STUDIES ON CRYOPRESERVATION OF MITHUN SEMEN

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OF

DOCTOR OF PHILOSOPHY

in

ANIMAL PRODUCTION AND MANAGEMENT

By

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DEPARTMENT OF ANIMAL PRODUCTION AND MANAGEMENT

SCHOOL OF AGRICULTURAL SCIENCES AND RURAL DEVELOPMENT

NAGALANO UNIVERSITY

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2014

Dedicated to:

My Beloved

Parents

DECLARATION

I, Dr. Bhaskar Bora hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to Nagaland University for the degree of Doctor of Philosophy in Animal Production & Management.

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All help received by him have been duly acknowledged. No part of this thesis has been reproduced elsewhere for any degree/diploma.

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CONTENTS

CHAPTER	43	TITLE	PAGE NO.
1.	INTR	ODUCTION	1-2
II.	REVI	EW OF LITERATURE	3-29
	2.1.	Cryopreservation of semen using different level of glycerol and its effect on different seminal parameters (progressive motility, sperm concentration, acrosome integrity)	3
	2.2.	Non-split & split method of glycerol addition, temperature of glycerol addition	14
	2.3.	Dual or triple staining for acrosomal integrity	18
	2.4.	Apoptosis of sperm	25
IIL	MAT	ERIALS AND METHODS	30-35
	3.1.	Location of the work	30
	3.2.		30
	3.3.	Semen collection, Evaluation, Dilution and	30
	3.3.	Preservation	-
	3.4.		33
	3.5.	Experiment 2. (Split method of glycerol addition)	33
	3.6.	Evaluation of acrosomal integrity and	33
	9,0.	morphological abnormality.	
	3.7.	Study of Apoptosis	34
	3.8.	Statistical analysis	35
	3.0.	Districtions district 210	
IV.	EXP	ERIMENTAL FINDINGS	36-69
	4.1.	Addition of glycerol in single dose (Non-split) 4.1.1. Progressive motility 4.1.2. Acrosomal Integrity 4.1.2.1. Live intact 4.1.2.2. Live damage 4.1.2.3. Dead intact	36 36 39 39 39 40 40
		4.1.2.4. Dead damage	40
		4.1.3. Sperm abnormalities	
		4.1.3.1. Head abnormalities	44
		4.1.3.2. Mid-piece abnormalities	44
		4.1.3.3. Tail abnormalities	45
		4.1.3.4. Total sperm abnormalities	45
	4.2	Addition of glycerol in split dose	49
		4.2.1. Progressive monliny	49
		4.2.2. Acrosomal integrity	49

		4.2.2.1. Live intact	49
		4.2.2.2. Live damage	52
		4.2.2.3. Dead intact	52
		4.2.2.4. Dead damage	56
	4.3	Sperm abnormalities	56
	ne.	4.3.1. Head abnormalities	56
		4.3.2 Mid-piece abnormalities	56
		4.3.3. Tail abnormalities	57
		4.3.4. Total sperm abnormalities	57
	4.4	Comparison of post freeze-thaw sperm qualities between glycerol addition methods	61
	4.5	Study on Apoptosis	61
	4,5	4.5.1. Non-split	61
		4.5.2. Split	62
		4.5.3. Comparison of apoptosis in non-split and split method of addition	62
V.	DISC	CUSSION	70-77
	5.1.	Addition of glycerol in single dose	70
		Addition of glycerol in split dose	72
		Double staining	74
	5.4.	Apoptosis of sperm	74
VL	SUM	MARY AND CONCLUSION	78-83
	BIBL	LIOGRAPHY	i-ix

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
4.1.	Variations (Mean ± SE) in progressive motility of Mithun spermatozon during different Stages of freezing in Non-split glycerol addition method.	37
4.2	Variations (Mean ± SE) in liveability and acrosomal integrity of Mithun spermatozoa during different stages of free zing in non-split glycerol addition method.	41
4.3.	Variations (Mean # SE) in head, mid piece, tail and total abnormality of Mittiun spermatozoa during different stages of freezing intron-split glycerol addition method.	46
4.4	Variations (Mean & SE) in progressive motility of Mithun spermatozoa during different stages of freezing in split glycerol addition method.	50
4,5,	Variations (Mean + SE) in liveability and acrossomal integrity of Mithun spermatozoa during different stages of freezing in split glycerol addition method.	53
4.6.	Variations (Mean # SE) in head, mid piece, tail and total abnormality of Mithun spermatozon during different stages of freezing in split glycerol addition method at different stages of ergopreservation	55
4.7	Comparison of seminal parameters between Non-split and split method of glycerol additionaliter freezing and thawing	63
4.8	Mean (±SE) Percentage of apoptotic sperm in Non-split glycerol addition method at different stages of eryopreservation	66
4.9.	Mean (#SE) apoptotic sperin percentage in split glycerol addition method at different stages of cryopreservation	66
4.10.	Comparison of apoptotic sperm percentage at different stages of eryopreservation in Non-split and split method of addition of glycerol.	68
4.11.	Comparison between the rocan apoptotic spenn percentage in the split and non split method or addition of glycerol in different stages of cry opreservation.	68

LIST OF FIGURES

Figure No.	CAPTION	PAGE NO.
4,1.	Variations (Mean ± SE) in progressive motility during different stages of freezing in non-split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05)	38
4.2	Variations (Mean ± SE) in live spermatozoa with intact acrosome during different stages of freezing in non-split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	42
4.3.	Variations (Mean ± SE) in live spermato7.00 with damage acrosome during different stages of freezing in non-split glycerol addition method. a b on error bar indicates a significant difference (P<0.05)	42
4.4.	Variations (Mean ± SE) in dead spermatozoa with intact acrossome during different stages of freezing in non-split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	43
4,5.	Variations (Mean ± SE) in dead spermatozoa with damage acrosome during different stages of freezing in non-split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	43
4.6.	Variations (Mean ± SE) in head abnormality of spermatozoa during different stages of freezing in non-split glycerol addition method, a hon error bar indicates (P<0.05)	47
4.7.	Variations (Mean ± SE) in mid-piece abnormality of spermatozoa during different stages of freezing in non-spht glyecrol addition method, a, b on error bar indicates (P<0.05)	47
48.	Variations (Mean ± SE) in tail abnormality of spermatozoa daring different stages of freezing in non-split glycerol addition method, a boa error bar indicates (P<0.05)	48
4.9	Variations (Mean ± SE) in total abnormality of spermatozoa during different stages of fireezing in non-split glyceror addition method, a b on error bar indicates (P<0.05)	48

4:10	Variations (Mean ± SE) in progressive motility during different stages of freezing in split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	51
A)TT	Variations (Mean ± SE) in live spermatozoes with intact acrosome during different stages or freezing in split glycerol addition method, a b on error bar indicates a significant difference (P<0.05)	54
4.12	Variations (Mean ± SE) in live spermatoz.on with damage acrosome during different stages of freezing in split glycerol addition method, a, b on error bar indicales a significant difference (P<0.05)	54
xi 13	Variations (Mean ± SE) in dead spermatozoa with intact acrosome during different stages of freezing in split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	55
4.14	Variations (Mean ± SE) in dead spermatozo a with damage acrosome during different stages of freezing in split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05)	55
4115	Variations (Mean ± SE) in lead abnormality of spermatozoa during different stages of freezing in split glycerol addition method, a, b on error bar indicates (P<0.05)	59
4:16	Variations (Mean ± SE) in mid-piece abnormality of spermatozoa during different stages of freezing in split glycerol addition method, a b on error bar indicates (P<0.05)	59
4.17	Variations (Mean ± Sl3) in tail abnormality of Spermatozoa during different stages of freezing in split glycerol addition method, a bon error bar indicates (1*<0.05)	60
4:18	Variations (Mean ± SE) in total sperm abnormality of spermalozoa during different stages of licezing in split glycerol addition method, a b on error bar indicates (P<0.05)	60
4,19	Comparison of seminal parameters between Non-split and split method of gly cerol addition after freezing and thawing.	64
A 201	Apoptotic sperm percentage in Non-split glycerol addition method at different stages of cryopreservation (* indicates p< 0.05). A. B. C. Don error bar indicates (P<0.05)	67
4,23	Apoptotic sperm percentage in split glycerol addition method at different stages of cryopreservation (* indicates p< 0.05), A. B. Con error bar indicates (P<0.05)	67

4.22 Comparison of apoptotic sperm percentage at different stages of cryopreservation in Non-split and split method of addition of glycerol
4.23 Apoptotic sperm percentage in split and non split method of addition of glycerol at different concentration in different stages of cryopreservation. a, b, c, on error bar indicates(P<0.05)

LIST OF PHOTOS

PHOTO NO.	CAPTION	PAGE NO.
A	Collection of semen by massage method.	30
В	Photographs showing four categories of spermatozoa with different acrosomal status.	After Page No.43
С	Photographs showing different spermatozoa abnormalities.	After Page No. 48
D	Photographs showing normal and apoptotic sperm cell stained with FITC and propidium iodide.	After Page No. 69

ABBREVIATIONS

- Degree centrigrade 10 -Microlitre nî. - Bovine serum albumin DEA 1.354 - Computer automated semen analyzer CH. - Contimeter - Carbondyoxide KAT. - Cumulus oucyte complex FIRE - Cryoprotectant ERA DD - Dead damage - Dead intact 131 - Denxynucleic acid DHA Ontis - Dubbeccos phosphate buffer saline Dex - Dibutyil phathalate xylene - Dead spermatozoa with damage acros ome unths: DELAS - Dead spermatozoa with live intact acrosome EHY - Fluorescein isothiocyanate GUY--Glycerul - Hour - Hydrochloric acid \$1611. - Hyponsmotic syselling test 1120 0011 - International unit 林三 - Liquid crystal display DUTT LD · Live damage - Live intact m IX. - Liquid Nitrogen - Live spermatozoa with dead acrosome:

-live spermatozoa with intact acrosome

- Milligram per millilite:

- Miliosmol per kilogram

Linea

my mi

mi/mag

MIS

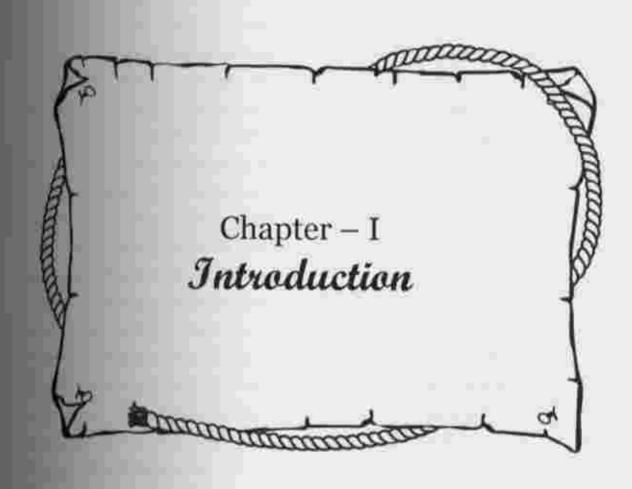
PVA

SYUR

WY.

AHES

- Phosphate buffer saline
- Phosphatidy Iserine
- Poly viny Lalcohol
- -Syber
- Volume by volume
- Microgram per mililitre
- Micro molar per second
- Second



INTRODUCTION

North-Fast India is the confluence of three major bio-geographical realm of the world and is extremely rich in florat and faunat biodiversity. Among the 7 northeastern states Nagaland is endowed with rich germptasm of different endemic species including mithun (Bax frontalis). Mithun, the 'eattle of mountains' probably originated more than 8000 years ago and is considered as the domesticated form of wild gaur. This rare species of boxine has a limited geographical distribution and mainty found in the 4 north-eastern states of Arunachal Pradesh, Nagaland, Mizoram and Manipur. However it is also found in small numbers in Myanmar, China, Bangladesh and Bhutan. This species is reared under the free range system at an elevation of 1000 to 3000 m above sea level. This unique species is primarily used for meat production and it also plays an important role in the socio economic life of its rearers (Simmons, 1984; Mondal and Pal, 1999). However, uncontrolled breeding in the existing free range rearing system may lead to geneficially inferior herd and crossbreeding with other bovine species may lead to decline in quality Mithun germplasm. Therefore an intense conservation and propagation programme is urgently required for this animal. Considering the importance of conserving this animal species modern biotechnological toots like semen cryopreservation coupled with A.I is necessary. The production performance of Mithun cows can be increased by breeding females with semen of the hot's of superior genetic merit. But due to non availability of a system to replace bulls at regular interval to maintain the genetic quality, there is a need to focus on some alternative way for breeding. This can be achieved easily through artificial insemination (AI) with the somen of proven Mithan hulls or by MOET (Multiple ovolation and embryo transfer). Artificial insemination (At) has become one of the most important techniques ever devised for the genetic improvement of farm animals like cattle, buffalo etc. Cryo-preservation of semen is used to reduce metabolism and to maintain sperm viability over an extended period of time.

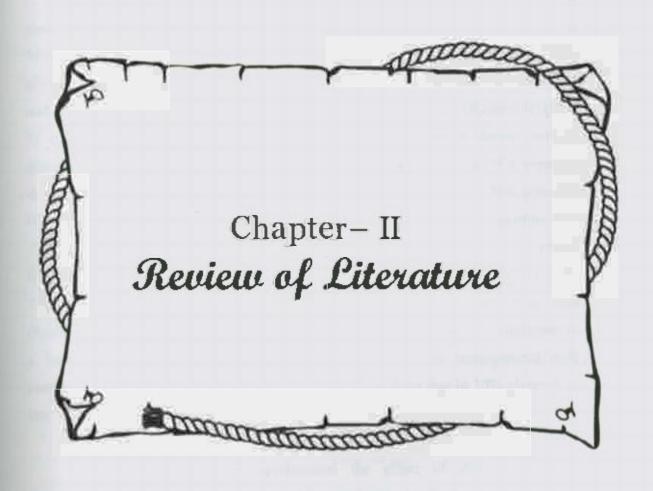
Semen cryopreservation offers the opportunity to improve the breeding potential of male animals. Semen storage technology was revolutionized more than 50 years ago by the discovery of the cryoprotective capacity of glycerol. The use of

glycerol can be toxic in certain concentrations. These specific concentration levels are species specific. Bull spermatozoa are routinely eryopre served using 4% to 8% glycerol (Fennessy et ed., 1990). However, there is very limited information in Mithuo, lo this context, it is very essential to standardize a suitable glycerol concentration in order to adopt A.1 in Mithun, II is reported that glycerol content of diluents can affect the post-thaw motility of spermatozoa (Rolt, 2000; Thun et al., 2002). The post thaw survival of spermatozoa can be influenced by the way in which glycerol is added prior to freezing (Fiser and Fairfull et al., 1989).

Morphology and acrosomal integrity are valuable markers of cryo-survival. The primary driving force dictating cryo-survival of spermatozoa is its initial prefreeze quality and morphology, especially having a morphologically normal, intact acrosome. The initial structural integrity of the acrosome is more important predictor of post-thaw sperm survivality than initial sperm motility score (Pukazhenthi & &d., 2007). The acrosomal integrity status can be used as a suitable marker for fertilization ability of spermatozoa (Jankovikova & &d., 2008).

Apoptosis is a physiologically programmed cell death characterized by shrinkage of cell, reduction of cytoplasmic volume, condensation of chromatin, DNA cleavage and nuclear fragmentation. A significant negative correlation was reported between the proportion of apoptotic cells and sperm viability or motility in ejaculated semen. Additionally the presence of apoptosis in sperm cells has been correlated with infertility. Evaluation of apoptosis in sperm cells would be a good indicative of the semen quality (Feitesa et al., 2008). Considering these facts the present research programme has been taken with the following objectives:

- To study the effect of different glycerol concentrations in the extender on post cryopreservation quality of mithun someo.
- To study the effect of different methods of glycerol addition in the extender or post cryopreservation quality of million semen.
- To study the effect of cryopreservation on acrosomal integrity and apoptosis
 of mithun spermatozoa.



REVIEW OF LITERATURE

2.1. Cryopreservation of semen using different levels of glycerol and its effect on different seminal parameters (progressive motility, sperm concentration, acrosome integrity)

Almquist (1962) incorporated 10 or 13% glycerol in skim milk diluents by gradual addition to partially diluted semen at 5°C and Spermatozoon livability during 14 days if stored at 5 °C was higher ($P \le 0.01$) than in the absence of glycerol. These glycerol levels prevented the sharp decline in motility observed between the fourth and sixth class of storage for semen diluted in skim milk glycerol. Glycerol Jevels of 16 to 25% did not significantly improve livability over semen diluted without glycerol. Four field trials involving 21.676 inseminations were conducted to compare the fertility of semen diluted in milk with that diluted in milk-10% glycerol. Homogenized milk was in two trials and fresh, unfortified slim milk in the other two. The combined data showed that addition of glycerol significantly ($P \le 0.01$) increased ficiality of semen used the second, third and fourth days after the day of collection by 7.4. 12.2. and 19.5 percentage units, respectively. The increase of 2.3 percentage units obtained with glycerolated semen used the day after collection was not significant, in a limited split-e jaculated trial, fertility of semen diluted in homogenized milk containing 15% glycerol was not significantly different from that in 10% glycerol on any of the three days of use.

Pursel et al. (1978) experimented the effect of different glycerol concentration on frozen boar sperm, in experiment it. Beltsville F3 extender containing 0 to 7% glycerol was added to boar sperm. Glycerol was either retained during freezing or removed by centrifugation before freezing. Whell glycerol was retained there was significant nogative linear relationship between the percentage of sperm acrosomes with a normal upiculi ridge (NAR) and the percentage of glycerol. When glycerol was removed before freezing, the percentage of NAR acrosomes did not differ among samples. The percentage of motile sperm and the percentage of glycerol in the original extender were linearly related regardless of whether glycerol was retained or removed before freezing, in experiment 2 four concentrations of

glycerol, three cooling times and two dilution rates were compared when semen was frozen in Beltsville F5 extender. The post-thaw results for percentages of NAR acrosomes and sperm motility were optimum with 1% glycerol and a 1:4 dilution rate. Cooling time had a minor affection on the freezing results. In experiment 3, the competitive fertilizing capacity of boar sperm frozen with 194 glycerol was compared with that frozen without glycerol. The number of ova fertilized by sperm frozen with 0 or 1% glycerol, 52 and 72 ova, respectively, were nearing statistically different from a 50:50 ratio (P < .070). These results indicated that the conditions of this study glycerol was of some positive value as a cryoprotectants for boar sperm.

Scheid *et al.* (1980) investigated the effect of four different glycerol concentrations in the deep freezing of boar semen in plastic tubes. The sperm-rich portion of 23 ejaculates was processed for freezing, the final extender containing 0, 2, 4 or 6% glycerol. Post-thaving morility for semen in the 4 diluent averaged 5.3, 31.5, 41.5 and 50.6% resp. (P \leq 0.001), and the proportion of acrosomes with normal apical ridge (NAR) 68.8, 48.2, 22.6 and 5.2% (P \leq 0.001). Preliminary results were available from an insemination trial. Nine ejaculates from 7 boars selected for good semen freezability were processed, glycerol levels of 0, 2 or 6% being used. Post thaving motility averaged 9.4, 35.0 and 51.2% resp., and NARs 69.1,56.3 and 22.3% of 7, 2 and 4 females inseminated, 6, 2 and 3 conceived, with an av. 8.8, 12.0 and 9.0 embryos and 16.2, 180 and 13.7 corpora lutea.

Almlid and Johnson (1988) conducted experiments (0 study the effect of glycerol concentration, equilibration time and temperature of glycerol addition on post thaw viability of boar spermatozoa after cryopreservation in straws. Semen (split ejaculate) in maxi-straws (6 mm o.d.) was frozen using a programmable freezing chamber. Three parameters for in vitro sperm evaluation were used: Motility (MOT) acrosome integrity (NAR) and flow cytometric analysis of sperm treated with carboxyfluorescein diacetate and propidium iodide to assess sperm plasma membrane integrity. No interactions were found among the three variables evaluated. Length of prefreeze exposure to glycerol, ranging from 0.5 to 75 min, had no effect on post thaw sperm viability. Exposure of to a glycerol containing extender medium at 5°C gave improved post-thaw viability over that exposed at o°C (p< o.o5). Glycerol at goncentration of 3 or 4% resulted in maximum post thaw motility. Acrosome integrity

values were greatest for 2 and 3% glycerol, whereas PMI was greatest when glycerol concentration was 4 to 6%. The primary cryoprotective: effect of glycerol on boar semen may be extracellular. It is concluded that 3 or 4% glycerol gives maximum viability of fruzen-thawed boar spermatozoa.

Zheng et al. (1992) studied the effects of two concentrations of glycerol 2% and 6% in a freezing diluent with respect to the in vitro fertilizing capacity of frozen-thawed boar spermatozoa which before exposure to oocytes were subjected to 3 methods of fractionation. These were 1) The upper fraction 2) The swim-up and 3) Percoll gradient-centrifugation. They found that by raising the glycerol concentration from 2.6% (v/v) during freezing decreased the proportion of spermatozoa with a normal apical ridge. They concluded that the glycerol level affected penetration and polyspermy only with spermatozoa separated in percoll gradient, where the higher level of glycerol increased oocytes penetration and polyspermy.

Rota et al. (1998) investigated to find out the best combination of extender and freezing thawing rate of dog spermatozoa. Four ejaculates were obtained from each other of five dogs and split samples were diluted in a Tris citrate glucose extender containing 0.5% Equex STM paste and either 3 or 5% glycerol. Two ejaculates from each dog were frozen at a slow freezing rate (10°C/min in the range 6° to 40°C) in 0.5mt straws using a programmable freezer. Prior to evaluation, the straws from each freezing rate and glycerol concentration were thawed in a water bath, either at 38°C for 1 min or at 70°C for 8 s. Samples of cryopreserved semen were evaluated for motility and for the proportion of spermatozoa having an intact plasma membrane, immediately after thawing and during 5 h of incubation at 38°c. The higher glycerol concentration and the faster thawing rate tested favored the recovery of living and motile spermatozoa and their survival during incubation While the tested freezing rates had no influence on sperm post-thaw long evity.

YI et al. (2002) conducted a series of experiments to determine the effects of N-acety l-D-glucosamine, glycerol concentration and equilibration time for the freezing of boar spermatozoa in 5ml maxi-straws. The final glycerol concentration in the first diluent was 2-3%, 11% lactose hydrate, 20% egg yolk, 0.05% N-acetyl D-glucosamine and glycerol equilibration time was 2-3 hrs. The glycerol concentration

in the second diluents was 4% with 11% lactose hydrate. 20% of egg yolk and 1% orwus as paste for the diluents of boar sperm freezing. They found the first diluent was better than the second.

