Characteristic and DNA Integrity of Spermatozoa Cryopreserved in the Presence of Glutathione and Hydrogen peroxide of Nilli Ravi Buffalo

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Naushaba Memon

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NAUSHABA MEMON

DEPARTMENT OF ANIMAL SCIENCES
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"In the Name of Allah, the most Beneficent, the most Merciful"

"It is He Who taught the Quran, He has created the human, He has taught him speech (and Intelligence), the sun and the Moon follow courses (exactly) computed and the herbs and the trees both prostrate in adoration, Then which of the favours of your Lord will ye deny"

Al-Rahman(chap:55)

DEDICATED TO

My Loving

HOLY PROPHET

(Peace Be Upon Him)

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Naushaba Memon

LIST OF ABBREVIATIONS

IVF	In vitro fertilization
IUI	Intra uterine insemination
ICSI	Intra cytoplasmic sperm injection insemination
CPA	Cryoprotectant agent
LN2	Liquid nitrogen
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
ROO^-	peroxyl radical
OH^-	Hydroxyl radical
NOS	Nitric oxide species
GSH	Reduced glutathione
GSSG	Oxidized glutathione
NADPH	Nicotinamide dinucleotide phosphate
G_6PD	Glucose 6-phosphate dehydrogenase
EET	Eosin Exclusion Test
WT	Water Test
HE-test	Hypoosmotic supravital staining test
PI	Propidium Iodide
PUFA	Polyunsaturated acids
HOS	hypo-osmotic swelling
DSB	Double-strand breaks
SSB	Single-strand breaks
ALS	Alkali-labile sites
TM	Tail moment
TD	Tail DNA
TL	Tail length
SCSA	Sperm chromatin structure assay

LIST OF TABLE

LIST OF FIGURE

Abstract

Cryopreservation affects (damages) sperm plasma membrane, acrosomal membrane and the DNA integrity and consequently reduces the sperm motility and fertilizing ability and also reduces the intracellular level of thiols specifically glutathione (GSH) which is an important antioxidant. The addition of GSH prior to cryopreservation for prevention of the sperms from these damages is gaining interest for researchers. Nili Ravi buffalo is an important breed for dairy industry and meat production that is why the semen production units produce semen commercially. The aim of this study is to investigate the effects of cryopreservation, preventive role of GSH addition and adverse role of H_2O_2 addition prior to cryopreservation on Nili Ravi buffalo bull sperm **c**haracteristics (Motility, plasma membrane integrity, acrosomal integrity, DNA integrity). The different combinations of these two additives were also used to determine the effect of GSH detoxification against H_2O_2 on sperm parameters. To conduct this study semen was collected from five bulls and diluted individually with EYTG extender and 10 groups were prepared from each semen sample. One group studied as fresh without any additives, the 2nd group cryopreserved without any additives and other groups were cryopreserved in the presence of additives (1 mM GSH, 5 mM GSH, 100 µM H_2O_2, 200 µM H_2O_2) and combination of these additives (1 mM GSH + 100 µM H_2O_2, 1 mM GSH + 200 µM H_2O_2, 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2). After thawing sperm parameters were observed such as total motility, motility gradation i.e. 4/4, 3/4, 2/4 1/4 by wet preparation method, sperm membrane integrity by HOS test, acrosomal activity i.e. halo formation rate, halo diameter and acrosin index by gelatin digestion test and DNA integrity by single cell gel electrophoresis (comet assay). The parameters of DNA comet i.e. comet length, height, head diameter, DNA percentage in head and tail, tail length, tail diameter, tail moment, olive tail moment were measured through tritek comet score soft ware.

Cryopreservation reduced significantly mean percentage sperm total motility from 80.4±0.84 to 71.28±2.65%. Additive 100 µM H_2O_2 maintained total sperm motility percentage at 71.51±2.18 whereas, both concentrations of GSH maintained total sperm motility at 56 and 51%. Combination GSH + H_2O_2 with different concentrations did not maintain sperm motility above 50% except additive 1 mM GSH + 200 µM H_2O_2 that supports sperm motility at 55%. In sperm motility gradation 4/4 represents the progressive motility that showed no significant difference in fresh semen, cryopreserved semen. All additives maintained motility at 50 – 69% except 5 mM GSH additive, showed significantly low 4/4 motility grade about 46%. This additive (5 mM GSH) showed significant increase (36.56%) in 3/4 motility grade which represents somewhat jerky and slow movement. Cryopreserved sperms also showed increased (26.84%) motility in grade 3/4 than fresh semen. No significant difference was observed in other two (2/4 and 1/4) categories. Cryopreservation maintained mean sperm membrane integrity at 75.35±1.74% which did not significantly decrease than fresh sperms (79.75±1.55%). The additive 5 mM GSH maintained sperm membrane integrity more than 50% i.e. 52.39±1.01. Sperm damaged membrane was observed 51-74% with all other additives.

Sperm acrosomal activity parameters were significantly lowered by cryopreservation than fresh, in cryopreserved semen mean sperm halo formation rate 38.84±2.85, mean

halo diameter 11.91±0.44 µm and acrosin index 4.6±0.34 were observed and in fresh these were 92.86±1.45%, 16.38±1.04 µm and 15.0±2.4. Additives 5 mM GSH, 5 mM GSH + 200 µM H_2O_2 increased sperm halo formation rate up to 5.85±1.58; 85±1.04 with mean halo diameter 12.78±0.58; 12.29±0.57 and acrosin index 10.1±0.1; 9.9±0.13 respectively. A highly significant increase 14.8±0.63 in halo diameter was observed with additive 1 mM GSH, whereas, halo formation rate and acrosin index decreased than that of 5 mM GSH additive 69.21±2.02 and 8.0± 0.23. All other additives significantly decreased halo formation rate and acrosin index, however, no significant increase was observed within all additives than cryopreserved sperms.

Cryopreservation significantly reduced sperm DNA integrity to 55.99±1.55%, in fresh sperm it was 85.63±0.65%. A dose dependent decrease in DNA damage %age was observed with additives 5 mM GSH, 1 mM GSH that was 83.43%; 60.01% respectively. Whereas, H_2O_2 additives increased DNA damage up to 45%, however, combinations showed better effect on DNA integrity percentage that was highest (89.12%) with additive 5 mM GSH + 200 µM H_2O_2 and other combinations also maintain DNA integrity up to (60-70%). DNA comet tail parameters (tail DNA, tail length, tail moment, olive tail moment); represent degree of DNA damage i.e. increase in all four tail parameters shows greater DNA damage.

The additive 1 mM GSH proved highly protective role to reduce significantly ($P<0.0001$) DNA damage compared to control. Additive 5 mM GSH + 100 µM H_2O_2 also reduced significantly ($P<0.0001$) DNA damage. Additives 200 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2 were highly significantly ($P<0.0001$) increased DNA damage compared to control in all four tail parameters.

It is concluded that the sperm cryopreservation of Nili Ravi buffalo bull decreased total motility, acrosomal activity and DNA integrity but did not decrease sperm functional plasma membrane integrity and intracellular sperm glutathione levels. Addition of antioxidant (GSH) either 1 mM or 5 mM prior to sperm cryopreservation showed protective effect on sperm DNA integrity and acrosomal activity but did not protect sperm total motility and plasma membrane.

Sperm cryopreservation, the storage of sperm cells at low temperature especially in the liquid nitrogen, is a widely used technique (Ozkavukcu *et al*., 2008) in human (Wright *et al*., 2006), nonhuman primates (Li *et al*., 2007), farm animals (Keshavarz, 2007), domestic, as well as in the wild and endangered species (Anel *et al*., 2005). It is an integral part of assisted reproductive techniques (Keshavarz *et al*., 2007) such as artificial insemination (AI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Dong *et al*., 2008). Cryopreservation of the gametes and particularly spermatozoa is an attractive approach that can be implemented for the breeding to maintain nonhuman primates that is otherwise not efficient by conventional breeding method (Li *et al*., 2007).

