the zona pellucida, fusion of the

outer membrane along with the plasma membrane occurs at multiple sites beginning at the tip of the acrosome and then moving down to the equatorial region (Johnson, 2007). This acrosomal reaction leads ultimately to complete loss of the cell membrane over the anterior half of the head.

The nucleosome is the basic chromatin element in cells, consisting of 146 base pairs of DNA packaged around an octamer of core histones (Luger *et al.*, 1997). They are assembled in a solenoid which forms the basic chromatin structure. During the final stage of spermiogenesis, protamines replace the normal somatic cell histones (Kierszenbaum, 2001; Boissonneault, 2002). These proteins are highly basic, about half the size of a typical histone. Arginine comprises 55-80% of their amino acid content and has a powerful DNA binding property. Another amino acid, cysteine, influences chromatin compaction during the final stages of sperm nuclear maturation by forming multiple inter- and intra-protamine disulfide cross-links within the nucleus (Balhorn, 1982).

These nucleoprotamines condense DNA ten times more efficiently than nucleohistones: such extreme compaction makes good biological sense because the spermatozoon, having no need to express its genome during the final stage of its development, needs to ensure that its genetic material is packed as efficiently as possible, to ensure safe delivery to the female gamete. As head shape and size are known to affect sperm motility and function (Ostermeier *et al.*, 2001, Malo *et al.*, 2006, Ausio *et al.*, 2007, Gillies *et al.*, 2009), it is likely that nuclear dimensions are an important factor with regard to the optimal shape of the head and efficient compaction of the paternal genome facilitates this. Interestingly, protamine insufficiency leads to a high incidence of sperm DNA strand breakage, as assessed by the Comet assay, suggesting that irreparable DNA damage is one cause of implantation failure in embryos derived from healthy ova fertilised by protamine-compromised sperm (Aoki *et al.*, 2005, Ramos *et al.*, 2008). Thus, the super-compaction of the genome afforded by these protamines confers on sperm a measure of protection against DNA damage that may be essential for successful fertilisation.

In most species, the sperm nucleus is surrounded by an envelope, forming a fold that extends for a variable distance back into the neck region. This is

unique in several respects: the entire area under the acrosomal cap and in the post-acrosomal region is devoid of nuclear pores; only in the most posterior aspect of the nucleus are pores found in close hexagonal array. Here, immediately caudal to the posterior ring of the sperm nucleus, the nuclear envelope is configured into scrolled sheets termed the “redundant nuclear envelope” (Fawcett, 1975). Haraguchi *et al.*, (2007) hypothesized that this is a site for degradation of nucleo-proteins such as histones and transition proteins; it may also act as an intracellular calcium store that releases Ca^{2+} directly to the axoneme for hyperactive motility without the active participation of mitochondria (Suarez and Ho, 2003; Costello *et al.*, 2009).

The nuclear lamina of the sperm head comprises a protein network that lines the inner surface of the nuclear envelope; it provides structural support for the inner acrosomal membrane and anchors chromatin to its inner surface. Proteins comprising the nuclear lamina include laminins which share extensive homology with those found in the intermediate filaments of somatic cells (Millette 1999). In *C. elegans* round worm these nuclear lamins are involved in chromatin organisation, cell cycle progression, chromosome segregation and distribution of nuclear pore complexes (Liu *et al.*, 2000) but their function in mammalian spermatozoa is unknown.

The flagellum

It is divided into four components: the connecting piece, the mid-piece, the principal piece, and the end piece (see Fig 1.3). The connecting piece is that portion which articulates with the implantation fossa of the nucleus in the sperm head. A fine filament called the axoneme, or axial filament complex, attaches the head to the flagellum and consists of two central microtubules surrounded by a ring of nine pairs of uniformly-spaced doublet microtubules. Seen in cross-section, the axoneme is composed of a rigid, characteristic 9+2 arrangement of microtubules and associated tubulin protein (Clermont *et al.*, 1990). Inner and outer dynein arms project from each of the outer nine microtubular doublets and are responsible for generating the motive force of the flagellum through ATPase activity. The axoneme doublets are themselves surrounded by nine pairs of outer dense

fibres, thus creating a cross-sectional 9 + 9 + 2 fibre-oriented pattern: a mitochondrial sheath encloses the outer dense fibres and the axoneme itself. The outer dense fibres provide passive elasticity to the motile flagellum (Fawcett, 1975).

The mid-piece originates from a point where each of the nine outer dense fibres is secured proximally to one of the segmented columns of the connecting piece. The mitochondrial sheath extends throughout the length of the mid-piece running longitudinally and covers the corresponding doublets of the axoneme. The mid-piece terminates at the annulus, about a quarter of the way down the flagellum (Fawcett, 1975). At this point, the surrounding mitochondrial sheath ends and the outer dense fibres - associated with numbers 3 and 8 outer axonemal doublets - are replaced by two longitudinal columns of a fibrous sheath that runs the entire length of the principal piece in a series of circumferentially - oriented ribs.

The principal piece consists of an inner axonemal doublet surrounded by seven outer dense fibres and a fibrous sheath. It constitutes approximately two thirds of the length of the flagellum. Near its distal extremity, the fibrous sheath and the outer dense fibres taper and terminate. It has an important mechanical role in sperm motility by providing a rigid support for the flagellum and determining its planar beat (Lindemann *et al.*, 1992). The remaining short region of the flagellum, consisting of only the axoneme surrounded by a plasma membrane, is the end piece (Turner, 2003).

Transport and maturation of spermatozoa

Epididymal transition

A functionally immature spermatozoon emerges from the testis that must undergo many changes during its passage through the epididymis, to develop into a mature sperm that is stored in the *cauda* epididymidis. Increased motility, zona binding and oolemma fusion are the capacities sperm acquire within epididymis and are necessary for those sperm that finally arrive at the site of fertilization (Cooper, 2007).

The epididymis is a convoluted tube that arises from the efferent ducts in the dorsal segment of the testis and emerging from the tail, it becomes the vas deferens. It is approximately 33-35 metres long and is divided into three

regions: the *caput* (head), *corpus* (body), and *cauda* (tail) (Salisbury *et al.*, 1978d).

Sperm are transported from the efferent ducts through the action of epithelial cilia, muscular contraction of the duct itself, and the flow of fluid from and within the seminiferous tubules to the caput of the epididymis. During this period, spermatozoa interact with proteins from epididymal epithelium and undergo maturation that involves the acquisition of progressive motility and the potential to fertilize ova (Sullivan *et al.*, 2007). Changes in sperm dimension and appearance of the acrosome and nucleus, and migration of the cytoplasmic droplet along the mid-piece, are also epididymis related events (Olson *et al.*, 2003).

The *caput* is the most active region secreting 70-80% of the total protein secretion found in the epididymal lumen. When spermatozoa enter this region, most of the fluid accompanying them from has been resorbed, resulting not only in a concentration of spermatozoa but other luminal contents such as sodium, chloride and low molecular weight organic compounds such as inositol. This fluid resorption in the epididymis is influenced by steroid hormones such as androgen and estrogen (Clulow *et al.*, 1998). Epididymal fluid does not consist of only soluble proteins but other aggregate proteins of varying molecular size: for example, amyloidogenic prion protein is found in insoluble exosome-like membranous vesicles known as epididymosomes (Ecroyd *et al.*, 2004), and in a soluble lipophilic complex associated with hydrophobic proteins (Ecroyd *et al.*, 2005). Epididymosomes are formed within the luminal epithelium by a process known as apocrine secretion, the rate being under androgenic control. This process involves formation of cytoplasm protrusions from principal cells that form blebs at their apex between microvilli, characterized by them having segregated cytoplasmic organelles, few endoplasmic reticulum cisternae, free ribosomes and cholesterol-rich small membranous vesicles and form epididymosomes of varying sizes (Aumuller *et al.*, 1999; Sullivan *et al.*, 2007). Similar types of vesicles, prostasomes, are produced by luminal epithelium within the prostate. These secretory products protect sperm against complement, enhance their motility and stabilise the sperm membrane during passage through the male tubular reproductive tract (Yanagimachi *et al.*, 1985).

Frenette *et al.*, (2002; 2003; 2005) investigated this process obtained vesicles from the caudal portion of the bovine epididymis and co-incubated them *in vitro* with spermatozoa collected from the caput. A secreted protein, macrophage migration inhibitory factor (MIF), was translocated to the intracellular compartment of the sperm and became incorporated in the acrosome and outer dense fibres. Other proteins, P25b and P26h, accumulated within the acrosomal cap of maturing spermatozoa that are associated with the sperm binding to the zona pellucida (Sullivan and Robitaille., 1989; Robitaille *et al.*, 1991).

Ubiquitin is another protein associated with bovine epididymosomes and transferred to spermatozoa during epididymal transit: it assists in eliminating defective spermatozoa by the phagocytic action of epididymal epithelial cells. Some defective sperm may escape phagocytosis and can be found in the ejaculate (Fraile *et al.*, 1996; Sutovsky, 2003). Ubiquitin is also involved in fertilization process and early cell division within the embryo when it is necessary for paternal mitochondria to be removed during the early cleavage divisions of the fertilised ovum (Sutovsky *et al.*, 2001; Sutovsky, 2003; Muratori *et al.*, 2005).

Female genital tract

As spermatozoa are expelled from the epididymis into the female genital tract at ejaculation, they encounter seminal fluid secreted from the male accessory sex glands: seminal vesicles, prostate, and bulbourethral (Cowper's) gland. Within the female genital tract, bovine sperm have to adapt momentarily to the acidic pH of vaginal secretions (Acott and Carr, 1984; Carr *et al.*, 1985). However, the pH of seminal plasma varies between 6.7 - 7.4 in common domestic species (Roberts, 1971) and may potentially neutralize vaginal pH.

After natural mating in the bull, sperm enter the cervical canal where they encounter cervical mucus which presents a significant barrier to sperm with poor motility, abnormal morphology or those with compromised hydrodynamic profile (Hanson and Overstreet, 1981; Barros *et al.*, 1984; Katz and Drobnis, 1990; Katz *et al.*, 1997). The viscosity of mucus is due to the large molecular size of mucins and its elasticity results from the entanglement of the molecules (Carlstedt and Sheehan, 1984, 1989;

Sheehan and Carlstedt, 1984; Sheehan *et al.*, 1986). These extended molecules become orientated by the flow of mucus in the mucosal grooves and thus serve to guide sperm. The mucosal folds of the cervical canal form channels that lead to the body of uterus (Mullins and Saacke, 1989). Under the influence of oestrogens, the mucus deep in these channels becomes less dense and different in composition compared to that found in the middle of the cervical canal. Sperm, entering these deep channels from the external os, migrate towards the uterine body, thereby avoiding the more viscous mucus whose function is to assist in removing uterine detritus. Mattner (1968) flushed the cervix of goats and cows 19–24 hours after natural mating: within that mucus were leukocytes, epithelial cells, and about half the sperm ejaculated during mating since the remainder were found in the deep cervical grooves. Thus, the cervix directs the onward movement of normal motile sperm towards the body of uterus whilst inhibiting the passage of microbes and sperm with abnormal form or motility. Normal, fresh, motile sperm avoid mucus containing neutrophils and are resistant to leukocytic phagocytosis.

Oxytocin stimulates the smooth muscle of the cervix and uterus and its release at the time of natural mating increases uterine motility. In cows and ewes, electromyography has shown that strong myometrial contractile activity occurs during estrus, while contractions are weak and localized during the luteal phase (Hawk, 1983). The physiological stimuli to the cow associated with natural mating is the physical presence and behaviour of the bull, non-copulatory mounting followed by copulation and ejaculation which results in increased uterine tone and cervical and uterine contractility thus rapid sperm transport. Artificial insemination techniques stimulate vulva and cervix of the cow thereby rapid transport of spermatozoa and increased uterine motility is similar to that observed through natural mating (VanDemark and Moeller, 1951; VanDemark and Hays, 1952).

Sperm capacitation and hyperactivated motility

The capacitation is associated with complex changes in the biochemical, physiological and cellular properties of sperm that confers on them the ability to develop new motility patterns as they progress towards the

oviduct and to undergo a zona pellucida-evoked acrosome reaction (Florman *et al.*, 2008). It is time-related, relative to the presence of sperm within the female reproductive tract before they are capable of fertilizing the ovum (Vadnais *et al.*, 2007). The physiological changes associated with capacitation include destabilisation of the plasma membrane, alterations of intracellular ion concentration and mitochondrial membrane potential, and tyrosine phosphorylation (Hunter and Rodriguez-Martinez, 2004; Vadnais and Roberts, 2007).

Hyperactivation occurs mostly during capacitation. Normally flagellar motion or activated motility is symmetrical, of low amplitude and high frequency and is characteristic of sperm in the anterior vagina (Smith and Yanagimachi, 1990; 1991). In the state of hyperactivity the flagellar motion becomes whip-like, asymmetrical, high amplitude and low frequency (Ho and Suarez, 2001).

The motility of sperm changes as they move into the oviduct, one stimulus for this being an increased bicarbonate ion concentration in oviductal fluid, that most likely depends upon increase activity of adenylyl cyclase and protein kinase A (Holt and Harrison, 2002; Prathalingam *et al.*, 2007). In boars, Satake *et al.*, (2006) found that exposure to bicarbonate affects individual spermatozoa differentially; some had progressive motility in the absence of bicarbonate while others were quiescent until activated. Differences in the proportion of bicarbonate sensitive and insensitive sperm in an ejaculate may accounts for differential fertility between boars (Satake *et al.*, 2006).

Intracellular Ca^{2+} stores increase during capacitation, encouraging sperm detachment from oviduct epithelium during the periovulatory period (Smith and Yanagimachi, 1991; Gualtieri and Talevi, 2000); un-capacitated sperm remain adhered to oviductal cells. These un-capacitated sperm drop some coating when they enter the oviduct, to reveal a fucose-binding molecule. Sperm then bind to the oviduct epithelium because fucose is expressed on the epithelial surface of oviduct (Lefebvre *et al.*, 1997; Revah *et al.*, 2000; Ignatz *et al.*, 2001; Kon *et al.*, 2009). Sperm receive unknown ovulation-associated signals, and their capacity to bind to the epithelium is lost because of removal or alteration of the fucose-binding molecule (Revah *et*

al., 2000). If the oocytes are present around the time of insemination then those sperm which do not bind to the oviduct epithelium would have a greater chance of fertilisation, but in delayed ovulation, sperm bound with epithelium and then released some hours later, would have an opportunity to fertilise oocytes (Satake *et al.*, 2006). The activated sperm flagellum continues to generate approximately equal forces at all viscosities, while in response to increased viscous loading, the sperm flagellae produces greater force, becoming hyperactive and capable of passing through the oviduct and ovum vestments (Suarez *et al.*, 1991; Stauss *et al.*, 1995). This motility transformation is supported by capacitation and intracellular Ca^{2+} .

The acrosome of mouse and the neck region of bovine sperm have been identified as storage sites for Ca^{2+} , gated by inositol 1,4,5-triphosphate (Ho and Suarez, 2001; Herrick *et al.*, 2005). The axoneme in the centre of flagellum is the target for Ca^{2+} which has an important role in sperm hyperactivation. The mechanisms remains unknown (Lindemann *et al.*, 1992; Ho and Suarez, 2001).

Calcium-ATPase has been located on the acrosome and mid-piece of spermatozoa and is involved in Ca^{2+} regulation (Fraser and McDermott, 1992; Dragileva *et al.*, 1999; Dorval *et al.*, 2003; Lawson *et al.*, 2007). Targeted removal of the CatSper1 gene, encoding a voltage-gated Ca^{2+} channel in the sperm principal piece causes termination of the cAMP-stimulated intracellular Ca^{2+} rise (Ren *et al.*, 2001). Similarly, disruption of the CatSper2 gene lowers sperm hyperactivity and subsequently lowers male fertility (Quill *et al.*, 2003). When bovine spermatozoa are attached to tubal epithelium *in vitro* and treated with heparin they are released following a moderate increase in intracellular Ca^{2+} (Gualtieri *et al.*, 2005). Heparin-like glycosaminoglycans are normally present in the bovine oviductal fluid, and are frequently used to induce bovine sperm capacitation *in vitro* fertilization (Parrish *et al.*, 1988, 1989).

An increase in intracellular cyclic adenosine mono phosphate (cAMP), along with a rise in pH may initiate hyperactivity during the capacitation (Yanagimachi, 1994). However, cAMP is not always required for flagellar movement if the activity of Ca^{2+} is reduced with W-13, a calmodulin inhibitor: hamster sperm became motile, although cAMP concentrations

remained low (Aoki *et al.*, 1999). Use of membrane-permeable analogues of cAMP or phosphodiesterase inhibitors enhances hyperactivated motility in the hamster (White and Aitken, 1989), human (Calogero *et al.*, 1998) and primate sperm (Yeung *et al.*, 1999). Ho *et al.*, (2002) treated bull sperm with Triton X-100, a de-membrating detergent, which disrupted plasma, acrosomal, and inner mitochondrial membranes but axonemes remained intact. The role of cAMP in switching on hyperactivation was examined using a reactivation solution containing 1 μ M Ca²⁺ and 2mM ATP. Most sperm exhibited a high amplitude and asymmetrical flagellar motion, similar to that of membrane-intact hyperactivated sperm. Thundathil *et al.*, (2006) showed that binding of ouabain to sperm plasma membrane, an inhibitor of Na⁺/K⁺ATPase, induces capacitation through membrane depolarization via the tyrosine phosphorylation pathway without a considerable increase in intracellular Ca²⁺.

Energy metabolism related to spermatozoa

Role of mitochondria

Mitochondria are localized within a mitochondrial sheath located in the mid-piece, and are arranged end-to-end to form a tight helix around the longitudinal fibrous elements of the tail (Fawcett, 1975). This helix is organised as three separate dextral -right-hand- spirals, each having about 24 turns (Bahr and Engler, 1970). The mitochondrial sheath comprises inner and outer membranes: between them is an intermembrane space. The outer membrane is enclosed in a keratinous structure formed by disulfide linking between cysteine- and proline-rich seleno-proteins such as the sperm-specific phospholipid hydroperoxide glutathione peroxidase (Ursini *et al.*, 1999). During spermatogenesis, this membrane provides enzymatic protection against lipid peroxidation and, as the major structural protein of the mitochondrial capsule, gives it rigidity: it is resistant to osmotic change within the changing micro-environment of maturing sperm within the male genital tract.

Mitochondria are responsible for synthesizing adenosine triphosphate (ATP) through aerobic respiration, a function similar to that in most somatic cells. Because of the unique anatomical structure of sperm, their mitochondria contain several sperm-specific proteins and isoforms associated with

carbohydrate metabolism: for example, in murine sperm these include lactate dehydrogenase C₄ and hexokinase-1, involved in lactolysis and glycolysis respectively (Burgos *et al.*, 1995; Travis *et al.*, 1998).

Mitochondria are a site for production of reactive oxygen species (ROS). ROS are remarkably reactive oxidizing agents that include hydrogen peroxide, superoxide and free radicals (Warren *et al.*, 1987). Within the inner mitochondrial membrane the electron transport chain (ETC) produces ROS at complex I and III following direct electron oxygen reaction or other electron accepters to generate super oxide anion (O_2^-) and hydroxyl radicals ($\cdot OH$) (Muller *et al.*, 2004; Grivennikova and Vinogradov, 2006). Another source of ROS in bovine semen is through oxidative deamination of aromatic amino acids from dead or damaged sperm (Shannon and Curson, 1982). In a rat model, Vernet *et al.*, (2001) showed that mitochondria leak electrons, thereby generating ROS. Human spermatozoa possess the enzyme NADPH oxidase 5 (NOX5) and coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) that generate ROS in the presence of Ca^{2+} (Aitken *et al.*, 1997; Banfi *et al.*, 2001).

Mammalian cells utilize 85-90% of their available oxygen for mitochondrial energy production of which up to 2% is converted to ROS. In spermatozoa the synthesis of low, regulated amounts of ROS is essential for capacitation, acquisition of hyperactivated motility, the acrosome reaction and interaction with oocytes (Chance *et al.*, 1979, Hansford *et al.*, 1997). Imbalance between its production and the antioxidant capacity of catalase and superoxide dismutase (SOD) creates oxidative stress (Sharma and Agarwal, 1996) damages spermatozoa: in mammalian semen, this balance is regulated efficiently by seminal antioxidants (Erenpreiss *et al.*, 2006). High concentrations of ROS disrupt the inner and outer mitochondrial membranes, thereby inducing release of cytochrome-c protein that activates caspases and induces apoptosis (Agarwal *et al.*, 2008). Sperm mitochondrial DNA (mtDNA) is susceptible to oxidative damage through the excessive production of ROS: alterations or deletions in mtDNA disrupt oxidative phosphorylation and cellular calcium homeostasis. Thus a dysfunctional mitochondrial respiratory chain creates more ROS (Wei *et al.*, 2001, Desai *et al.*, 2010) that consequently damages the mitochondria themselves (James and Murphy, 2002). Disruption of the mitochondrial

membrane can be assessed by measuring its membrane potential ($\Delta\Psi_m$) (Guthrie *et al.*, 2008). In a mouse model, a lowered $\Delta\Psi_m$ was associated with reduced NADPH and glutathione (GSH). Since mitochondria contain relatively high Ca^{2+} stores any reduction in mitochondrial functionality may affect Ca^{2+} flux that reduces their forward motility and lowers their potential for fertilizing the ovum (Perl *et al.*, 2006).

Energy production

Spermatozoa obtain energy by utilizing the specific substrates glucose, fructose, mannose, sorbitol and the disaccharide maltose through glycolysis and oxidative phosphorylation. Epididymal sperm have a glycolysis rate sufficient to maintain their motility under anaerobic conditions in the presence of suitable substrate (Lardy and Philips, 1941): the metabolism of fructose leads directly to lactate production (Kamp *et al.*, 1996).

In either aerobic or anaerobic glycolysis, glucose is converted to glyceraldehyde-3-phosphate by phosphorylation that requires two molecules of ATP and synthesizes four molecules of ATP when pyruvate is made. Thus, two molecules of ATP are produced by the oxidation of one molecule of glucose (Fig 1.4). Several glycolytic enzymes have been identified in the membrane of head, fibrous sheath and mitochondria, such as hexokinase, lactate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase (GAPD-S) (Bradley *et al.*, 1996; Westhoff and Kamp, 1997; Bunch *et al.*, 1998; Mori *et al.*, 1998). Those enzymes down-stream from GAPD-S and remain attached to the cytoskeleton even after membrane removal, which suggests that they are components of the fibrous sheath or the outer dense fibres (Storey and Kayne, 1975). Consistent with these observations, Kim *et al.*, (2007) studied the fibrous sheath of ejaculated human sperm and found seven glycolytic enzymes bound to it all being isoforms of aldolase A: triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase, a testis-specific isoform of lactate dehydrogenase-C, (LDH-C), the somatic isoform lactate dehydrogenase-A (LDH-A) and sorbitol dehydrogenase. A sperm-specific isoenzyme lactate dehydrogenase (LDH), LDH-X or LDH-4 has been located in mitochondria (McIndoe and Mitchell 1978; Gallina *et al.*, 1994) which catalyses lactate oxidation to pyruvate.

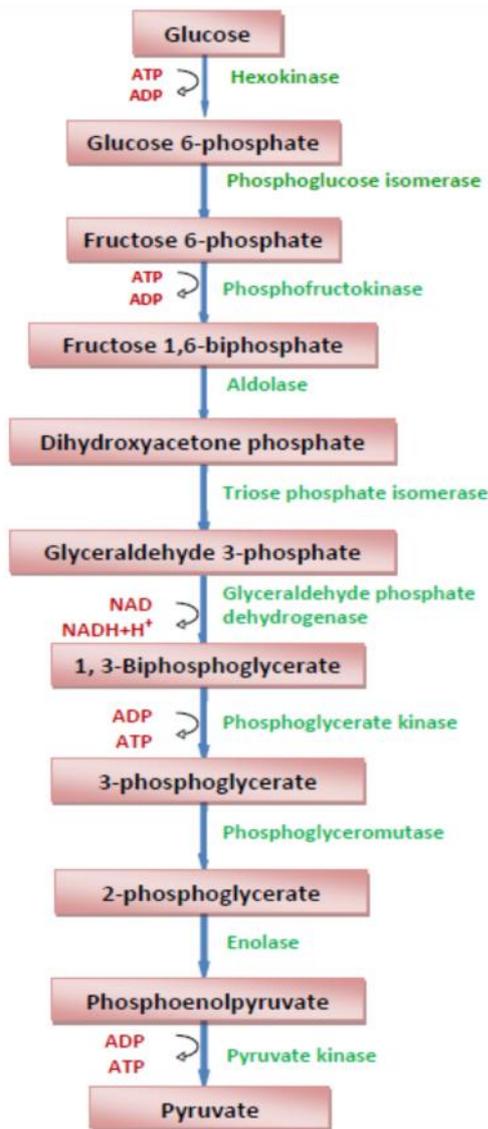


Figure 1.4: Outline of the glycolytic pathway.

ATP synthesis via oxidative phosphorylation occurs primarily in mitochondria. Electrons derived from the oxidation of substrate, are led through redox carriers in the inner membrane electron transfer chain (ETC) to a final acceptor that is molecular oxygen. Electron transfer is associated with proton pumping in the intermembrane space at complex I, II and IV (Fig 1.5). This establishes an H^+ -based electrical and chemical gradient used to drive the activity of the enzyme ATP synthase to produce ATP via complex V (Harris, 1995).

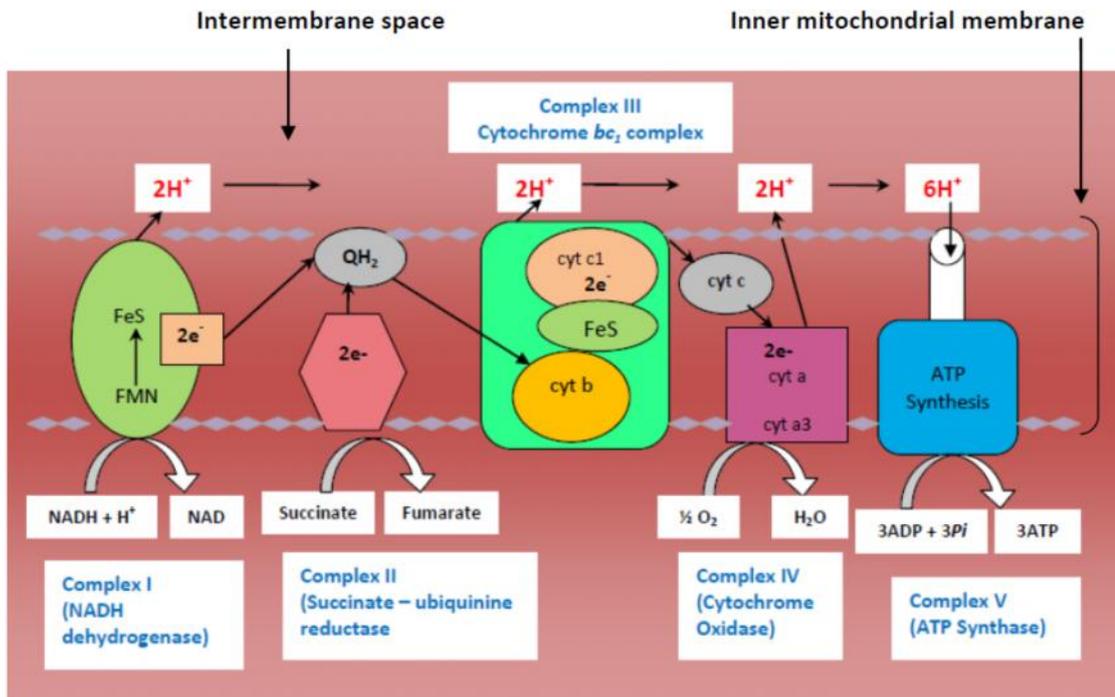


Figure 1.5: Electron transport chain (ETC) of the inner mitochondrial membrane involved in oxidative phosphorylation, including ETC complexes (I, II, III, IV and V), electron carriers' ubiquinone reductase (QH₂) and cytochrome c (cyt c). FMN = Flavin mononucleotide; Fe = Ferrum (Iron); Pi = Inorganic phosphate.

Spermatozoa in the female genital tract maintain most of their motility by endogenous respiration (Nevo, 1965), the basic sugars such as fructose and sorbitol being present in seminal plasma. Seminal plasma also has the buffering capacity to neutralise the acid pH of vaginal fluids, and may act as an agent through which molecules such as the reducing agents ascorbic acid, hypotaurine and ergothionine, protects spermatozoa against potential oxidation following the exposure to atmospheric oxygen (Johnson, 2007).

Energy and sperm motility

Spermatozoa can maintain their motility through glycolysis and/or, respiration provided an appropriate substrate is supplied (Krzyszosiak *et al.*, 1999). The axonemal dynein associated with flagellar motility, has a high demand for ATP. The flagellum of bull sperm is about 54µm long. Sperm of many species such as rat, mouse, honey possum, hamster, and guinea pig have even longer flagella. If the mitochondria, found only in the mid-piece, are the only source of ATP this molecule needs to migrate a relatively long distance -40-50µm- to meet the energy requirements of the axonemal dyneins located in the more distal segments of the flagellum. It is possible that the ATP utilised at the distal end of the flagellum is synthesised locally

by glycolysis (Turner, 2003). If sperm mitochondria are rendered dysfunctional after addition of the electron transport chain inhibitors rotenone and antimycin A, their motility remains satisfactory (Krzyzosiak *et al.*, 1999). Also sperm with functional mitochondria remained motile when placed in a glycolysis-inhibiting medium in aerobic conditions. ATP diffusion from the mitochondria may be satisfactory for species with smaller sperm such as the bovine and sea urchin. Rapid ATP delivery to dynein ATPase required by larger sperm may be achieved through an adenylate kinase shuttle (Ford, 2006): after adding α -chlorohydrin, an inhibitor of the glycolytic enzyme GAPDHs, to sperm he found that they remained motile for at least one hour without an external source of glucose. Earlier, Mukai and Okuno (2004) found that sperm remained motile when either pyruvate or lactate was added to their culture medium in the absence of glucose: motility ceased in the presence of carbonyl cyanide *m*-chlorophenylhydrazone an inhibitor of mitochondrial oxidative phosphorylation. All this research suggests that ATP generated by mitochondria can diffuse far enough within the flagellum to support normal motility. Whilst the glycolytic pathway is the primary source of energy for human sperm motility oxidative phosphorylation does not contribute sufficient ATP to sustain hyper motility (Nascimento *et al.*, 2008), suggesting that a mechanism exists for transporting mitochondrial energy to the distal flagellum.

The presence of a phosphocreatine shuttle, an active link between mitochondrial ATP and dynein-ATPases, has been found in human (Yeung *et al.*, 1996), sea urchin (Tombes and Shaprio, 1987) and rooster (Wallimann *et al.*, 1986) spermatozoa, but this mechanism has not been detected in the bovine (Brooks, 1971). When oligomycin, an inhibitor of the electron transport chain, was used to block oxidative phosphorylation in mitochondria, bovine spermatozoa produced 20-44% of their ATP requirement through glycolysis, suggesting that bovine spermatozoa rely on glycolysis to supply ATP to the distal end of the flagellum (Garrett *et al.*, 2008). Bovine sperm are highly motile when the enzymes adenylate kinase and 3-phosphoglycerate kinase are most active, both of which catalyse the reaction $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$ (Schoff *et al.*, 1989). Hence, sperm rely on the adenylate kinase shuttle for ATP transfer along the flagellum and this

perhaps participates in capacitated sperm by increasing the available energy (Schoff *et al.*, 1989).