Garde et al. (2003) tried to evaluate various diluents for the eryopreservation of spermatozoa from the 3 species of gazelles. The diluents compared were Tes [Ntris(hydrozymethyl) methyl-2 amionoethane sulfonic acid |- Tris with 596 egg yolk and 6% glycerol (TEST) and Triladyl, yolk-citrate. Tristrehalose and Tris-lactose-all of them with 20% egg yolk and 6% (Triladyl) or8% glycerol. Semen was obtained by electro e jaculation from 12 G. cuvieri. 12Gdama and 13Gdorcas males. Samples with less than 50% motile sperm, positive endosmosis, or acrosome integrity were not used. Diluted samples were loaded into 0.25-ml straws, eooled slowly to 5°C over 1,5 h(-0.16 degrees C/min), equilibrated at the temperature for 2 h frozen in nitrogen vapors for 10 min, and plunged into liquid nitrogen. Subsamples were assessed fresh, after refrigeration-equilibration, after freezing and thawing, and 2 h after thawing, Differences were soon between diluents, with best overall recovery rates after freezing and thawing found with Triladyl, TEST, and Tris-trehalose in G cuvieri, TEST in G.dama, and Trilady I and TEST in G.dorcas, Differences were observed between species in the ability to withstand freezing and thawing, with best result seen in G.dama, and worst results in G.cuvieri. These differences were inversely related to the average values of inbreeding of these populations. The underlying mechanism respotisible for these differences may be a differential resistance to osmotic shock.

Gil et al. (2003) compared the effect on post thaw ram sperm parameters of a milk based extender containing four different egg yok concentrations and a commercially available extender (Bioexcell) containing two different final glycerol concentrations (3,2% and 6,4%). In both experiments glycerol was added either at 5 degree Celsius or al 15 degree Celsius together with the second fraction of each extender. The sperm concentrations assessed were motility, membrane integrity (SYBR-14/PI) and capacitation status. The results indicate that when freezing ram semen, Bioexcell containing 6.4% glycerol may be used as an alternative extender to the conventional milk extender containing 5% egg yolk.

Hong et al. (2003) studied the effect of glycerol concentration on cryopreservation of blue faxes sperm. They used glycerol concentration of 2, 4, 6 and 8% for cryopreservation. The samples were equilibrated for 2h at 5°C and packed in 0.5ml straws. Freezing was done on a rack 5cm above liquid nitrogen and thawed at 70°C for 9s. Before and after freezing-thawing, the effects of different concentrations of glycerol on the changes in spermatozoa characteristics and the alterations in the ultrastructure of spermatozoa were studied by transmission electron microscopy. The sperm motility, plasma membrane integrity of spermatozoa and percentage of intact acrosomes were related to the dose of glycerol. All these characteristics were highest in the fraction, wherein the final glycerol concentration was 4% (41.8, 43.6 and 48.4% whereas the lowest appeared in the fraction added with 2% glycerol (24.5, 27.6 and 31.7%). Along with the extension of incubation, significant differences in characteristics of spermatozoa were observed between sperm fractions of 2 and 4%, whereas the differences between 2, 6 and 89 is sperm fractions were insignificant. After 6h, the motility of each fraction was not more than 10%, whereas the highest viability and the percentage of intact acrosomes were 11.8 and 12.7% respectively.

Wani et al. (2004) conducted experiments to study the effect of cryoprotectants, dimethyl sulfoxide (DMSO), ethylene glycol (EG), 1,2-propanediol (PROH), and glycerol at different concentrations (3.5, 4, 5, 6, and 7 M each with 0.5 M sucrose and 0.4% BSA in DPBS, on survival, in vitro maturation, in vitro fertilization, and post-fertilization development of vitrified-thawed immature bulfalo oocytes. The COCs were harvested from the ovaries by aspirating the visible follicles. The recovery of post-thaw morphologically normal oocytes was lower in 3.5 and 4 M DMSO, EG, and PROI-I compared to 5, 6, and 7 M. In all the concentrations of glycerol, an overall lower numbers of oocytes recovered were normal compared to other cryoprotectants. Less number of oocytes reached metaphase-II (M-II) stage from the oocytes cryopreserved in any of the concentrations of DMSO, EG, PROH, and glycerol compared to fresh oocytes. Among the vitrified groups, highest maturation was obtained in 7 M solutions of all the cryoprotectants. The cleavage rates of oocytes vitrified in different concentrations of DMSO, EG, PROH, and glycerof were lower than that of the fresh oocytes. The cleavage rates were higher in oocytes cryopreserved in 6 and 7 M DMSO. EG. PROLL and glycerol compared with oncytes cryopreserved in other concentrations. However, the percentage of morula and blastocyst formation from the cleaved embryos did not vary in fresh occytes and vitrified occytes. In conclusion, this report describes the first successful production of buffalo blastocysts from immature occytes cryopreserved by vitrification.

Wei si et al. (2004) studied the effect of glycerol and DMSO on cryopre-servation of the sus monkey sperm. They used different concentrations (2%) 5%, 10% and 15% of glycerol or DMSO) on thesus monkey spent cryopreservation. The results showed that the highest recovery of post-thaw sperm motility, and plasma membrane and acrosome integrity was achieved when the sperm was frozen with 5% glycerol. Spermatozoa cryopreserved with 15% DMSO showed the lowest post-thaw sperm motility, and spermatozoa cryopreserved with 15% glycerol and 15% DMSO showed the lowest plasma membrane integrity among the eight groups. The results achieved with 5% glycerol were significantly better for all parameters than those obtained with 5% DMSO. The functional cryosurvival of sperm frozen with 5% glycerol was further assessed by in vitro fertilization (IVF), Overall, 85.7% of the oncive were successfully fertilized, and 51,4% and 5.7% of the resulting zygotes developed into morulae and blastocysts, respectively. The results indicated that the type and concentration of the ponetrating cryoprotectants used can greatly affect the survival of rhesus monkey sperm after it is irozen and thawed. This suitable glycerol level for rhesus monkey spertn freezing is 5%, and DMSO is not suitable for rhesus monkey sperm cryopreservation.

Fiser and Fairfull (2005) studied the combined effect of glycerol concentration and cooling velocity and acrosomal integrity of boar spermatozoa. The interaction of glycerol concentrations of 0-10% and cooling rates from 1 to 1,500°C/min with boar spermalozoa motility acrosomal integrity (proportion of spermatozoa with normal local ridge) was studied after thawing 0.5ml straws at a constant rate. While increasing the glycerol concentration from 0 to 4% progressively improved motility, the percentage of spermatozoa with a normal apical ridge gradually decreased. The magnitudes of the respective changes depend on cooling rate. A peak value of 48.1% and rating 3.8 were obtained in semen protected with 4% glycerol, frozen at 30°C/min, increasing the glycerol levels above 6% resulted in a gradual decrease in motility. The proportion of spermatozoa with normal apical ridge was higher in semen protected with 0-1% glycerol after cooling at 30° C/min (64, 4%)

and 66.1% respectively), but at these glycerol concentrations the percentage of motile spermatozoa was low. At the 30°C/min cooling rate, the decline in the proportion of cells with normal apical ridge due to increasing glycerol levels to 3 and 4% was relatively slow (57.3% and 49.4%, respectively). Cooling at 1°C/min was detrimental to acrosomal integrity, which decreased with increasing glycerol concentration, in contrast to increasing motility, which even as its maximum, remained low. The direct plunging of straws into liquid nitrogen (1.500°C/min) resulted in damaged acrosomes in lift spermatozoa with the total loss of motility. Balancing motility and acrosomal integrity, freezilly boar semen protected with 3% glycerol by cooling at 30°C/min resulted in optimal survival for boaf semen frozen in 0.5 ml French straws.

Kashiwazaki w d. (2006) compared glycerol, lactamide, acetamide and dimethylsulfoxide (DMSO) as eryoprotectants in egg-yolk diluent of ejaculated Japanese white rabbit spermatozoa for improvement of sperm cryopreservation methods. Rilbblt semen was frozell with 1.0 m glycerol, lactamide, acetamide, or DMSO in plastic stratys. Forward progressive motility and plasma membrane integrity of the post-thaw spermatozoa were examined. The rate of forward progressive motile spermatozoa fil lactamide (37.8 \pm 3.0 %) was significantly (p \leq 0.05) higher than in glycerol (17.0 \pm 3.3%). In addition, the rates of sperm plasma membrane integrity in lactamide and acetamide (35.9 \pm 3.3% and 30.2 \pm 3.0%), respectively) were significantly (p \leq 0.05) higher than in glycerol (17.0 \pm 2.6%), the results indicate thall 1.0 m lactamide and acetamide have higher cryopreservative effects than 1.0 m glycerol for cryopreservation of Japanese white rabbit spermatozoa.

Martinez *et al.* (2006) tested two glycerol concentrations (4 and 8 %) and three extender osmolalities (320, 380 and 430 mOsm/kg; before adding cryoprotectants) for cryopreservation of epididymal and ejaculated spenn samples from Iberian red deer. All the extenders were based on Tes-Tris and fructose (for osmolality adjustment), and complemented with 20% egg yolk. Epididymal and ejaculated sperm samples were obtained from the cauda epididymis (Post-mortem) and using electroejaculation, respectively. Samples were diluted 1:1 with each extender and equilibrated for 2 h at 5 degrees C. Then, they were diluted down to 100 × 10(6) sperm/ml and frozen a -20 degrees C/min. Post-thawed samples were assessed for motility (CASA). HOS test, proportion of swollen (osmotically

challenged) eells in the untreated samples, viability and acrosomal status. For epididymal samples, 8% glycerol rendered a slightly higher proportion of intact acrosomes on viable spermatozoa than 4%; regarding extenders osmolality, 380 and 430 mOsm/kg rendered higher motility results, and the 430 mOsm/kg yielded the lowest proportion of swollen spermatozoa. For ejaculated samples, 4% glycerol yielded more viable spermatozoa than 8 %; for extender osmolality, 320 mOsm/kg rendered the highest percentages of progressively motile and viable spermatozoa, although 380 mOsm/kg extender was not significantly different. These results show that sample source influences extender suitability, and that extender should be isosmotic or rather slightly hyperosmotic. Future studies should test multiple glycerol concentrations and extender osmolalities in order to adjust them to these kinds of sample.

Santos et al. (2006) conducted two experiments to determine the effects of egg yolk (EY), glycerol, and eooling rate on the cryosurvival of red deer epididymal spermatozoa. The aim of Experiment 1 was to examine the effects of two EY types (clarified EY, CE, prepared by centrifugation, and whole EY, WE), and four EY concentration (0, 5, 10 and 20%) on ervosurvival of red deer epididymal spermatozoa. Sperm samples were diluted to a final sperm concentration of -200 × 106 spermatozoa/ml with a Tris-citrate-fructose-EY extender (TCF) prior to freezing. Sperm eryosurvival was judge in vitro by microscopic assessments of individual sperm motility, viable and plasma membrane (by means of HOS test and acrosome (NAR) integrities. Cryopreservation of red deer epididymal spermatozoa frozen in a clarified EY extender, and with a 20% EY resulted in more vigorous post-thaw and post-incubation motificies $(P \le 0.0001)$. Moreover, our results showed that regardless of the egg yolk concentration tested, the best sperm quality was obtained with the use of CE. Therefore, the objective of Experiment 2 was to explore the post-thaw effects of four clarified egg yolk concentrations (0, 5, 10, and 20%), two final giveerol concentrations (3 and 6%), and two cooling rates from 22 to 5°C (slovy.0.23°C/min; rapid: 4.2°C/min) on red deer epididymal spermatozoa. Al thawing, the effects of CE and glycerol concentrations, and cooling rate, all independently affected post-thaw sperm quality, while there were no effects of interaction on post-thawing sperm quality. Therefore, we studied each variable separately. Differences (P < 0.05) for most of the sperm parameters evaluated were found between the two final glycerol

concentrations tested, with the high values after thawing found with the use of 6% glycerol (58.8 \pm 1.4 versus 46.2 \pm 1.4 for sperm motility). Moreover, the cooling rate did not have an effect on the semen characteristics, except for NAR (P \leq 0.05), with the high values after thawing found with the use of the rapid protocol (64.5 \pm 1.4 versus 59.9 \pm 1.4). In conclusion, the use of 20% CE and 6% glycerol in combination with a rapid cooling rate, significantly improved red deer epididymal spermatozoa freezability.

Santos et al. (2006) with the aim of finding an ideal cryoptotectant (CPA) in a suitable concentration for red deer epididymal spermatozoa eryopreservation, they evaluated the effects of the 3 most commonly used CPAs, glycerul (GY1), ethylene glycol (EG), and propylene glycol(PG), on sperm cryo-resistance. The aim of Experiment 1 was to evaluate the influence of 3 different final concentrations (3%). 6% and 12%) of each CPA on sperm freezability. Sperm samples were diluted to a final sperm concentration of ~400 × 10° spermatozoa/ml with a Tris-citrate-fructose-EY extender (ICF) prior to freezing. Sperm cryosurvival was judged in vitro by microscopic assessments of individual sperm mofflity (SMI), viability, and plasma membrane (by means of the HOS test) and acrosome (NAR) integrities. Thawed samples were incubated at 37°C for 2 hours in the freezing medium. At the end of this incubation period, sperm suspension was again assessed. Our results showed that 12% of any CPA was toxic 10 red deer epididymal spermatozoa membrane integrity (PS .05). Moreover, regardless of the level of CPA, results indicated that the eryoprotective effects on red deer epididymal spermatozon of the 3 CPAs after thawing are in the following sequence: $GLY \ge EG \ge PG$ (higher symbols mean $P \le$ (001). Furthermore, our results also showed an Improvement in sperm parameters when the TCF diluents contained 6% of GLY. In Experiment 2 extenders were prepared using GLY 6%. This experiment was designed to investigate the effect of 2 different temperature of Cl. Y addition - 22°C (ambient temperature) and 5°C2 on sperm fre er ability. Our result showed a differential response (P≤ .05) of motility (smj) to temperature of GYL addition before freezing, the best being 22°C (81.94 ± 2.4% vs. 72.38 ± 2.4%). Although there were no significant differences (P≥ .05) between the 2 temperature of GYL addition after thawing in term of sperm quality. after 2 hours of incubation, results tended to be better when CPAs were added at 22°C. In conclusion, the work showed the efficacy of TCF diluents with 6 % of GYL

and its addition at 22°C, as an alternative to the more common 3% - 4% GYL and addition at 5°C in red deer senten freezing protocols.

Rasul et al. (2007) used 6% glycerol for cryopreserving buffalo semen. He found that glycerol (6%) in extender yielded better post thaw sperm motility, velocities (straight-line and average path) plasma membrane integrity and normal acrosomes than DMSO. The addition of glycerol (6%) at 37°C yielded better post-thaw sperm motility, plasma membrane integrity and velocities than addition at 4°C (P≤0.05).

Dhali et al. (2008) used 5.6% glycerol for cryopreserving mithun semen and got 55% post thaw motility, 65% intact acrosome and 75% spermatozoa were morphologically normat after cryopreservation.

Garde et al. (2008) studied the effect of egg yolk, cryoprotectants and various sugars on semen eryopreservation in endangered Cuvier's gazelle. Cryopreservation of spermatozoa from endangered species is a valuable tool for genetic management. Previous studies showed the feasibility of cryopreservation of spermalozoa from various endangered gaz elles but have also revealed difficulties with available protocols for semen freezing in Cuvier's gazelle (Gazelle cuvieri). Experiments were carried out to investigate the effect of (a) 5% or 20% egg yolk or 4% or 6% glycerol, and (b) addition of sugars (glucose, fructose, lactose and raffinose) on cryopreservation using Tes-Iris-based diluents (TEST). A diluent containing 13.5% raftinose, 5% or 20% egg yolk, and 6% glycerol (REYG) was also evaluated. Semen was obtained by electro ejaculation from 22 G cuvieri males. Diluted samples were baded into 0.25 ml straws, cooled to 5°C over 1.5h (-0.16 °C/min), equilibrated a the temperature for 2h, frozen in nitrogen vapors for 10 min and plunged into liquid nitrogen. Subsamples were assessed for mobility and acrosome integrity upon collection, after refrigeration-equilibration, after freezing and thawing, and 2h after thawing. Use of TEST with 20% egg yolk or with 4% glycerol led to worse motility preservation, whereas ITESI with 5% egg yolk and 6% glycerol led to better results. Addition of fractose or raffinose to TEST resulted in similar or worse preservation of motility than inclusion of glucose. On the other hand, use of a raffinose-based medium with 20% egg yolk and 6% giveerol (REYG) afforded better

afforded better preservation of motility than use of TEST. With REYG, 20% egg yolk was better than 5% egg yolk for motility preservation. Differences were noted between males in their responses to cryopreservation when using different egg yolk or glycerol concentrations. More over, spermatozoa from most males exhibited better cryopreservation with REYG although some were better cryopreserved in TEST. The raffinose-based diluents thus represent an improvement over previous result but more work is needed to better characterize cryopreservation conditions for future routine banking of gazelle spermatozoa.

Mapeka et al. (2009) studied the effect of different cryoprotectants on postthaw survival and motility of Kolbrock sperm. Semen from kolbrock boars was collected with the gloved hand technique. Ejaculates were diluted with Beltsville thawing solution (BTS) at a ratio of 1:1 prior to freezing. Semen was diluted with egg volk tris; thereafter, one of the three cryoprotectants (14% glycerol, 14% DMSO or 7% glycerol + 7% DMSO) was added. Diluted samples were then loaded into 0.5 ml straws and cooled with a programmable freezer. Thereafter the semen straws were plunged directly into liquid nitrogen (-196°C) and stored for 48 h. Frozen straws were thawed at 39°C for a minute and evaluated for sperm motility and survival at 0, 30, 60 and 90 min post-thaw. The post-thaw sperm survival frozen using glycerol as a cryoprotectants was significantly higher immediately after thawing compared to DMSO, however, similar to the combination of glycerol and DMSO. There was no significant difference on motitity rate immediately (0 min) post-thaw between the three cryoprotectants. Sperm cryopreserved with glycerol exhibited significantly higher percentage motility at 30, 60 and 90 min post-thaw than in the other cryoprotectants. Based on sperm motility, glycerot was a better cryoprotectants for eryopreservation of Kolbrock boar sperm.

Nur et al. (2011) investigated the effect of different freezing rates in postthaw sperm motility, acrosome defect, and sperm chromatin structure and apoptotic activity in ram semen. Collected semen was diluted at 1:5 (semen/extender) with Bioxel® (IMV terchnologies France) at 30°C and then cooled to 5°C within 1h Cooled semen was subjected to the equilibration for 2 hours. Equilibrated semen was frozen in 0.25 mt straw at two different cooling rates (slow: 0.5°C/min from 5 to -20°C and fast 5°C/min from 5 to -20°C). Both groups were frozen from -20 to -120°C at 25°C/min and stored in liquid nitrogen until use. Post-thaw (37°C for 30 min) sperm motility, defected acrosome (*Pisum satirum* agglutinith fluorescein conjugate, FTC PSA), sperm chromatin structure determined by Acridin Orange (AO) and apoptotic activity using terminal deoxy nucleotidyl transferase mediated dUIL? nick-end labeling (fUNEL) were evaluated. Post-thaw sperm motility, acrosome defect, AO and TUNEL for slow frozen semen were 428±38%, 31.6±12.9%, 2.9±24% and 2.8±1.6%, and for fast frozen semen were 36.5±9.9%, 24.7±11.1%, 3.3±2.2% and 6.3±3.4%, respectively. Post-thaw semen analysis showed that there was no significant difference between two freezing curves in terms of acrosome defect, sperm chromatin damage (AO). hobseset, a significant difference was found for post-thaw semen motility between two groups (P<0.05). In conclusion, while the slow freezing procedure improved post-thaw sperm motility, acrosome and chromatin integrities and apoptotic index in term spermatozoa did not show any significant difference between freezing rates.

2.2. Non-split & split method of glycerol addition, temperature of glycerol addition

Arriola and foote (1987) in an experiment added a standard egg-yolk-tris extender containing detergent mixture, sodium and friethanolamine lauryl sulfate in liuft sperm. Glycerol was added in one step to the initial extender or in three steps after cooling sernen to 4°C. The extender semen was packaged in .5-ml French straws and frozen over static hitrogen vapor. Thawing was at 4, 30 and 60°C, There were little differences due to the mediod of adding glycerol provided detergent was present. Percentages of motile sperm after freezing in the six concentrations of detergent, in ascerving order, were 31, 45, 52, 51, 48, and 36. Percentages of motile sperm following thawing at 4, 30, and 60°C were 36.5, 46.0 and 48.6. Acrosomes retention also was better preserves with the higher thaw temperatures. A second experiment, similar in design to the first one, was conducted with whole egg-tris extender. The best results were in whole egg-fris containing 0.125% detergent where post-thaw motile sperm of 70.4% greatly exceeded 30.7% without sodium and triethanolamine lauryl sulfate. Fertility of sperm frozen in yolk-Tris-detergent was equivalent to whole milk used as a control.

Fiser and fairfull et al. (1989) found that the post thaw survival of spermatozoa can be influenced by the way in which glycerol is added prior to freezing, but post thaw spermatozoa modility and acrosomal integrity can be maintained even after rapid decrease in glycerol concentration. In the experiment the concentration of glycerol was adjusted to 4°C prior to freezing in one step addition at 30°C (Method 1), by cooling the semen to 5°C and addition of the glycerol gradually over 30 min (Method 2), by one step addition of glycerol prior to equilibration for 2 hrs(Method 3), or by cooling to 5°C followed by a holding period of 2 hrs at 5°C and the one step addition of glycerol just prior to freezing (Method 4). After thaving the glycerol concentration of the semen was reduced by stepwise dilution from 4 to 0.4% over 15 or 30 min or by a one-step ten-fold dilution. When averaged over the method of glycerolation the post thaw percentage of motile spermatozon (43.7-44.2%) and the percentage of intact acrosomes (56.8-59.5%) did not differ significantly in semen subjected to gradual decrease in glycerol concentration and diluents osmolatity (over 15 and 30 min) or by a one-step, 10-fold dilution.

Pena et of. (1998) used four different concentrations of glycerol in a trisfractose citric acid extender for frozen dog semen and the effects of adding glycerol at 37°C or 4°C to the extender were studied by monitoring the post-thaw sperm longevity and acrosomal integrity during incubation at 39°C.

Morrier et al. (2002) used 7% glycerol to sludy the effects of method of glycerol addition in ram spermatozoa. The ram semen was divided into 2 aliquous and diluted with commercial extenders that were identical except that one contained 7% glycerol. In a second experiment ram semen was prepared for cryopreservation by a one step dibition with 7% glycerol or gradually with a 2 step protocol to test the hypothesis that the method and time of glycerol addition affects sperm quality after freezing and thaving. They found that the presence of glycerol did not affect the quality of fresh sperm. For cryopreserved sperm, the method of glycerol addition also did not affect the quality of thawed sperm. However a decrease in sperm motility and viability occurred due to the duration of time in extender.

Thun et al. (2002) used different temperatures for cryopreservation using fris egg yolk-citrate extender and Biophos-plus, in method A, they used Tris egg yolk as extender and packaged at 4°C. In method B, they used Tris egg yolk and packaged at room temperature between 18 and 22°C. In method C, they used Biophos-plus as extender and packaged at room temperature. They compared the three methods using post-thaw motility, viability, morphology and osmotic resistance as semen quality parameters. All the laboratory parameters investigated were significantly better in method A and B compared to method C. But there was no any difference between the cryopreservation done at 4°C and 18-22°C.