Main aspects of Sperm Cryopreservation

Establishing a cryobank to preserve the germplasm provides an effective tool for preserving the genetic biodiversity (Dong *et al*., 2008). Sperm cryopreservation is potential source of valuable genes for genetic resource banking to maintain genetic diversity in domestic as well as wild species populations (Critser and Russell, 2000; Wildt, 1992). Semen banking also provides a way to easily transfer genetic materials among colonies of animals and facilitate the distribution of "genetically superior" domestic species lines, because only a few straws of frozen semen must be transported (Dong *et al*., 2008) and for conservation of animal programs. Efficient freezing, archiving, and thawing of sperm are essential techniques to support the large scale research programs using mouse models of human disease (Yildiz *et al*., 2007) and captive primate populations for biomedical research as well as for conservation programs (Dong *et al*., 2008). Offspring production and study of gamete biology of farm animal species are frequently using posthumous gamete recovery procedure (Keshavarz *et al*., 2007).

AI and IVF programs are extensively using cryopreservation of human spermatozoa (Donnelly, 2001). Specially, human spermatozoa are widely cryopreserved in assisted conception units as a preservation strategy of male gametes. It provides the opportunity for future fertility, for example in the treatment of malignancy (Ozkavukcu *et al*., 2008;

Tournaye, 1999; Sanger *et al*., 1992). Testicular failure or ejaculatory dysfunction may be caused by cytotoxic chemotherapy, radiotherapy and some kinds of surgical treatment. Fertility insurance can be achieved by freezing of spermatozoa before initiation of treatment. The cryopreservation of ovarian tissue is gaining popularity with subsequent transplantation to preserve fertility in female cancer survivors (Bedaiwy, 2004). Pregnancies following reimplantation of thawed ovarian tissue were reported by Meirow *et al*., (2005) and Donnez *et al*., (2004) i.e. one after an IVF treatment cycle and other in a spontaneous cycle respectively.

Sperm cryopreservation became a major area of scientific investigation, when the dairy industry needed long-term storage methods for bull sperm after a pivotal discovery of glycerol as a cryoprotective agent (CPA) (Walters *et al*., 2009). Cryopreservation of mammalian sperms is a complex process that involves balancing of many factors in order to obtain the satisfactory results and insure even minimal success. The knowledge of the sperm physiology for the species is essential to maximize post-thaw recovery of sperm and consequently the fertility (Purdy, 2006). Fundamental cryobiology is quantitative study of biophysical and biochemical phenomena that occur during cryobiological procedures. These include the transmembrane fluxes associated with the addition and removal of CPAs, the change in chemical potentials during cooling and warming, both intracellular and extracellular ice formation, the effects of cooling and warming rates and storage temperatures, heat transfer in solutions and tissues, and, most importantly, the optimization of cryobiological procedures (Walters *et al*., 2009).

Cryopreservation protocols for the bull to be used for AI in the dairy industry started developing in 1950s. Various media formulations were investigated in terms of their ability and these were termed "extenders" (Walters *et al*., 2009; Purdy, 2006). The purpose of an extender or cryopreservation diluents is to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the spermatozoa to survive temporarily. Logically, each of the different components comprising the media had investigated separately, and in combination, to maximize the post-thaw sperm viability and fertility (Purdy, 2006).

It is well known that cryoprotectants are essential for the survival of cells during cryopreservation and the most commonly used cryoprotectants are sugars and glycerol

(Yildiz *et al*., 2007). Sugars have cryoprotective properties because they lower the Van der Waals interactions at the membrane hydrocarbon chains that enhance trans-membrane transfer especially phospholipids (Anchordoguy *et al*., 1987; Strauss *et al*., 1986). Polge *et al*., (1949) showed that the use of glycerol as a permeating solute could provide protection to cells at low temperatures. This is often cited as the defining moment in the establishment of modern sperm cryobiology. The exact mechanism of action for glycerol in protecting cells from freeze–thaw damage is not completely understood. However, it is known that glycerol is osmotically active and is slow to permeate membranes resulting in cell volume changes from both the addition of glycerol and water loss during the freeze–thaw cycle (Hammerstedt *et al*., 1990; Schneider and Mazur, 1984). Glycerol induces interdigitation of the two bilayer leaflets, by increasing the order of the interior fatty acyl chains (Boggs and Rangaraj, 1985), causes membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures, and therefore an increased ability to survive cryopreservation by reducing the likelihood of fracture (Holt, 2000; Watson, 1995). Classic cryobiology studies suggest the use of glycerol or any permeating additive to more efficiently protect cells from freezing injuries through colligative or solution effects (Mazur, 1970). That is proved by using permeating and non permeating such as raffinose with either permeating cryoprotectants that were glucose, fructose, propylene glycol, ethylene glycol, glycerol, or sodium pyruvate. These cryoprotectants are significant factors for improving progressive motility, plasma membrane integrity, DNA integrity, in vitro fertilization rate, and in vitro embryo development rate to blastocyst in cryopreserved mouse sperm (Yildiz *et al*., 2007). These cryoprotectants have also been tested (Kundu *et al*., 2000; Leboeuf *et al*., 2000; Singh *et al*., 1995; Tuli and Holtz, 1994; Ritar *et al*., 1990) but the most frequently used penetrating cryoprotectant is glycerol (Purdy, 2006). Glycerol is effective in protection against freezing membrane injuries in commonly used species of livestock (Curry, 1995; Parks and Graham, 1992) and a combination of sucrose and glycerol successfully protects embryos of various mammalian species and also bull sperm (De Leeuw *et al*., 1993; Honadel and Killian, 1988) during cryopreservation.

During early development of extender, it was found that bull sperm can be protected from "cold shock," the sensitivity of cells to reduced temperatures, with the help of lipids in egg yolk (Foote, 1998; Watson, 1975; Watson and Martin, 1975; 1973). For the bull sperm cryopreservation extender was aided by lipid in combination with glycerol due to its cryoprotective properties, and as a result Tris-egg yolk–glycerol (TEYG) method for freezing was established, which has now become a standard (Watson, 1975; Watson and Martin, 1975; 1973; Foote, 1970). Until the new methodologies have been thoroughly investigated (Bilodeau *et al*., 2000), use of the Tris–egg yolk cryopreservation diluents was recommended (Salamon and Ritar, 1982). TEYG extender is widely used for cryopreservation **(**Abdel-Khalek *et al*., 2009; Chatterjee *et al*., 2001). Tris–egg yolk diluents are beneficial because of their ease of use, in particular, because centrifugation of semen is not necessary (Ritar and Salamon, 1982). Sherman (1963) discovered the storage of human sperm at liquid nitrogen (LN_2) temperature (−196 °C) that was superior to storage at −75 °C. In addition, no loss of motility was observed when the sperms were stored in LN_2 for one year; however, there was a decline in motility when stored at −75 °C.

The sperm cell is compartmentalized in terms of structure and function, and these compartments have different sensitivities to cryoinjury (Li *et al*., 2007). Successful sperm cryopreservation can be obtained by maintaining post-thaw structural and functional integrity whereas, to maintain functional integrity, the compartments of the sperm need to be fully protected so that frozen–thawed sperm can undergo normal fertilization under in-vitro or in-vivo conditions (Agca *et al*., 2002; Guthrie *et al*., 2002; Willoughby *et al*., 1996). There are several features of a spermatozoon necessary for fertilizing an egg which must be conserved after cryopreservation. The most important of these features can be summarized as DNA content, acrosomal integrity, motility, and viability (Ozkavukcu *et al*., 2008).

A marked reduction in motility is the most commonly reported detrimental effect of cryopreservation on human spermatozoa (Yoshida *et al*., 1990; Critser *et al*., 1988). Sperm cryopreservation is a great challenge, since many sperm are irreversibly damaged or present altered functionality after the whole process (Moce and Vicente, 2009). Cryopreservation and/or thawing induce many changes in mammalian spermatozoa

(Medeiros *et al*., 2002). Diminished motility and membrane changes, including sperm capacitation or acrosomal reaction, are some of the main forms of damage brought out by cryopreservation. Cryopreservation definitely affects sperm viability (Martin *et al*., 2004).