More recently Garrett *et al.*, (2008) studied oxygen consumption (ZO_2) in spermatozoa using a novel oxygen biosensor plate system. They found ZO_2 to be higher in frozen/thawed sperm than that of fresh spermatozoa, although the difference was significant only in one of three bulls investigated. This increase in ZO_2 was due possibly to mitochondrial membrane damage during the freezing process, or to sperm becoming hyperactivated through the capacitation-like effects of the freeze/thaw process. Fresh spermatozoa with higher ZO_2 are more fertile than frozen/thawed spermatozoa. If cryopreservation was the only factor affecting sperm metabolic activity and individual bulls are affected by the capacitation-like effects of the freeze-thaw process (Green and Watson, 2001) that reflected in increased sperm ZO_2 , it is likely that the increase in ZO_2 in frozen semen might be due to the selection of motile sperm for freeze/thaw samples using Bovipure isolation of motile sperm which consume more oxygen as compared to membrane intact sperm separated on an Optiprep gradient for fresh samples.

The economic advantage of using AI and semen of genetically superior bulls depends on precise evaluation of the semen that reflects reasonably their fertility potential, especially when low-dose inseminations are the norm (Christensen *et al.*, 2011). For many bulls standing in commercial breeding centres, lower doses of semen can be used without compromising overall cattle fertility (Den Daas, 1997). The problem of selecting *in vitro* tests, capable of identifying those bulls which are fertile when low doses of sperm are used for inseminating without the need to conduct numerous field trials has been addressed in a number of ways, but none has yet proven reliable. The assessment of a bull's ejaculated is based on motility, which has rarely correlated well with fertility (Januskauskas, 1995; Rodriguez-Martinez, 2003; Holt and Van Look, 2004). Other criteria are the proportion of the sperm in the sample that resist osmotic stress (Revell and Mrode, 1994), the percentage with intact plasma membranes (Christensen *et al.*, 2005) and the proportion that remain motile after prolonged incubation (Correa *et al.*, 1997).

With artificial insemination in cattle, sperm are deposited in the body of the uterus and migrate from there to the oviduct via progressive motility. After arriving in the oviduct, spermatozoa are sequestered in the isthmus where they engage with the epithelium: their subsequent release from the isthmus comes about when sperm became hyperactively motile. This facilitates sperm-epithelium detachment and positions sperm in the ampulla prior to fertilization. Hyperactivated motility is an essential precursor to successful fertilization that requires increased ATP availability (Suarez and Ho, 2003; Quill *et al.*, 2003) through oxidative phosphorylation within mitochondria (Turner, 2003).

However, the freeze-thaw process affecting adversely the sperm plasma membrane integrity thereby decreases the number of viable sperm and their mitochondrial function. The aim of this entire study was to investigate aspects of sperm energy metabolism related to their mid-piece length and mitochondrial function. We hypothesize that sperm survival rates following the freeze-thaw process are related to robustness of mitochondria and their membranes which may be a heritable trait in bulls. If sperm from different bulls, incubated with JC-1, have variable mitochondrial membrane potential following the freeze/thaw process, this may be useful to predict commercial bull fertility. Furthermore, this approach may be developed to identify the optimal semen quality during the process of young bull selection for artificial breeding programme in the future: also, it may improve the accuracy for estimating semen quality and identify bulls with good fertility potential both in fresh and freeze/thaw sperm.

Chapter 2

General Materials and Methods

2.1. Preparation of artificial vagina (AV)

The AV comprises an outer heavy rubber cylinder (AV body), an inner latex liner and a semen receiving cone that is attached to a separate collecting glass or plastic tube (IMV, France) (see Fig 2.1). Before use, all components of the AV are cleaned, and sterilized by autoclave at 105°C temperature for 15 minutes. For its assembly, the inner liner is placed within the rigid AV outer body casing and both of its ends turned back over the outer body casing, thus forming a watertight space between these two components. The receiving cone together with the collecting tube attached to its apex, is slipped over one of the ends of the outer casing and secured tightly with rubber bands.

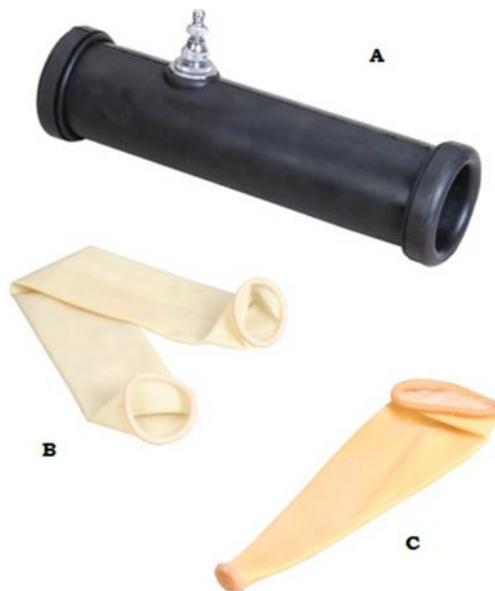


Figure 2.1: Parts of artificial vagina used for bull semen collection. A= AV body; B= inner liner; C= receiving cone.

Before semen collection, the inner liner and receiving cone are lubricated with petroleum jelly. The space between the outer casing and inner liner is filled with water at 42°C through the outlet provided in the outer casing which is then closed with watertight screw. Air is then introduced into this water-filled space via an air screw to create the desired pressure within the

AV lining that provides stimulation via the bull's penis for complete erection and ejaculation. The collecting tube is covered with a thermal jacket to minimize cooling of the semen during and after collection.

2.2. Teasing bulls and semen collection

Before collecting semen from them, all bulls were stimulated sexually by parading them around a teaser animal usually another trained castrated bull. At least three false mounts are allowed to enhance their libido. When the bull is judged suitably aroused, the collector stands to the right side of the teaser. The bull to be collected from mounts the back of the teaser, its prepuce is grasped, and the penis directed into the AV. After making several pelvic thrusts, the bull ejaculates into the AV.

2.3. Semen handling and assessment

After ejaculation, the AV is removed from the penis and held vertically with the cone at the bottom to allow the semen to flow from the cone into the collecting tube. Then, the collecting tube is detached from the cone and taken to the laboratory for assessment of the semen collected.

Colour

Semen with a white or slightly creamy colour is considered satisfactory. Yellow, red and brown coloured semen is indicative of presence of pus/urine/fresh or degenerative blood and is considered abnormal.

Semen volume

The weight of the collecting tube and its contents were measured and the known weight of the tube subtracted from it, in grams.

Sperm motility

A drop of semen is placed on a slide at around 39°C, spread gently and viewed using x10 objective lense under light microscopy (Nikon Labophot 2) for the presence of waves and eddies indicate a forward movement of sperm. A subjective assessment was made using the following criteria: many dark waves moving rapidly, graded +++ or very good, dark waves with moderate movement, graded ++ or good; clear waves with modest

movement, graded + or acceptable; sluggish or no wave movement, graded poor and unacceptable (Laing, 1945; Glover, 1968).

Sperm concentration

Using a nucleocounter (Chemometec A/S, Denmark) 25µl semen was added to 10ml solution S100, which disrupts the sperm plasma membrane. Thus, the sperm nuclei can be stained with propidium iodide impregnated in a nucleocounter SP-1 cassette. After shaking the suspension, approximately 60µl of the sample was drawn up into a disposable SP-1 cassette by immersing the tip of the cassette into the suspension and pressing its piston. The loaded cassette was placed in the nucleocounter, which displayed the total sperm nuclei count within 30 sec.

Sperm morphology

One hundred microlitres of raw semen was added to 250µl of eosin nigrosin stain. After mixing well and staining for five minutes, a drop of stained semen was placed at one end of a clean microscope glass slide and a thin smear created by drawing the drop out with the clean edge of another glass slide. The smear was dried at room temperature and then the slide was labelled with the bull's identity and date. The smear was examined under x100 oil immersion objective, to assess the percentage of primary and secondary morphological abnormalities present in 100 sperm. Primary morphological abnormalities of sperm were abnormal head and/or mid-piece, knobbed acrosome and coiled tails. Secondary abnormalities were detached heads, proximal and distal cytoplasmic droplet, and bent tails. Primary abnormalities are suggestive of testicular dysfunction, and secondary abnormalities were associated with either epididymal transport or handling the semen.

Semen dilution, packing and freezing

The raw semen was diluted in two stages. After initial dilution of semen in egg yolk/ tris/glycerol (EYTG) extender at the proportion of 1:1 the semen was cooled to 4°C. Then, an appropriate volume of EYTG extender was added depending on the semen concentration required and volume per straw. The dilute semen was then packed into 0.25ml polyvinyl chloride straws (IMV Technologies, L' Aigle, France) at 60×10^6 /ml sperm

concentration. The straws were labelled with the bull's identity, date and number of ejaculate, and then placed in a refrigerator for equilibration at 4°C for four hours. Following equilibration, semen was immediately frozen in liquid nitrogen vapour in a Digitcool Freezer (IMV Technologies, L' Aigle, France), using the recommended bovine freezing curve. The straws were then stored in liquid nitrogen.

Assessment of motility after freeze-thaw process

Three frozen straws from one ejaculate per bull were placed in a water bath at 39°C for 30 sec and pooled. The motility of individual sperm was assessed using a Hamilton Thorne Computer Assisted Semen Analyser (CASA) system (Hamilton Thorne, Inc. Beverly, USA). The sperm with 26.7µm/s average path velocity and 55% path straightness classified as progressive and were considered satisfactory: those moving in small circles or having side-to-side motion without forward progression were considered unsatisfactory. An ejaculate characterised by >40% of sperm with satisfactory movement was considered suitable for commercial cattle breeding and samples with ≤40% sperm with satisfactory movement was rejected.

Bull stud records

All semen collections from every bull standing at stud were recorded for ejaculate volume, sperm concentration, total sperm output, percentage of sperm with normal morphology and motility before and after freezing using Roneo cards and stored at the stud.

2.4. Non-return rates in bulls

Fertility of AI bulls is estimated by non-return rates (NRRs). It is obtained from the total number of cows inseminated by semen from a particular bull and the number of cows which were not re-presented for further insemination within a time period such as 28 or 56 days, or more commonly 60-90 days (Koops *et al.*, 1995): this percentage of cows that were not rebred is termed the NRR. In this study, the NRRs used by Genus Breeding Company, Ruthin, North Wales were estimated from cows that had not been re-inseminated within 49 days following first insemination (Revell, personal communication).

2.5. Preparation of reagents

All chemicals were purchased from (Sigma, St. Louis, USA), unless otherwise stated.

Nigrosin-eosin stain

Eight hundred millimetres of 2.9% Sodium Citrate solution (23.2g/800ml water) was heated to 80°C in a Quickfit flask, fitted with a reflux condenser. The warmed solution was stirred and 80g of nigrosin was dissolved in it using 10g increments. Then, 12.8g of eosin was added to the solution in two parts and stirred for 2-3 hours until thoroughly mixed: 2-3 drops of Bloat Guard was added to prevent foaming. Then, the solution was boiled for 4 hours, cooled, and filtered through Whatman filter paper. Quantities of 30ml were put in small stain bottles, capped loosely, and autoclaved at 105°C for 15 minutes: After bottles of stains were cooled, the bottle tops tightened and stored under refrigeration.

Phosphate buffered saline (PBS) extender

Eight grams, sodium chloride, 0.2g potassium chloride, 1.44g disodium hydrogen phosphate/sodium phosphate dibasic, and 0.24g (2mM) potassium dihydrogen phosphate/potassium phosphate monobasic was dissolved in 800ml of ultra-pure water. After mixing well, the pH was adjusted to 7.4 with hydrochloric acid or sodium hydroxide using a pH meter. The solution was made up to one litre by adding 200ml ultra-pure water. The osmolality of the solution was 290-310mOsm/kg. This solution was separated into aliquots and stored in disposable bottles at 4°C.

2-deoxy-D-glucose (DOG) suspension

This was purchased as powder in 250mg vials. 49.25mg of DOG, 0.09mg bovine serum albumin (BSA) and 59.4mg pyruvic acid was dissolved in 30ml PBS, to produce a 10mM solution. The solution was stored as one millilitre volume in microfuge tubes at -20°C.

Iodoacetamide (IAM) suspension

IAM was supplied as powder in 5g vials. 5.5mg of IAM, 0.09mg bovine serum albumin (BSA) and 59.4mg pyruvic acid was dissolved in 30ml PBS,

to provide a 1mM solution. The solution was stored as one millilitre volume in microfuge tubes at -20°C.

Valinomycin (VAL)

This was supplied as a 1mg/ml stock solution in dimethyl sulfoxide (DMSO) i.e. 0.9mM. One millilitre of stock solution was dilute with 100 millilitre of Ruthin extender to produce a 9000nM working solution. The solution was stored as 50µl volume in microfuge tubes at -20°C

Sodium sulphite (Na₂SO₃)

One millilitre fresh solution was prepared by adding 12.6mg Na₂SO₃ to 1ml of PBS, to give a 100mM solution.

JC-1 fluorescent dye

JC-1 (Invitrogen, Oregon, USA) was supplied as a 7.5mM stock solution at 5 mg/ml in dimethyl sulfoxide (DMSO). One hundred micro-litre of stock solution was diluted in 37.5 ml Ruthin extender to give a 20µM working solution. The solution was stored as 100µl volumes in microfuge tubes at -20°C.

Propidium iodide (PI) fluorescent dye

This was supplied as a 2.4mM at 0.5mg/ml solution in isotonic saline (Invitrogen, Oregon, USA) and stored as 50µl volumes in microfuge tubes at -20°C.

SYBR-14 fluorescent dye

SYBR-14 (Invitrogen, Oregon, USA) was supplied as 100µl (1 mM). Working solution was prepared by diluting the dye concentrate 10 fold volume in DMSO (100µl/ml). The working solution was stored as 50µl volume in microfuge tubes at -20°C.

Hoechst 33258 (H33258) fluorescent dye

This was supplied as a 100 mg powder. Working solution of H33258 was prepared by diluting the dye to 10µg/ml in 2.9% (w/v) sodium citrate solution. The working solution was stored as 100µl in microfuge tubes at -20°C.

Chapter 3

Mid-piece length of spermatozoa in different cattle breeds and its relationship to fertility

3.1. Introduction

Sperm are highly differentiated, specialised cells that transfer a haploid set of paternal chromosomes to the female gamete at fertilisation. To achieve this, they must traverse the length of the female genital tract, bind to oviduct epithelium to undergo capacitation, attach to the zona pellucida and finally penetrate the oocyte itself (Foote, 2003).

Spermatozoa obtain energy for their motility through glycolysis and/or respiration, provided that appropriate substrate is available (Krzyzosiak *et al.*, 1999). Rikmenspoel (1965) estimated that 70% of adenosine triphosphate (ATP) produced by bovine spermatozoa is used for motility, the precise proportion being related to the incubation temperature: for example, at 18°C and 37°C around 9% and 58% of sperm respectively showed progressive motility (Hammerstedt and Hay, 1980). Spermatozoa generate most of their energy by glycolysis rather than oxidative phosphorylation and a number of sperm-specific enzymes involved in the glycolytic pathways are located in the fibrous sheath (Miki *et al.*, 2004). Mitochondria that produce ATP through oxidative phosphorylation are located in the sperm mid-piece, found at the extreme anterior end of the flagellum (Turner, 2003; Ford, 2006). This function is crucial because it correlates with the energy status of spermatozoa, their motility and potential for achieving successful fertilisation (Casey *et al.*, 1993; Kasai *et al.*, 2002).

Hyperactivated motility in sperm is an essential precursor to successful fertilization that requires increased ATP (Suarez and Ho, 2003; Quill *et al.*, 2003). The immediate availability of mitochondrial ATP is crucial when spermatozoa become hyperactive: their flagellar movements become asymmetrical and whip-like, have high amplitude and low frequency, and this facilitates their detachment from the oviduct epithelium to position sperm in the ampulla prior to fertilization (Turner, 2003).

Biometric differences in sperm morphology between mammalian species were described by Cummins and Woodall, (1985), who measured lengths of mid-piece, caput or head and principal piece for most species except primates and bats. Gage (1998) found a correlation between mid-piece and flagellar lengths for the 179 species he investigated. More recently, Aggarwal *et al.*, (2007) observed a wide variation in the length of the mid-piece between buffalo bulls of different breeds and suggested that sperm biometric characteristics might be associated with male fertility. It is in this context that sperm competition has been considered in those species where two or more males compete for opportunities to mate with a single female, such as in canines, and fertilize ova. Sperm mid-piece volume may be indicative of a higher mitochondrial content, and is significantly larger in these species (Anderson *et al.*, 2005): there is also a positive correlation between it and testis size. Mid-piece volume is correlated positively with motility in domestic fowl, possibly dependent upon an endogenous substrate (Froman and Feltmann, 1998), and in primates that exhibit multiple-partner mating activity (Anderson and Dixson, 2002; Dixson *et al.*, 2004). Generally, sperm with longer flagella tend to swim faster to reach the ova sooner and achieve fertilization compared to those with shorter tails when ejaculates from different males intermingle in the same female tract (Gomendio and Roldan, 1991). There is a positive correlation ($r=0.6$; $p<0.01$) between sperm length and its maximum velocity (Gomendio and Roldan, 1991). By contrast, sperm from the Iberian red deer stag have long mid-pieces but swim relatively slowly (Malo *et al.*, 2006), suggesting that their activated spermatozoa with shorter mid-pieces may exhibit greater motility.

Several methods have been used to assess semen quality, using laboratory parameters to predict the fertilization potential of bulls and correlating these results with actual pregnancy rates achieved in the field. Post thaw motility is assessed most frequently as a measure of sperm viability and is correlated ($r=0.4-0.5$) with 56 day non-return rates in cows (Januskauskas *et al.*, 2003). Also used are the percentage of viable spermatozoa present in an ejaculate, computer-based estimates of motility characteristics, and acrosome integrity that are all correlated significantly with good fertility (Budworth *et al.*, 1988; Kjaestad *et al.*, 1993; Zhang *et al.*, 1998, Januskauskas *et al.*, 2003). Hence, bulls with apparently normal semen

that are subfertile are worthy of further investigation using more recently devised molecular techniques (Aggarwal *et al.*, 2007).

Froman and Feltmann (1998); Froman *et al.*, (1999) concluded that mitochondrial function and ATP content of avian sperm were the most important factors affecting their motility, and sperm motility was the primary determinant for optimum fertility. For example, the mean rate of oxygen consumption was 1.14 $\mu\text{l O}_2/\text{min}$ in fresh semen ejaculates of high motility compared with 0.62 $\mu\text{l O}_2/\text{min}$ in those presenting only moderate motility. Recently Garrett *et al* (2008) found a positive correlation between oxygen consumption (ZO_2) in bull spermatozoa and non-return rates and concluded that an increase in ZO_2 , characteristic of the freeze/thaw process, was possibly associated with mitochondrial membrane damage during this procedure: alternatively, sperm may be hyperactivated through the capacitation-like effects of freezing/thawing.

We hypothesize that the morphology of spermatozoa may be associated with their rate of ZO_2 and fertility: for example, sperm mid-piece length where mitochondria are located. Such a relationship has not been investigated before, particularly in context of commercial cattle breeding programmes and bull fertility characteristics. This present study investigates variation in sperm mid-piece biometry by breed, age, and between bulls used in current breeding programmes for dairy and beef herds in both intensive and semi-intensive cattle livestock farms, worldwide.

3.2. Materials and methods

Thirty four bulls from six different breeds were chosen for investigation: five mature and five immature Holsteins, and five bulls from each of the following breeds, Friesian, Belgian Blue, Charolais and Limousin. Only four Aberdeen Angus bulls were investigated. Five ejaculates were collected from each bull, their numbers (1, 2, 3 etc.) always relating to the order in which they were collected, and a prepared slide was examined for mid-piece length from each ejaculate. To prepare semen samples for microscopic examination, 100 μl of raw semen was added to 250 μl of Eosin Nigrosin stain (Hancock, 1952) and, after shaking well, drops of stained semen were placed at one end of a clean microscopic glass slide and a smear made by drawing them out with the clean edge of another glass slide. The slides were

labelled with bull identity and date, and allowed to dry at room temperature. The mid-piece length of forty morphologically normal sperm per ejaculate was measured, selected from various fields at random on each of five slides, using a x100 oil immersion objective with a Nikon Labophot 2 Microscope fitted with a digital CCD camera (Penguin-600 CL-CU) with imaging software within the Penguin/Pro application suite (Penguin/Pro Application Suite v.3.5.0, Pixera corporation USA) (see Fig 3.1).

Sperm with abnormal morphology of either head or tail and post-ejaculatory injury were excluded so that only those with normal morphology and a distinct mid-piece, either straight or curved, were measured (see Fig 3.2). Before taking the measurement, the captured digital image of the individual spermatozoon was magnified to a level where the mid-piece could be distinguished clearly from the principal piece. Measurements (in micrometers) were made of those sperm with straight flagellum by dragging the cursor from the distal point of the mid-piece to its anterior end at the base of head region: where the flagella were bent, mid-pieces were measured using a series of short straight lines that described their curve, the sum of these being considered as a single value.

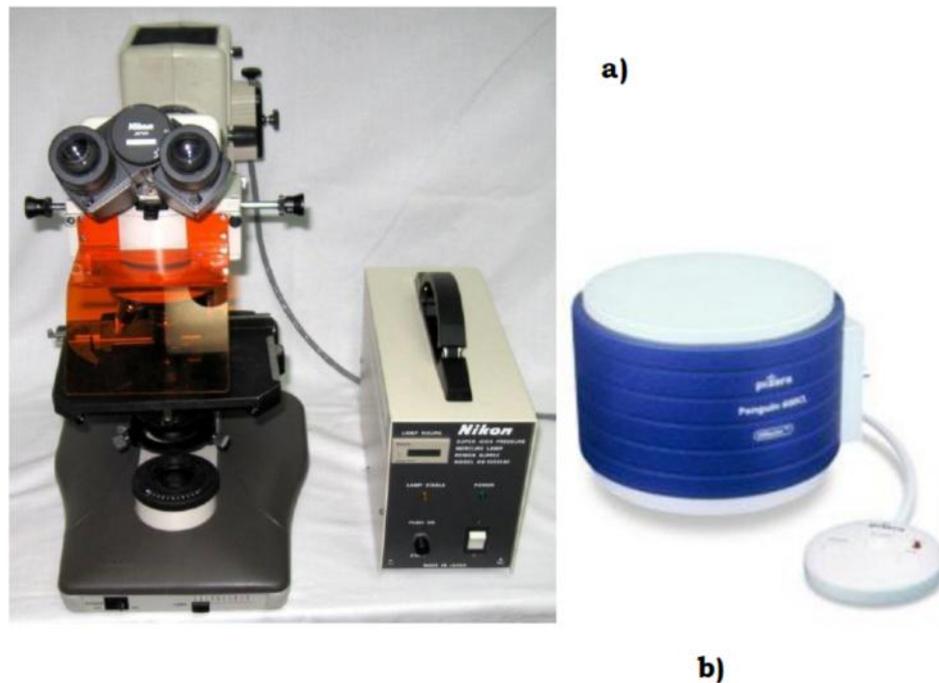


Figure 3.1: Microscope (a) and digital camera (b) used to measure sperm mid-piece length.

3.3. Data collection and statistical analysis

The estimated mid-piece lengths for 40 sperm from each ejaculate were recorded and organized into Excel files with the independent variables being breed and ejaculate number. After cleaning, differences in mid-piece length between and within bulls were investigated using one-way analysis of variance (ANOVA) in Minitab Relationships between mid-piece length and NRRs for all bulls except the five immature Holstein were investigated using linear correlation coefficients. Significance was accepted at a $p \leq 0.05$ and results presented as mean \pm SD, except where stated.



Figure 3.2: Spermatozoa with both straight and bent mid-pieces that were measured either as a single straight line or the sum of several measurements.

3.4. Results

Differences between ejaculates from the same bull

These are summarized in Fig 3.3 a, b and c. Of the 34 bulls examined, nine showed significant variation between their own ejaculates: Mature Holstein bulls 2, 3, 4 ($F = 2.48$, $p \leq 0.05$) and immature bull 1 had the greatest variation ($F = 3.75$, $p \leq 0.01$), Friesian bull 1 ($F = 2.92$, $p \leq 0.05$), Belgian Blues bull 1 and 5 ($F = 2.98$, $p \leq 0.05$), Aberdeen Angus bull 3 ($F = 2.54$, $p \leq 0.05$) and a Limousin bull 4 ($F = 2.71$, $p \leq 0.05$). The ejaculates obtained from Friesian bulls showed least variation between collections whereas Holstein bull 2 showed just over 2% variation ($F = 5.89$, $p \leq 0.01$). In mature

Holstein bulls the mid-piece length was always shorter in the later numbered ejaculates. By way of contrast, the later ejaculates of some bulls contained sperm of longer mid-piece length: for example, Holstein 5, Limousin 1, 2 and 5.

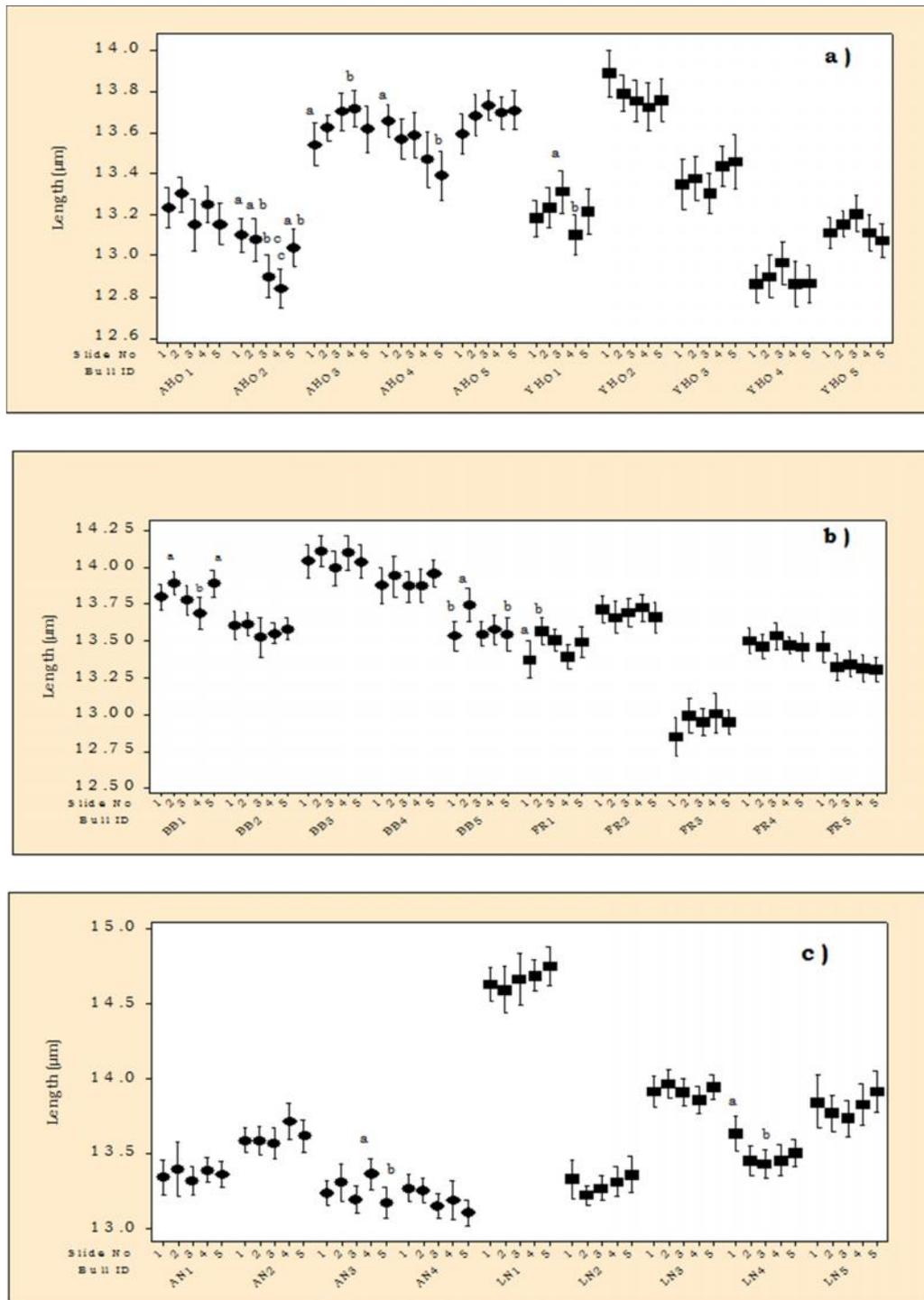


Figure 3.3: Variation in sperm mid-piece length of 40 sperm in each of five ejaculates for each bull within different breeds: a) juvenile (●) and mature (■) Holsteins, b) Belgian Blue (●) and Friesian (■), and c) Aberdeen Angus (●) and Limousin (■). Means that do not share a letter are significantly different between ejaculates.

Differences between breeds

There were significant differences ($p \leq 0.001$) between bulls within the same breed: for example, between juvenile Holstein bull 2 and the other four within that group, and two Belgian Blue bulls- 2 and 5 - that had significantly shorter mid-pieces than the other three (see Table 3.1). Despite this intra-breed variation, there was no significant difference in mid-piece length between the breeds. Sperm from Belgian Blue and Limousin bulls presented with mid-pieces of similar length that were longer than the other breeds investigated (see Fig 3.4): those with the shortest length were from juvenile Holstein bulls.

Table 3.1: Mean mid-piece length (SD) of 200 spermatozoa examined from five ejaculates, each obtained from either juvenile and mature Holstein or five other breeds of bull.

Breed of bull	Bull					F value (p)
	1	2	3	4	5	
Holstein (juvenile)	13.21 (0.08)	13.78 (0.06)	13.38 (0.06)	12.89 (0.05)	13.13 (0.5)	84.4 (≤ 0.001)
Holstein (mature)	13.22 (0.06)	12.99 (0.12)	13.64 (0.07)	13.53 (0.11)	13.68 (0.05)	
Friesian	13.46 (0.08)	13.69 (0.03)	12.95 (0.06)	13.48 (0.03)	13.35 (0.06)	115.7 (≤ 0.001)
Aberdeen Angus	13.36 (0.03)	13.61 (0.06)	13.25 (0.08)	13.19 (0.07)	--	42.6 (≤ 0.001)
Belgian Blue	13.81 (0.9)	13.58 (0.04)	14.06 (0.05)	13.91 (0.04)	13.59 (0.09)	51.8 (≤ 0.001)
Charolais	13.53 (0.04)	13.59 (0.05)	14.16 (0.09)	13.36 (0.03)	13.86 (0.03)	183.7 (≤ 0.001)
Limousin	14.67 (0.06)	13.30 (0.05)	13.93 (0.04)	13.49 (0.08)	13.82 (0.07)	231.2 (≤ 0.001)

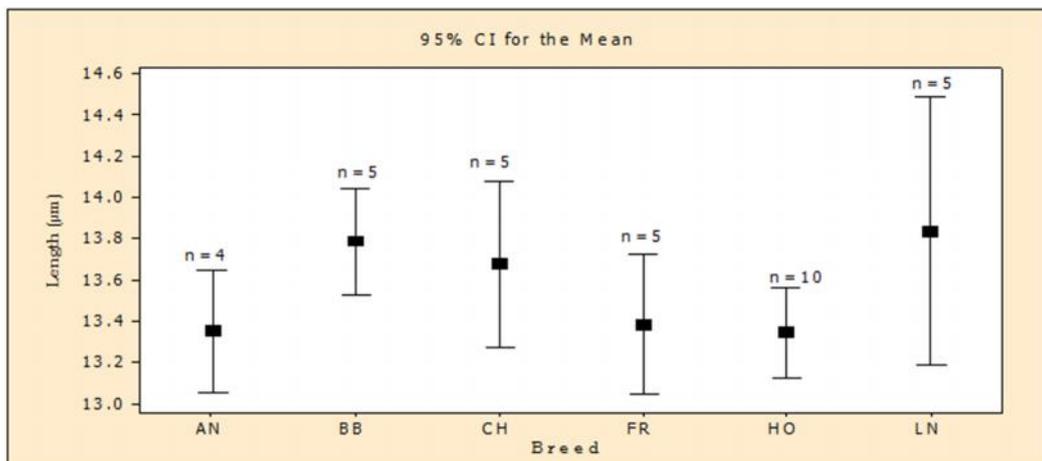


Figure 3.4: Variation in sperm mid-piece length between breeds: Aberdeen Angus (AA), Belgian Blue (BB), Charolais (CH), Friesian (FR), Holsteins (HO) and Limousin (LN).