Wessel and Ball (2004) investigated the effect of step-wise dilution for the removal of the permeating cryoprotectant, glycerol, from both fresh and cryopreserved equine spermatozoa on their motility and viability. There were significant (P < 0.05) effects of osmolality and dilution method on both total and progressive motility as well as viability in fresh spermatozoa. With the rapid, one-step dilution being significantly more detrimental to viability and motility, compared to the fixed molarity and fixed volume-dilutions. With frozen-thawed spermatozoa there were significant effects of stallion on total and progressive motility of spermatozoa after cryopreservation and lhawing; however, treatment (type of dilution after thawing) did not affect post-thaw motility. These data indicate that rapid, single-step removat of glycerol from fresh equine spermatozoa results in a post-hyperosmotic stress that is characterized by a reduction in both molility and membrane integrity. This post-hyperosmotic stress can be reduced by a step-wise dilution for removal of glycerol, which improved the maintenance of both motility and membrane integrity. However a similar benefit for step-wise dilution for removal of glycerot was not observed in cryopreserved equine Spermatozoa.

Santos et al. (2005) studied the effects of different cryoprotectants and the temperature of addition on the survival of red deer epididymal spermatozoa. They evaluated the effects of four most comtuouly used cryoprotectants (CPAs), Gly cerol(G). Ethylene glycol (EG). Propylene glycol(PG), and Dimethyl sulfoxide (DMSO), on the sperm survival. They also studied the effects of two temperatures of CPA addition 22°C (ambient temperature) and 5°C on sperm quality. For each temperature sperm samples were evaluated after 0, 15, 30 and 60 min of spermatozoa exposition to CPAs. The sperm qualities were judged in vitro by microscopic assessments of individual sperm motility (SMI), and of plastua membrane (viability)

and acrosome (NAR) integrities. Overall, DMSO showed the highest toxicity for red deer epididymal spermatozoa, and glycerol the lowest. Their results showed a differential response of acrosome membrane to temperature of CPAs addition. Regardless of the CPA used, statistically significant (p<0.05) were found between the two temperatures of addition of CPAs for acrosome integrity, the best being the 22°C (NAR=83,8% vs. 69.8%). Hence, the experiment indicated that sperm quality of red deer epididymal spermatozoa, in addition to be affected by the eryoprotectant, can also be influenced by the temperature at which CFAs are added prior to freezing.

Maria et al. (2006) compared different cryoprotectants and studied the effect of the temperature of addition on the survival of Red Deer Epididy mal Spermatozoa with the aim of finding an ideal cryoprotectant in a suitable concentration for red deer epididymal spermatozoa preservation. They evaluated the effects of four most commonly used cryoprotectants (CPAs). Glycerol (G), Ethylene glycol(EG), Propylene glycol (PG), and Dimethyl sulfoxide (DMSO), on the sperm survival. Besides, the effect of two temperature of CPA addition 22°C (ambient temperature) and 5°C-on sperm quality were also lested. For each temperature tested, sperm samples were evaluated after a 15, 30 and 60 min of spermatozoa exposition to CPAs. Thus, sperm quality was in vitro judged by microscopic assessments of individual spetm motility (SMI), and of plasma membrane (Viability) and acrosome (NAR) integrities. Overall, DMSO showed the highest toxicity for red deer epididymat spermatozoa, and glycerol the lowest. Thus, at 60 min of incubation SMI results showed that the toxicity to red deer epididy mal spermatozoa of the four CP As are in the following sequence: G - EG - PG ≤ DMSO ('less than' symbol mean P≤ 0.05 and approximately symbol means \$ =0.08). Furthermore, our result also showed a different response of acrosome membrane to temperature of CPAs addition. Regardles s of the CPA used, statistically significant variations (P≤ 0.05) were found between the two temperature of addition of CPAs for acrosome integrity, the best being 22°C (NAR=13.8% vs. 69,8%). These data indicated that sperm quality of red deer epididymai spermatozoa, in addition to be affected by the cryoprotectant, can also be influenced by the temperature at which CPAs are added prior to freezing.

2.3. Dual or triple staining for acrosomal inlegrity

Didion et al. (1989) explained a simple dual stain procedure (DS) for simultaneously determining sperm viability and acrosomal status. The DS includes the use of the vital stain trypan blue to detect live and dead spermatozoa and Giernsa to detect the presence or absence of an acrosome. For staining, spermatozoa are washed incubated with trypan blue, washed, dried onto slides, and subjected to Giemsa. Dead spermatozoa stain blue in the post acrosomal region while live spermatozoa remain unstained. The acrosome stains light purple-dark pink while acrosome free sperm remain unstained. This staining pattern enables differentiation of spermator by which have undergone a true acrosome reaction (TAR) from those which have undergone a false acrosome reaction (FAR). Incubation of bull, boar, ram, and stallion spermatozoa for 60 minutes at 37°C in the presence of calcium ionophore increased the proportion of spermatozoa undergoing a TAR in all species except the stallion. Incubation of bull spermatozoa for up to 24 hours at 37°C resulted in a decrease over time in the percentage of live acrosome intact spermatozoa and a simultaneous increase in the percentage of spermatozoa categorized as having undergone a TAR and FAR. The DS could be a useful technique in evaluating sperm viability and aerosomal status in fertilization and clinical studies.

Kovacs and Foote (1992) developed a practical and reliable staining procedure to distinguish the viability and acrosomal status of bull, boar and rabbit spermatozoa. The first stain with trypan blue or Congo red is rapid and avoids artifacts. This stain is precipitated by neutral red during the 2 min required for fixation. The precipitate gives a high contrast black color, resistant to the subsequent risings and persists during the time required for staining the acrosome with Giemsa. Ten classes of spermatozoa are distinguished (live or dead with intact acrosomes, bose acrosomes, damaged acrosomes, no acrosome, or with no acrosome and no postacrosomal ring). The intact acrosomes are purple, the loose acrosomes are dark lavender and the damaged acrosomes are pale lavender. The anterior part of the head of tive spermatozoa with no acrosome is white or light pink and same of dead spermatozoa is white or pale grey. The postacrosomal ring is red. The postacrosomal area of the head of live spermatozoa is white or light pink and the same part of dead

spermatozoa is black, dark violet or gray. The procedure did not give satisfactory results for stallion spermatozoa.

Sidhu et al. (1992) described a simple dual staining procedure for detecting the true acrosome reaction in dried smears of buffalo spermatozoa. Trypan blue was used first to differentiate live from dead spermatozoa and the dried smears which had been prepared were stained with Giemsa for acrosome evaluation. Four categories of spermatozoa were recognized: A) live, intact acrosome (acrosome pink, post nuclear cap clear); B) dead, infact acrosome (acrosome pink, post nuclear cap blue); C) live, detaded acrosome (acrosome clear, post nuclear cap clear); and D) dead, detached acrosome (acrosome clear, post nuclear cap blue). The procedure was simple, rapid and convenient for assessing true acrosome reaction in buffalo spermatozoa. Simultaneous assessment of sperm viability and its acrosomal status in dried smears makes this procedure attractive because the true acrosome reaction can be studied thoroughly at a later state after the incubation period.

Tamul i and Watson (1994) used two common staining techniques, nigrosineosin (NE) and the Giemsa stain in order to determine actosromal status in live spermatozoa. He found no significant difference between proportions of live boar spermatozoa determined by the NE technique and the nigrosin-cosin-Giemsa (NEG) technique, the also compared the proportion of live spermatozoa determined by NEG technique with the results of fluorescent vinbility stains, carboxyfluorescein diacetate and propidium hodide. The difference although significant (p< 0.01) was small, 87% (NEG) vs 80% (CFDA/PI). Acrosomal alterations, determined by differential interference contrast microscopy and by the NEG technique did not differ significantly for boar or ram spermatozoa. This novel staining technique is capable of determining four categories of spermatozoa: live acrosome-intact, live acrosome-reacted or damaged, dead acrosome-intact and acrosome reacted or damaged. He explained the method to be very simple to perform requires only basic equipment and appears extremely reliable.

Way et al. (1995) compared four staining procedures for simultaneously assessing viability and acrosomal integrity of bovine, ejaculated and cauda epididymal spermatozoa (CES) were evaluated. Fast green FCF/eosin B (FG/EB),

sativum agglutinin (PI/PSA) were compared with naphthol yellow and erythrosine B (NY/EB) and E-Ab as standards for acrosomal integrity and viability, respectively. Both ejaculated and CES samples were incubated with heparin (10 µg/ntl; 5 % C0₂/air; 39°C) for 4 h. Lysophosphatidylcholine was added to spermatozoa to induce the acrosome reaction in capacitated spermatozoa. The sperm cells were then stained by the different methods and evaluated as either intact live, intact dead, acrosome-reacted live, or acrosome reacted dead. The results indicated that FG/EB, E-Ab and PI/PSA stains provided an accurate assessment of both viability and acrosomal integrity for ejaculated spermatozoa. Although PI/PSA was a reliable method of determining viability and acrosomal integrity of ejaculated and cauda epididyntal sperm cells, it was not suitable for use with spermatozoa capacitated with heparin due to sperm agglutination. The E-Ab stain, which was originally used to determine viability only, was also found to be suitable for determining acrosomal integrity of both ejaculated and CES, when differential interference contrast microscopy was used.

Garde et al. (1997) described a procedure for simultaneous evaluation of sperm viability aild acrosomal status in fixed deer spermatozoa. This technique, which is a modification of the triple-stain technique (TST) developed for human sperm. utilizes trypan blue to identify the percentage of live sperm in culture and subsequent staining of fixed sperm to differentiate the acrosome. Four classes of deer sperm can be distinguished with the TST: (1) live unreacted sperm. (2) live acrosome-reacted sperm (lrue acrosome reaction), (3) dead unreacted sperm, and (4) dead acrosomereacted sperm (false acrosome reaction). The study shows that the acrosome status can be detected either by TST stain or by phase-contrast microscopy (-. 97), p< 001) and that TST does not differ from 0.4% trypan blue in its ability to identify live and dead sperm (r=.995, p<.001). This staining procedure enables differentiation of spermatozoa that have undergone a true acrosome reaction (TAR) front those that have undergone a false acrosome reaction (FAR). In this way, incubation of deer spermatoz oa for 30 min at 37 d'C in the presence of calcium ionophore A23187 increased the proportion of spermatozoa undergoing a TAR. Sperm incubated in the absence of A23187 undergo the TAR at a much lower rate (p< 001) than those incubated in the presence of ionophore. This stain procedure was also used to study the time course of the true acrosome reaction of deer sperm in vitro. Incubation of

the time course of the true acrosome reaction of deer sperm in vitro. Incubation of deer spermatozoa for up 24 h at 37°C resulted in a decrease in the percentage of tive acrosome-intact spermatozoa and a simultaneous increase in the percentage of spermatozoa categorized as having undergone a l'AR and FAR.

Nagy a d (1999) evaluated sperm tail membrane integrity by tight microscopy. During routine evaluation of tryp an blue-Giemsa stained semen smears, sperm cells can be found with unstained heads and with stained tails. It was hypothesized that these cells were immotile and should not be considered as alive. Sperm motility was determined in iso osmotic and presumably isotonic trypan blue-stained wet preparation. Bult, ram and boar scroen smears were stained with hypoosmotic trypan blue-Giemsa to compare the relationship between the percentage of stained sperm tails and the percentage of sperm tails reroaining straight under by po osmotic condition. Activity moving spermatozoa with unstained heads, but with tails were never observed in wet preparation. The correlation coefficient found between the percentage of sperm with stained tails and the percentage with straight tails was 0.81.

0.94 and 0.85 for bult, ram and boar spermatozoa, respectively. Results of this study show that sperm cells with an intact head membrane, but a stained and presumably membrane-damaged tail are not motile. Therefore these certs should be included in the dead category in their than a ive in the usual live-dead studies with vital stains.

Nagyet d. (2001) evaluated the membrane Integrity of frozen/thowed deer spermatozoa. A simultaneous live/dead and acrosome staining, originally described for domestic mammals, was successfully applied on red deer (cervus elaphus) and fallow deer (Dama dama) spermatozoa collected from the cauda epididymidis and vas deferens of shot stags. The staining is simple enough for routine application. Seven classes of spermatozoa were distinguished in the smears of frozen/thawed semen samples. Morphology, including cytoplasmic droptets, was evaluated as well.

Percentage of live cells with intact acrosomes and with no other morphological might be practical index of semen quality.

Rasul et al. (2001) studied motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa after different stages of cryopreservation (i.e., ditution, cooling to 4°C, equilibration at 4°C, and freezing and

diluted in tris-citric acid extender, cooled to 4°C over 2 hours, equilibrated at 4°C for 4 hours, dispensed into 0.5mL straws, and frozen in a programmable cell freezer before plunging into liquid nitrogen. Frozen semen was thawed at 37°C for 15 seconds. After completion of each stage, sperm motion characteristics, ptasma membrane integrity, and acrosomal morphology were determined using computer assisted semen analysis, hypo-osmotic swelling assay and phase-contrast microscopy. respectively. Data were presented as mean ± standard error of the mean. Visual and computerized mobility did not differ due to dilution, cooling, or equilibration (77.3% ± 2.3% and 90.5% ± 1.2%, respectively), but was reduced (P ≤ .05) after freezing and thawing (53.0% ± 4.6% and 48.6% ± 6.5% respectively). Linear motility of spermatozoa, was lower (P \le .05) after dilution or equilibration (56.2% \pm 2.4%) than after cooling or freezing and thawing (79.6%% ± 1.4%%). Sporm curvilinear velocity was reduced (P \leq 0.5) from 112.4 \pm 5.3 µm/sec after dilution to 96.0 \pm 5.8 µm/s after cooling, and from 87.6 ± 4.1 µm/s after equilibration to 69.4 ± 2.0 µm/s after freezing and thaving. Sperm lateral head displacement differed (P ≤ .05) after each stage (i.e., dilution, $3.9 \pm 0.2 \,\mu m$; cooling, $2.3 \pm 0.2 \,\mu m$; equilibration, $3.1 \pm 0.3 \,\mu m$; and freezing and thawing, 1.7 ± 1.2 \(\alpha\mathrm{m}\)). Spermatozoa with intacl plasma membranes were 80.2% ± 3.9% after dilution, reduced (P < .05) to 60.4 %± 5.6% after equilibration, and then to 32.6% ± 3.8% after freezing and thaveing. The percentage of spermatozos with normal acrosomes remained higher after dilution, cooling, or equilibration (73.2% ± 2.4%) than after freezing and that sing (61.8% $\pm 2.4\%$; P \leq .05). In conclusion, the maximal damage to the motility apparatus, plasma membrane, and acrosomal cap of buffalo spermatozoa occurs during freezing and thawing followed by equilibration.

Revay et al. (2002) the viability and sex of bovine spermatozoa were sintultaneously evaluated. After viability and acrosome staining with trypan blue/Giernsa, only live spermatozoa became decondensed by a modified papain-dithiothreitol method. Owing lo this specific effect, live sperm heads were easily distinguished by their enlarged size and dark violet color from small, light blue dead sperm lieads. In the same sperm sample, X- and Y-chromosome-bearing sperm were distinguished by their fluorescent signal, using fluorescence in situ hybridization (FISH) with an XY paint set and 4,6-diamino-2-phenylindole counterstaining. The combined staining provides a method for morphological and viability evaluation before FISH and permits identification of the proportions of X- and Y-chromosome-

containing live spermatozoa in a semen sample. However, only 25% of the undecondensed dead sperm express signals allowing detection of the sex of the chromosome. The method may be an effective tool in evaluating sex-oriented semen samples.

Nagy et al. (2003) demonstrated a triple stain flow cytometric method to assess acrosome integrity of cryopreserved bovine sperm. In this study a new triple stain combination was developed for the simultaneous evaluation of viability and acrosomal integrity of bovine sperm processed in egg yolk based extender by flow cytometer. SYBR-14 propidium iodide enabled the discrimination of sperm cells from egg yolk and debris particles, which was instrumental for the flow cytometric analyses of frozen thawed bovine sperm, because it implied that washing steps to remove egg yolk were no longer required. In addition, phycocrythrin-conjugated peanut agglutinin was used to discriminate acrosome-damaged/reacted sperm cells from acrosome intact cells.

Octde (2003) studied changes in acrosome morphotogy during cooling and freezing of dog semen. Sixteen semen samples, collected from purebred beagles over a period of 12 months, were diluted, frozen and thawed. In an attempt to minority any changes occurring to the acrosome during processing, smears made at various stages of the handling procedure were stained with Spermac stain and examined tinder 1000× magnification. Most acrosomal damage appeared to occur during the freeze/thaw processes. Sperm motility did not correlate well with acrosomal integrity.

Feng et al. (2004) investigates the efficacy of five extenders in contributing to the outcome of semen eryopreservation in Formosan Sika and Sambar deer. Pooled semen (n=4) of six males of each breed was used. In sika deer, semen collection rate was 96% (23/24) over all electro-ejaculations. Volume, sperm motility and sperm concentration of fresh ejaculation was 0.5 ± 0.4 ml., $77 \pm 6\%$ and $1471.3 \pm 940.0 \times 10^6$ ml's respectively. Post thaw motility in respective extender was A: $66 \pm 16\%$; b: $71 \pm 2\%$; c: $73 \pm 6\%$ D: $9 \pm 4\%$ and E: $26 \pm 12\%$ (mean $\pm 5.D$.). In extender C ($74 \pm 14\%$) more viable spermatozoa were preserved than in the other (A: $64 \pm 10\%$) B: $48 \pm 11\%$; D: $41 \pm 16\%$; E: $47 \pm 6\%$; $P \le 0.05$). Acrosomal integrity was not influenced by extender composition. Post thaw motility did not decrease during a 4-h incubation

period, irrespective of the extender used ($P \ge 0.05$). In Sam bar deer, semen collection rate was 88% (21/24) over all electro-ejaculations. Volume, sperm motility and sperm concentration of fresh ejaculates was 1.3 ± 0.5 ml, 82 ± 4 % and $379.1 \pm 252.2 \times 10^6$ ml⁻¹, respectively. Post thaw viability in extenders A ($70 \pm 7\%$), B ($76 \pm 7\%$) and C ($79 \pm 2\%$) was higher than that D ($25 \pm 19\%$) and E ($29 \pm 7\%$) ($9 \le 0.01$). Sperm acrosomal integrity was better preserved in extender B ($86 \pm 4\%$) and C ($83 \pm 4\%$) than in extender A ($54 \pm 13\%$), D ($39 \pm 22\%$) and E ($46 \pm 22\%$) ($9 \le 0.05$). Post-thaw sperm longevity in extender A reduced from 69 to 16% during incubation ($9 \le 0.05$) whereas only a slight decrease was observed in the other extender after 4 h in conclusion these data show that egg-yolk-Tris-Tes-glycerol based extender C containing Equex STM paste is optimal for freezing semen of Formosan Sika deer while egg-yolk-tris-citric acid-glycerol based extender B containing Equex and extender C are superior in semen cryopreservation to others for Formosan Sambar deer.

Kutvolgyi et al. (2006) narrated a simple trypan blue-neutral red-Giemsa staining procedure for simultaneous evaluation of acrosome, sperm head, and tail membrane integrity and morphology has been used to evaluate equine spermatozoa. Some special characteristics and problems have arisen in evaluating stallion semen, One problem was the differentiation of intact vs. damaged sperm tails primarily in frozen and thawed samples. After freezing and thawing, a high percentage of spermatozoa with an unstained head and stained tail were observed. These cells are considered immotile. Therefore, unambiguous differentiation of intact vs. damaged sperm tail membrane is very important for evaluating semen quality. The aim of our study was to develop a method especially for stallion sperm to distinguish more accurately the different cell types. We compared Chicago sky blue 6B (CSB) to trypan blue (TB) for viability staining. CSB/Giemsa staining showed good repeatability and agreement with TB/Giemsa measurements. For densitometry analysis, individual digital images were taken from smears stained by CSB/Giemsa and by TB/Giemsa, A red-green-blue (RGB) histogram for each area of spermatozoa was drawn. Differences of means of RGB values of live vs. dead tails and separate live vs. dead heads from each photo were used to compare the two staining procedures. CSB produced situilar live/dead sperm head differentiation and better tail differentiation. TB can be replaced by CSB and this results in more reliable

evaluation. After staining with 0.16% CSB and 4 min fixation, 2-4 h Giemsa staining at 25-40° Cis recommended for stallion semen.

Jankovicova et al. (2008) used two staining methods for evaluating acrosomal and viability status of bovine spermatozoa. Artificial insemination with frozen-thawed spermatozoa is commonly used in cattle breeding. A simple and fast procedure is needed for routine evaluation of the acrosomal status of frozen-thawed bovine sperm. Therefore, the purpose of this study was two staining procedures used to determine the viability and of acrosome of frozen-thawed bovine spermatozoa. Double staining and Hoechst/FITC-Pisum sativum agglutinin (FITC-PSA) labeling were tested for evaluating the viability and acrosome reaction induced by calcium ionophore of bull spermatozoa. In our experiments no significant differences were detected in the frequency of acrosome-reacted sperm either by double staining (37.98%) or by FITC-PSA labeling (39.33%). The viability of sperm stained by the double staining method was 67.17%, and a higher portion of viable sperm (82.67%) was observed by staining with the Floechst procedure (P \leq 0.01). On the basis of the result obtained it is concluded that both methods can be used for detecting the acrosome reaction of frozen-thawed bovine spermatozoa.

Rubio et at. (2009) explained that plasmatic and acrosomal membrane integrity can be used as valuable information for determining sperm quality because it is important to know the morphological and functional role as celtular detinctiation and in effective cell interactions. The study was aimed to determine cryopreservation effects on sperm percentage of vitality, number of damaged acrosomes (DAR), the structural and functional sperm plasma membrane integrity in five fresh ejaculates and 5 thawed straws of 5 bulls by using Fosin-Nigrosin stain. Significant differences were found on seminal quality parameters between thawed and fresh sperm. Freezing-thawing procedure had detrimental effect on the integrity.

2.4. Apoptosis if sperne

Anzar et al. (2002) studied the effect of cryopreservation on bull sperm and to test the relationship between apoptotic sperm and ferfality potential of semen. The study was conducted to detect sperm apoptosis in fresh and frozen semen and to

determine its relationship with bull fertility. Three ejaculates were collected from five breeding bulls with different fertility levels and were cryopreserved using standard methods. Two flow cytometric methods were employed to measure apoptosis: an assay for phosphatidylserine (PS) translocation across the plasma membranes using fluorescein-labeled Annexin V and propidium iodide (PI), and an assay for nicked DNA using bromodeoxyuridine (BrdU), terminal deoxynucleotidyl transferase, and fluorescein-labeled unti-Brdl monoclonal antibody. Both assays showed that fresh sperm contailled 10%-20% apoptotic sperm. Significant differences in the percentage of apoptotic sperm were observed among the bulls. Cryopreservation induced translocation of PS to the outer leaflet of the plasma membrane and caused most of he necrotic cells in fresh sperm to disintegrate. Bull fertility was significantly related to the percentage of necrotic or viable sperm in fresh semen as detected by the Annexin V/PI assay, to the number of apoplotic sperm in fresh semen as detected by the TUNEL assay, and to the level of chromatin or DNA condensation as dearcted by Pl slaining. The present study suggests that the presence of apoptotic spermatozoa in fresh semen could be one of the reasons for poor fertility in breeding bulls. His study revealed that eryopreservation causes fragmentation of spermatozoa, overcondensation of spermatozoa DNA and sperm apoptosis. All these changes likely to contribute to the general decline in fartility that is observed in senior samples after cryoprescryation.