The formation of intracellular ice is the primary cause of cellular damage during cryopreservation (Watson, 1995; Muldrew and McGann, 1990). Water is removed from the solution in the form of ice, whenever cells, or culture media, are cooled below their freezing point, and causing increase in the concentration of solutes remaining in the unfrozen fraction and depressing the freezing point (Brothernton, 1990) and increasing the osmotic pressure of the remaining solution. Hence, over a wide range of temperature, biological systems freeze progressively and gradually, the solute becomes more concentrated as the temperature falls (Brotherton, 1990). This leads to disturbance of cellular organelles and irreversible rupturing of plasma and nuclear membranes. During the thawing process cellular damage may be caused as the ice melts or re-crystallizes (Mazur, 1989). Generally the nucleus is considered to be a stable part of the cell, but studies have suggested that inappropriate chromatin condensation can occur (Royere *et al*., 1991; 1988) with freezing.

Human spermatozoa have unusual cryobiological behavior and improvements in their survival have not been achieved by the standard approaches of cryobiology. The biophysical basis of these different responses was examined using the cryostage of a scanning electron microscope and freeze substitution and it was found that, surprisingly, all samples of spermatozoa in the frozen state were neither osmotically dehydrated nor had any visible intracellular ice. Viability on thawing did not appear to correlate with conventional theories of cellular freezing injury, which suggests that for human spermatozoa other factors determine viability following freezing and thawing (Morris *et al*., 1999). However, freezing process produces physical and chemical stress on the sperm membrane which in turn reduces sperm viability and fertilizing ability. The cold shock of sperm cells during the freezing-thawing process is now associated with oxidative stress induced by free radicals (Sanocka and Kurpisz, 2004; Salvador *et al*., 2006).

Oxidative Stress

Oxidative stress is a condition associated with an increased rate of cellular damage induced by reactive oxygen species (ROS) (Sikka *et al*., 1995). Oxidative stress occurs when antioxidant defense mechanisms are overwhelmed by free radicals. This imbalance can cause either increased free radical formation or decreased antioxidant capacity (Agarwal *et al*., 2005; Sanocka and Kurpisz, 2004; Martin *et al*., 2004; Saleh *et al*., 2003; Sohal, 1996). The cause of the pro-oxidant–antioxidant shift may be an increase in ROS production, a decrease in antioxidant capacity, or possibly a combination of the two. For example, sperm preparation methods associated with assisted reproductive techniques involve the removal of seminal plasma and hence the antioxidant protection for spermatozoa, thereby increasing their susceptibility to oxidative stress. Sperm cells used in AI are exposed to oxygen and visible light radiation during various processing procedure or in semen stored by cooling or at room temperature, which could lead to formation of ROS, and negatively affect sperm cell motility and genomic integrity (Bilodeau *et al*., 2001; Aitken *et al*., 1998; Storey, 1997; Aitken and Clarkson, 1987).

Reactive Oxygen Species (ROS)

ROS are free radicals derived from oxygen metabolism. Free radicals are transient ionic species with high chemical reactivity that are produced during oxidation of organic molecules. The interactions between free radicals and biomolecules result in oxidative biomolecular modifications (Halliwell and Gutteridge, 1984; 1998). However, since ROS are highly reactive, they initiate molecular function defects in spectator biomolecules as well (Agarwal *et al*., 2004; Agarwal and Saleh, 2002).

As ROS are highly reactive oxidizing agents (with one or more unpaired electrons) therefore, ROS have a tendency towards chain reaction, in such a manner that "radical begets radical". Most common of those having potential implications in reproductive biology include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radical and the very reactive hydroxyl (OH-) radical (Koppenol *et al*., 1992).

ROS play an important positive role in many physiological functions such as phagocytosis. ROS also, have been recognized as fundamental participants in cell signaling and regulation mechanisms (Finkel, 1998).

Hydrogen Peroxide (H_2O_2)

H_2O_2 is of higher oxidant potential, it is relatively stable and being uncharged molecule can cross cell membranes freely (Halliwell and Gutteridge, 1984; 1998). H_2O_2 is produced by the dismutation of superoxide anion (O_2^-) either spontaneously or enzymatically (Halliwell and Gutteridge, 1984; 1998). Because, mammalian spermatozoa under aerobic conditions generate ROS due to normal metabolic activity, i.e. superoxide anion (O_2^-) (Aitken and Clarkson, 1987; Alvarez *et al*., 1987), which is low reactive and have short half-life (1 ms), therefore, it is not very harmful but when it reacts with its targets (enzymes responsible for tyrosine phosphorylation–dephosphorylation of sperm proteins are possible targets for ROS, it can produce more toxic reactive species such as hydroxyl radicals (Halliwell and Gutteridge, 1984; 1998).

High amounts of ROS, and mostly hydrogen peroxide (H_2O_2), were first shown to have deleterious effect on spermatozoa (Lamirande *et al*., 1995). However, it is now recognized that the generation of low and controlled levels of O_2^-, H_2O_2, and nitric oxide (NOS) (Flaherty, 2005; Herrero *et al*., 2000; Aitken *et al*., 1998; Lamirande *et al*., 1997; Lamirande and Gagnon, 1995**)** by human and bovine spermatozoa themselves is involved in the acquisition of fertilizing ability.

Antioxidants

The cytoplasm of somatic cells contains several antioxidants that neutralize ROS by an elaborate defense system. The system consists of enzymes such as catalase, superoxide dismutase, and glutathione peroxidase or reductase, and numerous nonenzymatic antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione (GSH), taurine, and hypotaurine (10). Sperm cells are devoid of most of this cytoplasm, so the antioxidant system in sperm cells of different species is weak (Li, 1975). However, Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Lewis *et al*., 1997). Under physiological conditions, ROS and antioxidants maintain a stable ratio. A shift toward ROS will give rise to oxidative stress.

Spermatozoa and seminal plasma possess a number of enzymes and low–molecular weight antioxidants that scavenge ROS in order to prevent possible cellular damage.

Together, the enzyme scavengers and low–molecular weight antioxidants make up the total antioxidant capacity of seminal plasma (Smith *et al*., 1979). However, the endogenous antioxidative capacity of semen may be insufficient during storage or dilution (Maxwell and Salmon, 1993). In vitro studies suggested that the addition of an antioxidant (GSH) to diluted semen could improve the motility and survival of bull spermatozoa in frozen semen (El-Nenaey *et al*., 2006). Ahmed (2008) found that addition of antioxidants (GSH or ascorbic acid) to extender of frozen buffalo semen improved sperm characteristics. Khalek *et al*., (2009) showed highest post thaw motility and best fertilizing capacity of buffalo spermatozoa frozen in Tris based extender containing catalase.

Glutathione

Glutathione belongs to a vital intra-cellular and extra-cellular protective antioxidant and is found almost exclusively in its reduced form. The reduced form is called reduced glutathione (GSH) and is involved in detoxifying processes of xenobiotics and scavenging of reactive oxygen species (ROS). In the process of scavenging of ROS two molecules of GSH are oxidized to form one molecule called oxidized glutathione (GSSG). Glutathione is found almost exclusively in its reduced form. This is mainly because the enzyme called glutathione reductase, which revert it from its oxidized form, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker for assessing cellular toxicity (Meister and Anderson, 1983; Carelli *et al*., 1997; Townsend *et al*., 2003).

The oxidation stress induced by xenobiotics can result in damages to DNA, RNA, membranes and other cell compartments. Spermatozoa have a high content of polyunsaturated fatty acids in their membranes (Lenzia *et al*., 2002) and also contain high amounts of DNA, which are both sensitive to oxidative stress (Peris *et al*., 2007).

Cryopreservation alters the membrane sulfhydryl status of spermatozoa and GSSG reduces the mobility of sulfhydryl-containing proteins in the sperm membrane. The fact that proteins bind with glutathione and form mixed disulphides (protein-S-S-glutathione). These disulphides can protect proteins against oxidative insult (Luberda, 2005).

Glutathione Redox System

The GSH/GSSG pair plays important role, both as a redox sensor and protector, against ROS induced damages in many cell types (Halliwell and Gutteridge, 1984; 1998). Sulfhydryl groups are under redox control and a change in the redox status of the membrane can be linked to the ROS production that occurs during cooling and freezing-thawing of spermatozoa (Mazur *et al*., 2000).

Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione, as the electron donor, removes peroxyl (ROO.) radicals from various peroxides including H_2O_2. Glutathione reductase then regenerates reduced GSH from GSSG as shown in the following equation:

$$GSH + H_2O_2 \xrightarrow{Se-GSH-P} GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GSH-Red} 2GSH + NADP^+$$

(Calvin *et al*., 1981)

Cyopreservation of spermatozoa reduced the GSH level 5-fold, without increasing the GSSG level, which could suggest that the GSH content of spermatozoa may be transported out of the sperm cells (Bilodeau *et al*., 2000). Therefore, upon addition to the extender, GSSG partially prevents the loss of sperm motility after a freezing/thawing cycle, whereas GSH does not. GSSG, but not GSH, prevents increase in the mobility of sulfhydryl containing proteins due to the freezing/thawing of spermatozoa (Chatterjee *et al*., 2001). GSSG also interacts with two vicinal free cysteine residues of the active site in protein tyrosine phosphates and inactivates these enzymes (Gabitta *et al*., 2000). Such a mechanism could explain the positive effects exerted by GSSG (Hammerstedt *et al*., 1990).

Reactive Oxygen Sspecies and Sperm Motility

Free radicals (ROS) are involved in lipid peroxidation as well as DNA and sperm membrane damages that may lead to decreased sperm motility or cell death. The balance between free radical production and their detoxification may be an important factor in

sperm survival and function before, during and after cryopreservation (Uysal and Bucak, 2007).

The increased formation of ROS has been correlated with a reduction of sperm motility (Lenzi *et al*., 1993; Armstrong *et al*., 1999). The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with reduction in membrane fluidity that is necessary for sperm oocyte fusion (Lamirande and Gagnon, 1995). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose 6-phosphate dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of NADPH which is used as a source of electrons by spermatozoa to fuel generation of ROS by an enzyme system known as NADPH oxidase (Aitken, 1997). Inhibition of glucose-6 phosphate dehydrogenase (G6PD) leads to decreased availability of NADPH and a concomitant accumulation of oxidized glutathione, which in turn can reduce the antioxidant defenses of the spermatozoa and peroxidation of membrane lipids (Grivaeu *et al*., 1995)**.**

Antioxidants play an important role in scavenging free radicals which may cause lipid peroxidation of sperm plasma membranes (Baumber *et al*., 2000). The addition of antioxidants is well known to improve viability and motility of liquid storage or cryopreserved sperm cells (Baumber *et al*., 2005; Maxwell and Stojanov, 1996). Sperm motility partially depends on functional integrity of the sperm membrane and other events included in the metabolism of sperm (Dobranić *et al*., 2005). The assessment of motility alone is inadequate for evaluation of sperm survival after thawing (Uysal *et al*., 2006). The integrity and functional activity of sperm membrane is also of major importance in the fertilization process and assessment of membrane function may be a useful indicator of fertilizing ability of spermatozoa (Uysal and Korkmaz, 2004),

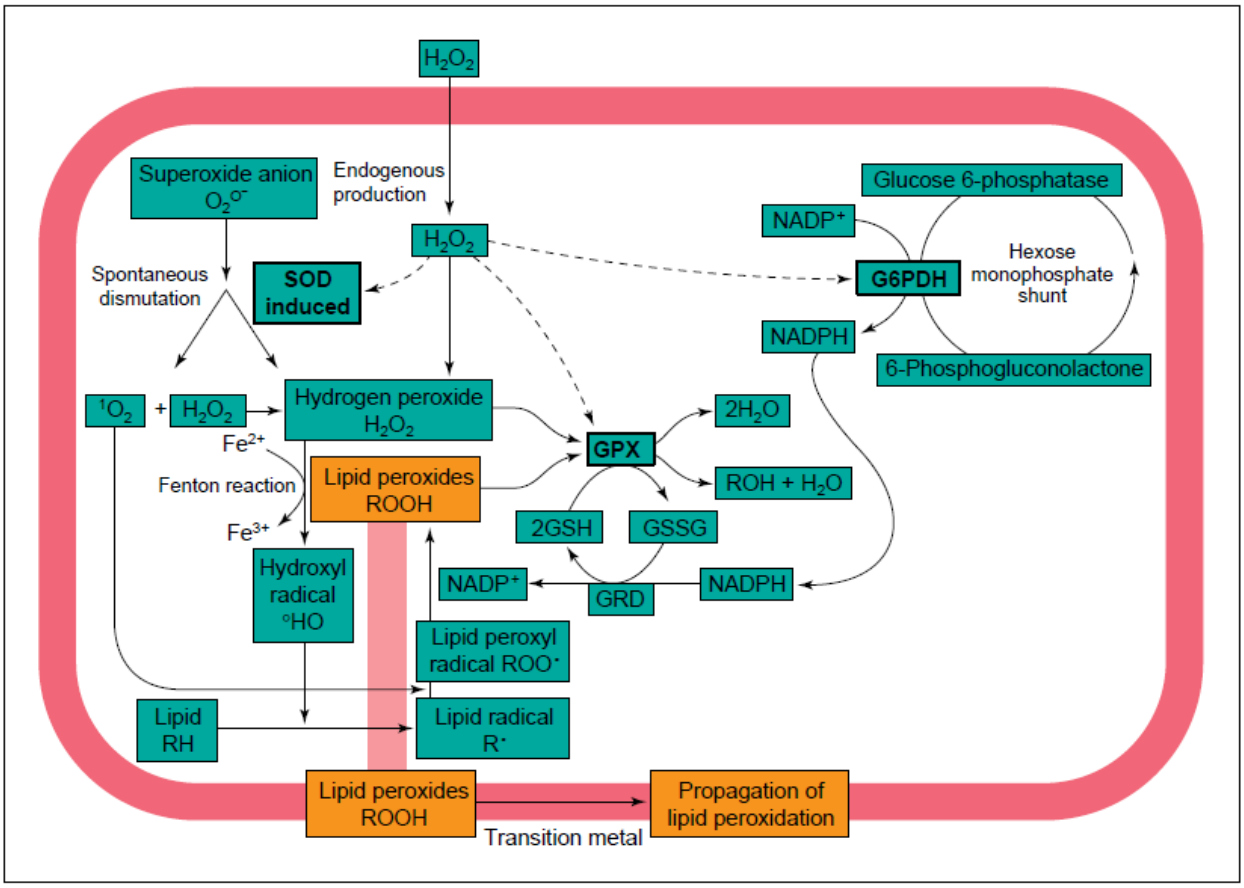


Figure 1: Lipid peroxidation and antioxidant enzymatic defense systems in the spermatozoon. Hydrogen peroxide may decrease the activity of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glucose-6-phosphate dehydrogenase (G6PDH) activities (- - - -), allowing the endogenous production of reactive oxygen species to result in the accumulation of toxic lipid peroxides and the development of lipid peroxidation (modified after Griveau *et al*., 1995). GRD: glutathione reductase; GSSG: oxidized glutathione; GSH: reduced glutathione.

because, highly motile cells can have damage in structures or functions which can be performed by combined (hypoosmotic-supravital staining) test (HE-test), evaluating head and tail membrane behavior. Conversely, highly non motile sperm cells can have intact plasmalemma and so viability. It is possible to evaluate, using Eosin Exclusion Test (EET), HOS, Water Test (WT) and fluorescent staining with Propidium Iodide (PI), the structural and functional membrane integrity and viability correlated with the in vitro fertilizing ability of sperms in frozen ram, boar and bull semen (Pintado *et al*., 2000; Uysal *et al*., 2005).

Reactive Oxygen Species and Sperm Membrane Integrity

The analysis of the fatty acid pattern of membrane phospholipids has shown the presence of significant amounts of polyunsaturated acids (PUFA), which are known to contribute membrane fluidity and flexibility that is a prerequisite for normal cell function (Lenzia *et al*., 2002). The fatty acid composition of cell membranes regulates the activity of different lipid-dependent membrane-bound enzymes and the membrane resistance to physical and chemical stress (Lenzia *et al*., 2002). Reactive oxygen species (ROS) are capable of triggering a spreading radical reaction sequence on the sperm membrane and the main anti peroxidative reactions protecting PUFA are the glutathione-dependent enzymatic scavenger mechanisms and a non-enzymatic one, based on vitamins E and C. The levels of oxidizable substances, such as PUFA, and the levels and activity of the free radical scavenger systems generally regulate cellular homeostasis (Lenzia *et al*., 2002).