Mid-piece length and 49-day non-return rates

The sperm mid-piece length (MPL) between breed from 29 beef and dairy bulls was found to be positively correlated ($r=0.27$; $p=06$) to their known 49 day NRR recorded by Genus but it was not significant (see Fig 3.5). When MPL of 29 individual mature dairy and beef bulls was compared with a known NRR expressed as variance (\pm) from the contemporary breed average NRR from Genus A.I. records (Appendix 3.1). A negative correlation ($r=-0.13$; $p=0.52$) was found between MPL of all bulls and their NRR (see Fig 3.6).

Beef breeders prefer to re-bred their cows naturally if they fail to conceive by artificial insemination as compare to dairy breeders (Garrett *et al.*, 2008), therefore to know this variation, the non-return rates (NRRs) of beef and dairy bulls are calculated separately. When MPL of individual bulls of dairy and beef breeds were compared to their NRR, the correlations were no different whether negative ($r= -0.53$) for black and white dairy breeds, or positively correlated for the beef breeds ($r=0.01$) (data not presented): the small numbers of bulls involved prevented meaningful statistical relationships being established.

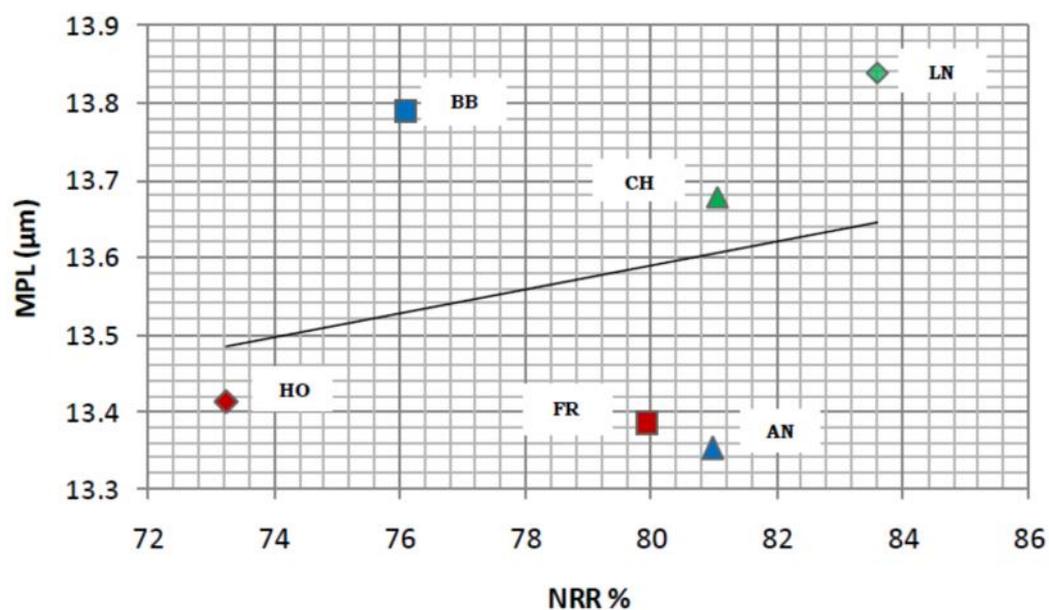


Figure 3.5: Sperm mid-piece length compared to 49 day non-return rate (NRR) between dairy and beef breeds (n=6), using Pearson correlation test. AA= Aberdeen Angus; HO= Holstein; BB= Belgian Blue; CH= Charolais; FR= Friesian; LN= Limousin.

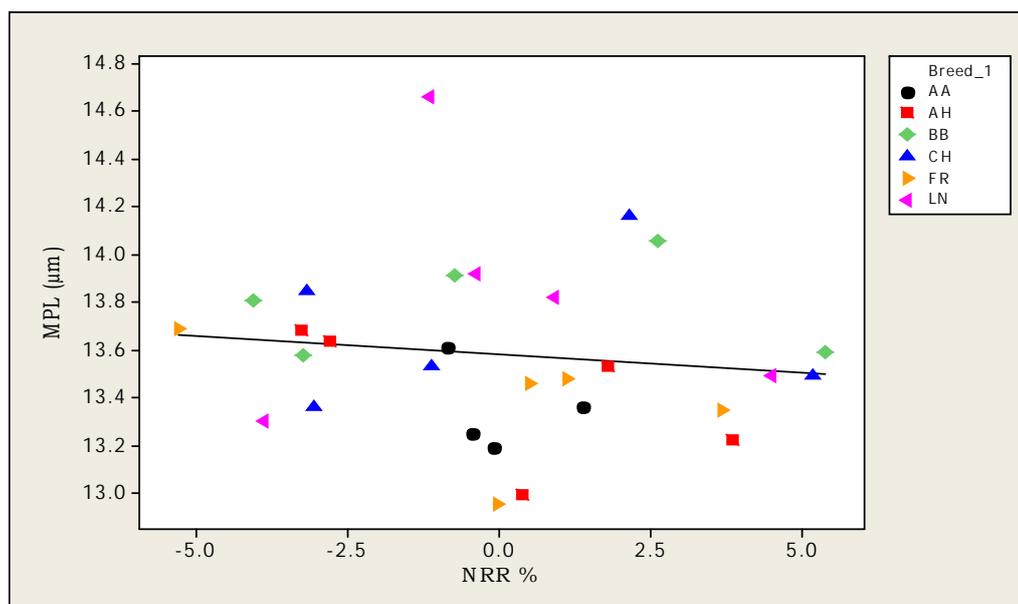


Figure 3.6: Sperm mid-piece length compared to 49 day non-return rate (NRR) of bulls from the dairy and beef breeds (n=29), using Pearson correlation test. AA= Aberdeen Angus; AH= Holstein; BB= Belgian Blue; CH= Charolais; FR= Friesian; LN= Limousin.

3.5. Discussion

Variations in the length of mammalian spermatozoa are due mostly to variation in tail length (Cummins and Woodall, 1985) and the size of the mid-piece contributes to such species' differences. Anderson and Dixson (2002) found that mid-piece volume was greater in primates whose normal mating patterns involved multiple rather than single partners. Earlier, Gomendio and Roldan (1991) had found similar differences between monoandrous and polyandrous species: in rodent and other species where males face sperm competition after mating with a single female, spermatozoa are longer than in species where only a single dominant male will mate with a female e.g. some wild cats and deer. The longer the sperm flagellum, the greater the forces generated by its motions (Katz and Drobnis, 1990) and its requirement for immediately available energy. This present study is the first to record variation in mid-piece length of a single species, the bovine, associated with collecting their ejaculates over a period of time. Whilst no significant differences were found between different breeds of bull, Aggarwal *et al* (2007) found such variation within eight various breeds of buffalo. Results from this study showed significant

variation in mid-piece length between ejaculates collected from nine of the 34 bulls investigated. The mid-piece lengths we obtained were similar to those found by Venkataswami and Vedanayagam (1962) who found an average mid-piece length of $12.41 \pm 0.85\mu\text{m}$ for buffalo and $14.19 \pm 1.57\mu\text{m}$ for domestic cattle. It is unlikely that the variation found in this present study occurred because of measuring technique since spermatozoa with straight tails could be measured as one entity and sperm with severely bent tails, due to cold shock, were positively excluded. In any case, the SD in this present study was ten-fold less than that recorded by the Indian researchers, suggesting this current computer-aided technique is robust. However, no attempt was made biochemically to distinguish between spermatozoa that were either non-apoptotic or undergoing programmed cell death: mid-piece morphology and mitochondrial volume is affected by apoptosis (Hendricks and Hansen, 2009) and this may have contributed to some variation found in this study.

Age influences mid- piece length in bulls. Cummins and Woodall (1985) found that sperm of bulls over 10 years old possess mid-pieces around 4% longer than younger fully mature males. In this present study, Holstein bulls under 18 months old had mid-pieces 1% shorter than older males, suggesting a slow, progressive lengthening of the mid-piece from puberty through to old age. Male reproductive potential generally falls with increasing age, associated with two factors. First, there is greater opportunity for mutations to adversely affect the mitochondrial genome and impair oxidative phosphorylation. The mid-piece is crucial for capacitation that involves tyrosine phosphorylated proteins, two of which are phospholipid hydroperoxide glutamate peroxidase and ATP synthase beta subunit: both have a primary role in sperm motility (Shivaji *et al.*, 2009) and disruption of their synthesis will reduce male fertility. Second, mitochondria are a source of reactive oxygen species (ROS) which is a normal product of cellular respiration: normally these are removed from cells by antioxidant defence mechanisms. In sperm, ROS can accumulate within the mid-piece and damage mitochondria: the likelihood of this occurring increase as males grow older (Desai *et al.*, 2010). Further preliminary data from this present study associated non-return rates (NRRs) in cows bred with semen collected from specific bulls whose mid-

piece lengths were measured: longer mid-pieces correlated with up to a 5% reduction in NRR in the dairy bulls investigated. The relationship between mid-piece length, its mitochondrial volume and the efficiency of oxidative phosphorylation should be investigated further.

There are several other factors that affect mid-piece length in bulls. For example, the mid-piece length of mature spermatozoa in pure-bred black-and-white cattle is 2% longer than in crossbred Friesian x Sahiwal animals (Sardar, 2005). Heterosis is well known as a positive influence for reproductive efficiency in cattle, particularly in reducing embryonic mortality (Heins *et al.*, 2008) but the contribution of the male gamete to such improved fertility is unknown. Also, sperm from unimproved Indo-Pak zebu breeds tend to have mid-pieces 2 – 9% longer than European cattle (Venkataswami and Vedanayagam, 1962): again, the significance of this in terms of breeding efficiency is not known.

The present study indicated significant variation in sperm mid-piece length between ejaculates within several bulls. If this is due to measurement imprecision, a larger sample might find it to be less significant. If it is real, it would seem unlikely to be due to short-term variation in spermatogenesis. Some effect of length of epididymal storage prior to ejaculation may be involved and this might be investigated by making a series of observations on ejaculates collected at varying interval. There was also a difference in mid-piece length between bulls within breed as well as among breeds. These preliminary results suggest that further work is necessary to relate mid-piece length with mitochondrial density and volume, and energy metabolism related to sperm motility, capacitation and fertility potential of known beef and dairy bulls.

Chapter 4

A method for estimating bovine sperm mitochondrial membrane potential

4.1. Introduction

Mitochondria in sperm are localized within a mitochondrial sheath located in the mid-piece. They are arranged end-to-end to form a tight helix around the longitudinal fibrous elements of the tail (Fawcett, 1975), and are responsible for producing adenosine triphosphate (ATP) for the cell to support their motility through oxidative phosphorylation. (Harris, 1995).

Mitochondrial membrane potential ($\Delta\Psi_m$) is related to cell energetics. It can be estimated using a range of laboratory reagents such as non-toxic membrane potential-sensitive dyes (Shi *et al.*, 2008) that are cationic fluorescent probes and accumulate in the inner membrane of either isolated or cellular mitochondria (Guthrie and Welch, 2008). Rhodamine 123 is one such probe which accumulates in mitochondria and fluoresces green (Evenson *et al.*, 1982): it is a cationic molecule that excites at 488nm and emits at 515-575nm, but cannot distinguish between mitochondria with a low or high membrane potential. A mitotracker dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) has been developed that can differentiate between sperm with low (monomers) or high (aggregates) mitochondrial activity. It is a lipophilic cationic fluorescent carbocyanine dye that enters mitochondria selectively: depending upon the concentration of stain within mitochondria, their colour changes from green to red/orange. The dye fluoresces green in all functioning mitochondria: it changes to red/orange in very active mitochondria when the dye molecules aggregate (Troiano *et al.*, 1998; Martinez-Paster *et al.*, 2004). Marchetti *et al.*, (2004) compared four fluorochromes suitable for characterising $\Delta\Psi_m$ in human sperm, chloromethyl-X-rosamine, tetramethylrhodamine ethyl ester, dihexyloxa carbo- cyanine iodide and JC-1 and found that the latter detected changes in inner mitochondrial membrane potential more specifically than the others.

Cells emitting light of different wavelengths can be recognised and counted using flow cytometry. Thus this method is suitable for evaluating sperm functional characteristics by passing them through a laser beam and quantifying the number of sperm with a particular characteristic (Troiano *et al.*, 1998; Petrunkina *et al.*, 2010). By labelling sperm mitochondria with JC-1, the number of sperm within an ejaculate with low and high $\Delta\Psi_m$ can be quantified: this may be of use to estimate and compare the potential fertility of bulls standing in AI studs in both fresh and frozen-thawed semen. Using this technique, bovine sperm can be categorised in two distinct populations (Garner *et al.*, 1997; 1999; Thomas *et al.*, 1998), similar to sperm obtained from sexually active rams when stained with JC-1. The sperm can be recognised by their different mitochondrial activity appearing as populations of both green and red-orange (Martinez-Pastor *et al.*, 2004). When stallion spermatozoa were separated by single-layer centrifugation using Androcoll-ETM and incubated with JC-1, they displayed three fluorescent staining characteristics (Garcia *et al.*, 2009): sperm fluorescing orange represented a high $\Delta\Psi_m$, green a low $\Delta\Psi_m$, and some fluoresced both orange and green simultaneously that suggested they were heterogeneous mitochondria.

The technique of flow cytometry depends on the ability of the instrumentation distinguishing between specifically bound fluorescent molecules and other intrinsic and non-specific background signals. All cells exhibit some intrinsic level of auto-fluorescence, the majority originating from mitochondria and lysosomes. Together with aromatic amino acids and lipo-pigments, the most important endogenous fluorophores are pyridinic (NADPH), riboflavin and flavin coenzymes (Aubin, 1979; Benson *et al.*, 1979). This emission is an intrinsic property of cells and must be distinguished from fluorescent signals obtained by adding exogenous markers. Cellular auto-fluorescence affects the sensitivity of this method since cells emit a low level of specific fluorescence (Mosiman *et al.*, 1997). Therefore, auto fluorescence is the fluorescence of material or other substance than the fluorophore of interest. It increases the background signal.

Recently, a new method to differentiate non-sperm particles and live sperm, has been described based on the osmotic intolerance of sperm when they

are immersed in water that causes damage to the cell membranes (Petrunkina *et al.*, 2010). When stained with propidium iodide (PI), which is absorbed by membrane-damaged cells, the nuclei of all dead sperm display a red fluorescence whereas non-nuclei particles remained unstained. The expected difference between PI positive sperm and negative non-sperm particles can be assessed quantitatively within an ejaculate, by comparing sperm samples diluted in an extender labelled with PI and the membrane-intact stain SYBR 14.

In our preliminary experiments to obtain maximum uptake of JC-1 in sperm population at minimum incubation time, variable results were observed with same bull examined on different days as well as fresh and post thaw semen. Selection of motile sperm by Bovipure showed maximum $\Delta\Psi_m$ at 30 minutes in fresh and post-thaw sperm labelled with JC-1, while maximum $\Delta\Psi_m$ was observed at 150 and 90min in corresponding fresh and post-thaw semen washed with Ruthin extender, a glycolysis supported medium (data not shown). Uprating of $\Delta\Psi_m$ in motile sperm of 3/4 bulls was seen in post-thaw semen. This was consistent with the results of Garrett *et al.*, (2008) who observed higher oxygen consumption rates in post-thaw semen. Whereas, semen washed in Ruthin medium: $\Delta\Psi_m$ has been increased with increase in incubation time in all bulls of fresh sperm and 2/6 bulls of post-thaw sperm respectively. Uprating of $\Delta\Psi_m$ in bulls might be sperm initially were dependent on ATP generated by glycolysis accumulating its end product pyruvate/lactic acid, later with increase in incubation time the sperm demonstrated capacitation like changes and became hyperactive (Watson, 1995a) which result in increase demand for ATP and relied on mitochondrial ATP for long time.

This study describes a novel method to validate the use of JC-1 for estimating $\Delta\Psi_m$ in bulls, by setting regional and logical gates to exclude dead sperm and other non-cellular components from live sperm present within an ejaculate and to determine the maximum mitochondrial membrane potential at minimum incubation time.

4.2. Materials and methods

Preparation of frozen-thawed and fresh semen

Ten straws of 0.25ml frozen bull semen were received in liquid nitrogen and thawed by immersing them in a water bath at 39°C for 30 seconds. The content of each straw was transferred into a centrifuge tube and Ruthin extender (IMV L' Aigle, France) was added to create a total volume of 10ml. The contents were centrifuged at 600g for 10 minutes at room temperature, the supernatant removed, and the resulting pellet transferred to a fresh test tube in a water bath at 39°C. Ruthin extender was then added to the pellet to give a suspension volume of 1ml. The resulting sperm concentration was estimated using a nucleocounter (Chemometec A/S, Denmark) and then adjusted to 12×10^6 /ml by adding warmed Ruthin extender separately in test tubes, in duplicate. The samples were rested for 15 minutes.

An aliquot of raw semen containing approximately 200×10^6 sperm was placed in a test tube shortly after collection and warm Ruthin extender added to give a total volume of 1ml. A similar procedure after sperm concentration estimation was applied as for the frozen-thawed semen above.

Fluorescent staining

From the 1ml of semen containing 12×10^6 /ml sperm, 360 μ l was placed in a test tube in duplicate; 40 μ l of JC-1 was added, approximately 2 minutes apart to accommodate running time on the flow cytometer and the tube agitated. It was incubated at 39°C for 40min. Then 40 μ l of the incubated semen was added to 995 μ l of warmed Ruthin extender and 5 μ l PI in a flow tube. The final stain concentrations of JC-I and PI in sperm suspensions were 0.1 μ M and 2.4mM respectively. Gains were set as FL1 316; FL2 550 and FL3 430.

Regional and logical gates

A Partec Cyflow Space flow cytometer (Partec GmbH, Gorlitz, Germany) was used for the analysis. Two dot-plots were set (see Fig 4.1). The first was a logical gate that excluded PI positive cells, defined as G10= (RN1 AND RN2) AND (RN3 OR RN5) NOT RN4, and the second included PI positive cells

defined as $G5 = (RN1 \text{ AND } RN2) \text{ AND } (RN3 \text{ OR } RN5)$. Here, RN1 was a regional gate in a one dimensional graphical presentation of forward scattered (FSC) light deflected on the cell population at a small angle to the exciting laser beam (see Fig 4.1) that counted spermatozoa but excluded small particulate matter. It related to the size of cells even though it depends on shape and does not increase continuously with size. RN2 was a regional gate representing the cell population in a one dimensional graphical presentation of side scatter light (SSC) detected at right angle to the exciting laser beam, that correlates with the internal complexity or granularity of cells (see Fig 4.1). This was set to include, as far as possible, only spermatozoa characterised by a twin peaked profile because their flat heads are highly reflective: the reflected light is very bright if the sperm are oriented at 45° to the laser beam. RN3 was a regional gate set in the green fluorescence spectrum that identified sperm population fluorescing green with low mitochondrial activity in channel FL1. RN4 was a regional gate set in the red fluorescence spectrum, representing those dead sperm population fluorescing red with PI in channel FL3, while RN5 was a regional gate placed in the greenish orange fluorescence spectrum that identified the sperm population fluorescing greenish orange with high mitochondrial activity in channel FL2. G10 selected the live sperm population with low and high $\Delta\Psi_m$ with a normal cell membrane but no membrane damaged, PI labelled dead sperm: G5 selected the sperm population with low and high $\Delta\Psi_m$ with either normal or damaged cell membrane. Such gates were set and a link made between RN1 and RN2 because few particles that were not spermatozoa would register in both of these gates in FSC and SSC. Thus, non-sperm specific materials were largely gated out.

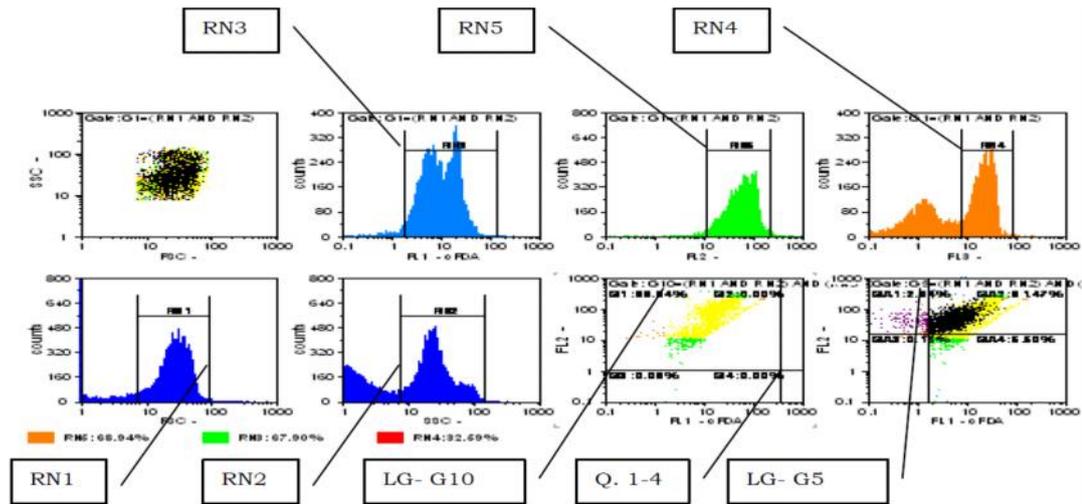


Figure 4.1: Arrangement of regional (RN) gates on different fluorescence histograms and logical (LG) gates on quadrants (Q).

Precision and trueness of regional/logical gates

To confirm the accuracy of the regional and logical gates to exclude correctly any dead cells and non-sperm material, we ran samples labelled with PI only, a combination of JC-1 with PI and PI and SYBR-14 (Invitrogen, Oregon, USA), which was a membrane-permeant nucleic acid stain labelling live sperm with green fluorescence in FL1 channel. For the spermatozoa stained with PI only, 40µl of unstained semen (concentration 12×10^6) was taken and added to 995µl Ruthin extender and 5µl PI was added shortly before flow cytometry. For SYBR-14, 5 µl of the working solution of SYBR-14 was added to the semen sample (40µl of JC-1 stained semen + 995µl Ruthin extender), left for 5 minutes to allow appropriate labelling of live sperm and 5 µl PI was then added shortly before flow cytometry.

Application of colour gates

JC-1 labelled spermatozoa were examined under a fluorescence microscope (Nikon Labophot 2 Microscope), whose mid-pieces exhibited both green and orange fluorescence but to varying degrees. A single orange vs green dot plot, plotted on quadrants Q1-Q4, was used to differentiate orange/green populations. The setting of logical gate G10 was changed to include and exclude the sperm within each regional gate RN3, RN4 and RN5 placed on fluorescence channels FL1, FL3 and FL2 respectively (see table 4.1). The selection of colours for logical gates was identical with the colour of fluorescence stain: green, orange and red represented low (RN3) and high

mitochondrial activity (RN5) and dead sperm (RN4) respectively. In addition those sperm population appearing simultaneously in (RN3 and RN5) or (RN3 and RN4) or (RN4 and RN5) were colour coded as represented below.

- **Yellow:** the population within RN3 and RN5, representing membrane-intact, live sperm labelled with JC-1 that fluoresced green and orange simultaneously: these sperm had heterogeneous mitochondria.
- **Blue:** the population within RN3 and RN4, PI labelled, that fluoresced green: these dead sperm had low $\Delta\Psi_m$.
- **Purple:** the population within RN4 and RN5 stained with PI that fluoresced orange: these dead sperm had high $\Delta\Psi_m$.
- **Black:** for the population within RN3, RN4 and RN5, labelled with PI that fluoresced green and orange simultaneously: these dead sperm had heterogeneous mitochondria.

Table 4.1: Logical gates set on quadrants 1-4.

Gate ID	Gate setting	Description
a	(RN3 AND RN5) NOT RN4	No dead; live sperm fluorescing green and orange simultaneously.
b	(RN3 OR RN5) NOT RN4	No dead; live sperm fluorescing green, orange and ratio of green and orange simultaneously.
c	(RN5) NOT RN4	No dead; live sperm fluorescing orange and ratio of sperm fluorescing green and orange simultaneously.
d	(RN3) NOT RN4	No dead; live sperm fluorescing green and ratio of green and orange simultaneously.
e	(RN3 OR RN4) NOT RN5	Dead and live sperm fluorescing green excluding orange.
f	(RN3 AND RN4) NOT RN5	Only dead sperm fluorescing green excluding orange.
g	(RN4 OR RN5) NOT RN3	Dead and live sperm fluorescing orange excluding green.
h	(RN4 AND RN5) NOT RN3	Only dead sperm fluorescing orange excluding green.

Auto-fluorescence

Auto-fluorescence in pre- and post-centrifuged fresh and frozen-thawed semen samples was determined by staining sperm with and without JC-1 and PI in Ruthin or PBS extender. The procedure for centrifuged fresh and frozen-thawed semen was the same as described in 4.2.1 but PBS extender was used instead of Ruthin extender throughout semen processing. A similar protocol of extenders was applied for un-centrifuged fresh and frozen-thawed semen. Fluorophore staining (except samples without JC-1 and PI) and flow cytometry settings were the same as described in 4.2.3 and 4.2.4 for both centrifuged and non-centrifuged semen either in Ruthin or PBS extender.

Incubation times

To evaluate maximum mitochondrial potential for a minimum incubation time, fresh and freeze-thawed samples were incubated at 39°C for 40 and 80 min after staining with JC-1. Preparation and staining method for fresh and freeze/thawed semen was the same as described above in 4.2.1. PBS extender was added instead of Ruthin extender only at the time of sperm washing since Ruthin extender contains sorbitol, a glycolytic substrate found in seminal plasma that we wanted to remove.

4.3. Statistical analysis

The real time data acquisition, analysis, and display was performed using FloMax®, the PC based FCM software. Mean fluorescence intensity was measured in relative fluorescence units (RFU) of green (low $\Delta\Psi_m$) and orange/red (high $\Delta\Psi_m$) appeared in log-x and log-y respectively, and stored in an Excel spreadsheet. After cleaning the data, differences between incubation times within fresh and frozen-thawed semen samples were investigated using a paired t-test in Minitab16 software. Significance of differences was accepted when $p \leq 0.05$.

4.4. Results

Precision and trueness of regional/logical gates

Sperm stained only with PI presented as a unified population in logical gate G5 PI positive; representing plasma membrane-damaged PI stained sperm but no population was identified on logical gate G10 PI negative (see Fig 4.2), suggesting that logical gate G10 was set correctly to eliminate dead cells. When JC-1 was used together with PI, a single population was observed on G10 PI negative showing that G10 selected the membrane-intact sperm stained only with JC-1 (see Fig 4.3). A single population was found in samples treated with JC-1/SYBR-14/PI on logical gate G10, which represented the plasma membrane-intact sperm stained with JC-1 and SYBR-14. Two populations were seen on logical gate G5, membrane-intact JC-1/SYBR-14 and membrane-damaged PI stained sperm (see Fig 4.4). These outcomes confirmed that the settings of the regional and logical gates were correct: dead sperm and non sperm-specific material was taken out.

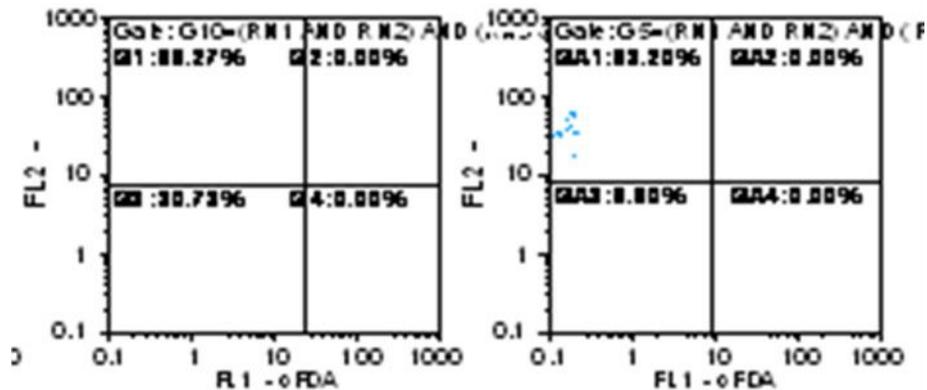


Figure 4.2: Spermatozoa labelled with PI presented a single population on logical gate G5.

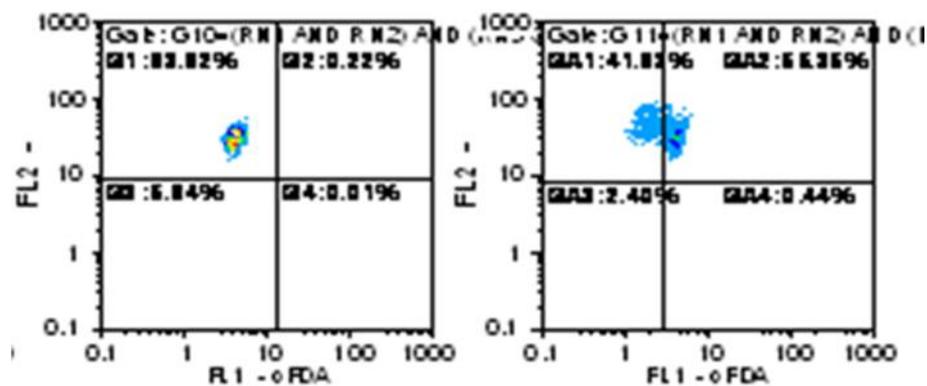


Figure 4.3: Spermatozoa labelled with JC-1 and PI presented unified population on logical gate G10.

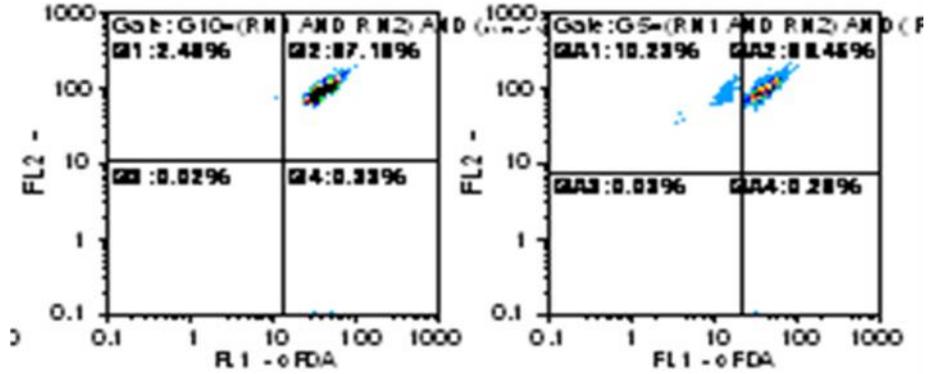


Figure 4.4: Sperm labelled with JC-1, SYBR 14 and PI presented unified population on logical gate G10.

Application of colour gates

Application of colour gating to quadrants Q1-Q4, showed a large proportion of yellow colour but little green and orange on logical gate G10 PI negative (see section 4.2.4); devoid of colours pertaining to dead cells. Within the yellow colour code, representing the sperm population whose mitochondria exhibited low and high $\Delta\Psi_m$ simultaneously, while green and orange colour describing sperms with low $\Delta\Psi_m$ ($l\Delta\Psi_m$) and high $\Delta\Psi_m$ ($h\Delta\Psi_m$) respectively when dead sperm had been excluded (see Fig 4.5). The resultant populations at altered logical gates were presented in Table 4.2.

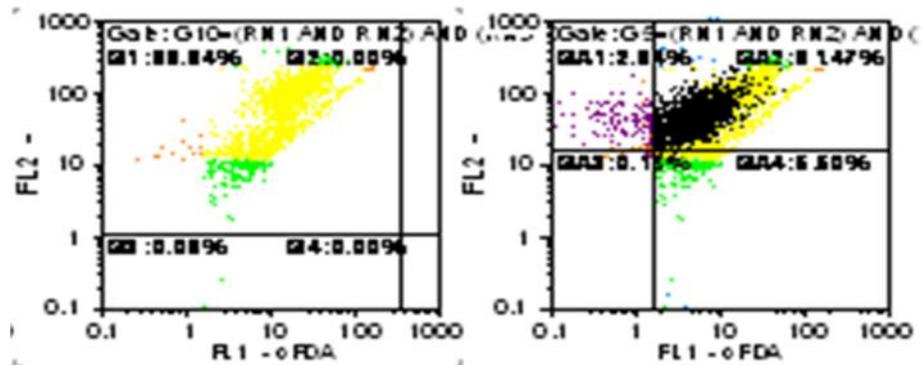


Figure 4.5: Grown populations on quadrants after application of colour gating.