Martin et al. (2004) reported that apoptosis can contribute to the fatal effect of eryopreservation on bovine sperm cells. A multiparametric study of effect of apoptosis on bovine sperm was conducted using flow cytometry, including mitochondrial membrane potential (DCm), caspase activation, membrane permeability, nucleus condensation, DNA fragmentation and phosphatidylserine (Ps) externalization. Cryopreservation and or thawing induced significant changes in all apoptolic markers in living bull sperm cells except those concerning the nucleus. They concluded that cryopreservation acts as an apoptotic mechanism inducer in bovine sperm cells, where the earliest but too the latest features of cells undergoing apoptosis occur.

Chaveiro et al. (2007) assessed sperm apoptosis in cryopreserved bull semen. The techniques used to prepared bovine spermutozoa for in vitro fertilization, to enhance the percentage of motile sperm cells include the swim-up (SU) method,

among others. The objective of the present study was to evaluate the phosphatidylserine (PS) translocation and plasma membrane integrity as the indicator of apoptosis and necrosis in post-thaw bull sperm after SU treatment using annex in V-fluorescein isothiocyanate (FITC)/propidium iodide (P) assay. A flow cytometric study was employed to measure apoptosis levels on frozen-thawed bull spermatozoa. The assay detects PS translocation across the plasma membrane using a fluorescein-labelled annexin-V and Pl. By using the annexin V/Pl assay four different subpopulations of sperm were observed (i) a population of apoptotic sperm, labeled with annexin V-FITC but now with Pl; (ii) a population of early necrotic sperm, labeled with Pl but now with annexin-FITC; and (iv) a population of fully viable sperm cells, sperm not labeled with annexin V-FITC and without Pl. Results clearly indicated that SU techniques itself could have an adverse effect on the spermatozoa metubrane stability. It had also been found, significant differences between bulls in the level of apoptotic sperm, after SU treatment.

Ortega et al. (2007) studied on apoptosis like changes in cryopreserved equine sperm. The kinematics of the apparition of apoptotic markers was studied flow cryometrically and by inmunoblot assays in equine spermatozoa subjected to freezing and thawing. Caspase activity, low mitochondrial membrane potential and increase in sperm membrane permeability were observed in all the phases of the cryopreservation producer. Freezing and thawing caused an increase in membrane permeability, and changes in the pattern of caspase activity; while decreases in tuitochondrial membrane potential were observed after centrifugation and cooling to 4°C and after freezing and thawing. It is proposed that the sperm mitochondria may be directly involved in the subtle damage that is present in most of the spermatozoa surviving freezing and thawing.

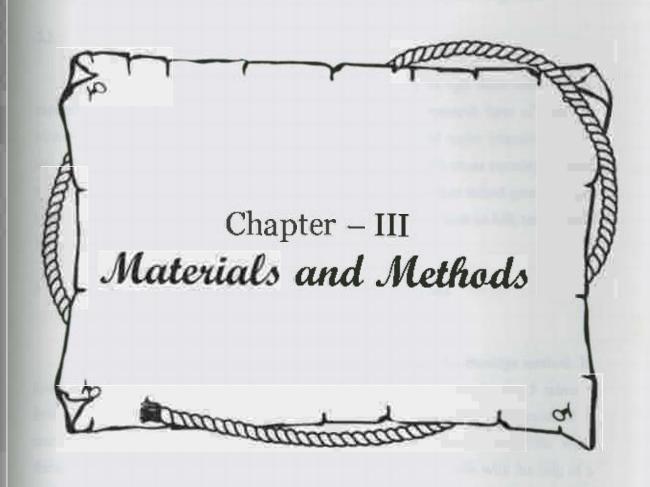
Feitosa et al. (2008) studied the kinetics of changes in plasma membrane related to apoptosis and necrosis in bovine sperm cells at different incubation times. Incubation time induce damages in sperm cells by necrosis and/or apoptosis. The aim of this study was the evaluation of changes in plasma membrane related to apoptosis and necrosis in bovine sperm cells through 2 hours of incubation. Sperm cells were incubated at 5% (v/v) CO₂ in air for 0, 30, 60, 90, and 120 minutes. After each period,

perm cells were incubated with fluorescent yo-pro and propidium iodide (PI) to deted change in plasma membrane related to apoptosis and necrosis respectively. Using yo-pro/PJ assay, three different subpopulations of sperm cells were detected by flow cytometry: a) necrotic sperm cells (PI* and Yo-pro* *); b) apoptotic sperm cells (Yo-pro* and PI*) and e) living cells (Yo-pro* and PI*). The percentage of live cells (plasma membrane integrity) significantly decreases over 2 hours of incubation, on the other hand, the percentage of necrotic and apoptotic cells increase during incubation. Changes in plasma membrane integrity were correlated to incubation time, while live cells were negratively correlated with the increase of incubation time, necrosis and apoptosis were positively correlated. It was also observe that necrosis was the main damage in sperm cells in all incubation time, in conclusion, incubation time induces changes in plasma membrane integrity related to necrosis and apoptosis, whether necrosis is present in higher quantity in alt incubation times.

Khan et al. (2009) studied apoptosis in fresh and eryopreserved baffalo sperm. The objectives of the study were (a) validation of annexin V/PI assay for estimation of sperm apoptosis in bulfalo (Experiment I) and (b) determining the effect of storages of cryopreservation on sperm apoptosis and its correlation with spemt motility and plasma membrane integrity (Experiment 2), in Experiment 1, different levels of apoptosis were artificially induced in buffino semen (100 × 106 sperm/uliquot) through graded doses of camptotheein (5, 10 and 20 µM/aliquot). Higher concentrations of camptothecin (10 and 20 μ M) successfully ($P \le 0.05$) induced apoptosis as compared to the lower (5 µM) dose and/or control in Experiment 2, semen samples (n=9), three pooled semen samples from each of the three buffalo bulls separately) were cryopreserved using vapor freezing. The mean percentage of apoptosis, necrotic and viable sperm did not differ between fresh and before freezing stages. However, freezing and thawing increased $(P \le 0.05)$ the percentage of apoptosis sperm (25.4 \pm 0.6 vs. 36.5 \pm 1.9) while decreased ($P \le 0.05$) the neerotic (35.1 \pm 1.2 vs. 29.7 \pm 0.7) and viable sperm (37.2 \pm 1.3 vs. 32.8 \pm 1.9. (P 0.07). Likewise, the mean percent motility and plasma membrane integrity. decreased (P \leq 0.05) (64 \pm 2.1 vs. 49.4 \pm 1.3) and (79.6 \pm 0.5 vs. 38.7 \pm 0.3) respectively, at post thaw compared to other stages. Coefficient of correlation, combined at all stages for each variable revealed that sperm apoptosis was inversely

correlated with sperm motility and plasma membrane integrity. It is concluded that (a) the annexin V/PI assay can be used as a tool to determine the buffalo semen apoptosis and (b) freezing and that wing induces apoptosis in buffalo sperm.

Bolan os et al. (2011) recognized apoptosis as a cause of sperm death during ervopreservation. They investigated the role of apoptosis and autophagy during cooled storage of stallion spermatozoa. Samples from seven stallions were splited: half of the ejaculate was processed by single layer centrifugation, while the other half was extended unprocessed, and stored at 5°C for five days. During the time of storage, sperm motility (CASA, daily) and membrane integrity (flow cytometry, daily) were evaluated. Apoptosis was evaluated on days 1, 3 and 5 (active caspase 3. increase in membrane permeability, phosphatidylserine translocation mitochondrial membrane potential) using flow cytometry. Furthermore, LC3B processing was investigated by western blotting at the beginning and at the end of the period of storage. The decrease in sperm quality over the period of storage was to a large extent due to apoptosis: single layer centrifugation selected non-apoptotic spermatozoa, but there were no differences in sperm motility between selected and unselected sperm. A high percentage of spermatozoa showed active caspase 3 upon e jaculation, and during the period of storage there was an increase of apoptotic sperinatozoa but no changes in the percentage of live sperm, revealed by the SYBR-14/PI assay. I.C3B was differentially processed in sperm after single layer centrifugation compared with native sperm. In processed Sperm more LC3B-II was present than in non-processed samples; furthermore, in non-processed sperm there was an increase in LC3B-li after five days of cooled storage. The results indicated that apoptosis played a major role in the sperm death during storage in refrigeration and autoptiagy plays a role in the survival of spermatozoa.



MATERIALS AND METHODS

3.1. Location of the work

The experiments were carried out at the mithun farm as well as in the laboratory of Animal Physiology Division of NRC on Mithun, ICAR, Jharnapani, Nagaland.

3.2. Experimental animals

A total of 5 healthy Mithun bulls of 4 to 6 years of age were used for the experiment. The bulls were maintained at individual pen at research farm of NRC on Mithun, Jharnapani, Nagaland, India. The animals were reared under intensive system and daily offered 4 kgs of concentrates (88% dry matter and 15% crude protein) fortified with mineral mixture and salt. The animals were also fed ad libitum mixed green forages (grasses, shrubs, herbs and tree leaves collected from the grazing area on hill, terrain with 19% dry matter and 11% crude protein) and drinking water.

3.3 Semen collection, Evaluation, Dilution and Preservation Semen collection:

A total of 80 semen samples were collected through rectal massage method. In this method seminal vesicles were massaged centrally and backwardly for 5 minutes followed by gentle milking of ampullas one by one for 3 to 5 minutes, which resulted in erection and ejaculation. During collection, the initial transparent secretions were discarded and neat semen drops were collected in a graduated test tube with the help of a sterilized funnel. (Dhali et al. 2008).



Photo.A. Collection of semen by massage method

The samples were kept in a thermo flask maintained at 37°C immediately after collection and evaluated for color, consistency, volume and mass activity. The sperm qualitative parameters viz. progressive motility, acrosomal integrity and morphological abnormalities were determined after initial dilution, after 4 hours of equilibration and after 7 days of freezing and thawing.

Evaluation of semen

Colour

Colour of semen was determined by visual inspection and was recorded immediately after collection. Any abnormal colored semen sample other than white and yellow colour was discarded.

Volume

The ejaculated volume was noted directly from the graduated semen collection tube and recorded in milli liter.

Mass Activity

Mass activity of fresh semen samples was determined by using a 5+ scale.(0-5: 0 → very poor, waves not present, sperm cell immotile; + → poor, waves not present, sperm cells motile; ++→ Fair, Barely distinguishable waves in motion; +++→ good waves apparent, moderate motion; ++++→ very good, dark distinct waves in rapid motion) as described by Bhosrekar (1990). A neat semen drop was placed in a pre-warmed glass slide and analyzed 5-6 fields by using bright field optics (Dewinter binocular microscope, magnification 100×).

After mass activity estimation the semen samples were initially diluted by adding I ml of pre-warmed (37°C) tris-egg yolk glycerol diluents tris- hydroxymethyl aminomethane 24.3 mg/ml, Sodium citrate 13.4 mg/ml, fructose 10mg/ml, penicillin G sodium salt 1000 IU/ml and dihydrostreptomycin 1 mg/ml. The partially diluted samples were maintained at 37°C for further processing. After initial dilution the

samples were brought to the laboratory for estimation of percentage of progressive motility and concentration.

Progressise motility

The semen samples were kept at 37°c in a water bath until cooling. A drop of semen was taken in a clean glass slide and put a cover slip and examine the drop first under low power (100*) and then turned onto high power magnification (400×). Sperm with straight forward movements were counted in each field. Altogether 5-6 fields were examined and the rating of intital mobility was done by visual estimation as described by Zemijanis (1970).

Sperm Concentration

The spermatozoa concentration was determined with the help of haemocy totaler.

Dilution and Preservation

Following the determination of spermatozoa concentration, final dilution of the semen samples was done with pre-warmed (37°C) tris egg york glycerol extender where the concentration of egg york was 20% and the glycerol was added in such a way that diluted samples comained 3,4,5,6, or 7% glycerol (v/v) and 60 × 10° spermatozoa pef 0,5 not straw. The entire quantity of glycerol was added in one step (non-split, EXPERIMENT-II) or split method (EXPERIMENT-II) as required. After the final dilution the samples were loaded into 0,5 ml straws (for one step addition of glycerol) and were scaled with PVA powder. The straws were then kept at 4°C for 4 hours for equilibration. After equilibration the straws were frozen in liquid nitrogen vapour (5cm above the liquid nitrogen level for 10 min and subsequently plunged into liquid nitrogen for storage. But for split method of addition of glycerol the semen was loaded into the straws for freezing and storage only after the completion of the equilibration step.

Experimental design (According to method of glycerol addition)

3.4. Experiment I (One step glycerol addition)

Semen samples were cryopreserved in tris-egg yolk glycerol diluent and sperm qualities were evaluated at different stages of cryopreservation. The total amount of required glycerol was added into the diluents in a single dose at 37°C after final dilution and kept immediately at 4°C for equilibration. The experiment was performed in three replicates.

3.5. Experiment II (Split method ofglycerol addition)

Semen samples were eryopreserved in tris-egg yolk glycerol diluent and sperm qualities were evaluated at different stages of cryopreservation. Glycerol was added into the diluents in split doses. The total amount of glycerol to be added was divided into four equal parts and a single part was added into the diluents after final dilution at the (immediately before equilibration, i.e. 37°C), 1h (4°C), 2h (4°C) and 3h (4°c) of equilibration. The experiment was performed in three replicates.

3.6. Evaluation of acrosomal integrity and morphological abnormality

The live spermatozoa count, morphological abnormalities acrosomal integrity status were evaluated simultaneously with the help of double staining technique using trypan blue and Giemsa stain (Kovacs and Foote, 1992).

Double staining procedure

Smears of a mixture of semen and 6.2% trypan blue were prepared and the slides were dried in the air at room temperature. The slides were then fixed in a fixative solution (86ml lin RCL + 14ml 37% formaldehyde + 0.2 g Neutral red, sigma St. Luis MO) for 2 minutes. After fixing, the slides were washed with distill water and stain with 7.5% Gientsa stain solution for 2-5 hours. After staining procedure the smears were rinsed in distill water. Then dried in the air and subsequently mounted with DPX. At least 300 numbers of spermatozoa per slide

were counted using a light microscope under 40 x objectives. Four different classes of spermatozoa were distinguished during the evaluation. These were:

- 1. Live, intact acrosome (acrosome pink, post acrosomal area clear)
- 2. Dead, intact acrosome (acrosome pink, post acrosomal area blue)
- 3. Live, damaged acrosome (acrosome clear, post nuclear cap, clear)
- Dead damaged acrosome (acrosome clear, post acrosomal area blue) (Sidhu et al, 1992).

The percentages of live intact, dead intact, live damaged and dead damaged were calculated separately (individually). The morphological abnormality percentages were counted separately for head abnormalities, mid-piece abnormalities and tail abnormalities and the total abnormalities were determined by adding the 3 abnormalities and aerosomal status were evaluated (magnificent 400 ×) with a Nikon microscope (Eclipse 80) attached with a liquid crystal display (LCD) viewer. The view of spermatozoa was further enlarged (2× or 4×) from the LCD viewer if required.

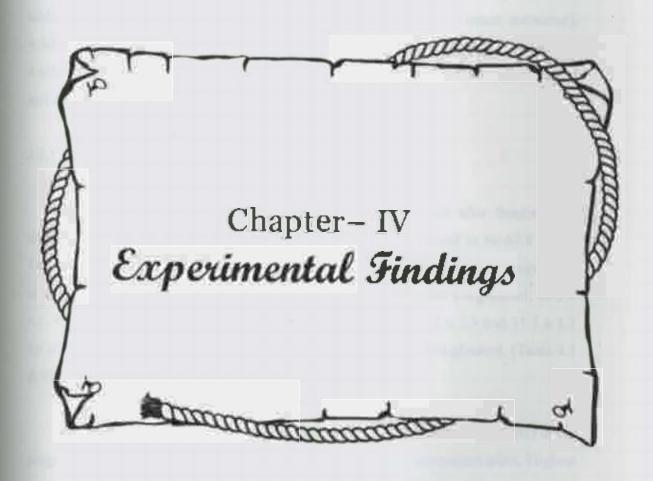
3.7. Experiment III (Study of Apoptosis)

Study of the apoptosis was carried out in the semeo samples just after collection, after equilibration and after 7 days of cryopreservation. The study of apoptosis was carried out by tuonel assay procedure using in Situ cell Death Detection Kit (Version 1t.0. Roche, catalogue no.)1684809910) according to manufacturer's instructions. Briefly, after preparation of the smears from the semen samples the slides were fixed with 100µ14% paraformaldehyde and kept for lhr. Then the slides were washed twice with washing solution (BSA in PBS). After washing, the slides were incubated in permeabilization solution (50µ1 triton- x-100 in sodium citrate and water) for 30 minute at room temperature. Preparation of the tunnel reaction mixture was done by adding 100 µl label solution and 50 µl enzyme solutions (provided in the kit). Then again the slides were washed twice and dried and 50 µl of tunnel reaction mixture was added and incubate for 1 hr in dark in humidified cliamber at 37°c. Washing was done with PBS. The slides were stained with working solution of propidium iodide and kept for 1 lir. After washing, the slides were counterstained with

FITC stain for the and were examined in the fluorescent microscope (Nikon Eclipse 90i, Germany) using red (wavelength- 598) and green filter(wavelength-488). A total of 200 number of cells were counted per slide and the percentage of apoptotic cells were calculated based on the color of the acrosome. (Green- normal; red-apoptotic.)

3.8. Statistical analysis

Statistical analyses were performed using the PASW 18.0. Software package (SPSS/1BM, Chicago, 1t, USA). For the analysis, acrisisme transformation was done for the result expressed in percentage. In each experiment, variation in semen parameters (Progressive motility, acrosome integrity, morphological abnormalities and apoptosis of sperm) among different glycerol concentrations at each stage of cryopreservation (final dilution, 4h of equilibration or post freeze thaw) were analyzed by ANOVA. Further, the post freeze thaw sperm qualities were compared between the single and split dose addition methods for each glycerol concentration by ANOVA. A probability value of less than 0.05 was considered significant, Pair wise multiple contparison procedure between means was conducted using student-Newman-kculs (SNK) test.



EXPERIMENTAL FINDINGS

Experiment-I

4.1. Addition of glycerol in single dose (Non-split)

Variations in sperm quality parameters (3-7% glycerol added in single dose) i.e. progressive motility, acrosomal integrity and sperm abnormalities are presented in table 4.1, 4.2 & 4.3 and fig 4.1(progressive motility), 4.2(live intact acrosome), 4.3(live damage acrosome), 4.4(dead intact acrosome), 4.5(dead damage acrosome), 4.6(head abnormality acrosome), 4.7(mid-piece abnormality), 4.8(tail abnormality) and 4.9(total spermatozoa abnormality) respectively.

4.1.1. Progressive motility

After initial dilution, after 4 hours of equilibration and after freezing and thawing the mean percentages of progressive motility were found to be 67.8 ± 2.8 . 43.2 ± 2.3 and 23.9 ± 2.0 for 3% glycerol. The subsequent percentages of progressive motility were found to be 67.2 ± 2.3 , 44.2 ± 2.4 and 26.5 ± 2.7 for 4% glycerol; 65.3 ± 4.1 , 49.8 ± 2.2 and 40.6 ± 1.7 for 5% grycerol; 66.1 ± 2.9 , 45.2 ± 2.5 and $3_1.7 \pm 1.1$ for 6% glycerol and 66.3 ± 3.7 , 45.1 ± 3.0 and 17.4 ± 1.3 for 7% glycerol. (Table 4.1 & Fig. 4.1)

Statistical analysis showed that there was significant variation (p< 0.05) in the progressive motility among the glycerol percentages after cryopreservation. Highest percentages of progressively motile sperm were seen in 5% glycerol concentration and lowest was observed in 7% glycerol after cryopreservation. The percentage of progressive motile sperm in 5% glycerol was significantly different to that of 3%, 4%, 6% and 7% glycerol.

Progressive motility

Table 4.1. Variations *(Mean ± SE) in progressive motility of Mithun spermatozoa during different stages of cryopreservation in non-split glycerol addition method.

Glycerol concentration (%)	Progressive motility(%)			
	After initial dilution	After 4 h equilibration	After freezing and thawing	
3	\$67.8 ± 2.8	†432±23	23.9±2,0 ⁶	
4	³ 67.2 ± 2.3	†442±2.4	26.5 ± 2.7 ^{AB}	
.5	² 65.3 ± 4.1	†49.8 ± 2.2	406± 1.7°	
6	[‡] 66.1±2.9	+45.2±25	31.7± 1.1 ^{ls}	
7	⁸ 66.3 ± 3.7	[†] 45.1 ± 3.0	17.4± 1.3 ^D	

A B indicates values with different superscript within column differ significantly (P<0.05)

"Noof observation-10

indicates values differ significantly (P<0.05) among the cryopreservation stages for the given glycerol concentration

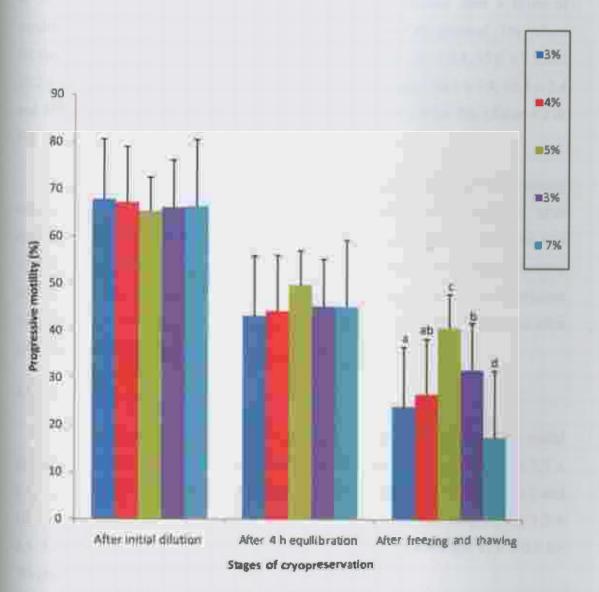


Fig. 4.1. Variations (Mean ± SE) in progressive motility during different stages of cryopreservation in non-split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

4.1.2. Acrosomal Integrity

4.1.2.1. Live intacl.

The mean percentages of live spermatozoa with intact acrosome were found to be 70.9 ± 2.7 . 46.7 ± 2.6 and 18.0 ± 1.7 after initial dilution, after 4 hours of equilibration and after freezing and thaveing respectively for 3% glycerol. The values for the subsequent percentages of glycerol were found to be $7i.3 \pm 2.4$, 45.6 ± 2.1 and 20.2 ± 2.2 for 4%; 69.2 ± 4.0 , 52.8 ± 2.2 and 43.4 ± 1.8 for 5%; 70.3 ± 3.3 , 49.4 ± 2.4 and $35.i \pm 1.0$ for 6% and 71.5 ± 4.1 , 49.9 ± 3.2 and 21.2 ± 1.4 for 7%. (Table 4.2 & Fig. 4.2)

Statistical analysis indicates that there is significant variation (p< 0.05) among the glycerol concentrations for live intact spermatozoa percentages after cryopreservation. Live intact acrosome percentage at 5% glycerol concentration was significantly different from 3, 4, 6 and 7% glycerol. There was no any difference among the values for 3 and 4% glycerol. Highest percentage of live intact acrosome was observed in 5% glycerol and lowest percentage was found in 3% glycerol after cryoprese rvation.