Plasma membrane integrity of sperm is of crucial importance for optimal sperm function. Only a sperm with an intact plasma membrane can undergo a series of complex changes in the female reproductive tract and can acquire the ability to fertilize an oocyte (Yanagimachi, 1994; Pintado *et al*., 2000; Uysal *et al*., 2005; Uysal *et al*., 2006). Therefore, plasma membrane is one of the most relevant spermatological parameters, the plasma membrane during cryopreservation, is exposed to sudden changes in osmotic pressure that exerts stress on it and can cause irreversible damage to its integrity (Jeyendran *et al*., 1984; Hammerstedt *et al*., 1990). Thus, along with other semen-quality

parameters (concentration, pH, motility), osmotic pressure plays a key role during the cryopreservation of sperm and eventually affects the quality of the frozen semen.

Sperm Membrane Integrity Evaluation

The most important mechanisms of fertilization, such as capacitation, acrosome reaction, and binding of spermatozoa to the egg surface are believed to depend on the functional integrity of the sperm membrane. Various tests of sperm function such as the hypo-osmotic swelling (HOS) test (Jeyendran *et al*., 1984), the zona-free hamster egg penetration assay (Yanagimachi, 1994), the triple stain technique for evaluation of the acrosomal reaction (Aitken *et al*., 1984), and others, have been proposed for measuring male fertilization potential (Henkle *et al*., 1995).

To evaluate plasma membrane integrity two simple basic tests are used: supravital staining (eosin/nygrosin) and hypoosmotic swelling test (HOS) assay (Curry and Watson, 1994; Curry, 1995). In hypoosmolar solution, fluid is transferred into the cell through the plasma membrane of spermatozoa. In attempting to achieve a balance between intracellular and extracellular spaces, a functionally intact membrane begins to swell starting at the tail of the spermatozoa. The swelling of the membrane leads to curling and invagination. The tail fibres changes are clearly visible under a phase-contrast microscope. Such spermatozoa are denoted as swollen or HOS reactive (HOS +ve), signifying functionally intact membranes. Spermatozoa with functionally defected membrane do not swell and their tails do not invaginate (Jeyendran *et al*., 1984). The osmolarity of the solution should be sufficient to provoke the best effect without lysing the sperm membrane (Rota *et al*., 1999). Fertilization of oocyte will not occur if the sperm membrane is biochemically inactive, even if it remains structurally intact. The HOS test is therefore a better indicator of fertilization potential than supravital staining (Tamuli and Watson, 1992). Supravital staining is based on the fact that the membrane of dead spermatozoa permits the passage of the red stain into the cytoplasm, but the membranes of live spermatozoa do not permit that. This means that all dead spermatozoa in ejaculate will be colored, while live spermatozoa will remain colorless (Herak, 1991). Check and Check (1991) concluded that evaluation of structural and functional integrity of membrane is a better indicator of fertilization than motility

evaluation alone. This test has been applied to a range of domestic and non-domestic mammals from the bull (Revell and Mrode, 1994) to the giant panda (Perez-Garnelo *et al*., 2004), and birds such as the turkey (Donoghue *et al*., 1996) and in emu (Malecki *et al*., 2005). The HOS test is an ideal method being quick, simple, and requiring minimal equipment, and has been shown to correlate well with supravital staining (Samardzija *et al*., 2008) and be a good predictor of fertility (Perez-Llano *et al*., 2001). The osmotic stress caused by the chosen hypo-osmotic medium must be sufficient to affect an influx of water into the cell to result in an increase in volume and hence curling of the tail, but to prevent lysis of the sperm membrane. Dobranić *et al*., (2005) showed that HOS test can be an easy method for routine evaluation of sperm quality.

Acrosomal integrity including acrosin activity has been shown to be predicting the fertilizing capacity of human spermatozoa (Henkel *et al.,* 1995). Acrosin is a trypsin-like serine proteinase that is exclusively found within the acrosome of mammalian spermatozoa (Harrison 1982). It is associated with the zona pellucida binding and zona pellucida penetration of spermatozoa (Polakoski and Siegel, 1986). Moreover, this enzyme has been found to be involved in capacitation, acrosome reaction, and chromatin decondensation during male pronucleus formation (Henkel *et al.,* 1995) The absence or reduced activity of acrosin in spermatozoa from patients with unexplained infertility (Mohsenian *et al*., 1982),

Due to important function of acrosin during the fertilization process, several techniques, including gelatinolysis, have been described to assess human sperm acrosin activity such as fluorometric assays (Harrison *et al*., 1982), radioimmunoassay (Mohsenian *et al*., 1982), or spectrophotometric assays using benzoylarginine ethyl ester (Schill, 1973, 1990) or N-a-benzoyl-DL-argmine p-nitroanilide (Kennedy *et al*., 1989; Tummon *et al*., 1991; De Jonge *et al*., 1993) as substrates (Henkel *et al.,* 1995).

Gelatinolytic technique is based on the ability of acrosomal enzymes to hydrolyze a high molecular weight protease like gelatin (Tavalaee *et al.,* 2007, Henkel *et al.,* 1995).

Normal halo formation rate as well as normal halo diameter (acrosin activity) indicates good fertilizing capacity of spermatozoa. Both of these parameters, that is, halo formation rate and halo diameter, were evaluated, subfertility could be detected because of the low acrosin activity index. An assay showing a halo formation rate <60% or a halo diameter <l0µm is indicative for subfertility. In cases where both parameters are

less than those values, that is the acrosin activity index is <6, a high probability of infertility can be suspected.

Effect of Cryopreservation on DNA Integrity

The integrity of mammalian sperm DNA is of prime importance for the paternal genetic contribution to normal offspring. (Yildiz 2007), the increasing popularity of assisted reproductive techniques (ART) including intracytoplasmic injection demands sensitive estimation of sperm DNA integrity in order to insure the genetic health of resultant offspring. Semen cryopreservation is an important part of assisted reproduction (Keshavarz, 2007; Cormier and Bailey, 2003) and is a potentially useful way of sperm banking until needed for experimentation or insemination. Long-term sperm preservation in liquid nitrogen is a subject of paramount interest because of extensive use of frozen semen for artificial insemination (AI) (Mossad, 1994; Donnelly *et al*., 2001).

Damaged DNA can have a significant negative impact on oocyte fertilization, embryo development rate, and live-birth rate. A significant correlation between the presence of nuclear DNA alterations in mature spermatozoa and poor sperm parameters or impaired reproductive efficiency is reported in both humans and animals (Hughes *et al*., 1996, Edwards and Beard 1999). To date, studies show that the cryopreservation process causes DNA damage to mammalian sperm in human (Royere *et al*., 1988, 1991, Donnelly *et al*. 2001, Hammadeh *et al*., 2001). Sperm DNA damage was significantly increased following cryopreservation, irrespective of the extender type and packaging material (Fraser and Strzeżek, 2006)**.** Freezing seems to affect chromatin structure and sperm morphology (Hammadeh *et al*., 1999), cryopreservation induced DNA damage in semen from infertile men has been detected using the alkaline comet assay (Donnelly *et al*., 2001).

Additionally, the sperm chromatin structure assay (SCSA) also revealed that the sperm quality deteriorated upon cryopreservation (Gandini *et al*., 2006). Cryopreservation of testicular spermatozoa by itself may reduce pregnancy rates (Thompson-Cree *et al*., 2003). The freezing–thawing process affects the DNA integrity of boar spermatozoa (Fraser and Strzezek, 2006) and SCSA (Hernandez *et al*., 2006).