Table 4.2: Outcome of populations at altered logical gates set on quadrant one to four.

Gate ID	Gate setting	Resultant population
A	(RN3 AND RN5) NOT RN4	Single yellow colour population represented sperm with low and high $\Delta\Psi_m$, excluding dead cells.
B	(RN3 OR RN5) NOT RN4	Three colour green, orange and yellow populations corresponded sperm with low, high and low and high $\Delta\Psi_m$ respectively, excluding dead cells.
C	(RN5) NOT RN4	Two colours orange and yellow populations corresponding to sperm with $h\Delta\Psi_m$ and low as well as high $\Delta\Psi_m$ excluding dead cells.
D	(RN3) NOT RN4	Two colours green and yellow populations corresponding sperm with $l\Delta\Psi_m$ and low as well as high $\Delta\Psi_m$ excluding dead cells.
E	(RN3 OR RN4) NOT RN5	Two colours green and blue populations corresponding with $l\Delta\Psi_m$ and dead sperm with $l\Delta\Psi_m$.
F	(RN3 AND RN4) NOT RN5	Single colour blue population representing dead sperm with $l\Delta\Psi_m$.
G	(RN4 OR RN5) NOT RN3	Two colour orange and purple populations corresponding to sperm with $h\Delta\Psi_m$ and dead sperm with $h\Delta\Psi_m$.
H	(RN4 AND RN5) NOT RN3	Single colour purple population representing dead sperm with $h\Delta\Psi_m$

Auto-fluorescence

Pre- and post-centrifuged fresh and frozen-thawed sperm placed either in PBS or Ruthin extender and labelled with JC-1 and PI presented as a unified population (see Fig 4.6 and 4.7, Appendix 4.1-4.6) and represented plasma membrane-intact sperm. No population was identified in un-stained samples with JC-1/PI (see Fig 4.8 and 4.9, Appendix 4.7-4.8) in pre- and post-centrifuged fresh and frozen-thawed semen containing either PBS or Ruthin extender. Sperm mitochondria did not exhibit any auto-fluorescence and appearance of populations in JC/PI samples was the only true JC-1 stained sperm population.

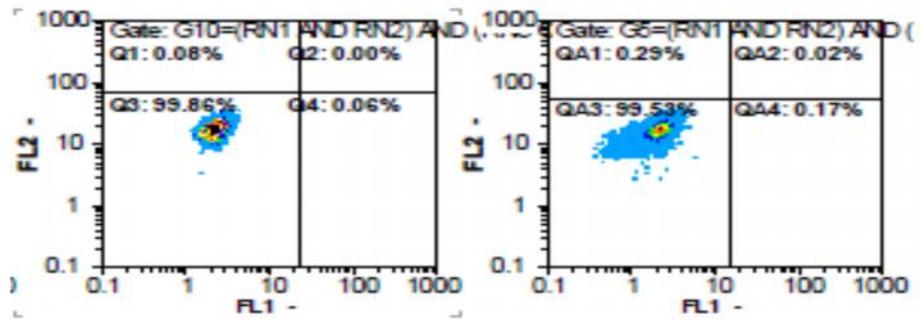


Figure 4.6: Fresh spermatozoa washed in PBS extender and labelled with JC-1 and PI.

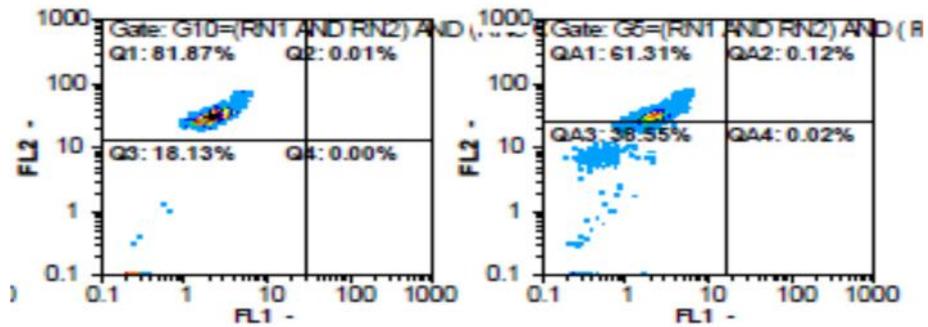


Figure 4.7: Frozen-thawed spermatozoa washed in PBS extender and labelled with JC-1 and PI.

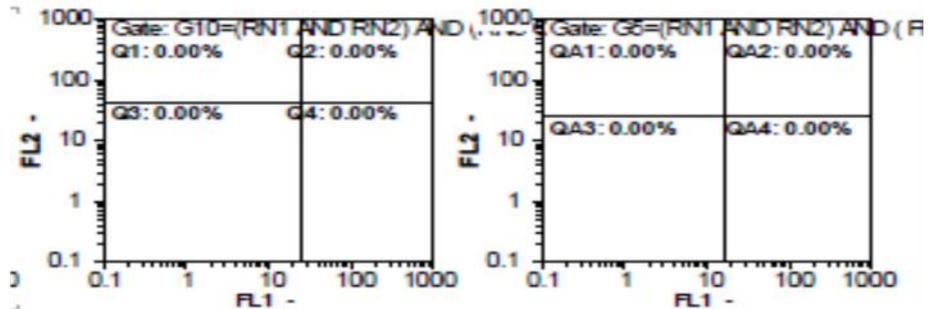


Figure 4.8: Fresh spermatozoa washed in PBS extender, unlabelled with JC-1 and PI.

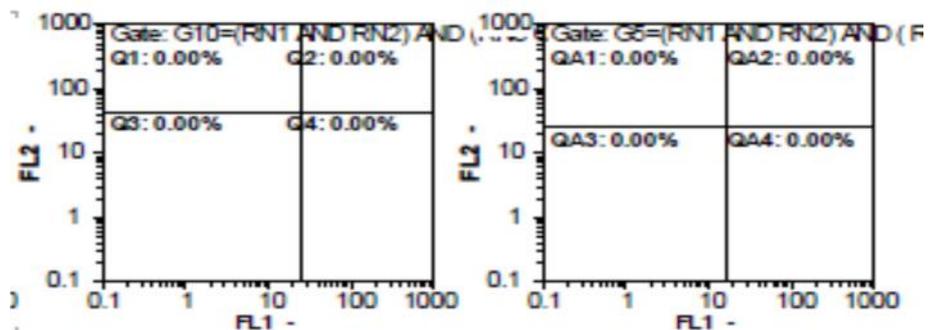


Figure 4.9: Frozen-thawed spermatozoa washed in PBS extender, unlabelled with JC-1 and PI.

Comparison of incubation times

Significant differences were observed in low and high $\Delta\Psi_m$ and high:low $\Delta\Psi_m$ ratio between 40 and 80 min incubation times in both fresh and frozen-thawed semen: this was not the case for high $\Delta\Psi_m$ in fresh and low $\Delta\Psi_m$ in frozen-thawed semen. Maximum high $\Delta\Psi_m$ and high:low $\Delta\Psi_m$ ratio were achieved after 40 min incubation time (see Table 4.3). After 80 min, the shift in proportion of the sperm moving from high to low $\Delta\Psi_m$ and *vice versa* was around 15%, the result of loss of membrane integrity or substrate of appropriate substrate.

Table 4.3: Mean Low and high mitochondrial membrane potential ($\Delta\Psi_m$) (\pm SD) and high:low $\Delta\Psi_m$ ratio of fresh and frozen-thawed semen after 40 and 80 minutes incubation time. (n=12).

Semen type	$\Delta\Psi_m$	Incubation time		t-value	Significance
		40 min	80 min		
Fresh	Low	11.4 \pm 4.3	13.9 \pm 6.1	-4.0	p \leq 0.01
	High	194.3 \pm 114.0	168.1 \pm 82.7	1.6	ns
	High:low ratio	16.3 \pm 5.7	13.0 \pm 5.7	2.5	p \leq 0.05
Frozen-thawed	Low	17.0 \pm 5.2	19.2 \pm 7.4	-1.48	ns
	High	248.6 \pm 108.6	175.1 \pm 71.0	5.5	p \leq 0.001
	High:low ratio	14.1 \pm 5.4	9.3 \pm 3.9	4.0	p \leq 0.01

4.5. Discussion

This is the first time that the relationship between maximum sperm mitochondrial membrane potential being expressed and duration of incubation time has been investigated. These results suggest this is achieved around 40 min incubation time for both fresh and frozen-thawed semen. It was apparent that as the incubation time was extended to 80 min, the fluorescence intensity in the green spectrum, representing low $\Delta\Psi_m$ increased whereas orange fluorescence representing high $\Delta\Psi_m$ and the ratio of high:low $\Delta\Psi_m$ decreased (figure 4.10). The increase in sperm with low $\Delta\Psi_m$ may be due to a shift from high to low $\Delta\Psi_m$ in sperm incubated at 80 min. Shift from high to low $\Delta\Psi_m$ has been reported by Thomas *et al.*, (1998) and Garner and Thomas (1999) in frozen-thawed bull sperm because of cryodamage to sperm mitochondria. At 40 min incubation the percentage of motile sperm is higher compared to 80 min incubation because there is the strong evidence that sperm high $\Delta\Psi_m$ is also

associated with the percentage of motile sperm in a sample (Garner and Thomas (1999), otherwise, there is equal opportunity that sperm might lose their organelle integrity as incubation time increases. Supply of metabolic substrate for a long period might be another concern related to mitochondrial function. After washing, sperm were diluted in Ruthin extender providing sorbitol as glycolytic substrate, which is reduced to fructose by the polyol pathway and fructose further reduced into pyruvate, providing respiratory substrate to fulfil the demand of sperm mitochondria. The reduction in $\Delta\Psi_m$ at 80 min incubation might be due to sperm having utilized all the respiratory substrate pyruvate as a marked reduction in seminal pyruvic acid has been observed after 60 min (Marden, 1961).

Flow cytometric dot plots of analyses for fresh and frozen-thawed spermatozoa incubated with JC-1 showed a single cell population of membrane intact spermatozoa, each displaying both green representing low $\Delta\Psi_m$ and orange fluorescence representing high $\Delta\Psi_m$ but to varying degrees. Two distinct populations have been observed in metabolically active ram (Martinez-Pastor *et al.*, 2004) and bovine (Garner *et al.*, 1997; Thomas *et al.*, 1998; Garner *et al.*, 1999) spermatozoa stained with JC-1 that appeared as populations of both green monomers and red-orange aggregates. When stallion spermatozoa separated by single-layer centrifugation using Androcoll-ETM were incubated with JC-1 for 40 min, they displayed three populations (Garcia *et al.*, 2009). Those fluorescing orange represented high $\Delta\Psi_m$, green fluorescence low $\Delta\Psi_m$, and both orange and green fluorescence heterogeneous mitochondria, with high and low $\Delta\Psi_m$. These heterogeneous mitochondria may represent dead sperm or non-sperm specific particles and are a significant confounding factor for interpretation of numbers of reactive cells in an ejaculate that display more than one staining characteristics. Cellular auto fluorescence is a common problem in interpreting data obtained by flow cytometry, interfering with detection of low level fluorescence (Mosiman *et al.*, (1997) and may be possible display more than one population. In this study, JC-1 and PI unstained spermatozoa samples were examined under flow cytometry, revealed no dot plot on the quadrants. This indicates that fluorescence developing in these labelled samples were true fluorescence signals without auto fluorescence. This study confirmed that a unified population was

present, and that setting of regional and logical gates effectively eliminated dead cells, seminal contents, egg-yolk particles and debris.

4.6. Conclusions

Maximum mitochondrial potential was found at 40 min incubation in fresh and frozen-thawed semen, suggesting that this incubation time is satisfactory for assessing mitochondrial membrane potential in similar research work in the future. Washing with PBS had no undesirable effect on sperm mitochondrial activity. Setting of regional and logical gates effectively eliminated dead spermatozoa, cells in seminal fluid, egg-yolk particles and other debris, indicating that this flow cytometric method determines adequately mitochondrial membrane potential using JC-1. It has a good precision and is satisfactory and suitable for further similar studies.

Chapter 5

Effects of some respiratory and glycolytic inhibitors on mitochondrial functionality in bovine semen

5.1. Introduction

Cryopreservation of bovine semen alters the integrity and functionality of some sperm through damage to the plasma and acrosomal membranes and associated disruption of mitochondrial function (Hammerstedt *et al.*, 1990; Parks and Graham 1992). Consequently, the percentage of fully functional sperm with intact cellular and organelle membranes is lowered after the freeze-thaw process (Holt, 1997). When sperm are frozen, ice crystals form in the extra-cellular medium that increases the osmolarity of the unfrozen solution. As intra-cellular water diffuses out of them, in response to this change in osmotic gradient, the cell and plasma membranes become dehydrated (Mazur, 1984). At thawing, this phenomenon is reversed as the extra-cellular ice crystals melt and water re-enters the sperm. The ionic permeability and enzyme activity within the plasma membrane are disrupted creating lipid phase transitions at around 17-36°C. (Holt and North, 1984; Drobnis *et al.*, 1993; Holt, 2000). Working with boar semen, Guthrie *et al.*, (2008) increased the extracellular extender osmolarity from 300 to 600mOsm/kg, and found a 50% reduction in mitochondrial membrane potential. After the freeze/thaw cycle had been completed, Bilodeau *et al.*, (2000) found that sperm glutathione (GSH) concentration was reduced by 78% and superoxide dismutase activity by 50%, suggesting that oxidative stress had occurred. Premature ageing and capacitation of sperm have been associated with acrosomal and/or mitochondrial membrane integrity that lowers ATP synthesis and reduces motility (Lindemann *et al.*, 1982).

Mitochondrial respiratory activity has been investigated *in vitro* using drugs that uncouple oxidative phosphorylation (OXPHOS), either by inhibiting the electron transport chain (ETC) or blocking the essential link between the respiratory pathways and phosphorylation. One such drug, carbonyl cyanide m-chlorophenyl hydrazone, markedly reduced mitochondrial

membrane potential ($\Delta\Psi_m$) in both mouse and boar spermatozoa (Mukai and Okuno, 2004; Guthrie *et al.*, 2008). A similar reduction has been observed in human sperm treated with either potassium cyanide that blocks cytochrome C oxidase within complex IV of the ETC or p-trifluoromethoxy carbonyl cyanide phenylhydrazone and valinomycin that both uncouple OXPHOS (Amaral and Ramalho-Santos, 2010).

ATP synthesis pathways may be investigated in sperm using a number of specific enzyme inhibitors. One example is 2-deoxy-D-glucose (DOG), a competitive inhibitor of glycolysis that targets hexokinase within the reaction glucose \rightarrow glucose 6-phosphate by competing with glucose during phosphorylation: the resulting product is DOG-6-phosphate which cannot be metabolized further (Appendix-5.1) (Hiipakka and Hammerstedt, 1978; Pasupuleti, 2007). When bovine sperm are re-suspended in a medium containing DOG and pyruvate and incubated at 37°C for 10 minutes, at least 70% of sperm remain motile, demonstrating that they maintain motility utilizing mitochondrial ATP without employing the glycolytic pathway at this level (Krzyzosiak *et al.*, 1999). By comparison, mouse sperm incubated similarly show a significant reduction in their motility, even in the presence of pyruvate and lactate (Mukai and Okuno, 2004; Pasupuleti, 2007): this suggests that glycolytic ATP plays a crucial role in murine sperm motility, or the decrease in motility might be due to utilization of mitochondrial ATP by DOG when phosphorylated with hexokinase. Investigating mitochondrial membrane potential labelled with JC-1, Mukai and Okuno (2004) found no change in $\Delta\Psi_m$ of mouse sperm when incubated with DOG and either glucose or pyruvate, suggesting that DOG had no effect on mitochondrial respiration.

Another glycolytic inhibitor is iodoacetamide (IAM) which acts on the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Appendix-5.1) by binding irreversibly to cysteine residues thereby inactivating the enzyme (Pasupuleti, 2007). IAM can deprive rat astrocytes of glutathione (GSH), inhibit cellular GAPDH activity, and reduce cell lactate synthesis. It can be responsible for the death of cell after a long incubation period (Schmidt and Dringen, 2009): this suggests that IAM should not be used to inactivate GAPDH and interrupt glycolysis since it quickly lowers the GSH content of cultured cells. IAM has no effect on sperm ATP concentrations

and motility if oxidizable substrate is provided but it does lower ATP concentrations and motility in the presence of glucose. It appears that IAM has no effect on mitochondrial ATP that sperm utilize to maintain their motility, but it interrupts glycolysis that reduces the supply of respiratory substrate to mitochondria thereby decreasing ATP synthesis and motility (Pasupuleti, 2007).

Another group of drugs are the K^+ ionophores one of which is valinomycin (VAL), a dode-cadepsipeptide that induces mitochondrial swelling especially in the presence of acetate (Harris, *et al.*, 1966) through an increased uptake of K^+ . This inhibits the ETC in mitochondria with a loss of inner membrane potential, oxidation of pyridine nucleotides, and induction of apoptosis (Saris *et al.*, 2009). In nature, dode-cadepsipeptide are found as a cellular toxins produced by several *Streptomyces* species of bacteria and have a similar effect on mitochondrial activity as VAL (Andersson, *et al.*, 1998). VAL is a passive carrier for K^+ , binding or releasing K^+ when it encounters the membrane surface: it transports these ions across cell membranes, thereby inhibiting oxidative phosphorylation by antagonizing the proton motive force. Whereas the molecular internal structure of the VAL- K^+ complex is polar, the surface of the complex is hydrophobic, and this allows VAL to enter the lipid core of the membrane bilayer and solubilize K^+ within this hydrophobic milieu (Ronald, 1978; Bekker and Allen 2010).

This study investigated the effect of freeze-thawing on sperm mitochondrial function using JC-1 after blocking glycolysis at several levels in its metabolic pathways. By using glycolytic inhibitors, this will force sperm to derive their energy from oxidative phosphorylation of pyruvate. To obtain a true measure of mitochondrial activity, sperm will be incubated with respiratory inhibitor to give a base fluorescence value that can be subtracted from, or compared with, a control sample.

5.2. Materials and methods.

Preparation of fresh and frozen/thawed semen

Ejaculates were collected from four bulls through an AV, as described in Chapter 2.1. After dilution with the same volume of EYTG, the semen concentration was $400-800 \times 10^6$ /ml. Then, PBS was added to an aliquot of 1-2 ml of this raw semen to give a volume of 10ml. The suspension was centrifuged at 600g for 10 minutes at room temperature, the supernatant removed, and the resulting pellet placed in a fresh test tube at 39°C. PBS extender was added to the pellet to give a new suspension volume of 2ml and the resulting sperm concentration estimated using a nucleocounter.

Thirty straws of frozen bull semen, each containing 0.25ml, were received in liquid nitrogen and thawed by immersing them in a water bath at 39°C for 30 secs. The contents of each straw were transferred into a centrifuge tube in water bath at 39°C and thereafter processed as for the fresh semen above.

Semen concentration related to different reagents

The concentration of sperm within this 2ml varied from sample to sample. A variable volume containing 24×10^6 sperm was now removed. For all the controls and to investigate the respiratory inhibitor, a variable volume of Ruthin extender was added to give a 1 ml suspension: for the glycolytic inhibitors, PBS was used instead. From this 1ml suspension, 250 μ l was removed to give a final concentration of 6×10^6 for each different treatment, in duplicate (Fig 5.1).

Addition of reagents and control

Now, 250 μ l of either Ruthin extender used for the control and VAL, or DOG and IAM reagents alone was added to the samples (Fig 5.1) that were labelled as control (C1, C2), valinomycin (VAL1, VAL2), 2-deoxy-D-glucose (DOG1, DOG2) and iodoacetamide (IAM1, IAM2) for each treatment respectively. They were rested for 15 minutes before staining.

For investigating VAL, the treatment was added after adding JC-1 (see next paragraph). For the control and VAL treatments sorbitol provided the

glycolytic substrate, and for DOG and IAM pyruvic acid was the respiratory substrate (see Chapter 2.5).

Fluorescence staining of sperm and flow cytometry

From the 500µl semen suspensions, 360 µl was placed in a fresh test tube. At an interval of 2 minutes, 40µl of JC-1 was added to the sample under investigation that coincided with the running time for the flow cytometer to measure mitochondrial membrane potential within the previous samples (Fig 5.1). Only for the VAL treatment, 4 µl of VAL was added at this stage. Each sample was incubated at 39°C for 40 min and 40 µl removed, added to 995 µl of warmed Ruthin/PBS extender as per treatment that was already present in flow tubes, together with 5 µl PI: the final concentrations of JC-I and PI were 0.1µM and 2.4mM respectively.

Samples were analyzed on a Partec Cyflow Space Flow Cytometer (Partec GmbH, Gorlitz, Germany). Regional and logical gates were set to select live sperm only, and exclude non-sperm specific events and dead cells (see Chapter 4).

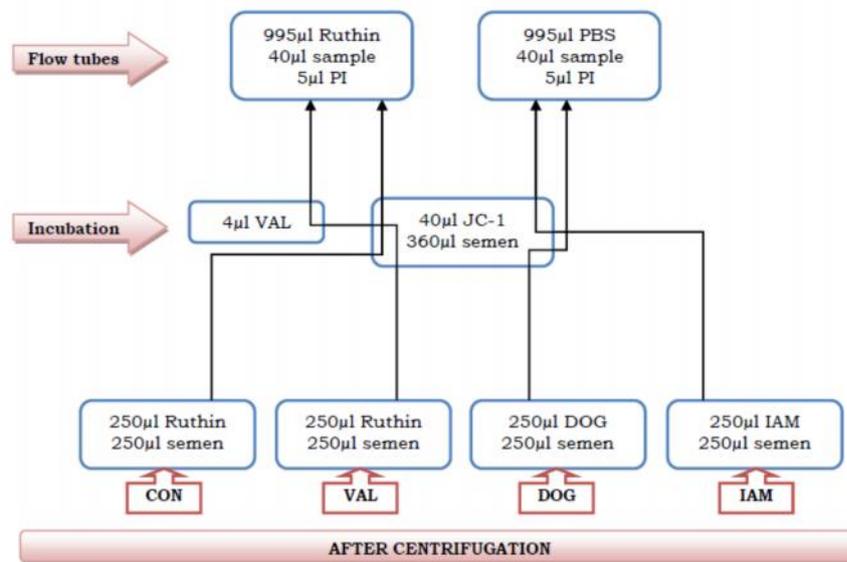


Figure 5.1: Flow chart for JC-I and PI protocol after treatment with control (CON), valinomycin (VAL), 2-deoxy-D-glucose (DOG) and iodoacetamide (IAM).

5.3. Statistical analysis

The real time data acquisition, analysis, and display was performed using FloMax®, the PC based FCM software. Mean fluorescence intensity was measured in relative fluorescence units (RFU) of green (low $\Delta\Psi_m$) and orange/red (high $\Delta\Psi_m$), the output appearing as log transformed data in the x and y axes respectively, and stored in an Excel spreadsheet. After cleaning the data, differences between fresh and frozen-thawed semen samples and bulls, and the control and treatments were investigated using paired t-test and ANOVA, applying Tukey and Dunnett's comparison test using a general linear models procedure in Minitab16 software. Significance of differences was accepted when $p \leq 0.05$.

5.4. Results

Fig 5.2 showed that there were only live sperm contributing to the flowcytometry output regarding sperm with high, low or both high and low $\Delta\Psi_m$. The $\Delta\Psi_m$ results appeared in the upper quadrant of the computer dotplot output because sperm exhibited combination of high and low $\Delta\Psi_m$ but to a varying degrees, at the same time with few sperm presenting high or low $\Delta\Psi_m$ only, this was common output for all three treatments as well as the control. The sperm with varying $\Delta\Psi_m$ were difficult to separate. Therefore, high:low $\Delta\Psi_m$ ratios were estimated to distinguish sperm with low and high $\Delta\Psi_m$ in fresh and frozen-thawed semen.

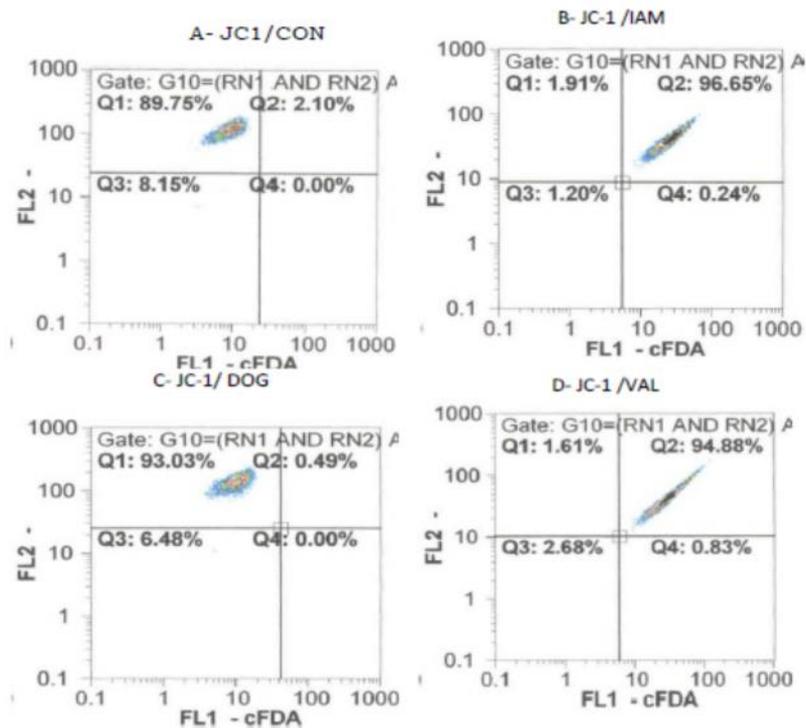


Figure 5.2: Dot plots resulting from flow cytometric analyses of spermatozoa stained with JC-1. Dot plot of sperm population with highly functional mitochondria displayed oval outline (A, C) and elongated in less functional mitochondria (B, D). A) population in presence of glycolytic substrate. B) population treated with IAM in presence of respiratory substrate. C) population treated with DOG in presence of respiratory substrate. D) population treated with VAL in presence of glycolytic substrate.

Comparison of control (CON) with treatment

To compare $\Delta\Psi_m$ of untreated spermatozoa in a medium supporting glycolysis (CON) with those treated after suspending their anaerobic (DOG, IAM) and aerobic (VAL) metabolic pathways, mean green fluorescence intensity (MFI) representing low $\Delta\Psi_m$ was significantly higher in IAM and VAL in fresh, and DOG and VAL in frozen-thawed spermatozoa as compared to the control (Fig 5.3a & 5.4a). In contrast orange MFI representing high $\Delta\Psi_m$ was significantly higher in controls compared to IAM for fresh, and including also VAL in frozen-thawed semen (Fig 5.3a & 5.4a). Shift in $\Delta\Psi_m$ from orange to green has been seen in samples treated with VAL and IAM. The high:low $\Delta\Psi_m$ ratio was higher in control and DOG treated samples representing more active mitochondria, while in samples treated with VAL and IAM the high:low $\Delta\Psi_m$ values were reversed to distinctly lower representing less active mitochondria at both incubation times in fresh and FT semen (Fig 5.3b & 5.4b). High:low $\Delta\Psi_m$ ratio was significantly higher in control than other treatments in both semen. To get

a true measure of JC-1, after subtracting the high:low $\Delta\Psi_m$ of VAL from the high:low $\Delta\Psi_m$ of control (data not presented), it was revealed that even though VAL causes a significant reduction in sperm mitochondrial activity, this has not been abolished completely.

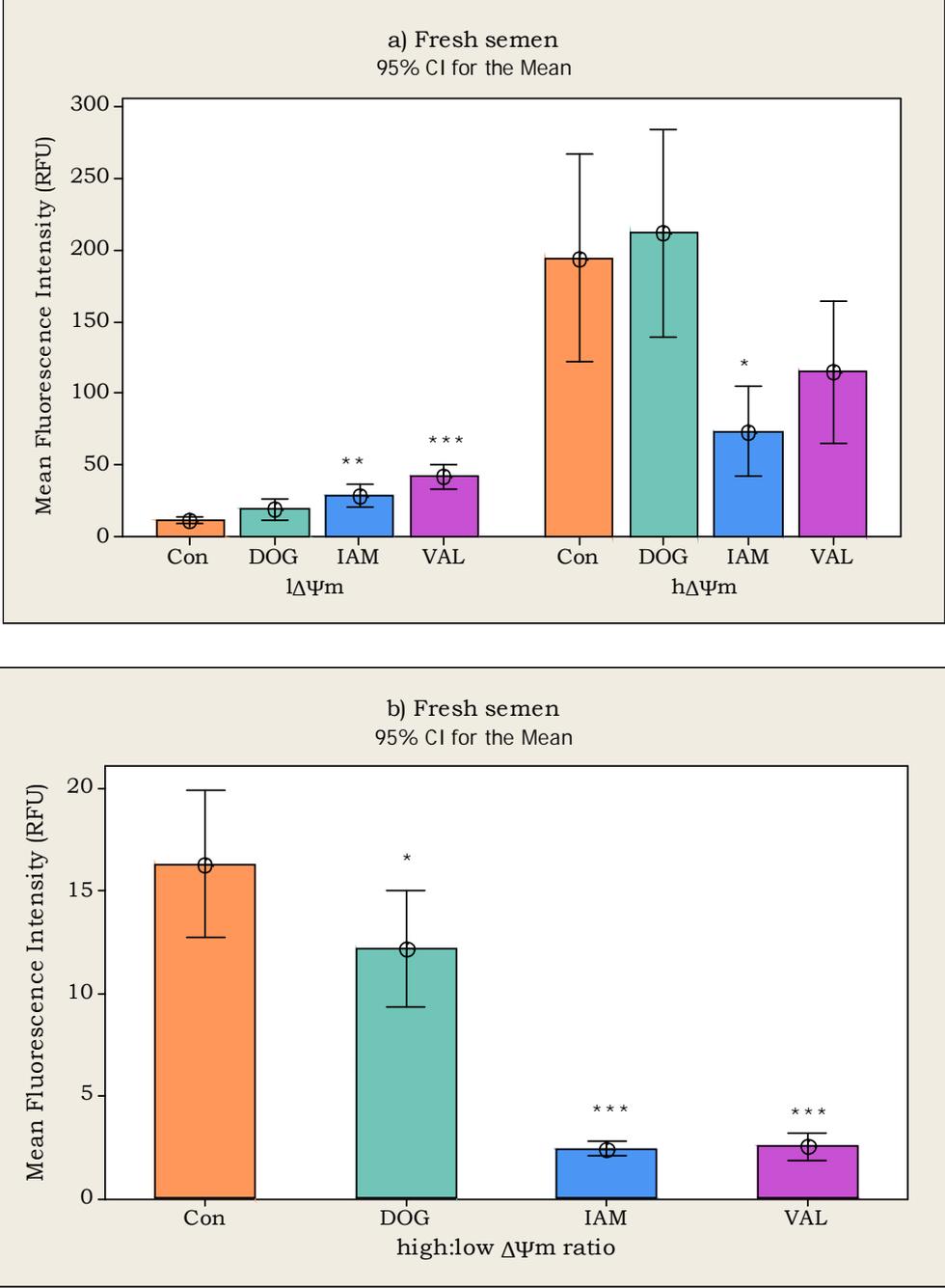


Figure 5.3 (a & b): Comparison of low and high mitochondrial membrane potential ($l\Delta\Psi_m$ and $h\Delta\Psi_m$) and high:low $\Delta\Psi_m$ ratio between controls and glycolytic and respiratory inhibitors for 12 semen samples. Bars with the superscripts are significantly different to control; * $p < 0.05$ and *** $p < 0.001$.