4.1.2.2. Live damage

The mean percentages of live spermatozoa with damage acrosome after initial dilution, 4 hours of equilibration and freezing and thawing were recorded to be 2.9 \pm 0.3, 4.7 \pm 0.5 and 10.7 \pm 0.5 for 3% glycerol respectively. 3.0 \pm 0.3, 5.0 \pm 0.7 and 10.5 \pm 0.5 for 4% glycerol; 3.0 \pm 0.6, 5.2 \pm 0.8 and 4.8 \pm 1.2 for 5% glycerol; 1.2 \pm 0.5, 5.2 \pm 0.7 and 7.0 \pm 0.5 for 6% glycerol and 2.0 \pm 0.5, 5.6 \pm 0.4 and 11.0 \pm 0.6 for 7% glycerol. (Table 4.2 & Fig 4.3)

Statistical analysis of the data showed that there was significant difference between the percentages of live damage acrosome al different glyceror concentration after cryopreservation, but there was no any difference among the glyceror percentages after initial dilution and equilibration. Evaluation of the data showed that the live damage acrosome percentages at 5% glycerol was significantly different from 3, 4 and 6% whereas there were no any difference between 3, 4 and 7% glycerol after cryopreservation. Highest percentages of five damage acrosome were seen in 7% glycerol and the lowest in 5% after cryopreservation.

The mean percentages of dead intact acrosome Spermatozoa were recorded to be 6.2 ± 0.8 , 14.4 ± 1.2 and 20.8 ± 2.3 after initial dilutions, 4 hours of equilibration and after freezing and thowing for 3% glycerol concentration. The subsequent reading were recorded to be 6.5 ± 0.9 , 15.1 ± 1.3 and 20.6 ± 2.3 for 4% glycerol: 6.9 ± 0.9 , 11.1 ± 1.1 and 10.3 ± 1.2 for 5%; 8.0 ± 0.7 , 13.0 ± 1.1 and 16.5 ± 1 for 6% glycerol and 7.6 ± 0.8 , 12.1 ± 1.0 and 17.9 ± 0.9 for 7% glycerol. (Table 4.2 & Fig. 4.4)

Statistical analyses revealed that there was significant difference between the glycerol percentages for the dead intact acrosome after freezing and thawing. There was no any difference between 3, 4 & 7% glycerol, but 5% glycerol showed significant difference to that of 3, 4, 6 & 7. From the data it was observed that 3% glycerol concentration showed the highest and 5% showed the lowest percentage of dead intact acrosome.

4.1.2.4. Dead damage

The mean percentages of dead spermatozoa with damage acrosome were found to be 21.3 ± 1.8 , 34.6 ± 20 and 50.8 ± 2.5 after initial dilution after 4 hours of equilibration and after freezing and thawing respectively for 3% glycerol. 20.6 ± 1.9 , 34.4 ± 1.5 and 49.5 ± 2.4 for 4% glycerol; 22.5 ± 2.8 , 31.9 ± 2.3 and 40.8 ± 2.7 for 5% glycerol; 22.1 ± 2.3 , 33.3 ± 3.2 and 41.6 ± 0.9 for 6% glycerol and 20.8 ± 2.1 , 33.4 ± 1.9 and 50.0 ± 2.4 for 7% glycerol concentration. (Table 4.2 & Fig. 4.5)

Statistical analyses showed that there was no any difference of values for dead intact acrosome percentages after initial dilution and equilibration. There was significant difference after cryopreservation. 5% and 6% glycerol was significantly different from 3, 4, & 7%, but in between 5 and 6% glycerol there was no significant difference. Highest dead damage percentage was seen in 3% followed by 7%, whereas 5% glycerol sliowed the lowest value.

Table 4.2. Variations *(Mean ± SE) in liveability and aerosomal integrity of Mithun spermatozoa during different stages of cryopreservation in non-split glycerol addition method.

Stage and glycerol	Stitus of spermatozoa			
concentratiun (%)	L-I	L-D	D-I	D-D
After initial dilution				
3%	370,9±27	\$2.9±0.3	16.2 ± 0.8	213418
4%	[‡] 71.3 ± 2.4	$^{2}3.0 \pm 0.3$	¹ 6.5 ± 0.9	\$20.6 ± 1.9
5%	² 69.2 ± 4.0	5.2 ± 0.8	$^{1}6.9 \pm 0.9$	\$22.5 ± 2.8
6%	70.3 ± 3.3	31.2±0.5	² 8.0 ± 0.7	122.i ± 2.3
7%	³ 71,5 ± 4.1	$^{3}2.0 \pm 0.5$	$^{2}7.65 \pm 0.8$	\$2 (1.8±2.1
After 4 h equilibration				2
3%	46.7 ± 2.6	4.7±0.5	*14.4 ± 1.2	$^{+3}$ 4.6 ± 2.0
4%	*456±2.1	*5.0 ± 0.7	115.1 ± 1.3	344± 1.5
5%	152812,2	4.8 ± 1.2	11.1 ± 1.1	*31.9 ± 2.3
01/0	149.4 ± 2.4	*5.2 ± 0.7	*13.0 ± L.1	*33.3 ± 3.2
71/0	*49.9 ± 3.2	*5.6 ±: 0.4	(2.1 ± 1.0)	33.4 ± 1.9
After freezing thawing				F 19
39/0	18,0±1.7 A	10.7±0.5 A	20,8 ± 2.3 _A	508±25
4%	20.2 ± 2.2 ^A	10.5±0.5 A	20.6 ± 2.3 [^]	495±24
5%	43.4 ± 1.8 ¹⁵	3.0±0 GH	(0,3±1,2 ft	40.8 ± 2.7
6%	$35.1 \pm 1.0^{\circ}$	7.0 ± 0,5 °	16.5±10 ^A	41.6±0,9
71/4	21.2 ± 1.4 [^]	11.0 ± 0.6^{A}	17.9±0.9 ^A	500±24

freezing stage differ significantly (P<0.05). Indicates values differ significantly (P<0.05) among the cryopreservation stages for the given glycerol concentration L-I: Live sperm with intact acrosome; L-D: Live sperm with damaged acrosome; D-I: Dead sperm with intact acrosome; D-I: Dead sperm with damaged acrosome.

* No of observation-10

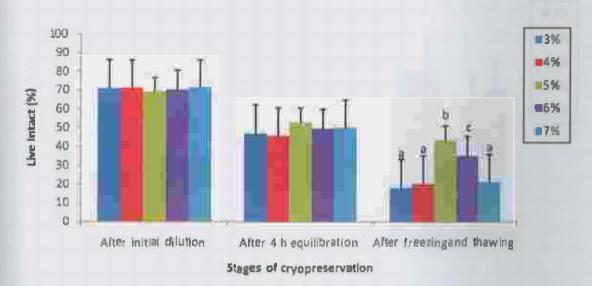


Fig. 4.2. Variations (Mean ± SE) in live spermatozoa with intact acrosome during different stages of cryopreservation in non-split glycerol addition method, a, b on error bar indicates a significant difference (P<0.05).

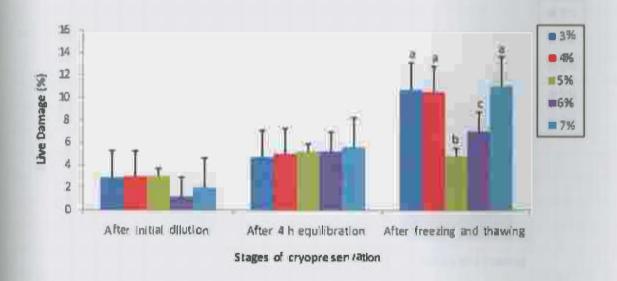


Fig. 4.3. Variations (Mean ± SE) in live spermatozoa with damage acrosome during different stages of cryopreservation in non-split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

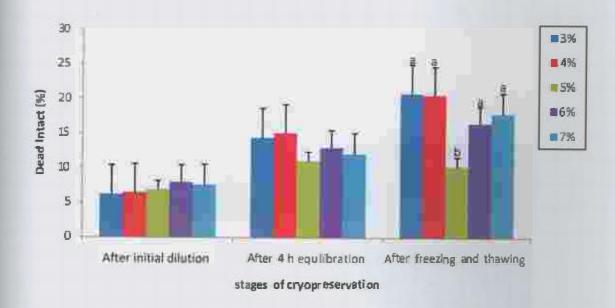


Fig. 4.4. Variations (Mean ± SE) in dead spermatozoa with intact acrosome during different stages of cryopreservation in non-split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

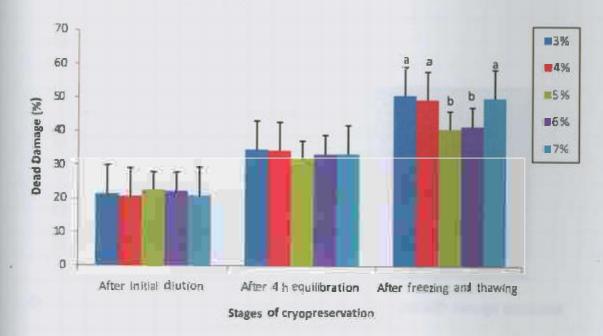


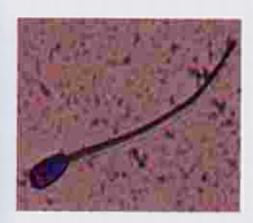
Fig. 4.5. Variations (Mean ± SE) in dead spermatozoa with damage acrosome during different stages of cryopreservation in Bon-split glycerol addition method, a, h on error bar indicates a significant difference (P<0.05).



Live spermatozoa with Intact acrosome



Live spermatozoa with damage acrosome



Dead spermatozoa with intact acrosome



Dead spermatozoa with damage acrosome

Photo.B. Photographsshowing four categories of spermatozoa with different acrosomal status.

4.1.3. Sperm abnormalities

4.1.3.1. Head alinormalities

The mean percentages of head abnormalities were found to be 6.9 ± 6.3 , 3.6 ± 0.3 and 6.1 ± 0.4 after initial dilution. 4 hours of equilibration and after freezing and thawing respectively for 3.6 glycerol. The corresponding values were 1.1 ± 0.3 , 2.8 ± 0.3 and 5.3 ± 0.6 for 4% glycerol; 1.2 ± 0.4 , 2.6 ± 0.4 and 2.8 ± 0.4 for 5% glycerol; 0.8 ± 0.3 , 2.0 ± 0.5 and 3.2 ± 0.4 for 6% glycerol and 0.6 ± 0.2 , 2.9 ± 0.3 and 6.2 ± 0.5 for 7% glycerol concentrations. (Table 4.3 & Fig 4.6)

From the table it was seen that 6% glycerol level showed the lowest percentage of head abnormalities whether 3% glycerol showed the blghest percentage. Statistical analyses revealed that there was significant difference among the glycerol percentages for the head abnormalities after eryopreservation. 5% glycerol was significantly different from 3, 4 and 7%. There was no any difference between 5 and 6% glycerol.

4.1 3.2. Mid-piesse atmormalities

The mean percentages of mid-piece abnormalities in the 3 stages i.e.; after initial dilution, after 4 liours of equilibration and after freezing and thawing were found to be 0.2 ± 0.2 , 1.2 ± 0.3 and 3.1 ± 0.3 respectively for 3% glycerol. For the subsequent concentrations the values were recorded as 0.4 ± 0.2 , 1.3 ± 0.3 and 2.9 ± 0.3 for 4% glycerol; 0.5 ± 0.2 , 0.5 ± 0.2 and 1.6 ± 0.2 for 5% glycerol; 0.2 ± 0.2 , 0.7 ± 0.2 and 1.5 ± 0.2 for 6% glycerol and 0.6 ± 0.2 , 2.9 ± 0.3 and 3.2 ± 0.2 for 7% glycerol. (Table 4.3 & Fig 4.7)

Statistical analysis of the data showed that there was no any difference in percentage of mid-piece abnormalities after initial dilution and equilibration. Significant difference was observed after cryopreservation. 5 & 6% glycerol were significantly different from 3, 4 and 7. Highest mid-piece abnormalities were seen in 7% and lowest was observed in 69% glycerol.

The mean percentages of tail abnormalities were found to be 4.7 \pm 0.3, 9.4 \pm 0.5 and 16.3 \pm 0.9 after initial dilution, after 4 hours of equilibration and after freezing and thawing for 3% glycerol; 3.4 \pm 0.5, 8.6 \pm 0.8 and 16.1 \pm 0.8 for 4% glycerol; 4.1 \pm 0.3, 6.5 \pm 0.3 and 9.7 \pm 0.5 for 5% glycerol; 4.7 \pm 0.5, 7.0 \pm 0.7 and 9.0 \pm 0.6 for 6% glycerol and 4.6 \pm 0.3, 9.5 \pm 0.4 and 16.6 \pm 0.9 for 7% glycerol. (Table 4.3 & Fig. 4.8)

Statistical analysis of the percentages tail abnormalities indicate that there were no significant difference after initial dilution, but significant difference was observed after equilibration and after cryopreservation. 5% glycerol was significantly different from rest of the percentages, but there was no difference between 3 and 7% after equilibration. After cryopreservation 5 and 6% were significantly different from 3, 4 and 7%. The highest percentage of tail abnormalities were observed with 7% glycerol, whereas the lowest percentage was observed with 6%.

4.1.3.4. Total sperm abnormalities

The mean percentages of total sperm abnormalities were recorded as 5.8 ± 0.5 , 13.6 ± 0.8 and 25.3 ± 1.0 after initial dilution, after 4 hours of equilibration and after freezing and thrawing for 3% glycerol; 4.9 ± 0.5 , 12.7 ± 1.1 and 24.2 ± 1.0 for 4% glycerol; 5.8 ± 0.3 , 9.6 ± 0.6 and 14.1 ± 0.8 for 5% glycerol; 5.7 ± 0.6 , 9.5 ± 0.9 and 13.7 ± 1.0 for 6% glycerol and 5.6 ± 0.4 , 13.6 ± 0.8 and 25.8 ± 1.2 for 7% glycerol. (Table 4.3 & Fig. 4.9)

The percentage of total morphological sperm abnormalities varies significantly among different glycerol percentages after equilibration and cryopreservation. After equilibration and cryopreservation, 5 and 6% glycerol were significantly different from 3, 4 and 7%. The highest percentage of total sperm abnormalities was found with 7% and the lowest was observed in 6% glycerol.

Table 4.3. Variations *(Mean ± SE) in head, mid piece, tail and total abnormality of Mithun spermatozoa during different stages of cryopreservation in non-split glycerol addition method.

Stage and glycerol	Sperma tozoa abnorma lities			
concentration (%)	Head	Mid piece	Tail	Total
After initial dilution				100
3%	0.9±0.3	10.2 ± 0.2	$^{1}4.7 \pm 0.3$	\$5.8 ± 0.5
4%	1.11±0.3	0.4±0.2	¹ 3.4 ± 0.5	\$4.9±05
5% 6%	12±0.4 0.8 ± 0.3	*0.5±0.2 *0.2±0.2	4.1 ±0.3 4.7 ± 0.5	5.8 ± 0.3
7%	10.6± 0.2	0.2 ± 0.2 0.4 ± 0.2	4.6 ± 0.3	$^{1}5.7 \pm 0.6$ $^{1}5.6 \pm 0.4$
After 4 h quilibration				
3%	3.0±0.3	+1,2 ± 0,3	†9.4±0,5 ^A	*13.6±0.8
4%	*28±0,3	+1.3±0.3	*8.6±08 AL1	*12.7 ± 1.1
39%	2,0±0.4	\$ 0.5± 0.2	*6.5 ± 0.3 °	19.65 ± 0.6 A
6%	[†] 2.0 ± 0.5	[‡] 0.7 ± 0.2	†7.0±0.7 BC	*9.5±0.98
7%	†2.9±03	†1,2±0,3	*9,5 ± 0,4 ^	13.6 ± 0.8
After Freezing thawing				
3%	6.1 ± 0.4 ^	3.1±0.3 A	16.3±0.9 ^A	253±1.0
4%	5,3 ± 0,6 A	2.9 ± ().3 ^	161±0.8 ^A	24.2 ± 1.0
5%	2.8±0.4 ^H	1.6 ± 0.2 ^B	9.7 ± 0.5^{B}	14.1 ±0.8
6%	3.2 ± 0.4 11	1.5 ± 0.2	9.0±0.6 B	1.3.7 ± 1.0
7%	6.2±0.5	$3.2 \pm 0.3^{^{^{^{^{^{^{^{^{^{^{^{^{}}}}}}}}}}}$	166±09	258±1.2

A.R.C indicates values differ significantly (P<0.05) among the glycerol concentration for the given stage of cryopreservation: indicates values differ significantly (P<0.05) among the cryopreservation stages for the given glycerol concentration. No of observation- 10

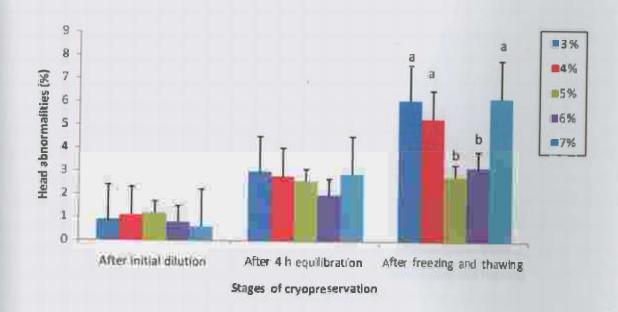


Fig. 4.6. Variations (Mean ± SE) in head abnormality of spermatozoa during different stages of cryopreservation in non-split glycerol addition method. a, b on error bar indicates P<0.05

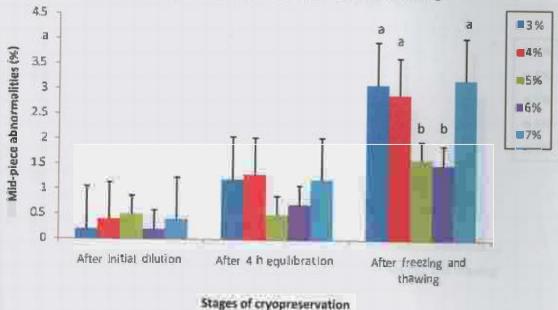


Fig.4.7. Variations (Mean ± SE) in mid-piece abnormality of spermatozoa during different stages of cryopreservation in non-split glycerol addition method. a, bon error bar indicates P<0.05.

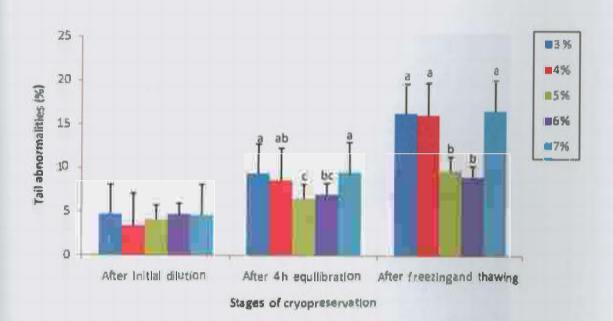


Fig. 4.8. Variations (Mean ± SE) in tall abnormality of spermatozoa during different stages of cryopreservation in non-split glycerol addition method. a, b on error bar indicates P<0.05

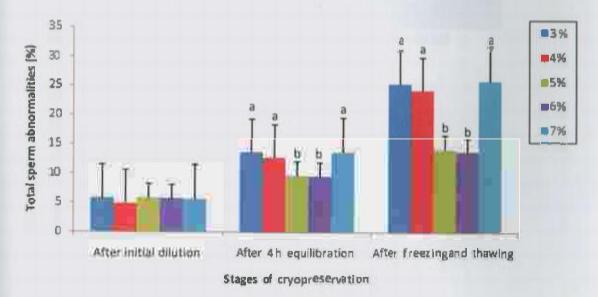
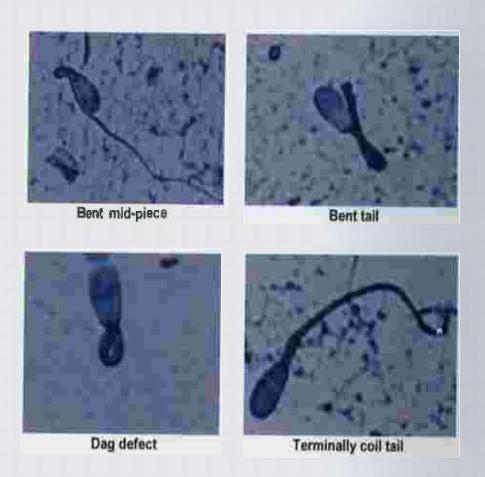


Fig. 4.9. Variations (Mean ± SE) in total abnormality of spermatozoa during different stages of cryopreservation in non-split glycerol addition method. a b on error bar indicates P<0.05





Detached head

Photo.C. Photographs showing different spermatozoa abnormalities.

Experiment-II

4.2. Addition of glycerol in split dose

Variations in sperm quality parameters (5-7% glycerol added in split dose) i.e. progressive mobility, acrosomal integrity and sperm abnormalities are presented in table 4.4, 4.5 & 4.6 and fig 4.10 (progressive mobility), 4.11 (live intact acrosome), 4.12 (live damage acrosome), 4.13 (dead intact acrosome), 4.14 (dead damage acrosome), 4.15 (head abnormality acrosome), 4.16 (mid-piece abnormality), 4.17 (tail abnormality) and 4.18 (total spermatozoa abnormatity) respectively.

4.2.1. Progressive motility

The mean percentages of progressive motility were found to be 98.8 ± 2.3 , 64.1 ± 1.5 and 50.2 ± 1.9 after initial ditulion, after 4 hours of equilibration and after freezing respectively for 5% glycerol. The corresponding values were 74.1 ± 2.9 , 58.0 ± 2.5 and 421 ± 1.5 for 6% glycerol and 74.5 ± 2.7 , 53.7 ± 2.0 and 33.9 ± 1.4 for 7% glycerol. (Table 4.4 & Fig 4.10)

Statistical analyses of the values indicate significant difference among different glycerol percentages after equilibration and eryopreservation. After equilibration 5% glycerol was significantly different from 6 and 7% but lafter eryopreservation all the glycerol percentages vary significantly among themselves. Highest percentage of progressively motile spermatozod was found at 5% glycerol, whereas lowest percentage was observed at 7% glycerol.

4.2.2. Acrosomal integrity

4.2.2.1. Live intact

The mean percentages of live intact acrosome spermatozoa were found to be 84.3 ± 3.1 , 67.5 ± 1.2 and 53.3 ± 1.8 after initial ditation, after 4 hours of eqdilibration and after freezing respectively for 5% glycerol. 78.3 ± 3.4 , 61.9 ± 2.2 and 45.8 ± 1.6 for 6% glycerol and 78.0 ± 2.9 , 57.7 ± 2.2 and 37.6 ± 1.2 for 7% glycerol. (Table 4.5 & Fig 4.11)

The data for live intact acrosome spermatozon were statistically analyzed and found that there was significant difference among different glycerol percentages after equilibration and cryopreservation. 5% glycerol was significantly different from 6 and 7%, whereas all the glycerol percentages vary significantly among themselves after

Progressive motility

Table 4.4. Variations *(Mean ± SE) in progressive motility during different stages of cryopreservation in split glycerol addition method.