The full sperm DNA integrity defined as the absence of DNA nicks or single strand breaks (SSB), double strand breaks (DSB) and chemical modifications DNA (Hoeijmakers, 2001). To detect integrity of DNA individual spermatozoa mostly used methods are: in situ nick translation, the terminal deoxynucleotidyl transferase assay (TdTA or TUNEL), the sperm chromatin structure assay (SCSA) and the single cell gell electrophoresis (SCGE) assay or comet assay. In situ nick translation utilizes DNA polymerase to incorporate labeled nucleotides in a template specific manner (Manicardi *et al*., 1995). The TdTA or TUNEL detects DNA strand breaks were 3'OH groups available (in SS and DS breaks). The SCSA indirectly measures DNA stability via the relative amount of acridine orange fluorescence indicating SS DNA (Evenson *et al*., 1980). The SCSA has been previously correlated with fertility in an ART setting (Evenson *et al*., 1999; Larson *et al*., 2000; Evenson 2002). The comet assay (SCGE) also detects SS and DS DNA breaks (Singh *et al*., 1989; Hughes *et al*., 1996; McKelvey Martin *et al* 1997). The comet assay has been previously used to correlate DNA damage with implantation success after ICSI (Donnelly *et al*., 2000; Morris *et al*., 2002). Aravindan and co-workers (1997) have compared the SCSA with aversion of neutral comet assay (NCA) and TUNEL assay and found the correlation between SCSA and comet assay to superior to that between SCSA and TUNEL assay.

It has an advantage over the other DNA damage-detecting methods, (1) It is a rapid, sensitive and reliable method to detect DNA damage and assess the DNA integrity of the genome within single cells is that of the comet or SCGE (Baumgartner *et al*., 2009), its demonstrated sensitivity for detecting low levels of DNA damage; (2) the requirement of small number of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) relatively small amounts of a test substance to conduct studies; and (7) relatively short time period required to complete an experiment (Tice *et al*., 2000).

Single Cell Gel Electrophoresis or Comet Assay

The comet assay has been widely used for genotoxic studies and cell biological studies and even in human biomonitoring studies (Fraser *et al*., 2006), since a method using micro-gel electrophoresis of immobilized cells lysed at high salt concentrations and embedded in agarose introduced by Ostling and Johanson (1984) and its independent

modification that when an electrophoretic field with pH conditions less than pH 10 was applied, tails were observed where the DNA migrated faster than the nuclear DNA. Later alkaline conditions for DNA unwinding and electrophoresis were incorporated to allow the detection of DSB, SSB and alkali-labile sites (ALS) at a pH of ≥13 by Singh *et al*., (1988).

Basic Principles of Comet Assay

The basic principles of the comet assay were based on previous results, which characterized the nuclear structure of lysed cells as containing super-helical DNA (Cook *et al*., 1976; Collins, 2004). Furthermore, cells being treated with high concentrations of salt in presence of a non-ionic detergent resulted in nuclear scaffold comprising of RNA and proteins together with the attached looped DNA (Rydberg and Johanson, 1978; Collins, 2004; Baumgartner *et al*., 2009).

An important step in detecting DNA damage is alkaline denaturation and separation of the DNA double helix (Singh *et al*., 1988). This allows detection of SSB and ALS. Collins *et al*., (1997) suggested that the formation of the comet tail seemed to originate predominantly from relaxation of super coiled loops, rather than alkaline unwinding. Nevertheless, unwinding occurs under alkaline conditions, and single-stranded DNA can be observed in the comet tail (Collins *et al*., 1997; Collins, 2004). A radically different kind of neutral comet assay was developed by Olive *et al*., (1991) to facilitate detection of DSB with out interference from SSB (Collins, 2004; Baumgartner *et al*., 2009).

Difference between Neutral and Alkaline Comet Assay

Under neutral (pH 8–9) conditions, mainly DSB are detected, some SSB might also be observed in the comet due to the relaxation of super coiled loops containing the breaks (Collins, 2004). Whereas, under alkaline conditions, DSB and SSB (at pH 12.3) and ALS (at pH≥13) can be seen, resulting in increased DNA migration in the electrophoretic field (Fairbairn *et al*., 1995; Collins, 2004). The amount of DNA damage seems to be reduced under these neutral electrophoresis conditions, when compared to alkaline conditions. This is probably due to either an alkaline environment required to reveal certain DNA lesions and/or the migration of damaged DNA being greater at a

higher pH (Angelis *et al*., 1999). AS DSB is induced by only a few agents besides ionizing radiation, therefore, the amount of DNA damage in the neutral assay might also be lower depending on the genotoxin (Lundin *et al*., 2005). In general, with the neutral comet assay, unless the DNA is unwound i.e., made single stranded under alkaline conditions, the vast majority of SSB will not be detected. High pH conditions (above pH 13) also allow detection of ALS. This alkaline version of the comet assay offers greatly increased sensitivity for identifying genotoxic agents. The first consensus made by an expert panel was that, in terms of a testing strategy for genetic toxicology, the alkaline version of the comet assay was the methodology of choice (Tice *et al*., 2000).

The comet assay has been widely used for genotoxic studies and cell biological studies, and even in human biomonitoring studies, several parameters of comet features have been developed. Bocker *et al*., (1997) summarized 10 measurement methods, and a new parameter, the tail profile, has been introduced by Bowden *et al*., (2003). Among many of its parameters, the tail parameters are the most frequently used such as the tail moment (TM), the tail DNA (TD), and the tail length (TL) (De Boeck *et al*., 2000). Singh *et al*., (1988) and Olive *et al*., (1990a, 1990b) developed the concepts of comet length or tail length as migrations of DNA. Olive *et al*., (1990a, 1992) and Muller *et al*., (1994) introduced the tail moment and the percentage of DNA in the tail (i.e. the tail DNA). Many researchers have used these parameters for genotoxic studies (Anderson *et al*., 2003; Bajpayee *et al*., 2002; Garaj-Vrhovac and Zeljezic, 2002; Kim *et al*., 2002; Schabath *et al*., 2003). With the increased use of computerized image analysis systems used to collect Comet data, a metric, based on the percentage of migrated DNA, such as the tail moment, has become popular (Tice *et al*., 2000).

Although the popularity of the comet assay in biomonitoring studies has increased (Moller *et al*., 2000), one of its shortcomings is a lack of agreement on a single appropriate comet parameter that adequately describes DNA damage (Kassie *et al*., 2000). Although there have been a few studies related to cell toxicity research, studies that compare comet parameters in human biomonitoring studies are rare. Bocker *et al*., (1997) reported that the tail moment and tail DNA showed more sensitivity than the tail length on the basis of an X-irradiation dose–response experiment. De Boeck *et al*., (2000) showed that tail DNA was a more appropriate parameter than tail length to

analyze induced DNA damage because of its smaller variation, based on their internal standard. Olive *et al*., (1992) have suggested that the tail DNA was more accurate for detecting DNA damages than the tail moment.

Prediction (Hypothesis)

The prediction is that cryopreservation damages sperm through increased production of free radicals and that hydrogen peroxide, as reactive oxygen species compound this damage, that glutathione, as an electron donor, reverses some of the effects of cryopreservation and hydrogen peroxide.

Objectives

Cryopreserved spermatozoa are used in assisted reproductive techniques, such as IUI, IVF and ICSI for human, dairy animals and non human primates etc. Therefore, this study was organized to find out effects of cryopreservation on sperm characteristics including DNA integrity and to determine the involvement of ROS during cryopreservation process.

Our primary aim was to determine the protective effect of glutathione on sperm parameters against the oxidative stress that can be produced during cryopreservation or can be induced prior to the cryopreservation process.

Our aim was also to determine the sperm motility, sperm membrane integrity, acrosomal activity and DNA integrity by inducing oxidative and antioxidative approaches during freezing-thawing process.

Experimental Design

The experiment was designed to evaluate the sperm characteristics of Nili Ravi buffalo bull, i.e. total sperm motility, motility grading, membrane integrity, acrosomal activity, sperm glutathione levels and DNA integrity in fresh semen, cryopreserved semen and cryopreserved semen with supplementations of glutathione (GSH), hydrogen peroxide (H_2O_2) and GSH plus H_2O_2 additives.

Preparation of Stock Solution

Before proceeding with the experiment, stock solutions for GSH and H_2O_2 (BDH) were prepared as given in Table 1.