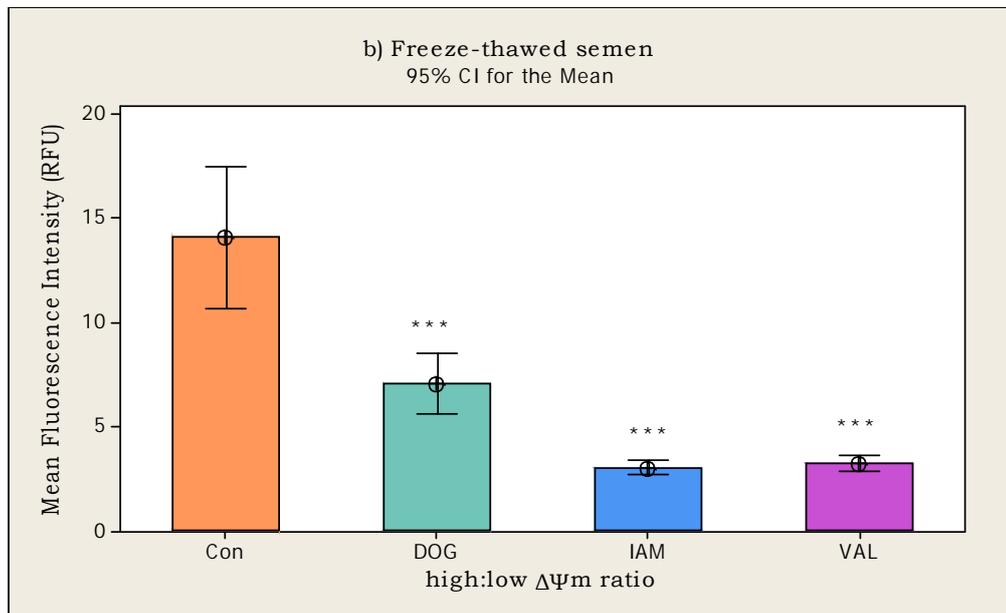
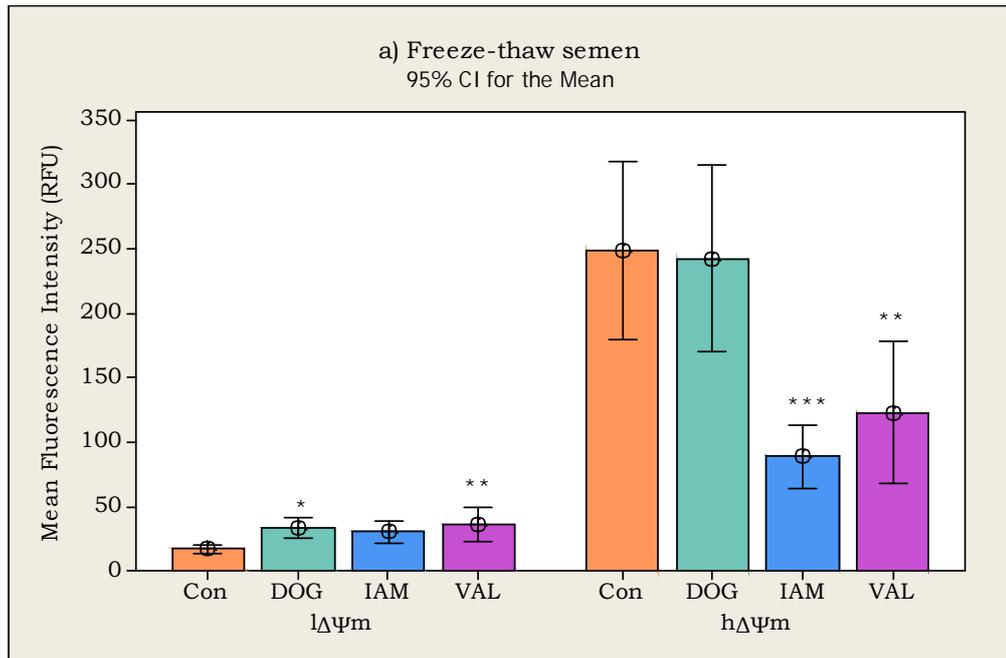


Figure 5.4 (a & b): Comparison of low and high mitochondrial membrane potential ($l\Delta\Psi_m$ and $h\Delta\Psi_m$) and high:low $\Delta\Psi_m$ ratio between controls and glycolytic and respiratory inhibitors for 12 semen samples. Bars with the superscripts are significantly different to control; *** $p < 0.001$.

Comparison between fresh and frozen-thawed semen.

The effect of cryopreservation on the ratio of high:low $\Delta\Psi_m$ in identical samples of sperm before and after cryopreservation in the presence of glycolysis supported medium (CON), glycolysis (DOG, IAM) and respiratory (VAL) inhibitors stained with JC-1 observed variable results (Table 5.1). Freeze/thawing resulted up to 15% decrease in high:low $\Delta\Psi_m$ of untreated sperm compared to fresh semen. In DOG incubated sperm this ratio fell by 40% in freeze-thaw compared to fresh semen. (Table 5.1). Freeze-thaw sperm had higher ratio than fresh when incubated with VAL (Table 5.1). Interestingly, similar change in high:low $\Delta\Psi_m$ close to VAL observed with glycolysis intermediate inhibitor IAM (Table 5.1). This variation in high:low $\Delta\Psi_m$ likely to be the IAM effect on mitochondria or freeze/thaw process that changed mitochondrial status, which lessen the JC-1 accumulation in sperm mitochondria, increase green fluorescence and ultimately reduced high:low $\Delta\Psi_m$ ratio.

Table 5.1: Comparison of high:low $\Delta\Psi_m$ ratio between fresh (FR) and freeze-thaw (FT) sperm. (n=12).

Treatment	Semen	Mean (\pmSD)	t-value	Significance
Control	FR	16.3 \pm 5.7	1.2	p \leq 0.05
	FT	14.1 \pm 5.4		
DOG	FR	12.2 \pm 4.5	5.9	p \leq 0.001
	FT	7.1 \pm 2.3		
IAM	FR	2.4 \pm 0.6	-2.3	p \leq 0.05
	FT	3.0 \pm 0.6		
VAL	FR	2.5 \pm 1.0	-2.9	p \leq 0.01
	FT	3.2 \pm 0.6		

5.5. Discussion

This study has investigated bovine sperm mitochondrial function following inhibition of specific glycolytic pathways in the presence of respiratory substrate for fresh and frozen-thawed semen. Using cytometry dot plots were made of analyses of washed fresh and frozen-thawed spermatozoa incubated with JC-1 and various treatments as stated in materials and methods. Each dot plot of treatments showed a single cell population of membrane intact spermatozoa and each population displayed both green

and orange fluorescence but to varying degrees. The orange and green populations in dot plots were difficult to separate. Therefore examination of orange:green fluorescence (high:low $\Delta\Psi_m$) ratio was performed to integrate green and orange fluorescence value in fresh and post-thaw semen. Therefore a profile of metabolically active sperm could be constructed for a sperm sample, whether fresh or freeze/thawed. This allowed comparison between fresh and freeze/thaw semen and between different bulls to be made. Also it enabled differences in the mitochondrial function between fresh and freeze/thawed to be investigated.

Bovine spermatozoa incubated with JC-1 exhibited significant differences between treatments at an outcome variable. An increase in the population with active mitochondria has been observed in samples either provided with only glycolytic substrate (sorbitol) or with glycolytic inhibitor (DOG) and pyruvate. Similar results have been observed by Mukai and Okuno, (2004) when mouse sperm were incubated with JC-1 in presence of either glucose or DOG and pyruvate. Consistency in $\Delta\Psi_m$ between treatments Control and DOG might be due to presence of sorbitol in the former treatment, which is reduced to fructose by the polyol pathway and fructose further reduced into pyruvate/lactate, providing respiratory substrate to sperm like the DOG treated sample. In addition the $\Delta\Psi_m$ slightly higher in DOG incubated sperm that may be due to inhibition of glycolysis which in turn may cause the sperm to force mitochondria to work at a higher level to generate energy.

Addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) reduced the mitochondrial inner-membrane potential as an uncoupler demonstrated as green emission from mitochondria (Mukai and Okuno, 2004). Change in state of JC-1 from aggregates to monomers has been observed in human sperm (Troiano *et al.*, 1998) and stallion sperm (Love *et al.*, 2003), using VAL to validate the JC-1 probe. In the present study, VAL reduced $\Delta\Psi_m$ in bull spermatozoa resulting in a shift in fluorescence from orange (high $\Delta\Psi_m$) to green (low $\Delta\Psi_m$). When subtracting the aggregate of valinomycin from control, the true value for mitochondrial potential was lower as compared to control (data not shown) indicating that valinomycin has not completely abolished mitochondrial activity and or the presence of aggregates fluorescence could be background fluorescence of JC-1 due to

the sensitivity of the fluorescence detectors; it is unlikely that sperm populations would ever show zero fluorescence. Increase in VAL concentration may help to differentiate the mitochondrial and fluorochrome activity.

A concentration of 1mM of IAM effectively inhibited the oxidation of pyruvate in rat heart mitochondria (Yang, 1957). Later, Hathaway (1963) observed that IAM completely reduced respiration in sea urchin spermatozoa within 45min. IAM, an inhibitor of GAPDH, did not reduce mouse sperm motility in presence of pyruvate and lactate but inhibited ATP produced from glycolysis with no effect on ATP produced by mitochondrial respiration (Pasupuleti, 2007). Recently Schmidt and Dringen (2009) found that IAM markedly reduced glutathione (GSH), cellular GAPDH and lowered cellular lactate production in astrocytes suggesting that such a decline in cellular GSH after exposure to IAM may be attributed to GSH alkylation, since IAM is used as alkylating reagents to modify thiol groups. Reduction in GSH level resulted in loss of mitochondrial membrane potential and fertility in mice sperm (Perl *et al.*, 2006). Sperm mitochondrial cysteine-rich protein (SMCP) and phospholipid hydroperoxide glutathione peroxidase are the proteins localized in the mitochondrial capsule and enhances sperm motility (Hawthorne *et al.*, 2006). Like GAPDH, IAM may alter SMCP, GSH level and decrease $\Delta\Psi_m$. SMCP gene knockout in mice sperm reduced sperm motility and fertility (Nayernia *et al.*, 2002). In our present study, IAM disrupted the mitochondrial activity in the presence of pyruvate and also shifted fluorescence from orange (high $\Delta\Psi_m$) to green (low $\Delta\Psi_m$). It reveals that IAM might be involved in the reduction of GSH level, thereby decrease in mitochondrial activity or restricting pyruvate oxidation, therefore depriving mitochondria of respiratory substrate as compared to samples treated with DOG.

The freeze-thaw cycle increases osmotic and oxidative stress, and sperm membrane permeability that decreases sperm viability and mitochondrial membrane potential (Garner and Thomas, 1999; Ortega-Ferrusola *et al.*, 2008). Using JC-1 probe to evaluate $\Delta\Psi_m$ in bovine sperm, Celeghini *et al.*, (2008) found that it was lower in frozen-thawed than fresh semen: when Bioxcell® extender as a cryopreservative 16.9% of sperm had high $\Delta\Psi_m$

compared to 23.9% when Botu-Bov® extender was used, the proportion in fresh semen being 76.8%. Similar results were found by Arruda *et al.*, (2005) in cattle semen after cryopreservation using a Tris-egg-yolk extender and another containing glycerol. Disappearance and significant reduction in high $\Delta\Psi_m$, a shift to low $\Delta\Psi_m$, and similar changes in high:low $\Delta\Psi_m$ ratio were recorded earlier by Thomas *et al.*, (1998) and Garner and Thomas, (1999), showing that some bulls have lower cryopreservation tolerance than others. In this study, washed frozen-thawed spermatozoa diluted in Ruthin extender providing sorbitol as glycolytic substrate showed significant decrease in high:low $\Delta\Psi_m$ ratio than that of the fresh spermatozoa. Changes in sperm membrane occur during the freezing process, similar to those that occur during capacitation that may further damage the mitochondria themselves (Watson, 1995a; Nagy *et al.*, 2003). Oxidative stress is another factor that influences adversely sperm mitochondrial function during freeze-thaw process (Bilodeau *et al.*, 2000): motility, mitochondrial membrane potential, and sperm viability are most likely interrelated (Garner *et al.*, 1997). Therefore reduction in the high:low $\Delta\Psi_m$ ratio in frozen-thawed sperm may be induced by oxidative stress associated with the freeze-thaw cycle.

5.6. Conclusions

Mitochondrial function was significantly reduced by the freeze-thaw process and in spermatozoa treated with the glycolytic inhibitor IAM and the respiratory inhibitor VAL. The physiological significance of these results may be investigated by other means: estimating the oxygen consumption of sperm incubated with these same inhibitors, the ATP production of sperm, or their motility investigated in Chapter 7.

Chapter 6

Oxygen metabolism and its relationship to sperm mitochondrial membrane potential

6.1. Introduction

Generation of adenosine tri-phosphate (ATP) in sperm mitochondria is through oxidative phosphorylation, provided that appropriate substrate is available. Oxygen is essential for this process, and thus its consumption is a key marker of sperm metabolic activity (Lopes *et al.*, 2010; Pena *et al.*, 2009). Mitochondria are located in the sperm mid-piece and are the predominant pathway for ATP synthesis. For motility it is necessary for ATP to diffuse, or be transported, to the distal end of flagellum via the axoneme to the dynein ATPases that creates the flagellar motion by sliding of the doublet microtubules (Schoff *et al.*, 1989) which helps detachment of sperm from oviductal epithelium and positioning at the site of fertilization (Ho and Suarez, 2001). Approximately 75% of ATP produced by spermatozoa is consumed for their motility (Bohnensack and Halangk, 1986).

Metabolic quotients are expressed by the Z notation ($\mu\text{l}/10^8$ spermatozoa/hr) (Redenz, 1933); thus, ZO_2 is μl oxygen consumed / 10^8 spermatozoa/hr. Investigating a human sperm, ZO_2 was inversely associated with sperm concentration in seminal plasma and higher in the artificial medium, regardless of sperm concentration. Oxygen consumption per sperm rose as the sperm concentration in an ejaculate fell below $20 \times 10^6/\text{ml}$ in seminal plasma (Deutch *et al.*, 1985). Similar cell concentration-dependent phenomena have been observed with human leukocytes (Hartman, 1952; Esmann, 1964) and lymphocytes (Hedeskov and Esmann, 1966). Using an oxygen biosensor system, Garrett *et al.*, (2008) found an increase in ZO_2 in frozen-thawed compared with fresh semen, suggesting that this increase could be associated with mitochondrial membrane damage during freeze/thaw process. Alternatively, sperm may become hyperactivated through the capacitation-like effects of freezing/thawing. It is likely that the increase in ZO_2 in freeze/thawed semen might reflect selection of motile sperm sample using Percoll gradient

that consume more oxygen in comparison with membrane-intact sperm isolated using Optiprep gradient centrifugation found in a fresh sample.

During the previous fifty years, a Clarke oxygen electrode has been used to measure oxygen consumption of various tissues and cells, including bovine spermatozoa (Schoff and First, 1995). Cells or tissue are placed in a suitable medium within a sealed incubation chamber, and stirred at high speed to ensure an even distribution of oxygen. Oxygen diffuses through a Teflon membrane separating the medium from the base of the incubation chamber. The oxygen is reduced by a cathode, thereby creating a small current to flow to the anode, which is proportional to the partial pressure of oxygen in the medium. This method has certain disadvantages, such as drift of calibration and due to the high speed at which the medium is stirred the spermatozoa is likely to be subjected to non-physiological forces which may inhibit their motility (Garrett *et al.*, 2008).

More recently an oxygen biosensor system has been developed that allows spermatozoa exhibit normal motility. It comprised a 96-well plate in which an oxygen-sensitive fluorescent compound, tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (II) chloride was embedded in a gas-permeable hydrophobic silicone matrix, permanently attached to the bottom of each well. Using pancreatic islet cells, Wang *et al.*, (2005) found that as the oxygen within each well became depleted, the fluorescence intensity increased in a concentration-dependent manner and decreased as cells died. Garrett *et al.*, (2008) obtained similar results using bovine sperm: fluorescence intensity was inversely proportional to oxygen concentration.

ATP synthesis via oxidative phosphorylation is dependent on the rate of oxygen consumption within sperm mitochondria: changes in oxygen consumption may indicate mitochondrial disruption. The aim of this study was to evaluate the rate of oxygen consumption in samples of live sperm obtained from both fresh and frozen-thawed semen and to compare both types of semen. Also to investigate whether oxygen consumption was correlated to mitochondrial membrane potential and if it differed between bulls for both semen types.

6.2. Materials and methods

First and second ejaculates were obtained from six bulls as either fresh (n=2) or freeze/thaw (n=2) samples: the first freeze/thaw ejaculate of bull four was not examined.

Preparation of raw semen

An aliquot containing 400×10^6 /ml sperm was added to 2ml Ruthin extender. The sperm concentration was adjusted to 12×10^6 /ml by adding Ruthin extender for investigating mitochondrial membrane potential and 100×10^6 /ml for assessing oxygen consumption.

Preparation of frozen-thawed semen

Centrifuge tubes and buckets were warmed and allowed to come to room temperature. Thirty straws of semen were thawed at 39°C and equally divided into two centrifuge tubes at room temperature. Semen was then diluted with 6ml warmed Ruthin extender and centrifuged at $600 \times g$ for 10 minutes. The supernatant was removed and the resulting pellet was transferred to a fresh tube at room temperature. Ruthin extender was added to the pellet up to the level of 1ml and sperm concentration was determined using a Nucleocounter. Sperm concentration was adjusted as described for fresh semen above.

Measurement of mitochondrial membrane potential

From the 1ml fresh and frozen-thawed sperm suspension of concentration 12×10^6 /ml, $360 \mu\text{l}$ was placed in a test tube and transferred to a water bath at 39°C : then $40 \mu\text{l}$ of JC-1 was added, approximately 2 minutes apart to accommodate running time on the flow cytometer and the tube agitated. This was left for incubation for 40min and $40 \mu\text{l}$ of incubated semen was then added to $995 \mu\text{l}$ of warmed Ruthin extender followed by $5 \mu\text{l}$ Propidium Iodide (PI) in a flow cytometer tube.

A Partec Cyflow Space flow cytometer was used for the analysis. Two dot-plots were set: the first was a logical gate using regional gates to select sperm but exclude PI positive cells, defined as $G10 = (\text{RN1 AND RN2}) \text{ AND } (\text{RN3 OR RN5}) \text{ NOT RN4}$, and the other included PI positive cells, defined as

G5= (RN1 AND RN2) AND (RN3 OR RN5). Dot plots were FL1 vs FL2. Gains were set as FL1 316; FL2 550 and FL3 430 (see Chapter 4.2).

Oxygen consumption in live sperm

Oxygen biosensor plate

This method was developed from Garrett and others (2008) with some modifications. A 96-well oxygen biosensor plate was allowed to equilibrate overnight at 39°C then placed in a Confocal Laser Scanning Microscope (LSM, Zeiss, Germany) also set to 39°C. Excitation of fluorescence was performed using an argon ion laser set at 488nm from the base of the plate. Emitted light was detected through a 650nm filter, also from the base of the plate. A blank reading of the plate was taken to allow each well to be referenced against its own initial signal (Fig 6.1). For the zero oxygen control, 310µl pre-warmed fresh 100mM sodium sulphite was added to a separate well in the plate. 210µl of Ruthin extender was added to all 'sample' wells and serial readings was then taken every two minutes for 18 min period. Then 100µl of re-suspended sperm concentrate 10×10^6 were added to the 'sample' wells and the plate was sealed using PCR foil seals. The serial readings were taken every two minutes for sixty minute period.

Sperm live:dead ratio

Previously in Chapter 4, sperm mitochondrial membrane potential was investigated using JC-1 and logical gates G10 and G5 in a flow cytometer. This produced results for live sperm with their cell membrane completely intact (at G10) and sperm with partially intact or damage membranes that were dead (at G5). Thus the live sperm percentage was determined as a proportion of the sperm population ($G10/G5 \times 100$).

Fluorescence intensity collection and normalization and calculation of oxygen concentration and oxygen consumption rate

Relative fluorescence intensity of each well was recorded and saved on a spread sheet. ZO_2 was calculated for each 'sample' well using the method of Timmins and Haq, (2002). Briefly each well's fluorescence value was first normalized by dividing by the fluorescence value of corresponding well's at the ambient condition (blank reading). Normalized relative fluorescence (NRF) = I/I_A , where I indicates fluorescence intensity, and the A subscript

represents 'ambient'. The oxygen concentration was calculated using NRF value by applying equation, $[O_2] = (DR/NRF-1)/K_{sv}$. DR is the dynamic range and will be a constant for a given set of experimental conditions (temperature, wavelengths, etc), which was determined using a positive control sodium sulphite (100mM). The sodium sulphite depletes oxygen at the well to zero, therefore maximum possible fluorescence intensity occurs at zero oxygen (Fig 6.1). DR is the ratio between the maximum fluorescence intensity (I_0) and fluorescence intensity at ambient conditions (I_A) for a given well, or $DR=I_0/I_A$. K_{sv} is the Stern-Volmer constant, which is determined as $K_{sv}=(DR-1)/[O_2]_A$. The $[O_2]_A$ is the ambient oxygen concentration that is determined as $[O_2]_A=(DR/NRF \text{ of blank well}-1)/K_{sv}$.

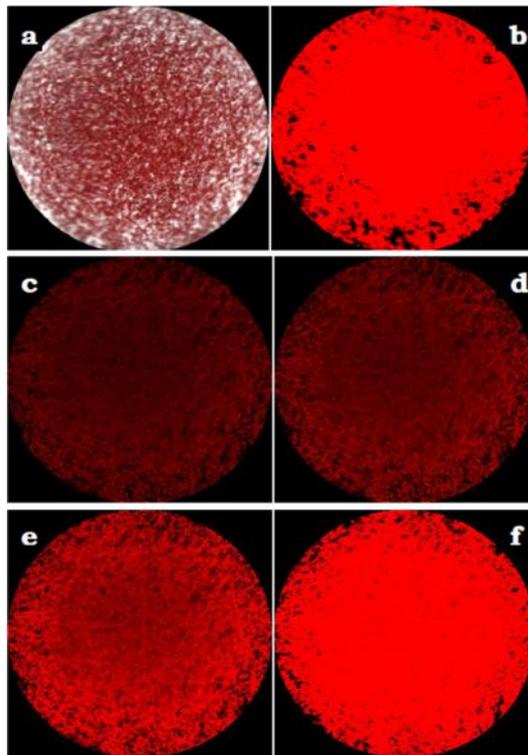


Figure 6.1: Confocal images of OBS wells **a**- blank well, the oxygen-sensitive fluorescent, compound tris (4,7-diphenyl-1,10 phenanthroline) ruthenium (II) chloride is embedded **b**- fluorescence intensity after addition of sodium sulphite in well, **c-f** fluorescence intensity after addition of sperm at 0, 15, 30 and 45 min.

Amplitude of lateral head displacement (ALH)

ALH is the indicative of sperm hyperactive motility and is defined as the mean width of head oscillation as the sperm swim. It was estimated using a Hamilton Thorne (HT) Computer-Assisted Sperm Analyser (CASA) system (Hamilton-Thorne Research, Beverly, USA) with an UV illumination system, described by Budworth *et al.*, (1988) and Holt (1995). This was estimated

for sperm collected at first and second ejaculates from the same bulls that provided sperm for estimating oxygen consumption and mitochondrial membrane potential.

6.3. Statistical analysis

The sperm oxygen consumption was calculated by applying Timmins and Haq, (2002) method as stated above. Comparison between ejaculates from each bull and between bulls and fresh and frozen-thawed semen was investigated using paired t-test and ANOVA, applying Tukey and Dunnett's comparison test using a general linear models procedure in Minitab software. Confidence intervals for the mean oxygen consumption rate were based the standard error of each measurement. Significance of differences was accepted when $p \leq 0.05$.

6.4. Results

Differences in ZO_2 between fresh and frozen-thawed semen

There was an increase of 20% in live sperm and a less significant (10%) increase in mean ZO_2 for fresh compared to frozen-thawed semen (see Table 6.1). ZO_2 was estimated from per live sperm only that has no direct relationship with the proportion of live sperm present in a sample. Therefore the reduction in ZO_2 of live sperm obtained from a freeze-thaw sample is associated specifically with the freeze-thaw process itself through changes in mitochondrial activity.

Table 6.1: Comparison of live sperm (%) and mean ZO_2 between fresh (FR) and frozen-thawed (FT) sperm. (n=33).

Variable	Semen	Mean (\pm SD)	t-value	Significance
Live sperm (%)	FR	62.8 \pm 24.1	2.3	p \leq 0.05
	FT	50.6 \pm 16.7		
ZO_2	FR	12.2 \pm 3.5	1.8	ns
	FT	11.1 \pm 1.4		

Differences in ZO_2 between ejaculates

Differences between 1st and 2nd ejaculates of fresh and frozen/thawed spermatozoa for five bulls are shown in Table 6.2. In fresh semen of bull 2,

there was 40% reduction in ZO₂ between 1st and 2nd ejaculates ($p \leq 0.001$) and a similar 16% reduction for bull 1 that was not significant because of small numbers. By comparison in freeze-thawed semen from bulls 2 and 3 it was the 2nd ejaculate that gave 10-20% higher ZO₂ results, again insignificant because of small numbers.

Table 6.2: Comparison of mean ZO₂ between the first (E1) and second (E2) ejaculates of fresh and frozen-thawed sperm. (n=3).

Bull No.	Ejaculate	Fresh Semen			Freeze-thaw Semen		
		Mean (\pm SD)	t-value	Significance	Mean (\pm SD)	t-value	Significance
BL1	E1	14.3 \pm 1.3	1.6	ns	10.8 \pm 0.2	0.5	ns
	E2	10.7 \pm 3.3			10.2 \pm 2.3		
BL2	E1	20.6 \pm 0.3	46.8	$p \leq 0.001$	10.5 \pm 1.1	-1.8	ns
	E2	12.6 \pm 0.2			12.9 \pm 1.4		
BL3	E1	10.8 \pm 2.5	0.4	ns	9.9 \pm 1.1	-1.2	ns
	E2	10.2 \pm 1.0			11.0 \pm 0.6		
BL4	E1	11.4 \pm 1.6	-3.4	ns	12.3 \pm 1.5	0.2	ns
	E2	13.4 \pm 2.1			12.0 \pm 2.0		
BL5	E1	10.4 \pm 2.1	-0.18	ns	11.5 \pm 0.9	1.44	ns
	E2	10.8 \pm 2.1			10.4 \pm 1.1		

Table 6.3: Comparison of ZO₂, high:low $\Delta\Psi_m$ ratio and sperm amplitude of lateral head displacement (ALH) between first and second ejaculates (mean \pm SD).

Variable	n	Fresh semen			Freeze-thaw semen		
		Eja-1	Eja-2	Significance	Eja-1	Eja-2	Significance
ZO ₂	12	14.3 \pm 4.5	10.6 \pm 1.5	ns	10.4 \pm 0.5	11.1 \pm 1.3	ns
High:low $\Delta\Psi_m$	12	31.2 \pm 3.7	18.1 \pm 3.0	$p \leq 0.05$	15.0 \pm 4.3	18.1 \pm 3.0	ns
ALH	8	6.5 \pm 1.0	6.3 \pm 0.8	ns	5.9 \pm 0.6	6.2 \pm 0.7	ns

ns= not significant

Differences between bulls

The mean oxygen consumption between bulls for fresh and frozen-thawed is summarised in Figure 6.2 (a & b). Generally, results between bulls were similar, around 10.4 – 16.6 μ l O₂/10⁸ sperm/hour. However, in fresh semen for bull 2 the ZO₂ was 16% higher than the rest ($p \leq 0.05$).

Adenosine-tri-phosphate (ATP) production due to oxygen consumption was calculated on the basis that one molecule of oxygen forms 4.8 molecule of ATP ($P/O_{\max} = 2.4$) (Guppy *et al.*, 2002; Garrett *et al.*, 2008). The ATP production in fresh semen was similar between bulls but as expected it was higher in bull 2: for frozen semen there was no difference (see Fig 6.3 a & b).

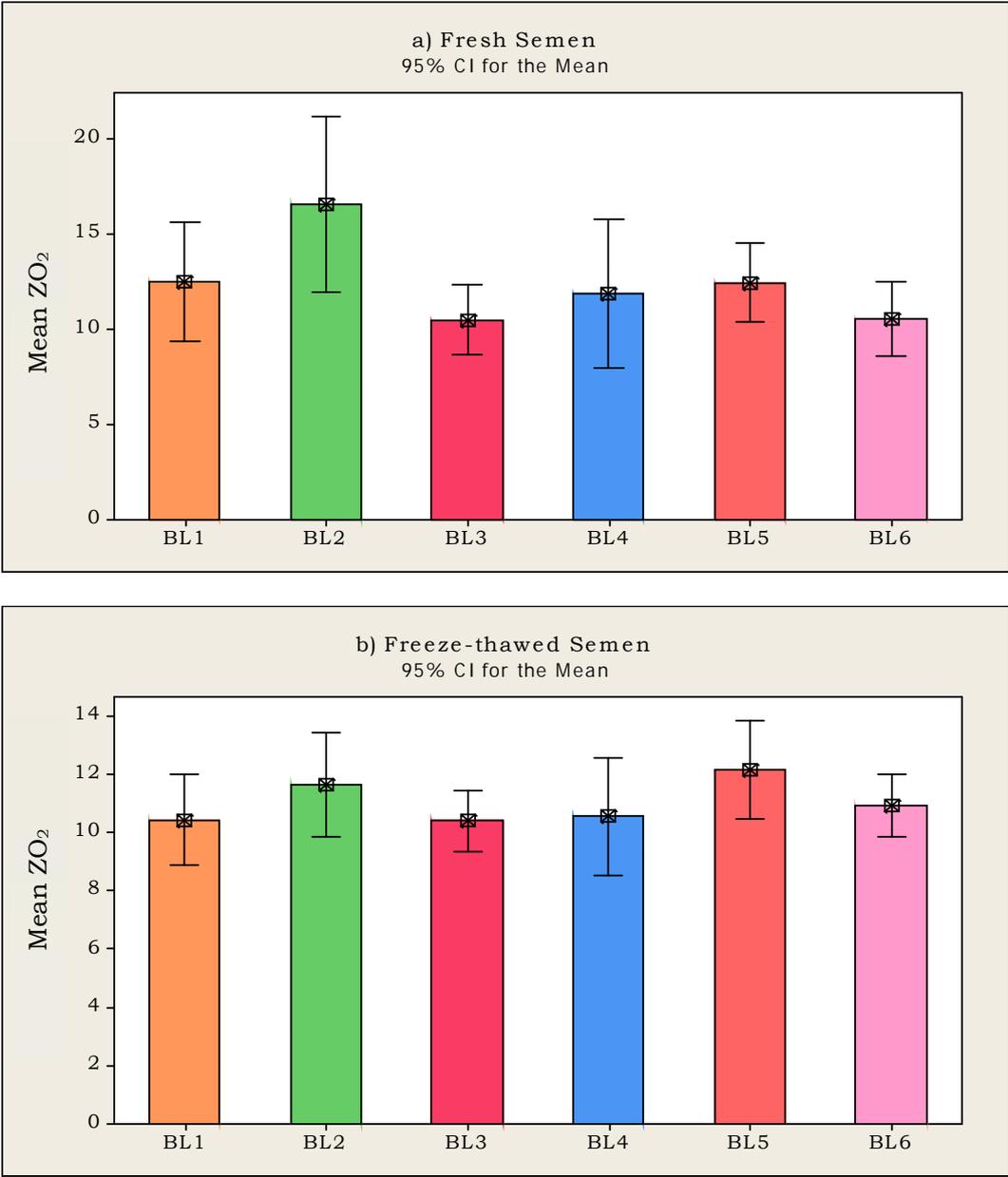


Figure 6.2 (a & b): Comparison of mean ZO_2 (μl oxygen consumed by 10^8 sperm/hr) between bulls of fresh and frozen-thawed spermatozoa. (n=6).