Glycerol concentration (%)	Progressive motility (%)			
	After initial dilution	After 4 h equilibration	After freezing and thawing	
5	³ 79.8 ± 2.3	164.4±1,5 ^A	50.2 ± 1.9 ⁶	
6	⁵ 74.1 ± 2.9	1580±251 ³	42.1±15 ^B	
7	174.5 ± 2.7	*537±20 ⁽¹⁾	33.9 ± 1.4°	

A indicates values with different superscript within column differ significantly (P<0.05)

indicates values differ significantly (P<0.05) among the cryopreservation stages for the given gly cerol concentration

^{*} No of observation-10

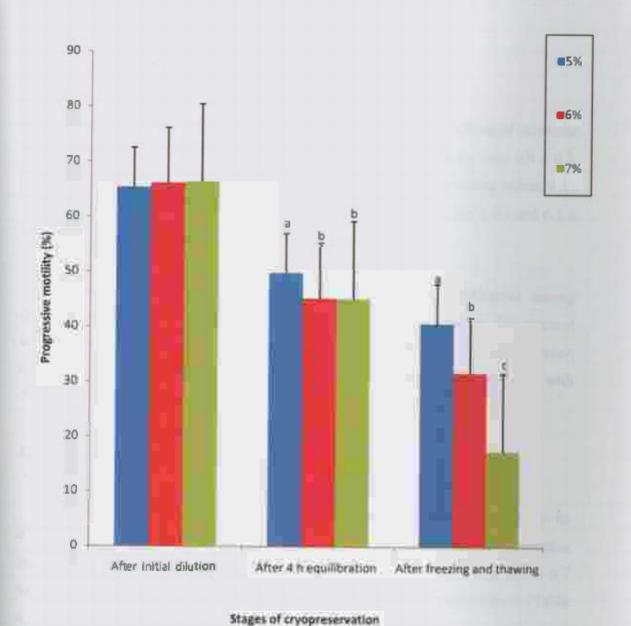


Fig. 4.10. Variations (Mean ± SE) in progressive motility during different stages of cryopreservation in split glycerol addition method, a, b on error bar indicates a significant difference (P<0.0).

eryopreservation. The highest percentage of live spermatozoa with intact acrosome was observed at 5% glycerol and lowest percentage was seen at 7%.

4.2.2.2. Live damage

The mean percentages recorded for live spermatozoa with damaged acrosome after initial dilution, after 4 hours of equilibration and after freezing were 0.9 ± 0.3 , 3.6 ± 0.5 and 3.8 ± 0.5 respectively for 5% glycerol. The corresponding values were 2.2 ± 0.6 , 5.9 ± 0.3 and 5.5 ± 0.3 for 6% glycerol and 2.5 ± 0.5 , 5.4 ± 0.3 and 6.1 ± 0.4 for 7% glycerol. (Table 4.5 & Fig 4.12)

Statistical analysis revealed that there was significant difference among different glycerol percentages after equilibration and cryopreservation. R6 glycerol was significantly different to that of 6 and 7% at both stages i.e., after equilibration and cryopreservation. Highest rive damage acrosome percentage was observed with 7% glycerol and lowest was seen with 5%.

4.2.2.3. Dead intact

The mean percentages for dead intact acrosome spermatozoa were found to be 4.7 ± 0.6 , 9.3 ± 0.5 and 13.3 ± 0.6 after initial dilution, after 4 hours of equilibration and after freezing respectively for 5% glycerol, 5.5 ± 0.5 , 12.0 ± 0.8 and 16.2 ± 0.7 for 6% glycerol and 5.7 ± 0.6 , 11.7 ± 0.9 and 19.3 ± 0.6 for 7% glycerol level, (Table 4.5 & 1 Fig. 4.13)

Statistical analysis of the dead intact percentages at different percentages showed significant variation after equilibration and cryopreservation. 5% glycerol was significantly different from 6 and 7%, but there was no any difference between 6 and 7% after equilibration. After cryopreservation it was observed that there were significant differences among all the glycerol percentages. Lowest percentages of dead intact acrossome were seen in 5% glycerol while highest percentage was found in 7%.

Table 4.5. Variations *(Mean ± SE) in liveability and acrosomal integrity of Mith un a perma tozoal during different stages of cryopreservation in split glycerol addition method.

Stage and glycerol concentration (%)	Status of spermatozoa					
	L-I	L-D	D-I	D-D		
After initial dilution						
5% 6% 7%	*8.4 3±3.1 *78.3 ± 3.4 *78.0 ± 2.9	\$0.59±0.3 \$2.2 ± 0.6 \$2.5±0.5	\$4.7±0.6 \$5.5 ± 0.5 \$5.7±0.6	\$136±1.7 \$16.4±1.6 \$16.3±1.4		
After 4 h						
5% 6% 7%	³ 67.5 ± 1.2 ^A ³ 61.9 ± 22 ^B ³ 57.7 ± 2.2 ^B	$3.6 \pm 0.5^{\circ}$ $5.9 \pm 0.3^{\circ}$ $5.4 \pm 0.3^{\circ}$	*9.3 ± 0.5 ^ *12.0±0,8 ¹¹ *11.7±0.9 ⁸	¹ 20.5 ± 0.9 ⁴ ¹ 20.9 ± 2.0 ⁴ ¹ 25.6 ± 1.3 ^B		
After freezing thasving						
5% 6% 7%	53.3 ± 1.8 ^A 45.8 ± 1.6 ^B 37.6 ± 1.2 ^C	3.8± 0.5 ^A 5.5±03 ^B 6.1±0.4 ^B	$13.3 \pm 0.6^{\circ}$ $16.2 \pm 0.7^{\circ}$ $19.3 \pm 0.6^{\circ}$	300±1.9 ⁴ 32.7±13 ⁴ 372±1.1 ⁸		

A. f. c indicates values with different superscript within column under a particular freezing stage differ significantly (P<0.05); f. f. indicates values differ significantly (P<0.05) among the cryopreservation stages for the given glycerol concentration L-l; Live sperm with intact acrosome; L-D; Live sperm with damaged acrosome; D-I; Dead sperm with damaged acrosome * No of observation-10

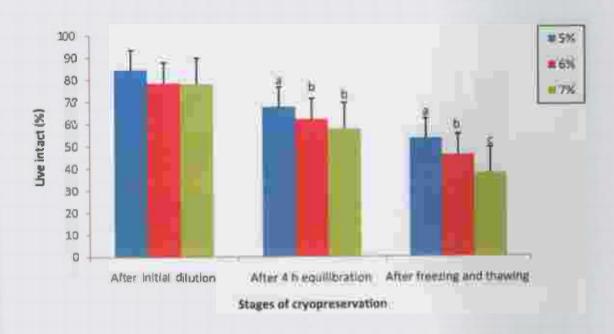


Fig. 4.11. Variations (Mean± SE) in live spermatozon with intact acrosome during different stages of cryopreservation in split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

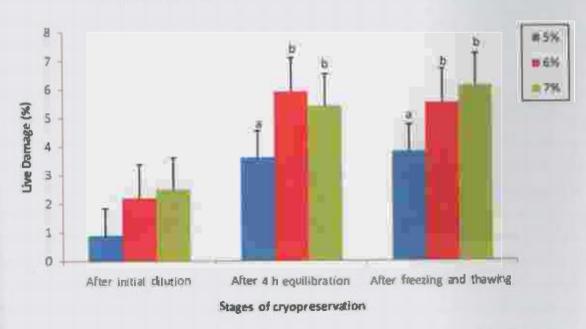


Fig. 4. 12. Variations (Mean ± SE) in live spermatozoa with damage acrosome during different stages of cryopreservation in split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

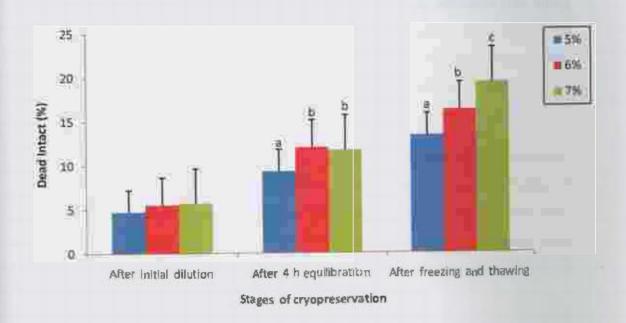


Fig. 4.13. Variations (Mean ± SE) in dead spermatozoa with intact acrosome during different stages of cryopreservation in split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

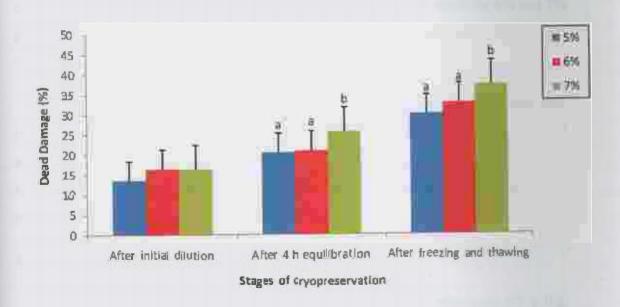


Fig. 4.14. Variations (Mean ± SE) in dead spermatozoa with damage acrosome during different stages of freezing in split glycerol addition method. a, b on error bar indicates a significant difference (P<0.05).

4.2.2.4. Dead damage

The mean percentages of dead spermatozoa with damage acrosome after initial dilution, after 4 hours of equilibration and after freezing and thawing were recorded as 13.6 ± 1.7 ; 20.5 ± 0.9 and 30.0 ± 1.9 respectively for 5% glycerol. The corresponding values were 1.4 ± 1.6 , 1.4 ± 1

Statistical analysis of the dead damage data showed that there was significant difference among the glycerol percentages after equilibration and cryopreservation. 5 and 6% was significantly different from 7% glycerol in both the stages i.e. after equilibration and cryopreservation. Highest dead damage percentage was found with 7% glycerol and lowest was seen with 5%.

4.3. Sperm abnormalities

4.3.1. Head abnormalities

The mean percentages of head abnormalities evaluated were found to be 0.7 \pm 0.3, 2.6 \pm 0.2 and 4.4 \pm 0.3 respectively after initial dilution, after 4 hours of equilibration and after freezing for 5% glycerol. The subsequent result for 6% and 7% glycerol levels were found to be 1.1 \pm 0.3, 2.9 \pm 0.2 & 4.2 \pm 0.4 and 1.0 \pm 0.3, 3.4 \pm 0.4 & 6.2 \pm 0.6 respectively. (Table 4.6 & Fig 4.15)

From the statistical analysis it was found that there was no any difference after initial dilution and equilibration, but after cryopreservation the values for head abnormality varies among the glycerol percentages. 5 and 6% glycerol was significantly different to that of 7%, Highest percentage of head abnormality was observed at 7% glycerol, whereas the lowest was seen at 6%.

4.332. Mid-piece abnormalities

The mean percentages recorded for mid-piece abnormalities were 0.7 ± 0.2 , 1.2 ± 0.2 and 2.9 ± 0.3 after initial dilution, after 4 hours of equilibration and after freezing respectively for 5% glycerol, 0.6 ± 6.2 , 1.6 ± 0.3 and 1.6 ± 0.2 for 6% glycerol and 0.4 ± 0.2 , 1.6 ± 0.2 and 2.7 ± 0.3 for 7% glycerol level. (Table 4.6 & Fig. 4.16)

Statistical analysis of the mid-piece abnormalities values showed significant difference after cryopreservation, 5 and 7% glycerol were significantly different to that of 6%. Highest value of mid-piece: abnormality was found at 3% and the lowest was seen at 6% glycerol after cryopreservation.

4.3.3. Tail abnormalities

The mean percentages of tail abnormalities after initial dilution, after 4 hours of equilibrration and after freezing were recorded as 4.1 ± 0.3 , 7.7 ± 0.7 and 12.3 ± 0.7 respectively for 5% glycerol. The corresponding values were 4.5 ± 0.3 , 8.4 ± 0.5 and 12.2 ± 0.8 for 6% glycerol and 5.0 ± 0.3 , 9.6 ± 0.5 and 11.8 ± 0.6 for 7% glycerol added in split dose. (Table 4.6 & Fig 4.17)

The data for tail abnormalities when analyzed statistically showed no significant difference among the glycerol percentages in all the 3 stages i.e., after initial dilution, after equilibration and after cryopreservation. Tail abnormalities increased after 4 hours of equilibration and showed the highest percentage after cryopreservation at 5% glycerol level and lowest was observed at 7% level.

4.3.4. Total sperm abnormalities

The mean percentages of total spermatozoa abnormalities were found to be 5.5 \pm 0.5, 11.5 ± 0.8 and 19.5 ± 1.0 after initial dilution; after 4 hours of equilibration and after freezing and thawing respectively for 5% glycerol level. The corresponding values were 6.2 ± 0.4 , 12.9 ± 0.8 and 17.9 ± 1.1 for 6% glycerol and 6.4 ± 0.5 , 14.5 0.5 and 20.6 ± 0.5 for 7% glycerol added in split dose. (Table 4.6 & Fig 4.18)

Statistical analysis of the total sperm abnormalities data revealed no difference after initial dilation and cryopreservation, but significant difference was observed after equilibration only. 5% glycerol was significantly different from that of 6 and 7% after equilibration. After cryopreservation highest total sperm abnormalities were shown by 7%, whereas lowest was observed at 6% glycerol level.

Table 4. 6. Variations *(Mean \pm SE) in head, mid piece, tail and total abnormality of Mithun spermatozoa during different stages of cryopreservation in split glycerol addition method at different stages of cryopreservation.

Stage and gly cerol codecentration (%)	Spermatozoa abnormalities					
	Read	Mid piece	Tail	Total		
After initial dilution 5% 6% 7%	\$0.74.03 \$1.1 ± 0.3 \$1.0 ± 0.3	\$ 0.7 ± 0.2 \$0.6±0.2 \$0.4±0.2	*41±0.3 *45±0.3 *5.0±0.3	\$5.5 ± 0.5 \$6,2±0,4 \$6,4±0.5		
After 4h equilibration 5% 6% 7%	2.6 ± 0.2 *2 O± 0.2 *3 4 ± 0.4	\$1.2±0.2 \$1.6±0.3 \$1.6±0.2	*7.7±0.7 *8.4±0.5 *9.6±0.5	*11.5±0.8 ^ *12.9 ±0.8 ^B *14.5 ± 0.5 B		
After freezing thaveing 5% 6%	4.4 ± 0.3^{A} 4.2 ± 0.4^{A} 6.2 ± 0.6^{B}	2,9±0,3 ^A 1.6± 0.2 ^B 2.7 ± 0.3 ^A	12.3 ± 0.7 12.2 ± 0.8 11.8±0.6	19.5 ± 1.0 17.9±1.1 206±0.5		

A II indicates values with different superscript within column under a particular freezing stage differ significantly (P<0.05); *** indicates values differ significantly (P<0.05) among the cryopreservation stages for the given glycerol concentration.

^{*} No of observation-10

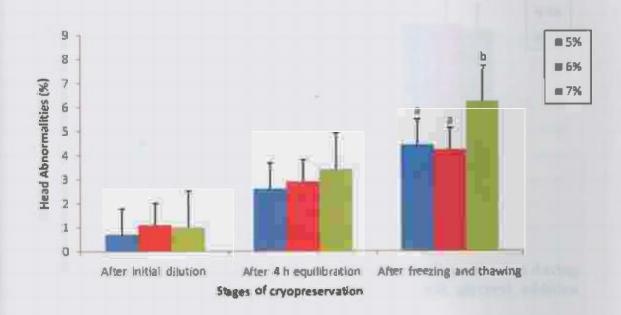


Fig. 4.15. Variations (Mean ± SE) in head abnormality of spermatozoa during different stages of cryopreservation in split glycerol addition method, a, b on error bar indicates P<0.05

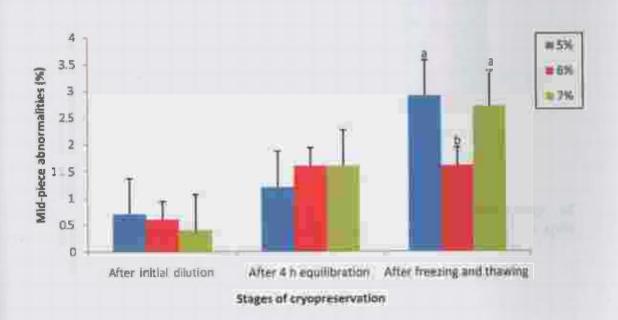


Fig. 4.16. Variations (Mean ± SE) in mid-piece abnormality of spermatozoa during different stages of cryopreservation in split glycerot addition method, a, b on error bar indicates P<0.05

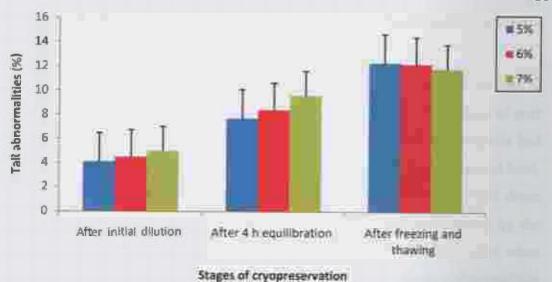


Fig. 4.17. Variations (Mean ± SE) in tail abnormality of spermatozoa during different stages of cryopreservation in split glycerol addition method. a, b on error bar indicates P<0.05

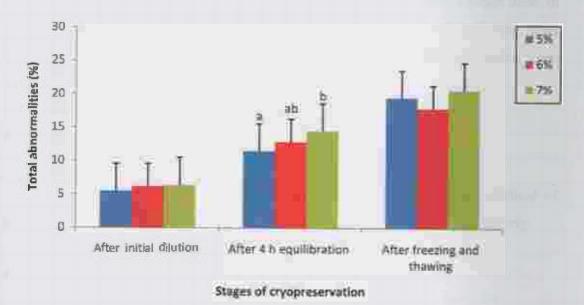


Fig. 4.18. Variations (Mean ± SE) in total sperm abnormality of spermatozoa during different stages of cryopreservation in split glycerol addition method, a, b on error bar indicates P<0.05

4.4. Comparison of post freeze-thaw sperm qualities between glycerol addition methods

The post freeze-thaw sperm qualities between non-split and split method of glycerol addition are presented in the table 4.7 & fig. 4.19. The comparison of post freeze-thaw qualities of Mithun sperm between the glycerol addition methods had revealed greater (P<0.05) progressive motility. LSIA and DSIA proportions and head, mid-piece, tail and total abnormalities in 5% glycerol when added in split doses compared to a single dose. In contrast, LSDA proportion was not affected by the methods, but lesser (P<0.05) DSDA proportion was observed in 5% glycerol when added in split doses. The proportions of DSIA and head and mid-piece abnormalities did not vary between the methods in 6 and 7% glycerol, in contrast, greater (P<0.05) motility and LSIA proportion and lesser (P<0.05) LSDA and DSDA proportions were recorded in 6 and 7% glycerol when added in split doses compared to a single dose. In addition, greater (P<0.05) and lesser (P<0.05) proportions of tail and total morphological abnormalities were recorded respectively in 6 and 7% glycerol when added in split doses.

4.5. Study on Apoptosis

The mean percentages of apoptotic sperm in non-split and split method of glycerol addition are presented in table 4.8 & 4.9 and fig 4.20 & 4.21 respectively.

4.5.1. Non-split

The mean percentages of apoptotic sperm after initial dilution were found to be 12.10 ± 1.29 , 12.20 ± 1.52 , 11.80 ± 1.39 , 12.70 ± 1.42 and 11.90 ± 1.29 for 3,4.5.6 and 7% gly cerol respectively. After equilibration the corresponding percentages were 36.00 ± 2.10 , 31.80 ± 2.19 , 24.00 ± 1.86 and 31.80 ± 1.68 ; whether after cryopreservation the values were found to be 51.80 ± 1.99 , 48.40 ± 1.58 , 33.40 ± 1.68 , 44.40 ± 1.72 and 48.00 ± 2.08 .

Statistical analysis of the apoptotic percentage data showed significant difference among the 3 stages of cryopreservation in all the glycerol percentages.

Significant difference was also observed in between the glycerol percentages after equilibration and cryopreservation. 5% was significantly different from that of 3, 4, 6 & 7% whereas there was no any difference in between 4 and 7% in both after equilibration and cryopreservation. The highest percentage of apoptotic sperm cell was observed in 3% and the lowest was seen in 5% glycerol concentration after cryopreservation.

4.5.2. Split

The mean percentages of apoptotic sperm after initial dilution were found to be 13.10 ± 1.53 , 13.40 ± 1.60 and 13.10 ± 1.42 for 5, 6 and 7% respectively. The corresponding value's were 20.10 ± 1.31 , 23.60 ± 1.74 and 30.90 ± 1.96 after equilibration and 34.90 ± 1.70 , 43.60 ± 1.77 and 47.70 ± 1.99 after cryopreservation.

Statistical analyses of the percentages of apoptotic sperm in split method of addition of glycerol revealed significant difference between the 3 stages of eryopreservation i.e. after initial dilution, after equilibration and after eryopreservation in all the glycerol percentages i.e.; 5, 6 & 7%. It was found that there was no any difference after initial dilution but statistically significant difference was observed after equilibration and after cryopreservation in between all the glycerol percentages. The highest apoptotic sperm cell percentage was seen in 7% whether the lowest was observed in 5% glycerol after cryopreservation.

4.5.3. Comparison of apoptosis in non-split and split method of addition

Comparative studies had been done between split and non-split addition methods regarding apoptotic sperm percentage which are presented in table 4.10 & 4.11 and fig 4.22 & 4.23 respectively.

Statistical analysis of the mean percentages of apoptotic sperm indicated non-significant difference between non-split and split method of addition of glycerol. But it was observed that percentage of apoptotic cell was slightly higher in non-split than split method of addition after equilibration and cryopreservation.

Table.4.7. Comparison of seminal parameters between Non-split and split method of gly cerol addition after freezing and thawing.