Preparation of Extender

Tris-homogenized egg yolk extender was prepared containing Tris (BDH) 24.20 g, citric acid (Sigma) 13.40 g, fructose (Riedel) 10 g, glycerol (BDH) 70 ml, egg yolk 200 ml, double distilled water (ddH_2O) 730 ml and procaine combiotic injection 1g, with pH adjusted to 7. All ingredients, except egg yolk and glycerol were dissolved in ddH_2O in a conical flask. Egg yolk was separated of albumen and yolk membrane was removed. Now 200 ml of egg yolk plus 70 ml glycerol was added in the solution and mixed well. The extender thus prepared was stored at –20°C. The extender was thawed at 37 °C for the dilution of semen samples.

Preparation of Aliquots

Sterilized eppendorf tubes (2 ml) were marked for respective groups and 1ml aliquots were prepared with and without supplementation of two different concentrations of GSH, H_2O_2 and combination of GSH plus H_2O_2 (Table 2).

Semen Collection

Semen of Nili Ravi Buffalo bull was collected from Semen Production Unit (SPU) Qadirabad, Sahiwal, Pakistan. Two consecutive ejaculates were collected from 5 bulls with the help of pre-warmed artificial vagina (42ºC).

Table 1. Stock solutions and Concentrations

Stock solutions	Concentrations
A	0.02 M GSH
B	0.1 M GSH
C	0.1 M H_2O_2
D	0.2 M H_2O_2

Table 2. Groups and Supplementations

Groups	Extender	Supplementations
1. Fresh 1	1 ml	no supplementation
2. Cryopreserved	1 ml	no supplementation
Supplementation prior to cryopreservation		
3. 1 mM GSH	0.9 ml	100 µl stock solution A
4. 5 mM GSH	0.9 ml	100 µl stock solution B
5. 100 µM H_2O_2	0.99 ml	10 µl stock solution C
6. 200 µM H_2O_2	0.99 ml	10 µl stock solution D
7. 1 mM GSH + 100 µM H_2O_2	0.89 ml	100 µl stock solution A+10 µl stock solution C
8. 1 mM GSH +200 µM H_2O_2	0.89 ml	100 µl stock solution A+10 µl stock solution D
9. 5 mM GSH +100 µM H_2O_2	0.89 ml	100 µl stock solution B+10 µl stock solution C
10. 5 mM GSH +200 µM H_2O_2	0.89 ml	100 µl stock solution B+10 µl stock solution D

The semen was transferred immediately to laboratory (SPU Qadirabad). Sperm mass motility was assessed microscopically. Total sperm concentration, total motility, progressive motility was assessed with the help of semen quality analyzer (SQA-Vb). Experiment was repeated for five times to confirm the results.

Mass Motility

Sperm mass motility was assessed by phase contrast microscope (Nikon) with closed circuit television (Graham *et al*., 1970). Mass motion was observed at (400 X) by taking a 5mm drop of semen on a clean, dry glass slide warmed at 37 ºC. The factors that affect mass motility of spermatozoa include concentration, percentage of progressive motility and speed/vigor of sperm motion. If one or more of these factors were compromised, the swirling of mass motion would be suppressed (Settergren, 1967). Descriptive assessment of mass motility for the presence of waves was evaluated following Baracaldo *et al*., (2006) as shown in Table 3.

Sperm Motility and Concentration

Before dilution of fresh semen 200 µl semen was mixed with 500 µl of media (provided with SQA Vb) in a small plastic beaker at 37 ºC on a slide warmer. This diluted semen was filled in a re-usable testing capillary tip and inserted into the SQA-Vb analyzer. Printed automated test results of fresh semen which include, total sperm concentration (TSC) motile sperm concentration (MSC), progressive motile sperm concentration (PMSC), % motility, morphology and velocity were obtained (Table 4). Five semen samples were taken in which sperm motility on average was 65% (the motility ranged 67% - 91%). The semen was given a holding time of 15 minutes at 37 ºC in water bath before the dilution.

Table 3. Description of Mass Motility wave pattern

Description	Scale	Wave Pattern
Very poor	0	Waves not present with immotile sperm cell
poor	1	Waves not present with motile sperm cell
Fair	2	Barely distinguishable waves in motion
Good	3	Waves apparent with moderate motion
Very good	4	Dark distinct waves with rapid motion

Table 4. Automated test results of fresh bull semen

Semen parameters	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5
Conc	614.3	737.8	702.2	706.5	369.3
Motality	81.1	70.5	67.7	76.1	91.5
p motality	78.2	68.1	65.2	73.5	88.1
Msc	498.2	520.2	475.4	537.6	337.9
Pmsc	480.1	502.2	457.7	519	325.3
Velocity	111	97	92	104	125
Morpho	89.9	86	84.9	88.1	93.7

Conc: is total sperm concentration $\times 10^{6}$ $ml^{-1.}$

Motility: sperm total percentage motility.

p motility: sperm progressive percentage motility.

Msc: motlile sperm concentration $\times 10^{6}$ $ml^{-1.}$

Pmsc: progressive motlile sperm concentration $\times 10^{6}$ $ml^{-1.}$

Velocity: sperm distance travel $\mu m\ S^{-1}$.

Morpho: sperm total percentage with normal morphology.

Semen Processing

Semen of each bull was extended in two steps; in the first step the undiluted fresh semen was diluted at 37 ºC to bring desired concentration approximately spermatozoa $\times 10^8$ ml^{-1} and temperature lowered down slowly at room temperature approximately 25ºC. In second step one ml of extended semen was added in each prepared aliquots with a 1:1 ratio and was mixed well at room temperature (semen processing and supplementations are given in Figure 2). The eppendorf with extended semen added were transferred to refrigerator at 5ºC and equilibrated for 4 hrs.

Cryopreservation

French polyvinyl straws (0.5ml) were marked for respective groups by computer attached digital marker. Equilibrated semen was loaded into straws and sealed with polyvinyl chloride. For each buffalo 10 eppendorfs were prepared for the ten experiment groups. From each eppendorf tube three straws were filled to perform different procedures. This procedure was carried out at 5ºC temperature. After preparation of 150 straws these were spread over wire gauze which was placed 5cm above the level of liquid nitrogen in a wide mouthed container for 7-minutes. Then these were plunged into liquid nitrogen (−196 ºC) and finally these were transferred in 35 L liquid nitrogen container.

Thawing

Thawing was carried out by immersing the straws in a water bath at 50 ºC for 40 seconds following Fraser and Strzezek (2005) and technique modified by Gadea *et al*., (2003; 2005). After thawing semen was transferred to 15 ml falcon tubes and were maintained at 37 ºC.