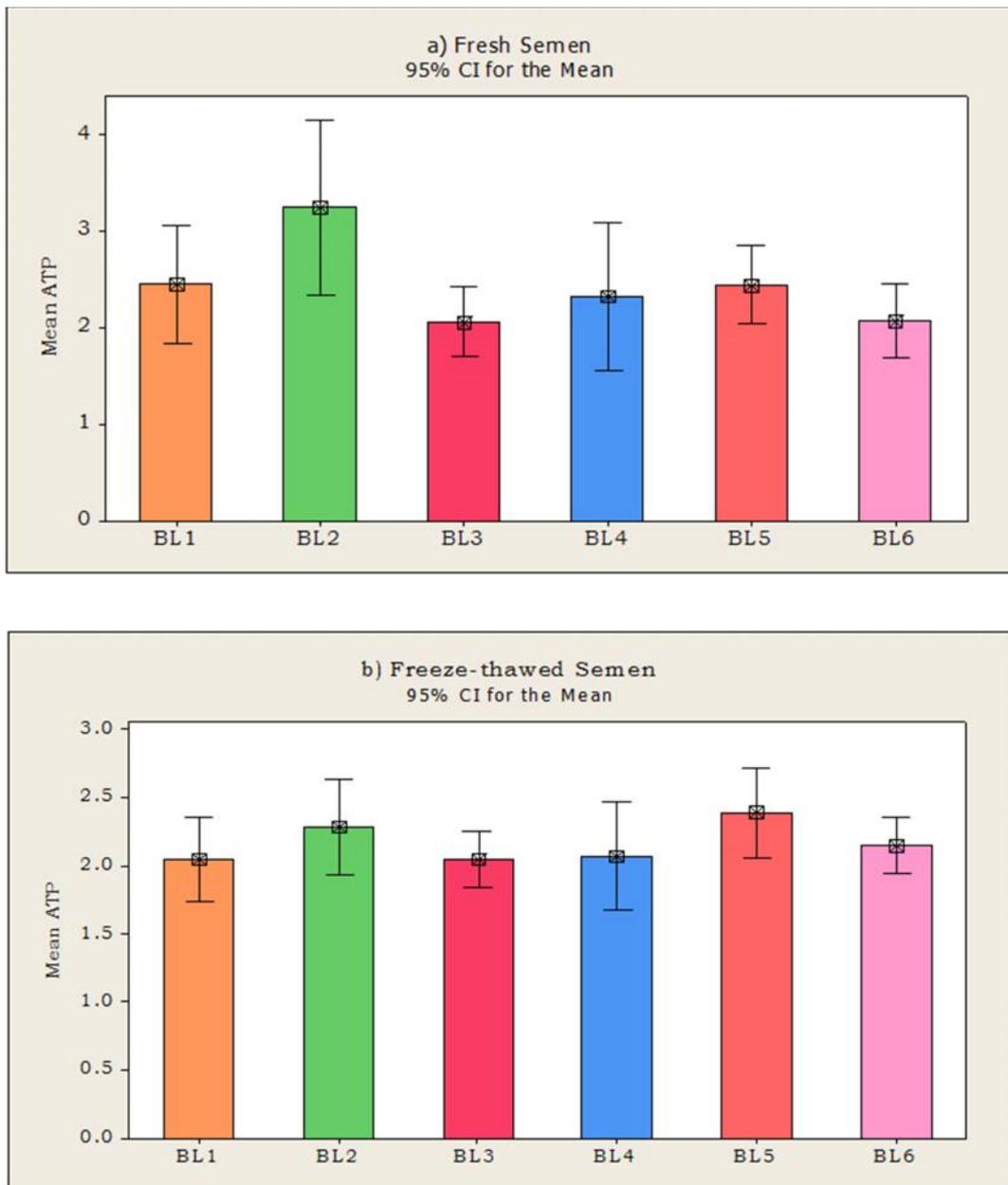


Figure 6.3 (a & b): Comparison of mean ATP ($\mu\text{l ATP from } 10^8 \text{ sperm/hr}$) between bulls of fresh and frozen-thawed spermatozoa. (n=6).

Oxygen consumption compared to mitochondrial membrane potential.

For fresh (Fig 6.4) and frozen/thawed (Fig 6.5) semen, the sperm oxygen consumption from six bulls was measured and correlated with their mitochondrial membrane potential. Correlation between ZO_2 and high:low $\Delta\Psi_m$ ratio varied, $r=0.82$ ($p\leq 0.05$) for fresh and $r= 0.49$ for frozen-thawed

samples that was not significant. The mitochondrial membrane potential was estimated on both live and dead sperm within a sample compared to the ZO_2 estimation based only on live sperm. Therefore the adverse effect of freeze/thawing was not reflected significantly within this correlation.

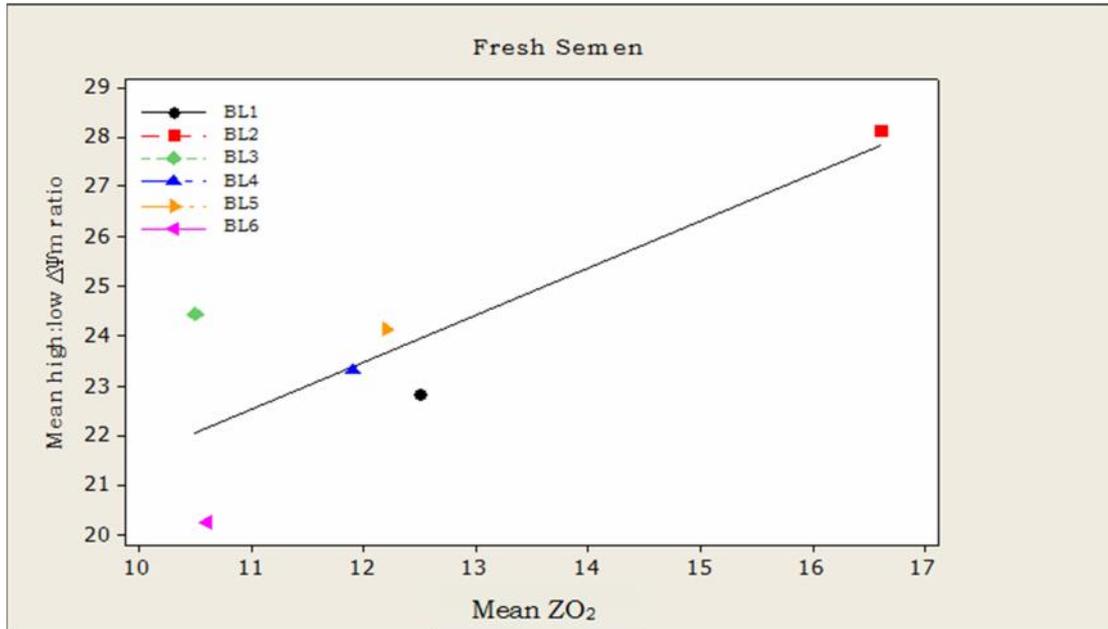


Figure 6.4: Correlation between ZO_2 and high:low $\Delta\Psi_m$ ratio of fresh spermatozoa from six bulls.

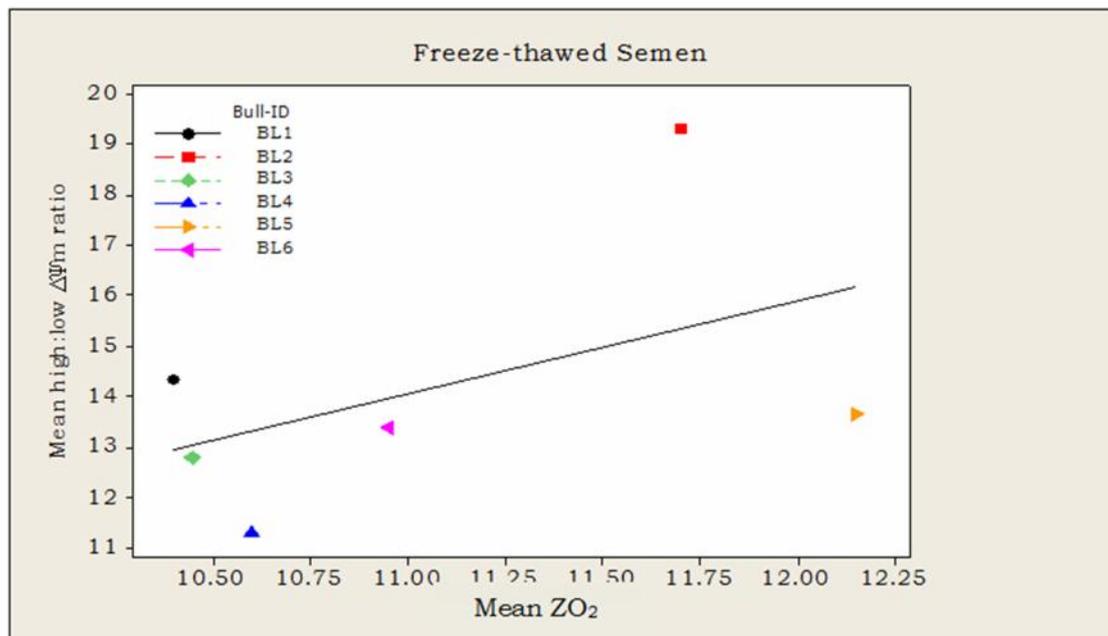


Figure 6.5: Correlation between ZO_2 and high:low $\Delta\Psi_m$ ratio of frozen-thawed spermatozoa from six bulls.

6.5. Discussion

The ZO_2 in fresh and frozen-thawed semen was calculated using the oxygen biosensor system. The fluorescence intensity was measured by applying the novel technique of Laser Scanning Confocal Microscopy. The data presented here (Table 6.1) showed higher ZO_2 in first ejaculate in 3/6 of bulls of fresh and frozen-thawed semen.

High ZO_2 in an ejaculate was associated probably with the function of sperm mitochondria related to hyperactive motility. In this present study when the ZO_2 increased, the high:low $\Delta\Psi_m$ and amplitude of lateral head displacement (ALH) also increased (see Table 6.3). Higher ALH is related to capacitation which transforms sperm motility from active to a hyperactive state (Mortimer and Mortimer, 1990; Ho *et al.*, 2002).

Inconsistency in ZO_2 was seen between bulls in both fresh and frozen-thawed semen ranging between 10.6 to 16.6 and 10.4 to 12.2 for fresh and frozen-thawed semen respectively. Using a similar technique OBS system ZO_2 in bull fresh and frozen-thawed spermatozoa was measured by Garrett *et al.*, (2008) by fluorescence plate reader. In fresh and frozen-thawed semen ZO_2 ranged between 13.0 to 35.8 and 26.6 to 39.9 respectively suggesting that variation in ZO_2 between bulls was possibly due to inter-breed variation rather than inter-bull variation (Garrett *et al.*, 2008).

Similarly comparing fresh or frozen-thawed semen mean ZO_2 appeared to be higher in fresh than frozen-thawed semen. These observations were consistent with White *et al.*, (1954) and Al-Taha and Strzezek, (1982) found higher ZO_2 in bull fresh semen than that of frozen-thawed semen. Contradictory results were observed by Garrett *et al.*, (2008) when comparing ZO_2 between fresh and frozen-thawed bull semen. Higher ZO_2 was seen in frozen-thawed semen suggesting that an increase in ZO_2 might be result of capacitation-like changes induced by the freeze-thaw process and sperm becoming hyperactivated (Garrett *et al.*, 2008). In our view this increase in ZO_2 with freeze/thaw was possibly due to the use of a different method of separation of live sperm for post-thaw samples such that fresh samples consisted of membrane intact sperm irrespective of motility, whereas post-thaw sperm were selected on the basis of good motility by the Percoll technique; therefore selection of motile sperm for post-thaw

samples, the sperm were dependent on mitochondrial ATP to fulfil their increase ATP demand and devoted more oxygen as compare to membrane intact sperm in fresh semen.

Bovine sperm produce 60-70% of adenosine-tri-phosphate from the metabolism of endogenous substrates (Hammerstedt, 1975) and approximately 70-80% of total ATP is converted into mechanical force by dynein ATPase found associated with the entire length of flagellum to support motility (Ford and Rees, 1990). Bovine epididymal sperm generated a range of 5-6 $\mu\text{mol ATP/h}/10^8$ sperm (Garbers *et al.*, 1973; Hammerstedt, 1975), $3\mu\text{m ATP/hr}/10^8$ sperm being required to drive motility (Hammerstedt *et al.*, 1988). The amount of ATP produced in the present study ranged between 2.1-3.3 $\mu\text{mol}/10^8/\text{hr}$ for fresh and frozen-thawed semen. These ATP values were 50% of those obtained by Morton and Lardy (1967) in bull epididymal sperm. They synthesize 5 μmol of ATP per μmol of oxygen consumed when using endogenous substrate. Production of mitochondrial ATP varied from 1.99 to 8.10 $\mu\text{mol ATP}/10^8$ spermatozoa/hour (Garrett *et al.*, 2008). The values of ZO_2 and high:low $\Delta\Psi_m$ ratio from identical fresh and frozen-thawed ejaculates from six bulls were found to be positively correlated, demonstrating that the mitochondrial membrane potential measured in this way by flow cytometry can be used as an indicator of ZO_2 .

2.6. Conclusions

The effect of cryopreservation reduced the proportion of live sperm in an ejaculate that was not reflected in the ZO_2 . The sperm of some bulls was less susceptible to this trait than others. Mitochondrial damage occurs during the freeze/thawing process, possibly through an increase in oxidative stress. Mitochondrial energy significantly contributes to sperm hyperactivity, therefore sperm oxygen consumption and mitochondrial activity may be a useful indicator of bull fertility.

Chapter 7

Effect of glycolytic and respiratory inhibitors on bovine sperm motility

7.1. Introduction

Artificial insemination (AI) is a first generation reproduction biotechnology technique that has increased significantly the genetic/economic efficiency of livestock, particularly in dairy cattle. This would not have been possible without successful freezing of semen from genetically superior bulls. Despite development of robust semen storage techniques to date, sperm viability may be reduced by 50% after freezing and thawing (Januskauskas and Zilinskas, 2002).

Spermatozoa obtained their energy for motility by glycolysis and/or respiration, provided an appropriate substrate is available (Krzyzosiak *et al.*, 1999). Approximately 70-80% of ATP synthesized by sperm metabolism is converted to mechanical force through the enzyme dynein ATPase, found in the flagellum, which can be altered by change in temperature (Rikmenspoel, 1965; Hammerstedt and Hay 1980; Bohnensack and Halangk, 1986; Ford and Rees, 1990). Mitochondria, the site of oxidative phosphorylation, are located in the sperm mid-piece at the extreme anterior end of the flagellum (Ford, 2006). Oxidative phosphorylation is the predominant pathway for ATP synthesis in bull sperm (Storey, 2008), resulting in 36 molecules of ATP being synthesised for each glucose molecule metabolised. The absence of mitochondria in the terminal portion of the flagellum and length of the flagellum could pose a potential problem related to the diffusion rate of mitochondrial ATP within the flagellum necessary to support motility. Therefore, sperm may utilise glycolysis to provide ATP within the flagellum for their motility: this suggests that mitochondrial ATP may be unnecessary to ensure optimal fertility in males (Mukai and Okuno, 2004).

The axonemal dynein associated with flagellar motility, has a high requirement for ATP. The flagellum of bull sperm is about 54µm long, but it

is even longer in sperm of other species such as rat, mouse, honey possum, hamster, and guinea pig. The head and principal piece lack any respiratory enzymes, relying on glycolysis as their source of ATP (Storey, 2008). If the mitochondria restricted to the mid-piece are the only source of ATP, this molecule needs to travel a relatively long distance of 40-50 μ m to satisfy the requirements of the axonemal dyneins located in the more distal portion of the flagellum. The localization of the glycolytic enzymes hexokinase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and others downstream from GAPDH on fibrous sheath of the principal piece support the hypothesis that the ATP utilised at the distal end of the flagellum is synthesised locally (Storey and Kayne, 1975; Westhoff and Kamp, 1997; Turner 2003; Kim *et al.*, 2007).

Hammerstedt *et al.*, (1988) reported no change in the motility and progressive velocity of bull sperm when exposed to either aerobic or anaerobic conditions. When sperm were incubated with electron transport chain inhibitors such as rotenone and antimycin A that render mitochondria dysfunctional, motility remained satisfactory since they can generate energy through glycolysis (Krzyszosiak *et al.*, 1999). At the end of incubation they observed that the sperm with functional mitochondria were motile in glycolysis inhibited medium in aerobic conditions. In species with shorter flagella such as cattle and sea urchins, ATP diffusion from mitochondria may be sufficiently rapid. ATP delivery required by longer sperm could be achieved by an adenylate kinase shuttle. Ford (2006) incubated sperm with α -chlorohydrin, an inhibitor of the glycolytic enzyme GAPDHs and found that sperm remain motile for at least one hour without an external source of glucose. Earlier, Mukai and Okuno (2004) found that the sperm remained motile with pyruvate or lactate in the medium in the absence of glucose: motility ceased in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone, an inhibitor of mitochondrial oxidative phosphorylation. This implied that ATP generated by mitochondria diffused far enough along the flagellum to drive normal motility. In human sperm the glycolytic pathway is the primary source of ATP necessary since oxidative phosphorylation does not contribute sufficient ATP to sustain motility (Nascimento *et al.*, 2008). Hence, there must be some mechanism

through which spermatozoa can utilize mitochondrial energy to support motility of the distal flagellum.

An active phosphocreatine shuttle for ATP, between mitochondria and dynein-ATPases has been found in human (Yeung *et al.*, 1996), sea urchin (Tombes and Shapiro, 1987) and rooster (Wallimann *et al.*, 1986) spermatozoa, but not in cattle (Brooks, 1971). When oligomycin, an electron transport chain inhibitor, was used to block oxidative phosphorylation in bovine sperm mitochondria, 20-44% of ATP requirement was produced through glycolysis. This suggests that bovine sperm rely more on glycolysis to supply ATP to the flagellum than oxidative phosphorylation in the mitochondria (Garrett *et al.*, 2008). This contradicts with earlier work by Schoff *et al.*, (1989) who reported that when adenylate kinase and 3-phosphoglycerate kinase were most active -both catalyse the reaction $2ADP \leftrightarrow ATP + AMP$. Hence, the sperm could rely on the adenylate kinase shuttle for ATP transfer along the flagellum and perhaps participating in capacitated sperm hyperactivity by increasing the available energy (Schoff *et al.*, 1989).

The objective of this study was to clarify these contradictions by investigating various characteristics of sperm motility using the sophisticated technique of Computer Aided Sperm Analysis (CASA) and the impact of glycolytic and respiratory inhibitors.

7.2. Materials and methods

Four different treatments were investigated: control (CON); valinomycin (VAL) a respiratory inhibitor, providing sorbitol as a glycolytic substrate; 2-deoxy-D-glucose (DOG) and iodoacetamide (IAM) both glycolytic inhibitors, together with pyruvic acid as a respiratory substrate (see Chapter 2.2).

Preparation of semen

Ejaculates were collected from four bulls (see Chapter 2.1). An aliquot of 200µl fresh semen was taken after final freezing dilution in Tris/citrate/fructose/egg yolk/glycerol (EYTG) extender in a test tube. The sperm concentration was adjusted to 60×10^6 /ml by adding Ruthin extender at room temperature and transferred to a water bath at 39°C. For frozen-

thawed semen: four 0.25ml semen straws (60×10^6 /ml) were plunged into a water bath at 39°C for 30 seconds and emptied into a tube.

From the 1ml (60×10^6) of fresh or frozen-thawed semen, duplicate quantities of 200µl were placed in tubes for each treatment, 200µl of Ruthin extender were then added to CON and VAL (along with 4µl valinomycin) and same volume of DOG and IAM suspension were added to semen as per treatment. Samples were labelled Control (C1, C2), Valinomycin (VAL1, VAL2), 2-deoxy-D-glucose (DOG1, DOG2) and Iodoacetamide (IAM1, IAM2). To compare the effect of long-term ambient extender Ruthin (CON) and EYTG extender on sperm motility parameters the equal amount of freezing extender EYTG was added to semen and labelled as FCON1 and FCON2.

After semen preparation 10µl of DNA-specific Hoechst 33342 stain (Sigma Chemical Co., St. Louis, Missouri) was then added to all samples, the contents were mixed well and the samples were left for 15 minutes to allow for staining.

Assessment of sperm motility

Sperm motility analysis was carried out using a Hamilton Thorne (HT) Computer-Assisted Sperm Analyser (CASA) system (Hamilton-Thorne Research, Beverly, USA) with an UV illumination system. Motility was estimated using the characteristics described by Budworth *et al.*, (1988) and Holt (1995) (see Table 7.1).

Table 7.1: Sperm motility characteristics and their description.

Motility characteristics	Initials	Definition
Total motility (%)	TM	The proportion of motile sperm in a sample.
Progressive motility (%)	PM	Sperm with 26.7 µm/s average path velocity and 55% STR classified as progressive.
Curvilinear velocity (µm/s)	VCL	A measure of the total distance travelled by a given sperm divided by the time elapsed, including all deviations of sperm head movement.
Straight line velocity (µm/s)	VSL	Velocity calculated using the straight line distance between the beginning and end of the sperm track divided by the time taken.
Amplitude of lateral head displacement (µm)	ALH	The mean width of head oscillation as the sperm swim.
Average path velocity (µm/s)	VAP	Velocity over a calculated, smoothed, path in: a shorter distance than that used for calculating VCL.
Path straightness (%).	STR	The average value of the ratio VSL/VAP.
Linearity (%)	LIN	The average value of the ratio VSL/VCL.

Around 2-5 μ l of stirred semen was placed in a pre-warmed (39°C) disposable Leja counting chamber slide (Orange Medical, Brussels, Belgium), transferring it to the HT slide stage and recordings were obtained by acquiring range of 7 fields for motility analysis. To avoid inaccuracy due to drift, each sample was given approximately one minute equilibration time after loading: drifting cells may be recorded as slow motile sperm, giving an inaccurate estimate of the true motile sperm percent.

7.3. Data handling and statistical analysis

Means of sperm motility characteristics provided by the HT semen analyzer, were stored in a spreadsheet. After cleaning data, differences between the control and treatment groups, fresh and frozen-thawed semen and between bulls were investigated using ANOVA, applying Tukey and Dunnett's comparison test using a general linear models (GLM) procedure in Minitab16 software. Significance of differences was accepted when $p \leq 0.05$.

7.4. Results

Relationship between sperm motility parameters

The relationship between motility variables of frozen-thawed sperm were investigated and results summarised in Table 7.2. Percentage of total motile spermatozoa and percentage of progressive motile spermatozoa, as well as curvilinear velocity and amplitude of lateral head displacement, were highly correlated. A positive correlation existed between total and progressive motilities and all motility parameters excluding curvilinear velocity and amplitude of lateral head displacement. Spermatozoa with a high curvilinear velocity, straight line velocity and average path velocity had a greater amplitude of lateral head displacement.

Differences between fresh and frozen-thawed semen

Sperm total and progressive motilities and curvilinear velocity were lower in all bulls as expected for frozen-thawed semen but were significant in 3/4 bulls (see Table 7.3). The effect of cryopreservation on sperm viability was to reduce it in bull 1, more so in bull 2 and more extreme in bull 3 and 4 reducing the percentage of motile sperm by approximately 20%, 35% and 50% respectively. Cryopreservation reduced sperm amplitude of lateral head displacement in bull 1, 2 and 4 whilst it increased in bull 3 compared

Table 7.2: Correlation between sperm motility parameters in 16 frozen-thawed spermatozoa samples obtained from four bulls.

Variables	Relationship (r)						
	Total motility	Progressive motility	Curvilinear velocity	Amplitude of lateral head displacement	Path straightness	Linearity	Average path velocity
Progressive motility	1.0*						
Curvilinear velocity	-0.06 Ns	-0.13 ns					
Amplitude of lateral head displacement	-0.08 Ns	-0.17 ns	0.99*				
Path straightness	0.2 Ns	0.3 ns	-0.57 ns	-0.7 ns			
Linearity	0.3 Ns	0.38 ns	-0.84 ns	-0.91 ns	0.91 ns		
Average path velocity	0.25 Ns	0.2 ns	0.9 ns	0.8 ns	-0.18 ns	-0.5 ns	
Straight line velocity	0.21 Ns	0.2 ns	0.5 ns	0.4 ns	0.4 ns	0.04 ns	0.8 ns

*= $p \leq 0.05$

to fresh semen. Moreover, mean of all bulls for fresh and frozen-thawed semen was compared, substantial decrease in percentage of sperm with total and progressive motilities and their curvilinear velocity was seen in frozen-thawed semen that was more than 20% as compared to fresh semen (see Table 7.3). In the same way, there was a reduction in sperm amplitude of lateral head displacement (8%) but it was not significant.

Table 7.3: Differences in mean motility parameters (SE of mean) between four bulls and their fresh and frozen-thawed semen. (n=4).

Variables	Semen sample	Bull identification				Mean
		1	2	3	4	
Total motility (%)	Fresh	68.5 (5.2)	65.3 (4.6)	56.5 (1.8)	57.0 (2.0)	61.8 (4.3)
	Frozen-thawed	^a 56.3 (4.9)	^{ab} 41.5 (3.9)	^b 28.5 (1.9)	^b 28.0 (1.5)	38.6 (6.7)
	Significance*	ns	$p \leq 0.01$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$
Progressive motility (%)	Fresh	58.8 (4.2)	61.5 (4.7)	54.0 (1.6)	51.3 (2.0)	56.4 (3.7)
	Frozen-thawed	^a 48.5 (3.9)	^a 37.8 (3.5)	^b 23.5 (1.9)	^b 24.0 (1.1)	33.4 (6.0)
	Significance*	ns	$p \leq 0.01$	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$
Curvilinear velocity ($\mu\text{m/s}$)	Fresh	^b 159.8 (3.4)	^b 146.4 (6.6)	^b 144.3 (5.5)	^a 183.8 (4.9)	158.6 (9.3)
	Frozen-thawed	^a 143.1 (1.8)	^b 122.2 (2.5)	^a 136.2 (2.4)	^a 146.3 (3.6)	136.9 (5.3)
	Significance*	$p \leq 0.01$	$p \leq 0.05$	ns	$p \leq 0.001$	$p \leq 0.001$
Amplitude of lateral head displacement (μm)	Fresh	^a 7.0 (0.2)	^b 5.9 (0.2)	^b 5.4 (0.13)	^a 7.3 (0.3)	6.4 (0.5)
	Frozen-thawed	^a 6.4 (0.1)	^b 5.1 (0.1)	^a 6.1 (0.1)	^a 6.5 (0.2)	6.0 (0.3)
	Significance*	$p \leq 0.05$	$p \leq 0.01$	$p \leq 0.01$	ns	ns

Means that do not share a letter are significantly different between bulls.

*= difference between fresh and frozen-thawed semen.

Comparison of sperm motility characteristics between bulls

Fresh and frozen-thawed semen from four different bulls, diluted in Ruthin extender, presented varying total and progressive motilities but differences between bulls were only significant for frozen-thawed semen (see Table 7.3). In both fresh and frozen-thawed semen of bulls 1 and 2 had the best motility. The higher sperm motion of bull 1 and 2 is suggestive of bulls with superior semen. Bulls 3 and 4 with lower sperm motion ranked lower. The curvilinear velocity and amplitude of lateral head displacement of bull 4 were significantly higher in fresh and frozen-thawed semen as compared to most other bulls (see Table 7.3). Spermatozoa are considered to be in a state of hyperactivation when their flagellar motion becomes whip-like, asymmetrical with high amplitude and low frequency (Ho and Suarez, 2001). This can be assessed using CASA if the sperm curvilinear velocity is $\geq 100\mu\text{m/s}$, linearity $< 60\%$ and amplitude of lateral head displacement $\geq 5\mu\text{m}$ (Mortimer and Mortimer, 1990). Therefore the increased amplitude of lateral head displacement and decreased linearity (see Appendix 7.2) of any bull indicated sperm hyperactive motility associated with an increase curvilinear velocity.

Comparison of motility characteristics of fresh and frozen-thawed spermatozoa diluted in EYTG and Ruthin extender

The identical ejaculates of fresh and frozen-thawed samples from each bull were examined under Computer-Assisted Sperm Analyser (CASA) to investigate whether adding either the EYTG or Ruthin (RUTH) extender had any effect on sperm motility. No differences were observed in sperm total and progressive motilities although these estimates were relatively higher in RUTH compared to EYTG extender (see Table, 7.4). All other CASA parameters such as average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, path straightness and linearity were significantly lower in the EYTG than in RUTH extender for fresh and frozen-thawed spermatozoa (see Table, 7.4). This was either related to EYTG extender being of a medium-to-high density that lowered sperm motility parameters through the extender's higher viscosity, or the effect of the extenders' chemical composition since sperm motility was improved in an extender with a less complex chemical composition (Vera-Munoz *et al.*, 2009).

Table 7.4: Effect of egg-yolk/tris/glycerol (EYTG) and Ruthin (RUTH) extenders on mean sperm motility parameters (SE of mean) for fresh and frozen-thawed bull semen. (n=16).

Variable	RUTH	EYTG	Significance	RUTH	EYTG	Significance
	Fresh	Fresh		Frozen-thawed	Frozen-thawed	
Total motility (%)	61.8 (2.2)	59.2 (2.1)	ns	38.6 (3.3)	36.8 (3.2)	ns
Progressive motility (%)	56.4 (1.8)	53.3 (2.1)	ns	33.4 (3.0)	32.6 (2.8)	ns
Average path velocity ($\mu\text{m/s}$)	96.2 (2.2)	69.8 (5.2)	$p \leq 0.001$	80.6 (1.1)	68.1 (3.7)	$p \leq 0.01$
Straight line velocity ($\mu\text{m/s}$)	82.9 (2.2)	56.2 (4.2)	$p \leq 0.001$	67.5 (1.1)	56.1 (3.9)	$p \leq 0.01$
Curvilinear velocity ($\mu\text{m/s}$)	158.6 (4.7)	124.2 (9.0)	$p \leq 0.01$	136.9 (2.7)	115.7 (3.5)	$p \leq 0.001$
Amplitude of lateral head displacement (μm)	6.4 (0.2)	6.0 (0.3)	ns	6.0 (0.2)	5.5 (0.1)	$p \leq 0.05$
Path straightness (%)	84.8 (1.0)	80.1 (0.8)	$p \leq 0.01$	81.1 (0.8)	80.6 (0.8)	ns
Linearity (%)	53.4 (1.4)	46.0 (0.8)	$p \leq 0.001$	49.8 (1.0)	48.0 (1.3)	ns

Sperm motility in presence of glycolytic and respiratory inhibitors

Motility characteristics of fresh and freeze-thawed spermatozoa incubated with 2-deoxy-D-glucose, iodoacetamide and valinomycin were compared with untreated spermatozoa (CON), expressed as variance (\pm) from the control mean (0) (see Fig 7.1 a & b, 7.2 a & b). The raw data is shown in Appendix 7.2 and 7.3. Based on values estimated in Table 7.1, the percentage of total and progressive motilities, curvilinear velocity, amplitude of lateral head displacement, path straightness and linearity were useful parameters and are described below.

2-deoxy-D-glucose

Fresh and frozen-thawed spermatozoa were diluted in a medium containing 2-deoxy-D-glucose (DOG), a competitive inhibitor of glycolysis that targets hexokinase within the reaction $\text{glucose} \rightarrow \text{glucose 6-phosphate}$ by competing with glucose during phosphorylation: the resulting product is DOG-6-phosphate which cannot be metabolized further. Consequently sperm were allowed to produce energy entirely from mitochondrial activity. By utilizing energy through oxidative phosphorylation, the total and progressive motilities in fresh spermatozoa were increased by up to 4% ($p > 0.05$) compared to the control but curvilinear velocity was reduced by over 20% ($p \leq 0.001$) and path straightness and linear motility tended to be

reduced, whereas amplitude of lateral head displacement was similar (see Fig 7.1 a & b). From the fresh or frozen-thawed semen results it is apparent that the degree of sperm total and progressive motilities should be similar if they were dependent exclusively on mitochondrial energy after restricting glycolytic pathways.