Seminal parameters	5%		6%		7%	
	Non-split	Split	Non-split	Split	Non-split	Split
Progressive motility	40.6±1.7	50,2± 1,9	31.7±1.1	42.1 ± 1.5	17.4± 1.3	33.9 ± 1.4
Live intact	43,4 ± 1.8	53.3 ± 1.8	35, I ± 1,0	45.8 ± 1.6	21.2± t.4	37,6±1.2
Live damage	4.8 ± 1.2	3.8 ± 0.5	7.0±0.5	5.5 ± 0.3	11.0±0.6	6.1 ± 0.4
Dead intact	10.3 ± 1.2	9.3 ± 0.5	16.5 ± 1.0	16.2±0.7	17.9±0.9	193±0.6
Dead damage	40.8 ± 2.7	30,0±1.9	41.6 ± 0.9	32.7 ± 1.3	50.0 ± 2.4	37.2±1.1
Total morphological abnormalities	14.1±0.8	19,5 ± 1.0	13.7± 1.0	17.9± 1.1	25.8 ± 1.2	20.6±0.5

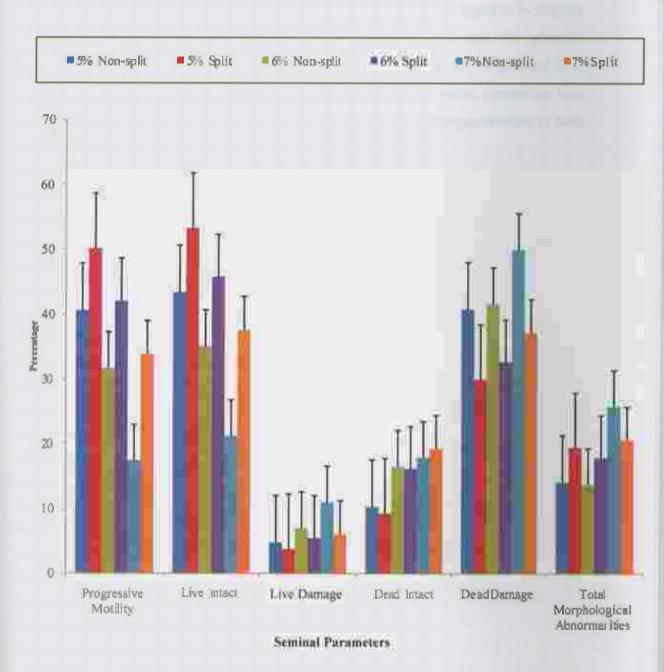


Fig. 4.19. Comparison of seminal parameters between Non-split and split method of glycerol addition after freezing and thawing.

Statistical analysis of the mean apoptotic sperm percentage data for the glycerol percentages (5, 6 & 7) showed non-significant difference between non-split and split method of addition. The apoptotic sperm percentage was found to be slightly higher in non-split than the split method of addition in 6 and 7% glycerol concentration after equilibration and cryopreservation, whereas at 5% glycerol it is slightly lesser in non-split than split. The lowest apoptotic sperm percentage was observed at 5% glycerol and the highest was seen in 7% after cryopreservation in both the addition methods.

Table.4.8. Mean (±SE) percentage of apoptotic sperm in Non-split glycerol addition method at different stages of cryopreservation.

Stages of cryo preservation	3%	4%	5%	6%	7%
After Initial Dilution	12,1 ± 1.29 A	12.2± 1.52°A	11.8±1.39"A	12.7 ± 1.42°A	11.9 ± 1.29 A
After Equilibration	36 ±2J0 bD	31.8±2.19 ^h C	19.3± 1.63 ^b A	24 ± 1,86 B	31.8± 1.68°C
After Cryopreservation	51.8± 199°D	48.4- ± 1.58°C	33,4±168° A	44.4± 1,72°B	48±203°C

Figures with same superscript (a, b, c) do not differ significantly in columns Figures with same superscript (A, B, C, D) do not differ significantly in rows

Table. 4.9. Mean (±SE) apoptotic sperm percentage in split glycerol addition method at different stages of cryopreservation.

Stages of cryopreservation	Glycerol Concentration					
	5%	6%	7%			
After Initial Dilution	13.10± >53 ^{aA}	13.40± 1.60 ^{±A}	13,10± 1.42 HA			
After Equilibration	20.10± 1.31 ^{bA}	23.60± 1.74bB	30.90 ± 1.96 bC			
After Cryopreservation	34.90± 1.70°A	43.60± 1.77°B	47,70± 1.99°C			

Figures with similar superscript (a, b, c) do not differ significantly in columns Figures with similar superscript (A, B, C) do not differ significantly in rows

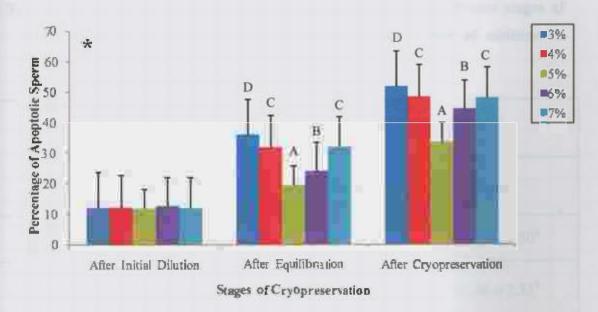


Fig. 4.20. Apoptotic sperm percentage in Non-split glycerol addition method at different stages of cryopreservation (* indicates p< 0.05). A. B. C. Don error bar indicates P<0.05.

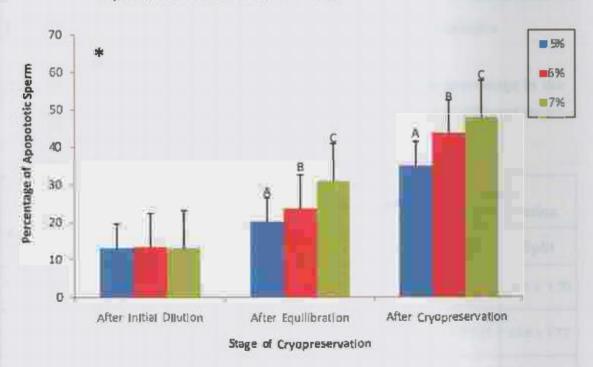


Fig. 4.21. Apoptotic sperm percentage in split glycerol addition method at different stages of cryopreservation (* indicates p< 0.05). A, B, C, on error bar indicates P<0.05.

Table. 4.10. Comparison of apoptotic sperm percentage at different stages of eryopreservation in Non-split and split method of addition of glycerol.

Stages of cryopreservation	Glycerol addition method			
oring of the young that the same	Non-Split	Split		
After Initial Dilution	12.14± 1.37*	13.20± 1.50°		
After Equilibration	28.58 ± 2.66 ^b	24.90 ± 2.33 ⁶		
After cryopreservation	45.20 ± 2.68°	42.07 ± 2.51°		

Figures with same superscript (a, b, c) do not differ significantly in columns

Table.4.11. Comparison between the mean apoptotic sperm percentage in the split and non split method of addition of glycerol in different stages of cryopreservation.

Glycerol concentration	After Initiat Dilution		After Equilibration		After cry opreservation	
	Non- Split	Split	Non- Split	Split	Non- Split	Spilt
5%	11.8± 1.39	13.1 ± 1.53	19.3± 1.63	201± 1.3[33,4 ± 1.68	34.9 ± 1.70
6%	12.7±142	13.4 ± 1.60	2 4 ± 1,86	23.6±1.74	444±1,71	436±177
7%	11,9 ± 1,2 9	13.1 ± 1A2	31,8 ± 1,68	3Q9± 1.96	48±203	47.7± 1.99

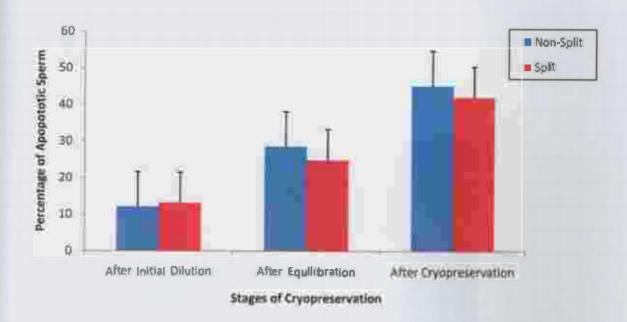


Fig. 4.22. Comparison of apoptotic sperm percentage at different stages of cryopreservation in Non-split and split method of addition of glycerol

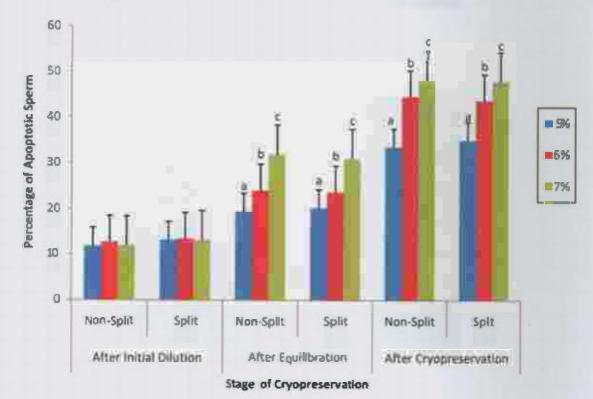
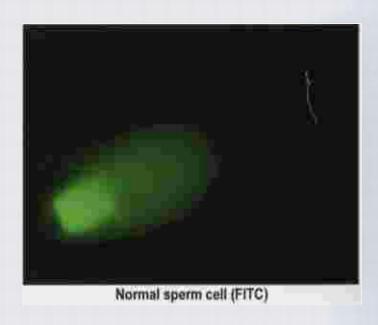
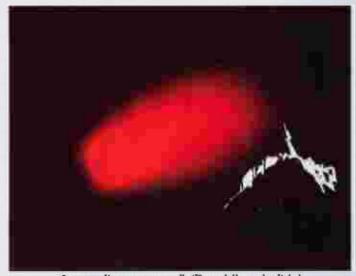


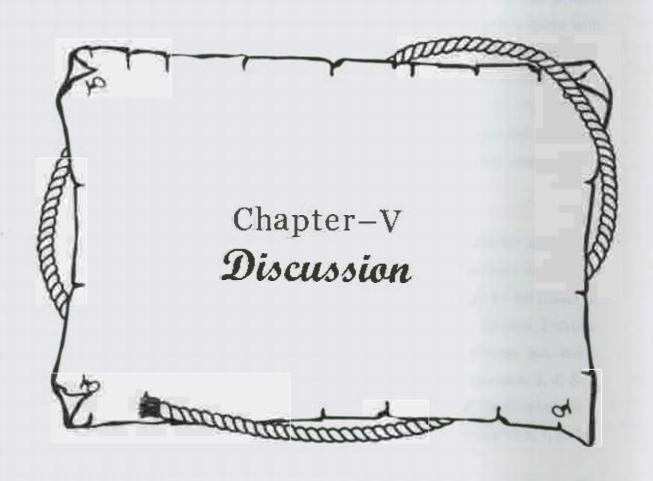
Fig.4.23. Apoptotic sperm percentage in split and non split method of addition of glycerol at different concentration in different stages of cryopreservation, a, b, con errol bar indicates P<0.05.





Apoptotic sperm cell (Propidium lodide)

Photo,D. Photographs showing normal and apoptotic sperm cell stained with FITC and propidium iodide.



DISCUSSION

5.1. Addition of glycerol in single dose

In the experiment-I, Progressive motility and the proportion of live sperm with intact acrosome (LSIA) were decreased (P<0.05) but, the proportion of dead sperm with damaged acrosome (DSDA) and mid-piece, tail and total morphological abnormalities were increased (P<0.05) after freeze-thawing compared to final dilution or 4-h equilibration in all glycerol concentrations. The proportion of live sperm with damaged acrosome (LSDA), dead sperm with intact acrosome (DSIA) and head abnormalities were increased (P<0.05) after freeze-thawing compared to final dilution or 4-h equilibration in all of glycerol concentrations except 5%. In 5% glycerol, LSDA proportion was not different among the cryopreservation stages and DSIA and head abnormalities portions were lesser (P<0.05) after dilution compared to equilibration or freezing.

After dilution the sperm quality parameters were not different among the glycerol concentrations. Similarly, after equilibration, no differences were observed in sperm quality parameters among the glycerol concentrations except for tail (lesser in 5% glycerol; P<0.05) and total abnormalities (lesser in 5 and 6% glycerol; P<0.05). After freeze-thawing, greater (P<0.05) motility and LSIA proportion but, lesser (P<0.05) LSDA and DSIA proportions were observed in 5% compared to 3, 4, 6 or 7% glycerol. Further, lesser (P<0.05) proportions of DSDA and all the morphological abnormalities were observed after freeze-thawing in 5 and 6 compared to 3, 4, or 7% glycerol.

During cryopreservation, sperms are exposed to severe stress due to cold shock, which affects the livability and structural and functional integrity of preserved sperms. The degree of cryo-injuries strongly depends on the species-specificity of sperm physiology (Thurston et al., 2002) that can differ among the related species (Fickel et al., 2007). Therefore, species specific optimized cryopreservation protocols are required for obtaining best results. In this study, we tried to optimize the concentration and addition method of glycerol into diluents for cryopreserving mithun

sperm using tris-egg yolk diluents. The results indicated that the post freeze-thaw qualities of mithun sperm were significantly affected by the concentration and addition method of glycerol.

Bult sperms are routinely cryopreserved in the diluent containing 4 to 8% glycerol and acceptable results have also been obtained in wild ruminants using this range (Hopkins et al. 1988: Garland 1989: Fennessyet al. 1990). In the present study. in single dose of addition of glycerol the percentages of progressive motility and live intact acrosome were found to be 40.6±1.7 and 43.4±1.8 in 5% glycerol concentration. These percentages were significantly greater than that obtained by cryopreserving in 3, 4, 6 and 7% glycerol. Additionally, the other sperm qualitative parameters were found better when 5% glycerol was used. The qualitative parameters were comparable with previous studies in mithun (Dhali et al., 2008; Karunakaran et al., 2006). Dhali et al., 2008 found 43% progressive motility and 55% live intact acrosome and 25% abnormal spermatozoa by using tris-egg yolk diluent containing 5.6% glycerol. In this study the percentages of progressive motility and live intact spermato20a in cryopreserved semen samples were found to be comparatively lower than cattle bull. Previous reports in cattle indicate that semen retains approximately 50-70% molality and 60-70% live; intact acrosome when cryopreserved in tris-egg yolk glycerol diluents (Vyas et al. 1992; Thun et al., 2002; Januskauskas et al., 2005). But similar results were found in case of spermatozoa abnormalities in cattle i.e. 70-80% remain morphologically normal (Somfai et al. 2002; Nagy et al. 2004). The total head abnormalities were found to be lesser in 5% glycerol added in single dose than the other glycerol percentages.

Glycerol is widely used permeating cryoprotectant (Watson, 1990; Holt, 2000) for sperm cryopreservation. Nevertheless, the optimal glycerol concentration in diluents varies among species (Holt, 2000; Agea and trister, 2002) and depends on the rate during the cryopreservation process (Fisher and Fairfull, 1989; Watson, 1990). The cryopreservation method used in the present study was simple and easy to perform and the method excluded the use of a controlled cooling device. In this method, after dilution sperms were equilibrated at 4°C for 4h, then kept in liquid nitrogen (LN₂) vapour for 10 minutes and finally plunged into LN₂ for storage. Various authors had found better results with different glycerol concentration. Hong

(2003) compared 2, 4, 6 & 8% glycerol for cryopreserving blue foxes spermatozoa and found 4% glycerol to be optimum for cryopreserving. Santos (2006) used two glycerol concentrations i.e. 6% and 3% for cryopreserving red deer epididymal sperm and found that 6% glycerol with rapid cooling rate, significantly improved spermatozoa freezability. Rasul (2007) found that the addition of glycerol (6%) at 37°C yielded better post thaw sperm motility, plasma membrane integrity and velocities than addition at 4°C. In this study the results indicated that using 9% glycerol was most suitable for eryopreserving mithun semen. The poor post thaw sperm qualities with 3 and 4% glycerol might be due to the fact that the cooling rate of the used cryopreservation method was not probably sufficient for freezing mithun sperm as lower glycerol concentrations generally requires faster cooling rate (Watson, 1990). In contrast, the post freeze thaw sperm qualities with 6 and 7% glycerol might be attributed to the toxic effects of such higher glycerol concentrations (Holt, 2000; McLaughlin et al., 1992).

5.2. Addition of glycefol in split dose

In the experiment-II, 5, 6 & 7 % glycerol was selected based on the results of the experiment-I to study the effect of addition of glycerol in split doses. 3% and 4% glycerol concentrations were not included because of the poor results shown in the experiment-I. An altempt had been made to improve the quality of preserved sperm by adding these glycerol concentrations in divided doses so that the toxic effects of glycerol does not affect the sperm quality, in all glycerol concentrations, motility and LSiA proportion were reduced (P<0.05), but the proportion of DSIA, DSDA and head, tail and total abnormalities were increased (P<0.05) after freeze-thawing compared to dilution or equilibration. The proportion of LSDA was found lesser (P<0.05) after dilution compared to equilibration or freeze-thawing in all glycerol concentrations. The proportion of mid-piece abnormalities was lesser (P<0.05) after dilution compared to equilibration or freeze-thawing in 6 and 7% glycerol. In 5% glycerol, although similar mid-piece abnormality was found after dilution and equilibration, it was greater (P<0.05) after freeze-thawing.

All the sperm quality parameters after dilution and the proportion of head, mid-piece and tail abnormalities after equilibration were found similar among the

glycerol concentrations. After equilibration and freeze-thawing, greater (P<0.05) motility and LSIA proportion and lesser (P<0.05) LSDA and DSIA proportions were observed in 5 compared to 6 and 7% glycerol. Lesser (P<0.05) proportion of DSDA after equilibration and freeze-thawing and lesser (P<0.05) total abnormalities after equilibration were found in 5 and 6 compared to 7% glycerol. The proportions of tail and total abnormalities after freeze-thawing were not different among the glycerol concentrations. In contrast, after freeze-thawing, lesser (P<0.05) head abnormalities in 5 and 6 compared to 7% glycerol and lesser (P<0.05) mid-piece abnormalities in 6 compared to 5 and 7% glycerol were observed.

The split dose of addition of glycerol was done at 4°c to compare the effects of temperature on addition of glycerol. Lower glycerol toxicity to ram spermatozoa had been found when it was added at 0°C (Colas, 1975), but glycerol addition at 22°C had improved the post thaw motility of stallion sperm (Vidament, 2000). Better post freeze-thaw quality of goat sperm had been recorded when 7% glycerol was added at 37°C contpared to 5°C. In contrast similar post thaw qualities of ram sperm had been observed when glycerol was added at room temperature or 5°c (Morrier et d., 2002). Similarly, no differences had been observed for bovine sperm glycerolaged at 5, 10, 20 or 35°C (Salisbury et al., 1978) and the quality of cryopreserved dog sperm was not influenced by glycerol addition at either 37 or 4°C (Pena et al., 1998), in the present study a significant increase in post thaw motility (50.2 \pm 1.9) and proportion of live intact acrosome (53.3 ± 1.8) was observed and most of the other qualitative paranteters were found better when 75% of total required glycerol (5, 6 or 7%) was added in split doses at 4°C compared to the single dose addition of entire amount at 37°C. There was no any previous information regarding the use of glycerol in split dose in mithun semen cryopreservation hence the results were not comparable to any other worker.

In both the experiments (single and split dose addition). 9% glycerol was found to be better than the rest of the concentrations in respect of all the qualitative parameters i.e., higher percentages of progressive motility, live intact acrosome and lower percentages of live damaged, dead intact and dead damage acrosomes. But in case of total abnormalities it was found that 5% glycerol showed slightly higher percentages than 6% which might be due to more tail and mid-piece abnormalities.

This might be due to individual variation and prolonged exposure to environmental temperature during collection or processing.

5.3. Double staining

The current study revealed that the Trypan blue and Giemsa staining technique can be used efficiently for simultaneous evaluation of viability, morphological abnormality and acrosomal integrity of mithun sperm. Different workers had used this double staining technique i.e. Trypan blue to differentiate live from dead and Giemsa to stain the acrosome. Sidhu et al. (1992) used this double staining procedure for detecting true acrosome of buffalo spermatozoa. Jankovicova et al. (2008) applied this double staining to study the acrosomal integrity in cattle spermatozoa. Didion et al. (1989) described double staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species.

5.4. Apoptosis of sperm

Apoptosis, a physiological programmed cell death is a complex phenomenon that can be divided into 3 phases: induction, execution and degradation. Mitochondria are known to play a central role during the execution phase. After induction of apoptosis, mitochondrial pores are opened, characterized by decreased mitochondrial membrane potential, Opening of the mitochondrial pores leads to the release of proapoptotic factors from mitochondria (Martin et al., 2004). In the cytoplasmic compartment, the proapoptotic factors, for example, different proteases related to the caspases family (cysteine proteases with aspartate specificity) are subsequently activated, leading to the degradation phase. During this phase, changes at both the cell surface and the nucleus occur. Phosphatidylserine (PS), ordinarily sequestered in the plasma membrane inner leaflet, appears in the outer leaflet, where it triggers non inflammatory phagocytic recognition of the apoptotic cell (Bratton et al., 1997).

The most significant changes related to apoptosis are the externalization of the phosphatidylserine (PS). DNA fragmentation, caspase activation, loss of mitochondrial membrane potential, and increase in Sperm membrane permeability (Bratton et al., 1997; Glander & Schaller, 1999; Martin et al., 2004; Khan et al.,

2009). Several pathways are reported for mammalian cell apoptosis. These include the intrinsic, extrinsic, and apoptosis-inducing factors. During the early phases of disturbed membrane function, asymmetry of the membrane phospholipids occurs, before the integrity of the plasma membrane is progressively damaged (Martin et al., 1995). When the cell membrane is disturbed, the phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane (Desagher & Martinou, 2000). Sperm cryopreservation is a widely used procedure in the context of assisted reproductive techniques. Apoptosis occurs in the freshly ejaculated sperm cells, but eryopreservation acts is an apoptotic mechanism inducer. The rate of apoptosis varies in individual bulls which could be regarded as a marker of fertility for selection of good quality breeding bulls. Cryopreservation and thawing is a procedure that inflicts irreversible injury on spermatozoa. Cryopreservation reduces the no. of live spermatozoa in the semen. There could be several reasons for the induction of apoptosis in frozen semen. During cryopreservation, the sperm plasma membrane is destabilized due to low temperatures and high salt concentrations, leading to marked changes in the lipid components of sperm plasma membranes in ram, boar, and butl. In addition, chemical toxicity due to glyceroi and immune responses due to egg yolk in semen extender cause alterations in the plasma membrane of spermatozoa. Under normal physiological conditions, cell membrane asymmetry is maintained by the phospholipid translocators (known as flippa.ses). Freezing and (hawing might lead to damage of the flippase present in the plasma membrane, causing translocation of PS from the inner to the outer leaflet, an early sign of apoptosis. Like somatic cells, spermatozoa live in an aerobic environment and face reactive oxygen species that cause fatty acid peroxidation of membrane pltospholipids and thus, affect sperm function. Lipid peroxidation of plasma membrane increases after freezing and thawing of bull and human sperm, which may cause apoptosis in these cells. (Anzar, 2002).