Iodoacetamide

Iodoacetamide (IAM) is a glycolytic inhibitor which inactivates the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by binding irreversibly to cysteine residues and allowing sperm to be dependent on mitochondrial energy. Addition of IAM to fresh and frozen-thawed semen reduced total and progressive motilities by up to 45% ($p \leq 0.001$) and their sperm curvilinear velocity was reduced by up to 71% ($p \leq 0.001$) (see Fig 7.1 a & b, 7.2 a & b). Values for sperm path straightness and linearity were lowered by 36% ($p \leq 0.001$) in fresh semen whereas these fell by over 25% ($p \leq 0.001$) for frozen-thawed semen. In both fresh and frozen-thawed sperm their amplitude of lateral head displacement was reduced ($p \leq 0.001$) by up to 30% and over 20% respectively compared to control (see Fig 7.1 a & b, 7.2 a & b). Reduction in all motility parameters in spermatozoa of both fresh and frozen-thawed semen indicates that IAM which is known as inhibitor of glycolytic pathways has also significant affect on sperm mitochondria (see Chapter V) possibly by modifying its cysteine similar to GAPDH enzyme in glycolysis. Hence like glycolysis, IAM effect on mitochondrial function is noticeable and revealed as mitochondrial inhibitor.

Valinomycin

Valinomycin (VAL) is a passive carrier for K^+ , binding or releasing K^+ when it encounters the membrane surface: it transports these ions across cell membranes, thereby inhibiting oxidative phosphorylation by antagonizing the proton motive force. Spermatozoa of fresh and frozen-thawed were diluted in a medium containing VAL as to confine sperm to produce energy exclusively by glycolysis. Addition of VAL increased sperm total and progressive motilities and sperm straightness and linear motility in both fresh and frozen-thawed semen. The estimate of curvilinear velocity was lowered by 13% ($p \leq 0.05$) in fresh semen compared to 5% ($p > 0.05$) for

frozen-thawed semen. Amplitude of lateral head displacement tended to be lower also (see Fig 7.1 a & b, 7.2 a & b). These results suggest that after inhibition of oxidative phosphorylation, spermatozoa can maintain their motility at same level as if using glycolysis as source of energy.

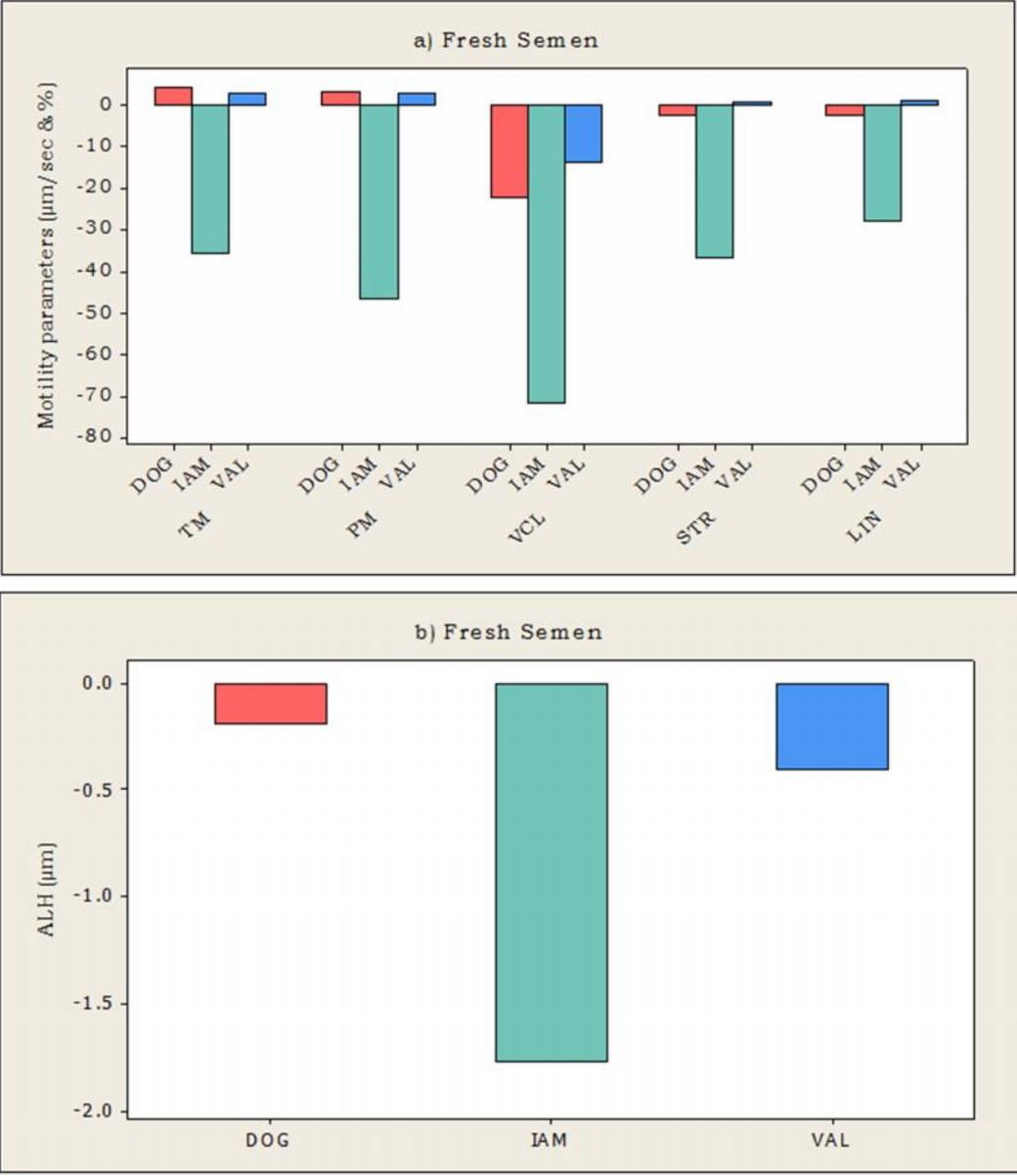


Figure 7.1 (a & b): Motility characteristics of fresh sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors compared with control, shown as variance (\pm) from the '0' mean control value. (n=16).

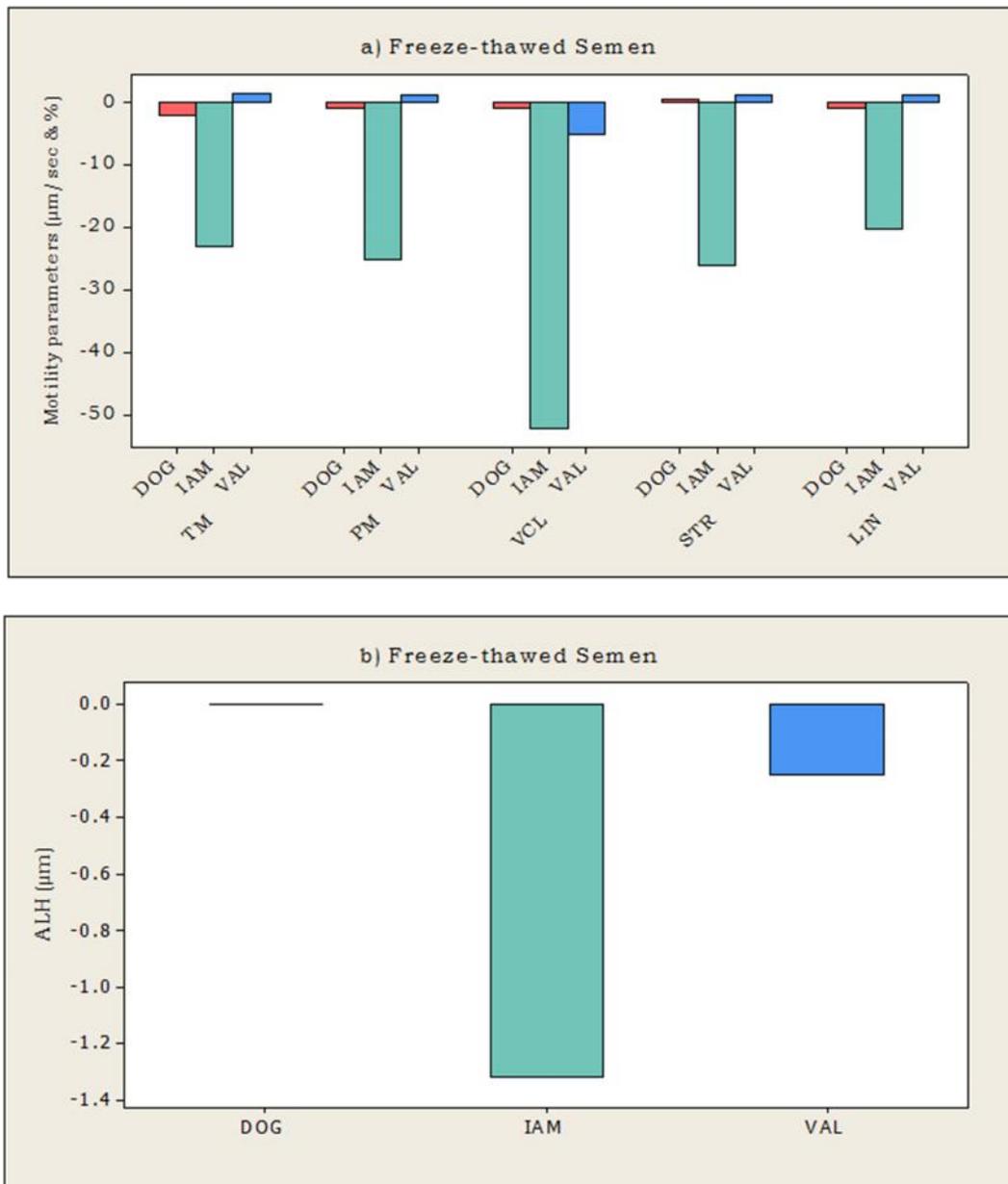


Figure 7.2 (a & b): Motility characteristics of frozen-thawed sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors compared with control, shown as variance (\pm) from the '0' mean control value. (n=16).

Sperm progressive motility, oxygen consumption and mitochondrial membrane potential compared to bull non-return rate (NRR) and sperm mid-piece length (MPL).

The sperm PM, ZO_2 and high:low $\Delta\Psi_m$ ratio of frozen-thawed spermatozoa was compared with the 49 day NRR from respective bulls: although these correlations were found to be not significant, they were positive with PM, ZO_2 and high:low $\Delta\Psi_m$ (see Table 7.5). Similarly certain motility characteristics were positively correlated with MPL, but only significantly in curvilinear velocity and amplitude of lateral head displacement (see Table 7.5).

Table 7.5: The relationship of CASA assessed sperm motility parameters, oxygen consumption (ZO_2) and high:low $\Delta\Psi_m$ ratio of frozen-thawed semen with 49 day bull non-return rates (NRR) and sperm mid-piece length (MPL), using Pearson correlation test.

Sperm parameters	Bull NRR (n=4)		MPL (n=3)	
	r	p-value	r	p-value
Total motility	0.3	ns	0.3	ns
Progressive motility	0.3	ns	0.2	ns
Average path velocity	-0.73	ns	0.8	ns
Straight line velocity	-0.9	ns	0.1	ns
Curvilinear velocity	-0.6	ns	1.0	0.05*
Amplitude of lateral head displacement	-0.5	ns	1.0	0.03**
Path straightness	-0.2	ns	-0.8	ns
Linearity	0.2	ns	-0.9	ns
ZO_2	0.2	ns	-1.0	ns
high:low $\Delta\Psi_m$ ratio	0.5	ns	-0.9	ns

* $p \leq 0.05$; ** $p \leq 0.05$
 ns= not significant

Sperm progressive motility compared to oxygen consumption and mitochondrial membrane potential

The sperm progressive motility from 4 bulls from the beef breeds Limousin, Aberdeen Angus, Charolais and Simmental were assessed and compared to their rate of oxygen consumption (ZO_2) and High:low $\Delta\Psi_m$ ratio in fresh and frozen-thawed semen (see Table 7.6). The higher correlation of PM with ZO_2 and High:low $\Delta\Psi_m$ ratio was seen in fresh spermatozoa compared to frozen-thawed spermatozoa but it was not significant.

Table 7.6: The relationship between sperm progressive motility and ZO_2 and high:low $\Delta\Psi_m$ ratio of fresh (FR) and frozen-thawed (FT) spermatozoa using Pearson correlation test.

Variables	Relationship		
	Progressive motility (%)		
	FR	FT	number
ZO_2	0.8	0.2	4
High:low $\Delta\Psi_m$	0.6	0.5	4

7.5. Discussion

Sperm motility characteristics have been carefully evaluated using glycolytic (DOG and IAM) and respiratory (VAL) inhibitors to limit the sperm to generating their energy either from glycolysis or mitochondria. In this study percentage of sperm total motility and progressive motility were at same level in either fresh or frozen-thawed sperm incubated with DOG or VAL compared to control, suggesting that sperm motility was supported either by glycolysis or mitochondrial energy. These results agree with a report which showed that sperm can use both metabolic pathways to support their motility (Bar-Sagie *et al.*, 1980; Krzyzosiak *et al.*, 1999). No change in motility was seen when sperm were exposed to medium containing either DOG or respiratory inhibitor Antimycin A, but motility completely terminated after addition of either Antimycin A to DOG or vice versa (Bar-Sagie *et al.*, 1980). Similarly, bull and human sperm were motile when suspended in medium that does not support glycolysis (DOG); their motility terminated after addition of respiratory inhibitors rotenone and antimycin A (Krzyzosiak *et al.*, 1999, Nascimento *et al.*, 2008).

Sperm velocity was significantly higher only in un-treated fresh sperm compared to DOG, and VAL. Krzyzosiak *et al.*, (1999) observed slightly higher velocities in bull sperm when examined under anaerobic than aerobic conditions suggested that this increase was probably due to the experimental conditions and/or greater change in the shape of sperm tracks in the aerobic state reflecting transition to a hyperactivation. Sperm can generate ATP through either glycolysis or respiration and the rate of ATP generation via these metabolic pathways may limit sperm motility. In the case of DOG a significant decrease in ATP level has been reported by Pasupuleti, (2007) in mouse sperm treated with DOG in presence of pyruvate and lactate suggesting that drop in ATP might be due to DOG inhibiting hexokinase and being phosphorylated to DOG-6-phosphate using a large amount of mitochondrial energy. Since marked variation in sperm velocities was seen only in fresh samples, it is unlikely that DOG depletion of mitochondrial ATP for phosphorylation was associated with sperm velocities. It is more likely that untreated sperm have shown capacitation-like changes and became hyperactive that might change the pattern of sperm track as the amplitude of lateral head displacement in DOG samples

was lower compared to control. In the case of VAL treated fresh sperm provided with sorbitol as a glycolytic substrate, the spermatozoa were exclusively dependent on ATP generated via glycolysis. The sorbitol turned into fructose via polyol pathways and fructose was further reduced into lactate/pyruvate (Sorbitol + NAD \leftrightarrow Fructose + NADH⁺) and then lactate was unable to be metabolized further due to mitochondrial inactivation. This accumulation of lactic acid causes decrease in intracellular pH (Storey, 2008) and finally a decline in sperm velocity.

In mouse sperm, IAM is known to inhibit glycolysis but did not reduce motility and ATP levels in presence of pyruvate and lactate (Pasupuleti, 2007), indicating that mouse sperm can remain motile with energy generated from either glycolysis or mitochondrial respiration. Studies in several cell lines have shown that IAM has significant effect on respiration of rat heart mitochondria, sea urchin spermatozoa and corneal endothelium (Yang, 1957; Hathaway, 1963; Laing *et al.*, 1992) but the consequences of IAM on bovine sperm have not been reported yet. There is also some evidence that IAM significantly reduced glutathione (GSH) metabolism in brain cells by modifying thiol groups (Schmidt and Dringen, 2009) and the cell is dependent on GSH for the rapid clearance of peroxides to prevent oxidative stress (Dringen *et al.*, 2005). Loss of mitochondrial activity associated with reduced GSH level has been reported by Perl *et al.*, (2006) in mouse sperm. In the previous chapter we demonstrated the effect of IAM on bull sperm mitochondrial activity (Chapter V) and indicated that IAM has also a deleterious effect on mitochondrial function possibly linked with oxidative stress or modification of mitochondrial cysteine. IAM inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by irreversibly modifying its cysteine residue (Schmidt and Dringen, 2009) and type two glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH2) protein has been found on the surface of mid-piece, principal piece and acrosome region of mouse and hamster spermatozoa. The GAPDH2 was found to be associated with sperm motility, regulation of reactive oxygen species (ROS) and sperm capacitation (Kota *et al.*, 2009; 2010). In this study a significant decrease has been demonstrated in all motility parameters in IAM treated fresh and frozen-thawed sperm confirming the IAM inhibitory effect on mitochondria possibly associated with oxidative stress or alteration of mitochondrial

protein and GAPDH2 cysteine residue but the exact mechanism of the IAM effect on bovine sperm respiration and associated motility remains to be investigated.

Cryopreservation has the remarkably adverse effect on sperm viability of reducing the viable population by up to 50% (Hammerstedt *et al.*, 1990). In this present study fresh and frozen-thawed sperm were compared for motility parameters: sperm total motility and PM decreased by 20% in frozen-thawed compared with fresh semen. Budworth and others (1988), studied frozen thawed semen and found 34% and 31% sperm with total motility and progressive motility respectively. In this study 39% and 33% of sperm demonstrated total and progressive motility respectively in frozen-thawed sperm.

In the present study objective sperm motility analysis by means of CASA demonstrated inconsistency between bulls in overall sperm motility parameters: the percentage of total motility and progressive motility spermatozoa were higher in bull 1 with high velocity. On the other hand bull 4 demonstrated low total motility and progressive motility with high sperm velocity. Sperm of bull 1 and 4 showed considerably higher amplitude of lateral head displacement and lower path straightness and linearity (see Appendix 7.1) indicating capacitation-like changes causing transition in motility pattern towards hyperactivation. Sperm morphology can considerably influence sperm velocity potentially such as length of flagellum. Relationship of tail length with sperm motility has been reported in spermatozoa of primates and rodents (Froman and Feltmann, 1998) and red Iberian deer (Malo *et al.*, 2006). Sperm with longer flagella tend to swim faster to reach the ova sooner and achieve fertilization compared to those with shorter tails when ejaculates from different males intermingle in the same female tract. Gomendio and Roldan, (1991) demonstrated positive correlation between sperm length and its maximum velocity. By contrast, sperm from the Iberian red deer stag have long mid-pieces but swim relatively slowly (Malo *et al.*, 2006), suggesting that their activated spermatozoa with shorter mid-pieces may exhibit greater motility. When sperm mid-piece length (MPL) was compared between breeds: MPL was longest in breed Limousin- bull 1 (13.83 μ m) followed by Belgian Blue (13.78 μ m), Charolais-bull 3 (13.67 μ m) and Aberdeen Angus-bull 2

(13.35 μ m) (Shahani *et al.*, 2010). In this study curvilinear velocity was higher in bull 1 (except bull 4 that is Simmental) followed by bull 3 (except fresh semen) and bull 2. Thus we observed that sperm MPL is significantly correlated with sperm curvilinear velocity and amplitude of lateral head displacement. Furthermore, the effect of sperm with abnormal morphology such as abnormal acrosome, abnormal mid-piece and tail, proximal and distal droplets on sperm velocity have been addressed by Blom (1977), Barth and Oko (1989), Johnston *et al.*, (1995), Mahmoud *et al.*, (1998) and Amann *et al.*, (2000).

Sperm motility is one of the characteristics that must be considered when evaluating male fertility potential, since a decrease is associated with male infertility (Auger *et al.*, 1989; Liu *et al.*, 1991). In the laboratory, optical methods are used most commonly to evaluate the percentage of motile sperm in a sample, the outcome varying between technicians working in laboratories (Budworth *et al.*, 1988). However, using a computer assisted semen analysis (CASA) method reduces human error and gives a better prediction of fertility than more subjective methods (Saacke, 1982). Sperm motility recorded either by visual or CASA has been correlated significantly to 56 day bull non-return rate (NRR) (Januskauskas *et al.*, 2000) but not sperm linearity (Januskauskas *et al.*, 2001). Later on, in one study they found a no correlation between bull fertility and CASA assessed sperm motility and linearity (Januskauskas *et al.*, 2003). Similarly, correlation of progressive motility (0.86), curvilinear velocity (0.68), straight line velocity (0.70) and linearity (0.60 and 0.59) and negative correlation of amplitude of lateral head displacement (-0.05) of frozen-thawed semen and field fertility has been reported by Amann, (1989) and Zhang *et al.*, (1998). More recently, Garcia-Macias *et al.*, (2007) found no correlation between field fertility and CASA assessed sperm total motility and progressive motility and velocity parameters but they observed the velocity parameters were highest in the high-fertility group. In this present study, progressive motility, sperm oxygen consumption and mitochondrial membrane potential of frozen-thawed spermatozoa were compared with the 49 day non-return rate (NRR) from four commercial breeding bulls. No correlation was found between any assessment of sperm motility and bull NRR. Unfortunately, no relationship was found between sperm oxygen

consumption and high:low $\Delta\Psi_m$ ratio of frozen-thawed spermatozoa with bulls' NRR. This result is likely related to the small number of proven bulls with relatively high fertility potential examined in this study.

When sperm become hyperactive, dramatic changes occur in pattern and sperm track. High amplitude of lateral head displacement and curvilinear velocity and decrease path straightness, linearity and slow or non-progressive motility are considered as indicative of hyperactivation (Kathiravan *et al.*, 2011). In this study significant correlation between sperm amplitude of lateral head displacement and curvilinear velocity and negative correlation of these values with sperm path straightness and linearity suggesting that considerable sperm population in sample may be demonstrated capacitation-like changes because of freeze-thaw process.

7.6. Conclusions

Bull sperm can maintain similar level of motility when controlled to generate their energy either from glycolysis or mitochondria or by both. Sperm hyperactive motility was associated with mitochondrial function. Iodoacetamide which is a known glycolytic inhibitor has been recognised as a respiratory inhibitor in this study. As a method of assessing unproven bulls, the relationship between sperm progressive motility, ZO_2 and high:low $\Delta\Psi_m$ ratio with NNRs may be useful to predict bull fertility.

Chapter 8

General discussion and conclusion

Artificial insemination (AI) is a universal method for breeding cattle in high-producing commercial dairy herds. One reason for lowered pregnancy rates is using semen of poor quality from a bull; another is the unavoidable issue of cryopreservation that causes lethal or sub-lethal differential damage to spermatozoa and reduces considerably the number of functional sperm that can fertilise the ovum (Watson, 1995a; Holt, 1997). The economic advantage of using AI and semen of genetically superior bulls depends on precise evaluation of the semen that reflects reasonably their fertility potential, especially when low-dose inseminations are the norm (Christensen *et al.*, 2011). For many bulls standing in commercial breeding centres, lower doses of semen can be used without compromising overall cattle fertility (Den Daas, 1997). The problem of selecting *in vitro* tests, capable of identifying those bulls which are fertile when low doses of sperm are used for insemination without the need to conduct numerous field trials has been addressed in a number of ways, but none has yet proven reliable. The assessment of a bull's ejaculate is based on motility, which has rarely correlated well with fertility (Januskauskas, 1995; Rodriguez-Martinez, 2003; Holt and Van Look, 2004). Other criteria are the proportion of the sperm in the sample that resist osmotic stress (Revell and Mrode, 1994), the percentage with intact plasma membranes (Christensen *et al.*, 2005) and the proportion that remain motile after prolonged incubation (Correa *et al.*, 1997). In this study, we investigated differences in sperm metabolic activity relative to their motility that may reflect better the fertility of bulls from their non-return rate (NRR).

Basic sperm morphology was described initially. Variations in the length of mammalian spermatozoa are due mostly to variation in tail length (Cummins and Woodall, 1985) and size of the mid-piece, according to each species' different morphology. Anderson and Dixson (2002) found that mid-piece volume was greater in primates whose normal mating patterns involved multiple rather than single partners. Earlier, Gomendio and

Roldan (1991) had found similar differences between monoandrous and polyandrous species: in rodent and other species where males face sperm competition after mating with a single female, spermatozoa are longer than in species where only a single dominant male will mate with a female such as in some wild cats and deer. The longer the sperm flagellum, the greater the forces generated by its motions (Katz and Drobnis, 1990) and its requirement for immediately available energy. In Chapter 3, differences in mid-piece length were considered between bulls of different breeds and within ejaculates collected at different times from the same bull. The hypothesis was that in sperm with a longer mid-piece, they have more active mitochondria that generate more energy, so motility will be greater, thus improving the likelihood of such sperm gaining access to the oviduct and achieving fertilization, compared to bulls with shorter sperm mid-piece. When ejaculates from different males intermingle in the same female tract, there is a positive correlation between sperm length and its maximum velocity (Gomendio and Roldan, 1991).

No significant differences were found in this present study between different breeds of bull, although Aggarwal *et al.*, (2007) found variation between eight breeds of buffalo (*Bubalus bubalis*). However, there was significant variation in mid-piece length between ejaculates collected from nine bulls investigated. Whilst the method of measurement may have been imprecise - spermatozoa with straight tails could be measured as one entity and sperm with severely bent tails, due to cold shock, were positively excluded - the differences were real enough. This variation was unlikely to result from short-term dysfunction in spermatogenesis although the length of epididymal storage prior to ejaculation may account for some variation. The preliminary data from this study was compared to non-return rates in cows bred with semen collected from the same bulls: longer mid-pieces correlated with a 5% reduction in NRR in the dairy bulls investigated. In bulls of beef breeds, this correlation was positive but very low: the small numbers of bulls investigated prevented meaningful statistical relationships being established.

Chapter 4 described a novel method to validate the use of JC-I for the assessment of sperm mitochondrial membrane potential ($\Delta\Psi_m$) by flow cytometry in bulls, by setting regional and logical gates to exclude dead

sperm and other non-cellular components from live sperm present within an ejaculate, and to determine the maximum mitochondrial membrane potential at minimum incubation time. By labelling their mitochondria with JC-1, the numbers of sperm in an ejaculate with low or high $\Delta\Psi_m$ can be estimated and this used to compare the potential fertility of bulls in both their fresh and frozen-thawed semen. A pilot study confirmed that spermatozoa in both fresh and freeze-thawed semen exhibited maximum high:low $\Delta\Psi_m$ ratio after 40 min incubation. Flow cytometric dot plots of analyses of fresh and frozen-thawed spermatozoa incubated with JC-1 identified a unified sperm population of membrane-intact cells, each population characterised by both low and high $\Delta\Psi_m$ but to varying degrees. This flow cytometric method was a simple robust method for determining of mitochondrial membrane potential, and serves double purpose: first to evaluate sperm $\Delta\Psi_m$, and the second to estimate the proportion of live:dead sperm within an ejaculate.

Chapter 5 investigated differences in $\Delta\Psi_m$ following incubation of fresh and freeze-thaw sperm with glycolytic and respiratory inhibitors. Whilst the glycolytic inhibitor 2-deoxy-D- glucose (DOG) has no detectable effect on mitochondrial function, mitochondrial activity was reduced significantly by iodoacetamide (IAM) and valinomycin (VAL). This increase in $\Delta\Psi_m$ in sperm incubated with DOG may be due to inhibition of glycolysis which up-regulated the mitochondria to generate energy. VAL reduced $\Delta\Psi_m$, probably inducing mitochondrial swelling through an increased uptake of K^+ . As a result, there may be disruption of electron transport chain (ETC) in mitochondria and loss of inner mitochondrial membrane (Saris *et al.*, 2009).

IAM, inhibits GAPDH by binding irreversibly to cysteine residues thereby inactivating the enzyme. Sperm mitochondrial cysteine-rich proteins (SMCP) are localized in the mitochondrial capsule and enhance sperm motility (Hawthorne *et al.*, 2006). SMCP gene knockout in mouse sperm reduced sperm motility and fertility (Nayernia *et al.*, 2002). IAM down regulated mitochondrial activity by decreasing pyruvate oxidation (Yang, 1957) and glutathione (GSH) level (Perl *et al.*, 2006). In this study, IAM disrupted mitochondrial activity in the presence of pyruvate and also shifted fluorescence from orange (high $\Delta\Psi_m$) to green (low $\Delta\Psi_m$). Disruption

of mitochondrial activity may be due to IAM action on altering SMCP, GSH level or restricting pyruvate oxidation: this requires further clarification.

The freeze-thaw cycle increases osmotic and oxidative stress on sperm, leading to increase membrane permeability thus decreasing sperm viability and mitochondrial membrane potential (Garner and Thomas, 1999; Ferrusola *et al.*, 2008). Using JC-1 probe to evaluate the functional potential of mitochondria of bovine sperm, Celeghini *et al.*, (2008), observed that for the percentage of spermatozoa with greater mitochondrial function, potential was considerably less in frozen-thawed semen than in fresh semen. Similar results were found by Arruda *et al.*, (2005). In this study, high:low $\Delta\Psi_m$ ratio of un-treated frozen-thawed spermatozoa decreased significantly compared to fresh sperm: perhaps the mitochondria encountered oxidative stress produced by the decrease in antioxidant level during a freeze/thaw cycle.

Chapter 6 described a novel method for investigating oxygen consumption in fresh and frozen-thawed semen. Using an oxygen biosensor system (OBS), the fluorescence intensity was measured by using Laser Scanning Confocal Microscopy. Oxygen consumption (ZO_2) was no different between fresh and frozen-thawed semen, but spermatozoa from fresh semen consume more oxygen than frozen-thawed spermatozoa. These observations were consistent with White *et al.*, (1954) and Al-Taha and Strzezek, (1982). Garrett *et al.*, (2008) found a higher ZO_2 in frozen-thawed semen suggesting that an increase in ZO_2 might be result of capacitation-like changes induced by the freeze-thaw process and sperm becoming hyperactivated (Garrett *et al.*, 2008). In our view this increase in ZO_2 with freeze/thaw was possibly associated with a different method for separating live sperm for post-thaw samples such that fresh samples consisted of membrane intact sperm irrespective of motility, whereas post-thaw sperm were selected on the basis of good motility by the Percoll technique. Motile sperm in post-thaw samples were dependent on mitochondrial ATP to fulfil their increased ATP demand and required more oxygen compared to membrane-intact sperm in fresh semen.

The mean oxygen consumption from the same fresh and frozen-thawed ejaculates were positively correlated with ratio of high:low $\Delta\Psi_m$, indicating

that the mitochondrial membrane potential measured in this way by flow cytometry can be used as an indicator of ZO_2 . Oxygen biosensor system (OBS) for technique of measuring oxygen consumption under Confocal microscopy was easy to use, time efficient and allowed evaluation of semen from more than one bull to be carried simultaneously. Since mitochondrial energy contributes significantly to sperm hyperactivity and their oxygen consumption and mitochondrial activity may be a useful indicator to prove young bulls and allow unsuitable bulls to be eliminated at an earlier stage.

In Chapter 7, sperm motility was measured using a Computer Assisted Semen Analyzer. Sperm motility was at the same level as control in fresh and frozen-thawed sperm that were exclusively supported either by glycolysis or mitochondrial energy after incubation with valinomycin or 2-deoxy-D-glucose respectively. These results agree with the hypothesis that sperm can utilize both metabolic pathways to support motility (Bar-Sagie *et al.*, 1980; Krzyzosiak *et al.*, 1999). No change in motility was recorded when sperm were exposed to medium containing either DOG or respiratory inhibitor Antimycin A, but motility completely terminated after addition of either Antimycin A to DOG or *vice versa* (Sagie *et al.*, 1980). Glycolytic enzymes bound to the fibrous sheath could provide energy along the flagellum at any point that is required. Therefore delivery of mitochondrial energy along the flagellum may be uncertain because it needs to travel long distance along the flagellum to support motility (Ford, 2006). This occurrence is likely to be appropriate for species having longer flagella as in mouse sperm: they become immotile after knock out of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mukai *et al.*, 2005). However, in the bovine species the rate of energy diffusion couldn't be a problem as their sperm flagellum is relatively shorter than rodents. Another mechanism which transports energy is adenylate kinase and 3-phosphoglycerate kinase and both enzymes are most active in bovine sperm (Schoff *et al.*, 1989). Hence, sperm rely on the adenylate kinase shuttle for ATP transfer along the flagellum and this perhaps participates in capacitation of sperm by increasing the available energy (Schoff *et al.*, 1989).

Iodoacetamide (IAM) significantly reduced sperm motility in both fresh and frozen-thawed semen. In Chapter 5, we demonstrated this effect on bull

sperm mitochondrial activity and indicated that IAM has a deleterious effect on mitochondrial function possibly linked with oxidative stress or modification of mitochondrial cysteine and glutathione level. IAM inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by irreversibly modifying its cysteine residue (Schmidt and Dringen, 2009) and type two glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH2) protein has been found on the surface of mid-piece, principal piece and acrosome region of mouse and hamster spermatozoa. The GAPDH2 was found to be associated with sperm motility, regulation of reactive oxygen species (ROS) and sperm capacitation (Kota *et al.*, 2009; Kota *et al.*, 2010). The exact mechanism of the IAM effect on bovine sperm respiration and associated motility remains to be investigated.

At the end of Chapter 7, progressive motility, sperm oxygen consumption and mitochondrial membrane potential of frozen-thawed spermatozoa were compared with the 49 day non-return rate (NRR) from respective bulls. There was a positive correlation of sperm progressive motility, sperm oxygen consumption and high:low $\Delta\Psi_m$ ratio with bull NRR. Furthermore sperm progressive motility from 4 bulls, all beef breeds Limousin (BL1), Aberdeen Angus (BL2), Charolais (BL3) and Simmental (BL4) were compared to their rate of oxygen consumption (ZO_2) and high:low $\Delta\Psi_m$ ratio in fresh and frozen-thawed semen investigated in (Chapter 6). A high correlation of progressive motility with ZO_2 and high:low $\Delta\Psi_m$ ratio was assessed in fresh spermatozoa compared to frozen-thawed spermatozoa but it was not significant.

Conclusions

The extensive assessment of bull's reproductive potential prior to breeding is highly important and includes examination of general physical soundness, external and internal genitalia, and semen quality. Commercial breeding success depends on the efficient use of bulls with high breeding value but semen quality limits the use of some of these bulls. Several parameters have been used to assess quality of either fresh or frozen-thawed semen. Among a variety of traditional parameters, sperm concentration, sperm raw and post-thaw motility and sperm morphology are commonly used for routine semen assessment in the laboratory. In

human reproduction sperm plasma membrane and mitochondrial membrane integrity, and DNA stability are all assessed prior to *in vitro* fertilization and assisted reproductive technique.

This thesis describes some novel techniques that could more precisely describe and predict bull fertility. Mitochondrial function is interesting because of its significant contribution to ATP synthesis and sperm hyperactive motility. When sperm are in the oviduct, they bind with the oviduct epithelium and then they require a high level of ATP to support hyperactivation that facilitates their escape from the oviductal reservoir and positioning at the site of fertilization and penetration of oocyte and its vestment. Sperm motility in fowl (Pizzari *et al.*, 2002) and human (Kao *et al.*, 1998) is highly heritable. There is a strong maternal genetic effect on sperm motility, independent from the autosomal effect, suggesting that mitochondrial genes not only contribute to male fitness but also sexual selection. In bovine sperm, the paternal mitochondrial DNA (mtDNA) is destroyed and tagged by ubiquitin in the cytoplasm of the ovum shortly after fertilization (Sutovsky *et al.*, 1996). In chickens, sperm with low motility contained more than tenfold abnormal mitochondria compared to those with high motility (Froman and Kirby, 2005). In the human, alteration in mitochondrial DNA gene, defects in internal structure and organisation of mitochondria are associated with the efficiency of mitochondrial energy production, reduced motility and male fertility (Rao and Martin, 1989; Kao *et al.*, 1998; Ruiz-Pesini *et al.*, 2000). When investigating subfertile and infertile human males, the proportion of deleted sperm mtDNA was higher with poor motility and reduced fertility compared to sperm with good motility and fertility (Kao *et al.*, 1998). In addition, they found a high frequency of multiple deletions of mtDNA in spermatozoa from patients with primary infertility associated with low sperm count and reduce sperm motility. Therefore, identification of such mitochondrial genes in bovine sperm influencing their motility could be useful in future research.

Even though most of the fresh and frozen-thawed semen parameters in this thesis, such as sperm mitochondrial membrane potential, sperm oxygen consumption and sperm motility were positively correlated with the bull NRR, this was not significant. Identification of such indicators in bovine

sperm influencing their fertility could form the basis for estimating bull fertility, rather than NRRs, thereby shortening the time required to select young bulls in a commercial breeding programme. Furthermore, these indicators may be of use in assisted reproduction techniques or/and to differentiate true fertility potential of sperm collected from human beings with either proven fertility or those with clinical subfertility and infertility.

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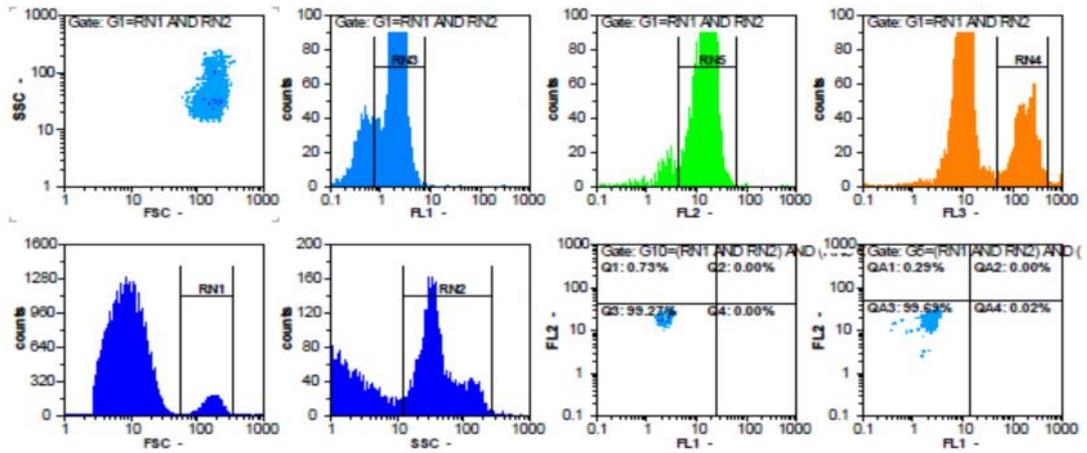
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Appendices

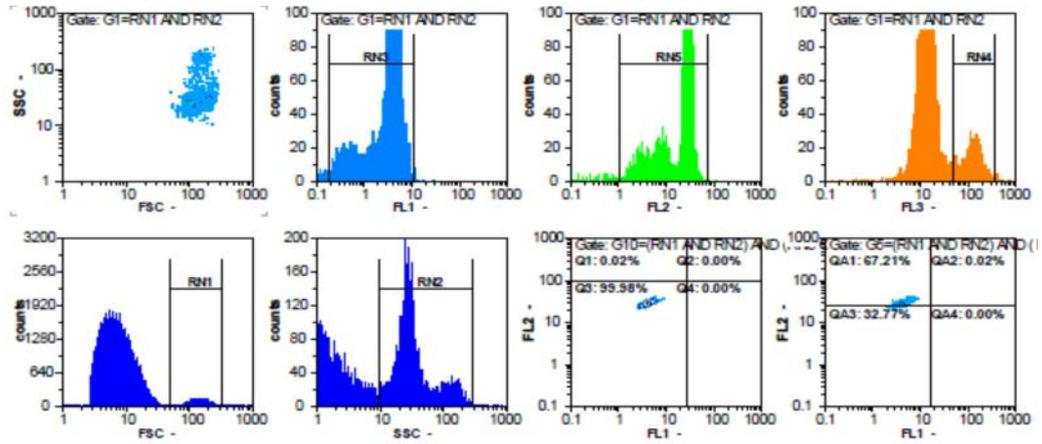
Appendix- 3.1. Representing number of mature bulls from different breeds, their mid-piece length (MPL) measurement and percentage of non-return rates (NRR %).

Breed /No. of bulls	No. of cows inseminated	No. of cows held	*NRR %	Mean NRR %	NRR % (e-d)	MPL (µm)
a	b	c	d	e	f	g
AHO1	2609.3	1997.7	77.11	73.25	3.86	13.22
AHO2	1691.0	1245.0	73.63	73.25	0.38	12.99
AHO3	7930.0	5588.0	70.47	73.25	-2.78	13.64
AHO4	6529.3	4900.0	75.06	73.25	1.81	13.53
AHO5	504.7	333.7	69.99	73.25	-3.26	13.68
Mean	38529	2812.9	73.25			
FR1	506	407	80.43	79.93	0.50	13.46
FR2	7768	5798	74.6	79.93	-5.29	13.69
FR3	156	124.5	79.9	79.93	-0.02	12.95
FR4	759	598	81.1	79.93	1.12	13.48
FR5	796	663.7	83.59	79.93	3.66	13.35
Mean	1997.0	1518.2	79.93			
AN1	425.0	350	82.35	81.0	1.38	13.36
AN2	714.0	572	80.11	81.0	-0.86	13.61
AN3	14369.0	11581	80.53	81.0	-0.44	13.25
AN4	2462.7	1949.3	80.89	81.0	-0.08	13.19
Mean	4492.7	3613.1	81.0			
BB1	1341.0	966.7	72.05	76.11	-4.06	13.8
BB2	3024.0	2204.0	72.88	76.11	-3.23	13.6
BB3	1071.3	817.7	78.7	76.11	2.61	14.1
BB4	3419.3	2578.7	75.4	76.11	-0.72	13.9
BB5	597.3	462.7	81.5	76.11	5.38	13.6
Mean	1890.6	1405.9	76.11			
CH1	2242.0	1802.3	79.95	81.1	-1.10	13.53
CH2	305.0	263.0	86.23	81.1	5.18	13.49
CH3	377.7	315.0	83.19	81.1	2.14	14.16
CH4	374.3	290.7	78	81.1	-3.05	13.36
CH5	265.0	206.5	77.88	81.1	-3.17	13.85
Mean	712.8	575.5	81.1			
LN1	2518.0	2075.7	82.43	83.6	-1.15	14.66
LN2	587.7	472.7	79.7	83.6	-3.88	13.3
LN3	21989.3	18292.3	83.21	83.6	-0.37	13.92
LN4	453.0	399.0	88.08	83.6	4.50	13.49
LN5	205.3	171.0	84.49	83.6	0.91	13.82
Mean	5150.7	4282.1	83.6			

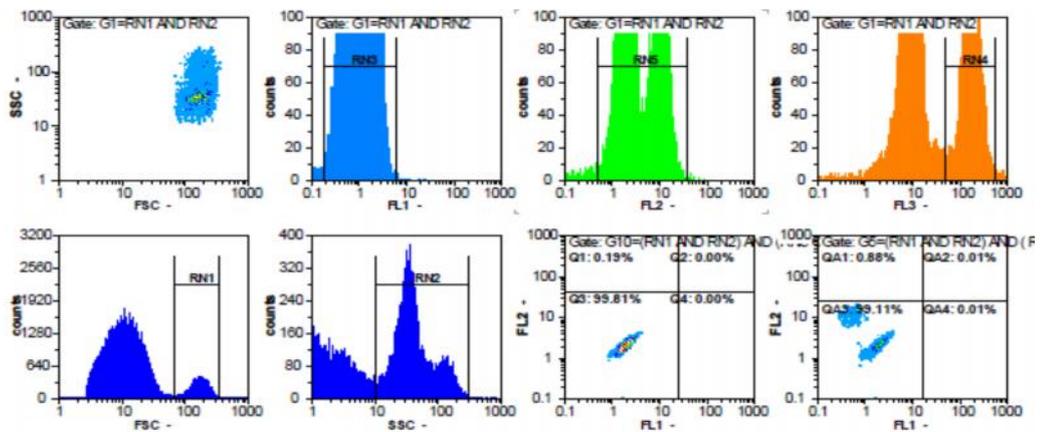
*= percentage of cows that do not require reinsemination. AHO, Holstein (mature); FR, Friesian; AN, Aberdeen Angus; BB, Belgian Blue; CH, Charolais; LN, Limousin.



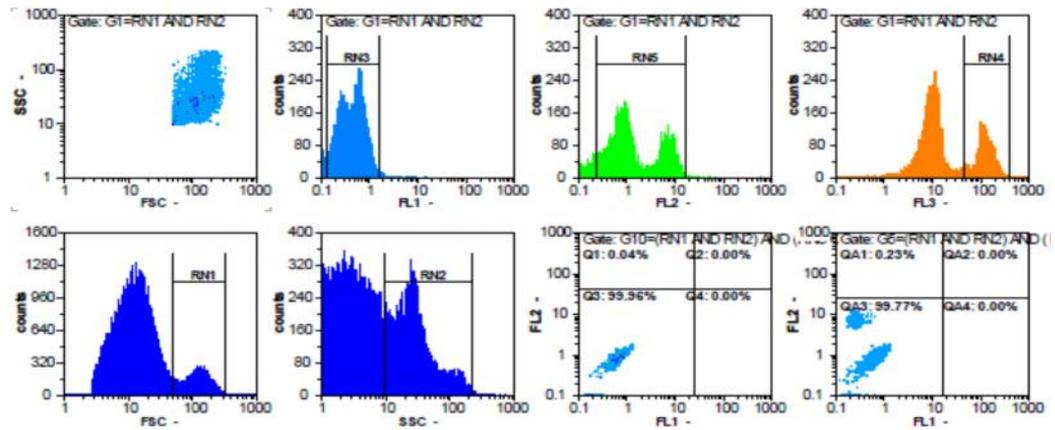
Appendix- 4.1. Fresh spermatozoa washed in Ruthin extender and stained with JC-1, and PI.



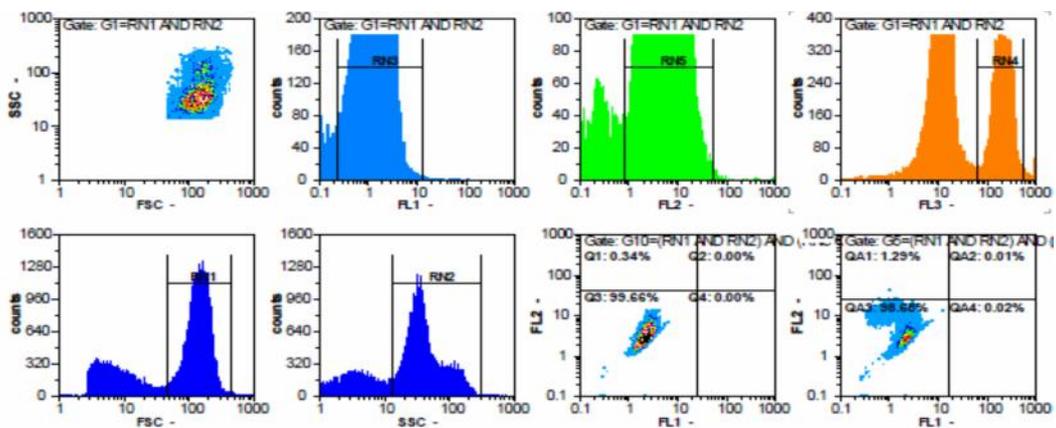
Appendix- 4.2. Frozen-thawed spermatozoa washed in Ruthin extender and stained with JC-1, and PI.



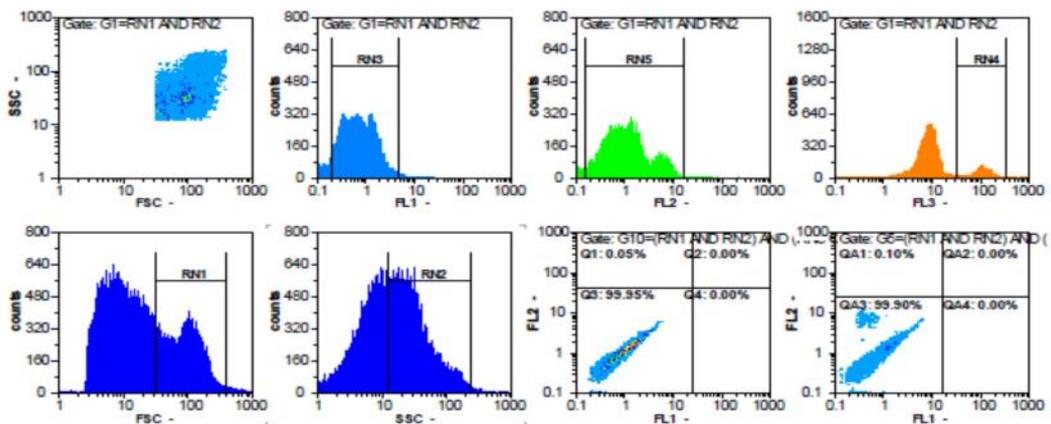
Appendix- 4.3. Fresh unwashed spermatozoa in Ruthin extender and stained with JC-1, and PI.



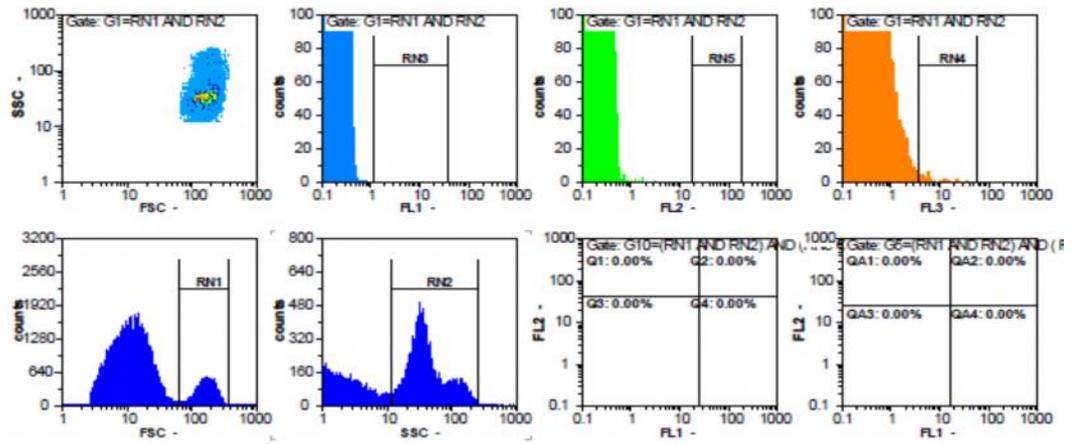
Appendix- 4.4. Frozen-thawed unwashed spermatozoa in Ruthin extender and stained with JC-1, and PI.



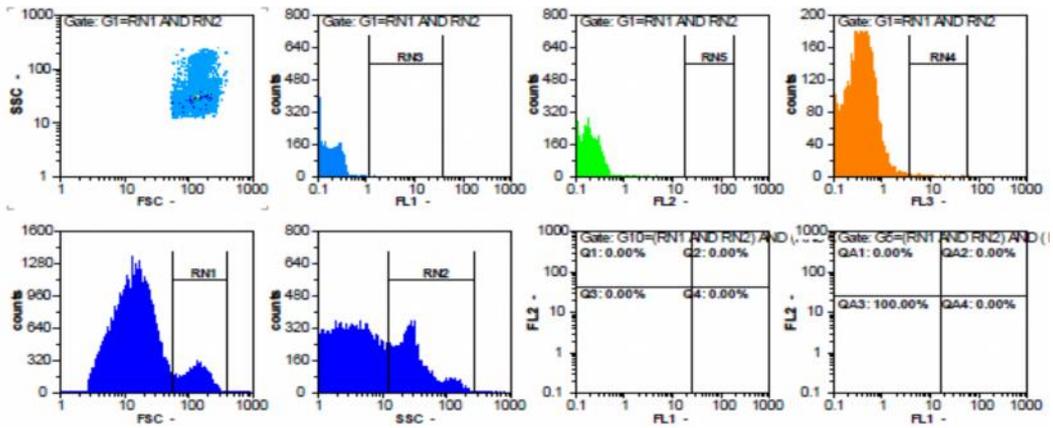
Appendix- 4.5. Fresh unwashed spermatozoa in PBS extender and stained with JC-1, and PI.



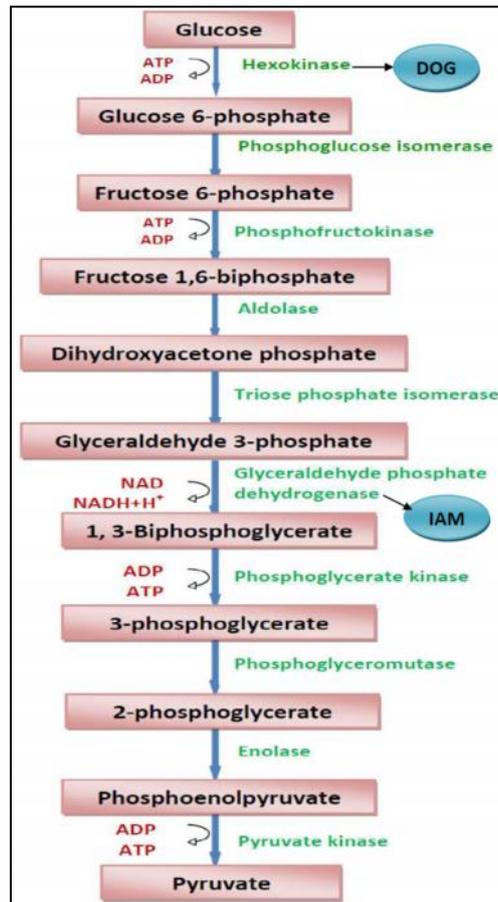
Appendix- 4.6. Frozen-thawed unwashed spermatozoa in PBS extender and stained with JC-1, and PI.



Appendix- 4.7. Fresh spermatozoa washed in Ruthin extender unstained with JC-1, and PI.



Appendix- 4.8. Frozen-thawed spermatozoa washed in Ruthin extender unstained with JC-1, and PI.



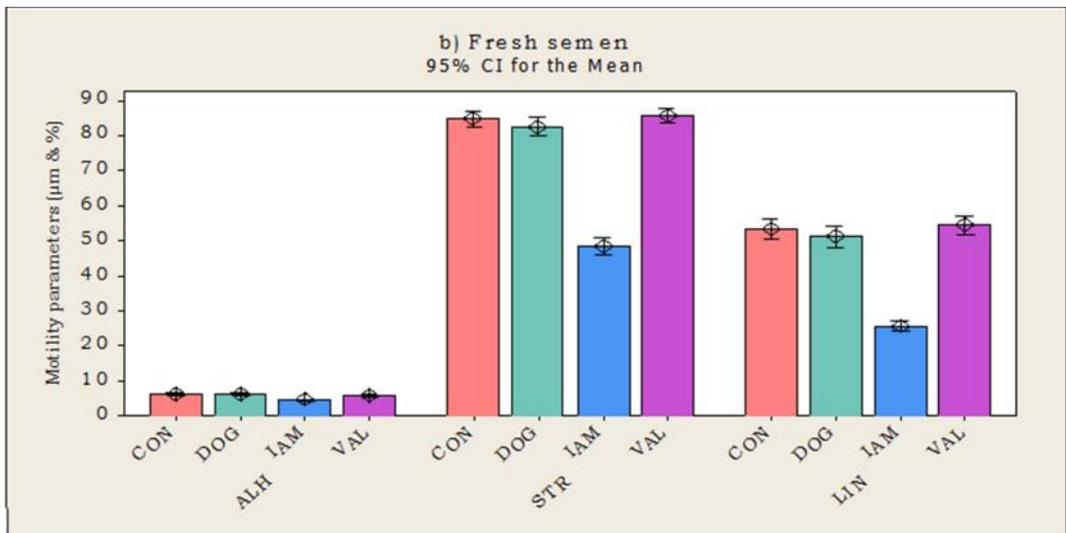
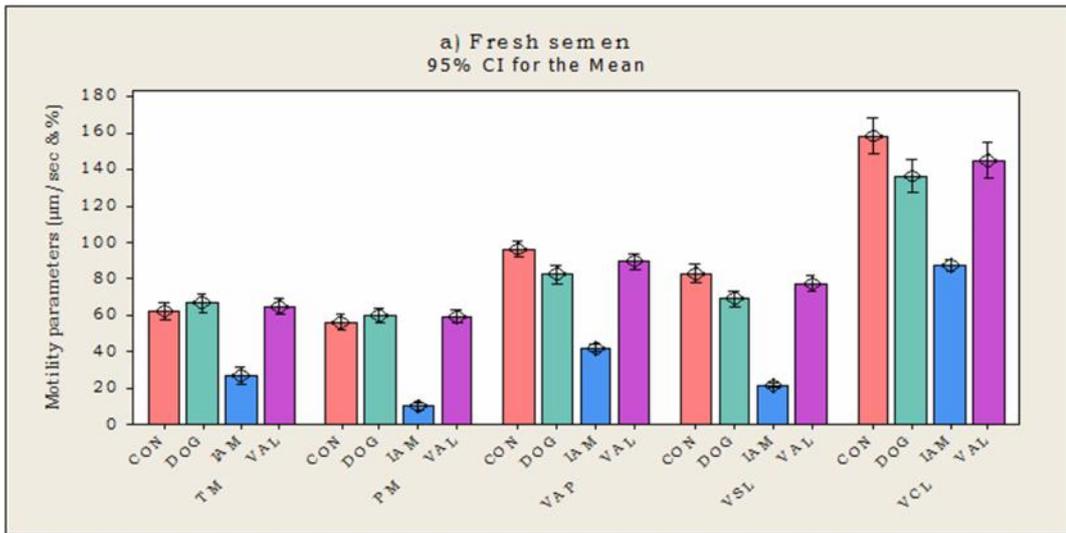
Appendix-5.1. Schematic presentation of how different modes of actions of 2-Deoxy-D-glucose and iodoacetamide affect glycolytic pathways.

Appendix 7.1. Differences in motility parameters (mean \pm SD) between the bulls and fresh and frozen-thawed semen.

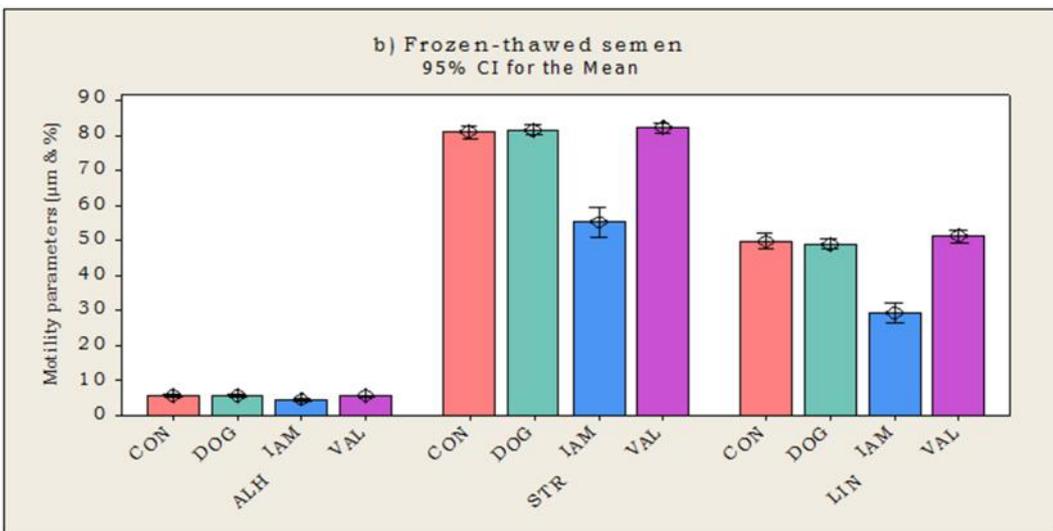
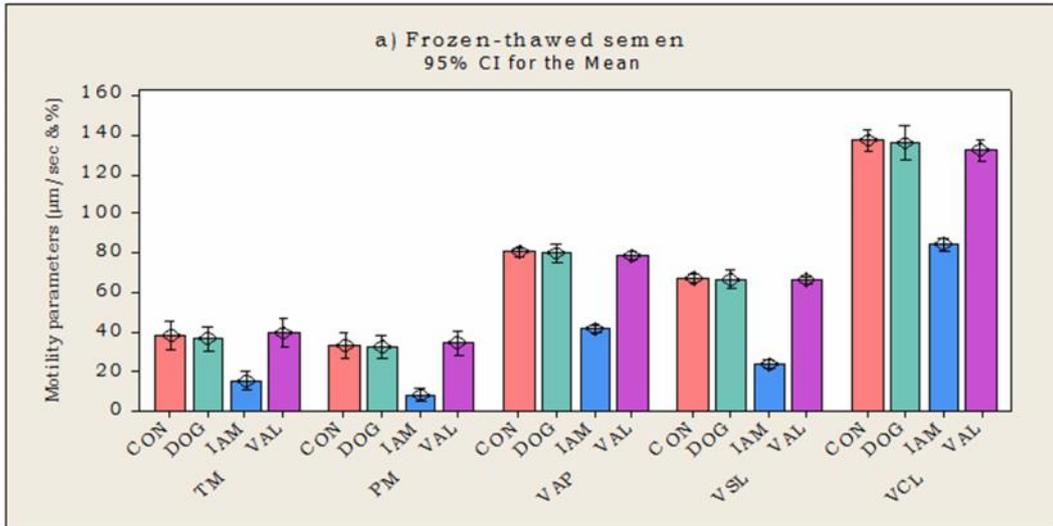
Variables	Semen sample	Bull identification				Mean
		1	2	3	4	
Average path velocity ($\mu\text{m/s}$)	Fresh	^b 90.9 \pm 2.1	^b 90.8 \pm 9.0	^{ab} 95.3 \pm 6.1	^a 107.8 \pm 4.7	96.2 \pm 9.0
	Frozen-thawed	^a 83.8 \pm 2.1	^a 76.8 \pm 3.0	^a 77.8 \pm 3.1	^a 84.0 \pm 4.6	80.6 \pm 4.5
	Significance*	p<0.01	p<0.05	p<0.01	p<0.001	p<0.001
Straight line velocity ($\mu\text{m/s}$)	Fresh	^b 73.7 \pm 2.3	^b 79.4 \pm 8.6	^{ab} 85.9 \pm 4.8	^a 92.7 \pm 4.7	82.9 \pm 8.9
	Frozen-thawed	^{ab} 68.9 \pm 1.8	^{ab} 66.8 \pm 2.8	^b 63.1 \pm 3.0	^a 71.2 \pm 5.8	67.5 \pm 4.5
	Significance*	p<0.05	p<0.05	p<0.001	p<0.01	p<0.001
Path straightness (%)	Fresh	^c 79.5 \pm 2.4	^{ab} 86.3 \pm 1.3	^a 89.5 \pm 1.0	^b 84.0 \pm 0.8	84.8 \pm 4.0
	Frozen-thawed	^{ab} 80.3 \pm 1.7	^a 84.5 \pm 1.3	^b 78.0 \pm 2.9	^{ab} 81.5 \pm 3.4	81.1 \pm 3.3
	Significance*	ns	ns	p<0.001	ns	p<0.01
Linearity (%)	Fresh	^d 46.8 \pm 2.2	^b 55.3 \pm 1.3	^a 60.5 \pm 0.6	^c 51.0 \pm 0.8	53.4 \pm 5.4
	Frozen-thawed	^b 48.8 \pm 1.3	^a 55.3 \pm 1.5	^b 47.0 \pm 2.7	^b 48.3 \pm 3.4	49.8 \pm 3.9
	Significance*	ns	ns	p<0.001	ns	p<0.05

Means that do not share a letter are significantly different between bulls.

*= difference between fresh and frozen-thawed semen.



Appendix 7.2 (a & b). Motility characteristics of fresh sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors and their comparison with control (CON).



Appendix 7.3 (a & b). Motility characteristics of frozen-thawed sperm incubated in diluents containing glycolytic (DOG and IAM) and respiratory (VAL) inhibitors and their comparison with control (CON).