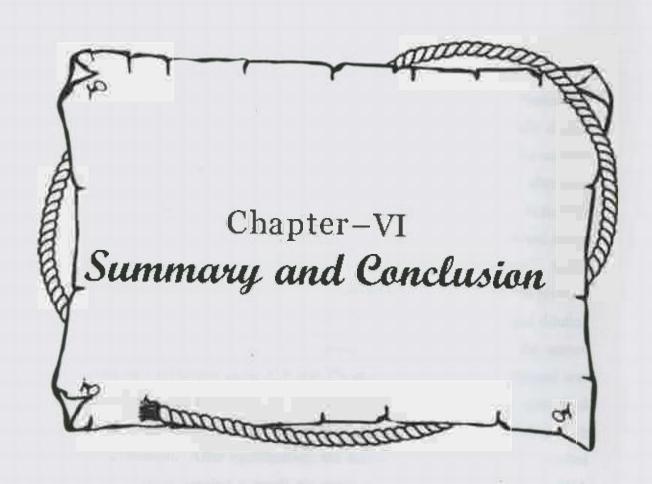
During conventional freezing, water precipitates as ice and often leads to tissue damage, acts on cytoskeleton or genome related structures. Ice crystallization induces an unregulated and mechanical damage in the spermatozoa leading to death which may be related to apoptosis like phenomena. It is widely known that the cryopreservation usually causes sub tethal cryo damage to spermatozoa, decreasing post-thaw cell viability. The freezing-thawing of human (Glander & Schaller, 1999),

bull (Martin *et al.*, 2004), boar (Pena *et al.*, 2003), stallion (Ortega Ferrusola *et al.*, 2007), and dog (Kim *et al.*, 2010) spermatozoa induces membrane PS translocation, what demonstrates that cryopreservation leads to apoptosis. Therefore, detecting early phases of membrane dysfunction, or initial phases of apoptosis of viable spermatozoa, would be important when evaluating stressed spermatozoa, such as those subjected to freezing and thawing, and would be useful for controlling freezing procedures in semen. Several workers had recognized apoptosis as a cause of sperm death during cryopreservation like Chaveiro *et al.* (2007). Feitosa *et al.* (2008) and Martin *et al.* (2004) in bovine sperm; Bolanos *et al.* (2011) and Ortega *et al.* (2007) in equine sperm and Khan *et al.* (2009) in buffalo sperm.

In the present study apoptotic cells were found in all the 3 stages of cryopreservation i.e. after initial dilution, after equilibration and after freezing and thawing. The apoptotic sperm percentages were increased significantly after equilibration and freeze-thaving. Similar results were obtained by Martin et al. (2004) where the apoptotic sperm percentage were 11.3 \pm 10.6% and 44.9 \pm 17% before and after cryopreservation respectively. Ortega et al. (2007) found apoptotic sperm with higher membrane permeability in fresh semen (7.2 \pm 3.30), cooled semen (10 \pm 9.66) and frozen (18.7 \pm 9.15) after stained with yo-pro and ethidium bromide. Khan et at. (2009) found increased apoptotic sperm percentage after freezing and thawing in buffalo sperm (25.4 \pm 0.6 % vs. 36.5 \pm 1.9%). In the present study the apoptotic sperm percentage were higher after equilibration and freeze-thawing in both split and non-split method of glycerol addition. But the apoptotic sperm percentage was slightly higher (non-significant) in non-split than split method of glycerol addition. There were no any difference in the apoptotic sperm percentage after initial dilution but difference was found after equilibration and freeze-thawing in both the addition methods. In the present study it was noticeable that the apoptotic sperm percentage were increased in all the glycerol concentrations except 3% where the apoptotic sperm percentage were comparatively lesser than rest of the concentrations in both split and non-split method. This establishes the role of adjact concentration of glycerol in controlling apoptosis by sperm damage due to cryopreservation. Proper dose of glycerol can reduce the percentage of apoptotic sperm by limiting the tissue damage due to cryopreservation. The glycerol addition method has no direct effect on apoptosis of mithun sperm.

The sensitive tunnel assay that was used to study the apoptosis in mithum sperm was based on the principle of detection of single and double stranded DNA breaks occurring at early stages of apoptosis. After an enzymatic reaction, the fluorescein label incorporated at the damage sites of DNA is visualized by fluorescence microscopy using FITC/PI stain. In the present finding the apoptotic sperm appear red with propidium iodide (PI), while the normal non-apoptotic sperm appear green with FITC stain. In the present experiment it was observed that the percentages of cells that stained with PI after cryopreservation was more than before cryopreservation. Similar result was found by Martin et al. (2004) with the PI assay and got $50.8 \pm 14.2\%$ eells stained with PI after cryopreservation than against $28 \pm 12.7\%$ before cryopreservation. The permeability of the PI was higher in the dead cells because of the opened mitochondrial pores due to apoptosis.

The present experiment was conducted with the hypothesis that cryopreservation was associated with the activation of apoptotic process in spermatozoa and with the tunnel assay procedure it was established that there was presence of apoptotic sperm in mithun semen in both fresh as well as cryopreserved state. The viability of the sperm cells reduced remarkably after freezing and thawing. But this tunnel assay method was not sufficient to establish apoptosis in mithun sperm as there need to be detailed studies on mitochondrial membrane potential, caspase activation and increase membrane permeability to explain this complex process of apoptosis.



SUMMARY

The present investigation was carried out for a period of 2 years in the Institute's (NRC on Mithun, Nagaland) furm at Jharnapani. Semen samples were collected from 5 adult healthy Mithun bulls aged between 4-6 years through rectal massage method and the semen samples exhibiting a mass activity score of 3 or more were only processed further for freezing.

After collection, the samples were partially diluted by adding I ml prewarmed (37°C) tris-egg yolk diluents (Tris-hydroxymethyl aminomethane 24.3 mg/mj, sodium cirate 13.4 mg/ml, fructose 10 mg/ml and egg yolk 20% Penieillium G sodium salt 1000 JU/ml and dihydrostreptomycin 1 mg ml). The partially diluted samples were maintained at 37°C for further processing. Final dilution of the samples was done with pre-warmed (37°C) tris-egg yolk diluents in such a way that after final dilution and glycerol addition each ml of sample contained 60×106 sperm. Following the final dilution, the samples were equilibrated at 4°C for 4-h and glycerol was added into the samples in a single dose (Experiment-1) or split doses (Experiment-2). In the experiment-I, the semen samples were cryopreserved using 3,4,5,6, and 7% glycerol and glycerol was added into the diluents in a single dose at 37°C after final dilution and kept immediately at 4°C for equilibration. In the experiment-II, the semen samples were cryopreserved using 5, 6 and 7% glycerol and the total glycerol was divided into four equal parts and a part was added into the diluents after final dilution at 0h (immediately before equilibration, 37°C), Ih (4°C), 2h (4°C) and 3h (4°C) of equilibration. After equilibration, the samples were loaded into pre-cooled (4°C) 0.50 ml straws leaving a small air space at the end and sealed with PVA powder. The straws were then frozen in liquid nitrogen vapour (5 cm above the liquid nitrogen level) for 10 min and subsequently plunged into liquid nitrogen for storage. In both the experiments, the interval between the partial dilution of semen samples and beginning of equilibration did not exceed 60 min. Semen volume, mass activity in the fresh samples and sperm concentration in partially diluted samples were determined. The sperm qualitative parameters (progressive motility, livability, aerosomal integrity, morphological abnormalities and apoptosis) were determined after final dilution, 4-h equilibration and after freeze-thawing. The frozen samples

were evaluated after minimum 7 days of storage in liquid nitrogen. The objective of this study was to find out the suitable glycerol concentration for cryopreserving mithun semen, study the effect of addition of glycerol in split and non-split method and the effect of cryopreservation on acrosomal integrity and apoptosis.

In the experiment-like, the addition of glycerol in non-split method, the mean percentages of progressive motility were found to be 67.8 ± 2.8 , 43.2 ± 2.3 and 23.9 ± 2.0 for 3% glycerol after initial dilution, after 4 hours of equilibration and after freezing and thawing. The subsequent percentages of progressive motility were found to be 67.2 ± 2.3, 44.2 ± 2.4 and 26.5 ± 2.7 for 4% glycerol; 65.3 ± 4.1. 49.8 ± 2.2 and 40.6 ± 1.7 for 5% glycerol; 66.1 ± 2.9 , 45.2 ± 2.5 and 31.7 ± 1.1 for 6% glycerol and 66.3 ± 3.7 , 45.1 ± 3.0 and 17.4 at 1.3 for 7% glycerol. The percentage of progressive motile sperm is 5% giveerol was significantly (P<0.05) different to that of 3%, 4%, 6% and 79 aglycerol. The mean percent of live intact were found to be 70.9 ± 2.7, 46.7 ± 2.6 and 18.0 ± 1.7 after initial dilution after 4 hours of equilibration and after freezing and thawing for 3% glycerol. The live intact percentages for 4% glycerol were found to be 71.3 ± 2.4, 45.6 ± 2.1 and 20.2 ± 2.2; for 5% 69.2 ± 4.0. 52.8 ± 2.2 and 43.4 ± 1.8; for 6% 70.3 ± 3.3, 49.4 ± 2.4 and 35.1 ± 1.0; for 7% 71.5 ± 4.1, 49.9 ± 3.2 and 21.2 ± 1.4. Live intact acrosome percentage at 5% glycerol concentration was significantly different from 3, 4, 6 and 7% glycerol. The mean percentages of live damage after initial dilution, 4 hours of equilibration and freezing and thiswing were recorded to be 2.9 \pm 0.3, 4.7 \pm 0.5 and 10.7 \pm 0.5 for 3% glycerol; 3.0 ± 0.3, 5.0 ± 0.7 and 10.5 ± 0.5 for 4% glycerol; 3.0 ± 0.6, 5.2 ± 0.8 and 4.8 ± 1.2 for 5% glycerol; 1.2 ± 0.5, 5,2 ± 0.7 and 7.0 ± 0.5 for 6% glycerol and 2.0 ± 0.5 , 5.6 ± 0.4 and 11.0 ± 0.6 for 7% glycero). Evaluation of the data showed that the five damage acrosome percentages at 5% glycerol was significantly different from 3, 4 and 6% whereas there were no any difference between 3, 4 and 7% glycerol after ory opreservation. The mean percentages of dead intact were recorded to be 6.2 ± 0.8, 14.4 ± 1.2 and 20.8 ± 2.3 after initial dilutions. 4 hours of equilibration and after freezing and thaveing for 3% glycerol concentration. The subsequent readings were recorded to be 6.5 \pm 0.9, 15. $l \pm$ 1.3 and 20.6 \pm 2.3 for 4% glycerol; $f_19 \pm 0.9$, 11. $l \pm$ 1.1 and 10.3 \pm 1.2 for 5%; 8.0 ± 0.7 , 13.0 ± 1.1 and 16.5 ± 1 for 6% glycerol and 7.6 ± 0.8 , 12.1 ± 1.0 and 17.9at 0.9 for 7% glycerol. There was no play difference between 3, 4 and 7% glycerol, but 5% glycerol showed significant difference to that of 3, 4, 6 & 7. The mean percentages of

dead damage were found to be 21.3 ± 1.8 , 34.6 ± 2.0 and 50.8 ± 2.5 after initial dilution after 4 hours of equilibration and after freezing and thawing for 3% glycerol; 20.6 ± 1.9 , 34.4 ± 1.5 and 49.5 ± 2.4 for 4% glycerol; 22.5 ± 2.8 , 31.9 ± 2.3 and 40.8 ± 2.7 for 5% glycerol; 22.1 ± 2.3 , 33.3 ± 3.2 and 41.6 ± 0.9 for 6% glycerol and 20.8 ± 2.1 , 33.4 ± 1.9 and 50.0 ± 2.4 for 7% glycerol concentration. There was significant difference after cryopreservation. 5% and 6% glycerol was significantly different from 3, 4, & 7%, but in between 5 and 6% glycerol there was no significant difference.

The mean percentages of head abnormalities were found to be 0.9 ± 0.3 , $3.0 \pm$ 0.3 and 6.1 ± 0.4 after initial dilution, 4 hours of equilibration and after freezing and thawing for 3% glycerol; 1.1 ± 0.3 , 2.8 ± 0.3 and 5.3 ± 0.6 for 4% glycerol; 1.2 ± 0.4 , 2.6 ± 0.4 and 2.8 ± 0.4 for 5% glycerol; 0.8 ± 0.3 , 2.0 ± 0.5 and 3.2 ± 0.4 for 6% glycerol and 0.6 ± 0.2 , 2.9 ± 0.3 and 6.2 ± 0.5 for 7% glycerol concentrations, 5% glycerol was significantly different from 3, 4 and 7%. The mean percentages of midpiece abnormalities in the 3 stages i.e.; after initial dilution, after 4 hours of equilibration and after freezing and thawing were found to be 0.2 ± 0.2 , 1.2 ± 0.3 and 3.1 ± 0.3 for 3% glycerol. For the subsequent concentrations the value were recorded as 0.4 ± 0.2 , 1.3 ± 0.3 and 2.9 ± 0.3 for 4% glycerol: 0.5 ± 0.2 , 0.5 ± 0.2 and 1.6 ± 0.2 for 5% glycerol; 0.2 ± 0.2 , 0.7 ± 0.2 and 1.5 ± 0.2 for 6% glycerol and 0.6 ± 0.2 , 2.9 ± 0.2 0.3 and 6.2 ± 0.5 for 7% glycerol. 5 & 6% glycerol were significantly different from 3, 4 and 7. The mean percentages of tail abnormalities were found to be 4.7 ± 0.3 , 9.4± 0.5 and 16.3 ± 0.9 after initial dilution, after 4 hours of equilibration and after freezing and thawing for 3% glycerol: 3.4 ± 0.5 , 8.6 ± 0.8 and 16.1 ± 0.8 for 4% glycerol; 4.1 ± 0.3 , 6.5 ± 0.3 and 9.7 ± 0.5 for 5% glycerol; 4.7 ± 0.5 , 7.0 ± 0.7 and 9.0 ± 0.6 for 6% glycerol and 4.6 ± 0.3 , 9.5 ± 0.4 and 16.6 ± 0.9 for 7% glycerol, 5% glycerol was significantly different from rest of the percentages, but there was no difference between 3 and 7% after equilibration. After cryopreservation 5 and 6% were significantly different from 3, 4 and 7%. The mean percentages of total sperm abnormalities were recorded as 5.8 ± 0.5 , 13.6 ± 0.8 and 25.3 ± 1.0 after initial dilution, after 4 hours of equilibration and after freezing and thawing for 3% glycerol; 4.9 ± 0.5 , 12.7 ± 1.1 and 24.2 ± 1.0 for 4% glycerol; 5.8 ± 0.3 , 9.6 ± 0.6 and 14.1 ± 1.1 0.8 for 5% glycerol; 5.7 ± 0.6 , 9.5 ± 0.9 and 13.7 ± 1.0 for 6% glycerol and 5.6 ± 0.4 ,

 13.6 ± 0.8 and 25.8 ± 1.2 for 7% glycerol. After equilibration and cryopreservation, 5 and 6% glycerol were significantly different from 3, 4 and 7%.

In the experiment-II i.e. addition of glycerol in split doses the mean percentages of progressive motility were found to be 98,8 \pm 2.3, 64.1 \pm 1.5 and 50.2 \pm 1.9 after Initial dilution, after 4 hours of equilibration and after freezing for 5% glycerol; 74.1 \pm 2.9, 58.0 \pm 2.5 and 42.1 \pm 1.5 for 6% glycerol and 74.5 \pm 2.7, 53.7 \pm 2.0 and 33.9 \pm 1.4 for 7% glycerol added in split dose. After e-quilibration, the mean progressive motility percentages at 5% glycerol were significantly different from 6 and 7%, but after cryopreservation all the glycerol percentages vary significantly among themselves. The mean percentages of live intact acrosome were found to be 84.3 ± 3.1 . 67.5 ± 1.2 and 53.3 ± 1.8 after initial dilution, after 4 hours of equilibration and after freezing for 5% glycerol: 78.3 ± 3.4 . 61.9 ± 2.2 and 45.8 ± 1.6 for 6%glycerol and 78.0 \pm 2.9. 57.7 \pm 2.2 and 37.6 \pm 1.2 for 7% glycerol. The racan live intact percentages at all the glycerol concentrations vary significantly among themselves after cryopreservation. The racan percentages recorded for live damaged spennalozoa after initial dilution, after 4 hours of equilibration and after freezing were 0.9 ± 0.3 , 3.6 ± 0.5 and 3.8 ± 0.5 for 5% glycerol; 2.2 ± 0.6 , 5.9 ± 0.3 and 5.5 ± 0.3 for 6% glycerol and 2.5 \pm 0.5, 5.4 \pm 0.3 and 6.1 \pm 0.4 for 7% glycerol level added in split. The racan live damage acrosome percentages at 5% glycerol were significantly different to that of 6 and 7% at both stages of cryopreservation i.e, after equilibration and cryopreservation. The mean percentages for dead intact spermatozoa were found to be 4.7 \pm 0.6, 9.3 \pm 0.5 and 13.3 \pm 0.6 after initial dilution, after 4 hours of equilibration and after freezing 5% glycerol; 5.5 ± 0.5 , 12.0 ± 0.8 and 16.2 ± 0.7 for 6° glycerol and 5.7 \pm 0.6, i1.7 \pm 0.9 and 19.3 \pm 0.6 for 7% glycerol level. The mean dead intact percentages at 5% glycerol were significantly different from 6 and 7%, but there was no any difference between 6 and 7% after equilibration. eryopreservation it was observed that there were significant differences among all the glycer of percentages. The mean percentages of dead damages after initial dilution. after 4 hours of equilibration and after freezing were recorded as 13,6 ± 1.7; 20.5 ± 0.9 and $3.0.0 \pm 1.9$ for 5% glycerol: 16.4 ± 1.6 . 20.9 ± 2.0 and 32.7 ± 1.3 for 6% glycerol and 16.3 ± 14 , 25.6 ± 1.3 and 37.2 ± 1.1 for 7% glycerol. The mean dead damage acrosome percentages at 5 and 6% glycerol were significantly different from 7% in both the stages i.e., after equilibration and cryopreservation.

The mean percentages of head abnormalities in the split method, evaluated were found to be 0.7 \pm 0.3, 2.6 \pm 0.2 and 4.4 \pm 0.3 for 5% glycerol after initial dilution, after 4 hours of equilibration and after freezing. The subsequent result for 6% and 7% glycerol levels were found to be 1.1 \pm 0.3, 2.9 \pm 0.2 and 4.2 \pm 0.4 and 1.0 \pm 0.3, 3.4 \pm 0.4 and 6.2 \pm 0.6 respectively. The mean head abnormality percentages at 5% and 6% glycorol were significantly different to that of 7%. The mean percentages recorded for mid-piece abnormalities were 0.7 ± 0.2 1.2 ± 0.2 and 2.9 ± 0.3 after initial dilution, after 4 hours of equilibration and after freezing for 5% glycerol; 0.6 ± 6.2, 1.6 \pm 0.3 and 1.6 \pm 0.2 for 6% glycerol and 0.4 \pm 0.2 1.6 \pm 0.2 and 2.7 \pm 0.3 for 7% glycerol level. The mean mid-piece abnormality percentages at 5% and 7% glycerol were significantly different to that of 6%. The mean percentages of tail abnormalities after initial dilution, after 4 hours of equilibration and after freezing were recorded as 4.1 ± 0.3 , 7.7 ± 0.7 and 12.3 ± 0.7 for 5% glycerol; 4.5 ± 0.3 , $8.4 \pm$ 0.5 and 12.2 \pm 0.8 for 6% glycerol and 5.0 \pm 0.3, 9.6 \pm 0.5 and 11.8 \pm 0.6 for 7% glycerol added in split dose. There was no significant difference among the glycerol percentages for the mean tail abnormality percentages in all the 3 stages i.e. after initial dilution, after equilibration and after cryopreservation. The mean percentages of total spermatozoa abnormalities were found to be 5.5 \pm 0.5, 11.5 \pm 0.8 and 19.5 \pm 1.0 after initial dilution; after 4 hours of equilibration and after freezing for 5% glycerol level; 6.2 ± 0.4 , 12.9 ± 0.8 and 17.9 ± 1.1 for 6% glycerol and 6.4 ± 0.5 , 14.50.5 and 20.6 ± 0.5 for 7% glycerol added in split dose. Statistical analyses of the total sperm abnormalities data revealed no difference after initial dilution and cryopreservation, but significant difference was observed after equilibration only, 5% glycerot was significantly different from that of 7% glycerol after equilibration.

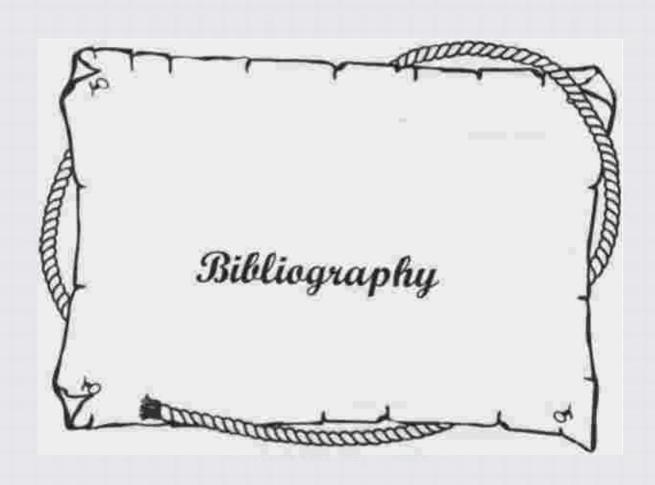
In the experiment-III, the mean percentages of apoptotic sperm in non split method, after initial dilution were found to be 12.10 ± 1.29 , 12.20 ± 1.52 , 11.80 ± 1.39 , 12.70 ± 1.42 and 11.90 ± 1.29 for 3, 4, 5, 6 and 7% glycerol respectively. After equilibration the percentages were 36.00 ± 2.10 , 31.80 ± 2.19 , 24.00 ± 1.86 and 31.80 ± 1.68 ; while after cry opreservation the values were found to be 51.80 ± 1.99 , 48.40 ± 1.58 , 33.40 ± 1.68 , 44.40 ± 1.72 and 48.00 ± 2.03 . The mean percentages of apoptotic sperm at 5% glycerol was significantly different from that of 3, 4, 6 & 7%,

whereas there was no any difference between 4 and 7% in both after equilibration and cryopreservation.

The mean percentages of apoptotic sperm in the split method of addition of glycerol after initial dilution were found to be 13.10 ± 1.53 , 13.40 ± 1.60 and 13.10 ± 1.42 for 5, 6 and 7% respectively. The corresponding values were 20.10 ± 1.31 , 23.60 ± 1.74 and 30.90 ± 1.96 after equilibration and 34.90 ± 1.70 , 43.60 ± 1.77 and 47.70 ± 1.99 after cryopreservation. Statistical analyses of the percentages of apoptotic sperm revealed significant difference between the 3 stages of cryopreservation i.e.; after initial dilution, after equilibration and after cryopreservation in all the glycerol percentages i.e.; 5, 6 & 7%.

CONCLUSION

- 1. The concentration and addition method of glycerol significantly affected the quality of cryopreserved mithun spermatozoa. The post freeze-thaw qualities i.e.; progressive motility, liveability, acrosomal integrity (percentage of live intact, live damage, dead intact & dead damage), morphological abnormalities of mithun spermatozoa were found superior with 5% glycerol compared to that of 3, 4, 6 and 7% when added in a single dose at 37°C.
- 2 The post freeze-thaw qualities of mithun sperm were further improved when 5% glycerol was added in split doses during equilibration at 4°C. Therefore for efficient cryopreservation of mithun sperm in tris egg yolk extender, it is suggested to add 5% glycerol in split doses during equilibration.
- 3. Cryopreservation had induced apoptosis in inlithun sperm. Addition of glycerol at 5% showed less numbers of apoptotic sperm than rest of the glycerol concentration. The method of glycerol addition showed no effect on the apoptosis of mithun sperm. Further studies are required to explain this complex process of apoptosis in the areas like DNA fragmentation and chromatin condensation, lobulation of acrosomal membrane, mitochondrial distension, caspase activation, and increase sperm membrane permeability of the inithun spermatozoa.



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