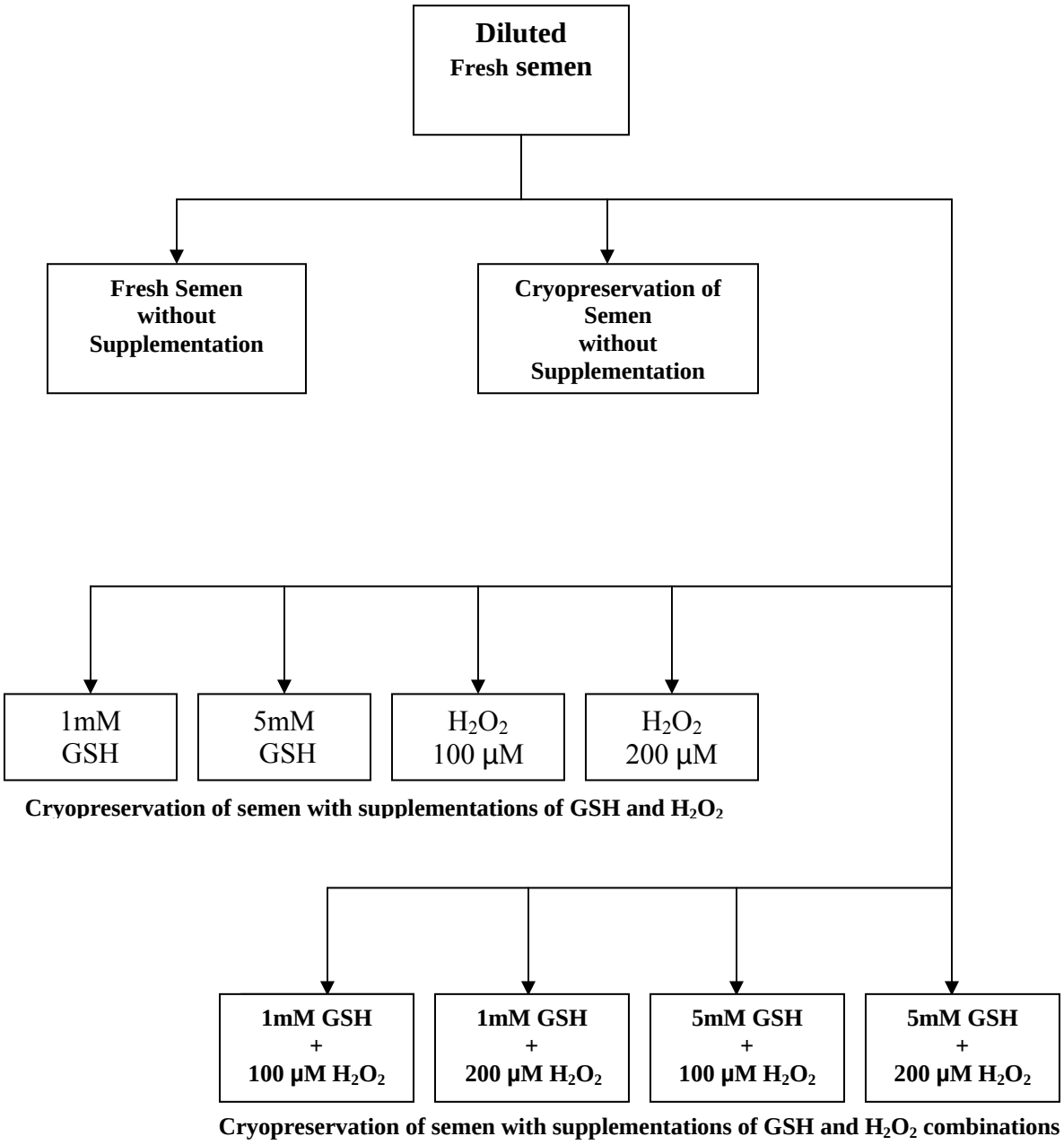


Figure: 2 Schematic division of semen samples used for the study of fresh semen cryopreservation and cryopreservation with different concentrations of supplements

Sperm Motility

Percentage sperm total motility was determined by using the following formula

$$\text{Percentage motility} = \frac{\text{Average number of motile sperm}}{\text{Average number of total sperm}} \times 100$$

The total motile sperms and the total number of sperms were counted with the help of Horwel chamber. A drop 8 µl of sperm suspension (with no air bubble) at 37°C was placed in the Horwel chamber and covered with the cover glass of 18 × 22 mm. The motile number of sperm was counted in 10 squares of grid under phase contrast microscope at 400 X magnifications. The same grid was used to count the total number of sperms× 10^{-6} ml^{-1}.Three observations for total sperm motility as well as for total number of sperm were recorded. Average of these observations was used for calculation of percentage sperm motility.

Assessment of Sperm Motility Gradation

The sperm motility gradation was based on total motile sperm count. The total motile sperms were categorized into four grades (WHO, 1999 criteria for human) by wet method as given below.

4/4: The rapid progressive, i.e. sperms motile in a straight line and cross field rapidly (Good to excellent progression or Fast forward movement).

3/4: The slow progressive, i.e. sperms motile in a straight line and cross field in slow, jerky motion (Sluggish progression or sluggish forward movement)

2/4: Non-progressive: i.e. sperms motile in a close circle. (Non directional movement)

1/4: Non motile: sperms motile on the spot either with tail or head (Non motile with no forward movement).

Sperm motility gradation was assessed by categorization into four groups of motility at 37˚C under phase contrast microscope at 400 X magnification. Of the extended semen 10 µl was placed on a dry clean slide at 37 ºC covered with 22 × 22 mm cover slip (giving a preparation depth of ~ 20 µm) This wet preparation was

immediately examined to avoid any artifact due to decrease in temperature and dehydration of the preparation. First all rapid and slowly progressive sperms were counted lying in the field of vision, thereafter, non progressive and immotile sperms in the same field were counted. Counting was carried out in three different fields.

According to motility categories the gradation of sperms was done (4 / 4, 3 / 4, 2 / 4 and 1 / 4) as has been describe earlier. Sperm motility percentage was calculated for further calculations.

Sperm Membrane Integrity

The functionally intact plasma membrane is permeable to hypo-osmolar solution and influx of fluid achieves a balance between intracellular and extra cellular spaces. The functional integrity of sperm membrane was evaluated by the hypo-osmolar solution (HOS) test following by Dobranić *et al*., (2005).

HOS solution was prepared by dissolving sodium citrate (0.735 gm) and fructose (1.351 g) in distilled water 100 ml osmolarity (150 mOsmkg−1). 50 µl of sperm suspension was incubated in 500 µl of HOS solution at 37°C for 1 hr. 10 µl of incubated sperm in HOS solution was placed on a clean and dry microscope slide and covered with 22 × 22 mm cover slip. It was examined under the phase contrast microscope at 400 X, spermatozoa with invaginated (curve) tail were considered with functionally intact plasma membrane (HOS +ve cells). Spermatozoa in which no change (straight) had occurred in tail these were considered to have defective membrane (HOS –ve cells) as shown in figure 3. Percentage of HOS +ve spermatozoa was calculated out of 300 sperm cells.

Acrosomal Activity

Acrosomal activity is the ability of spermatozoa to undergo exocytosis (acrosin) and was evaluated by the gelatin digestion test (Fiscor *et al*., 1983, Henkel *et al*., 1995).

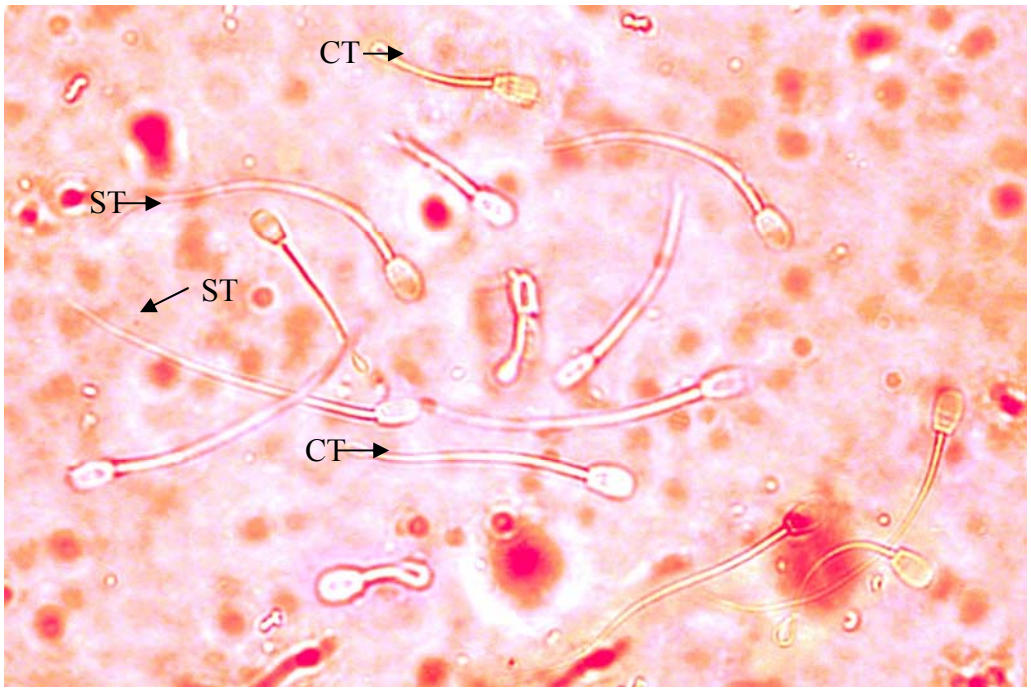
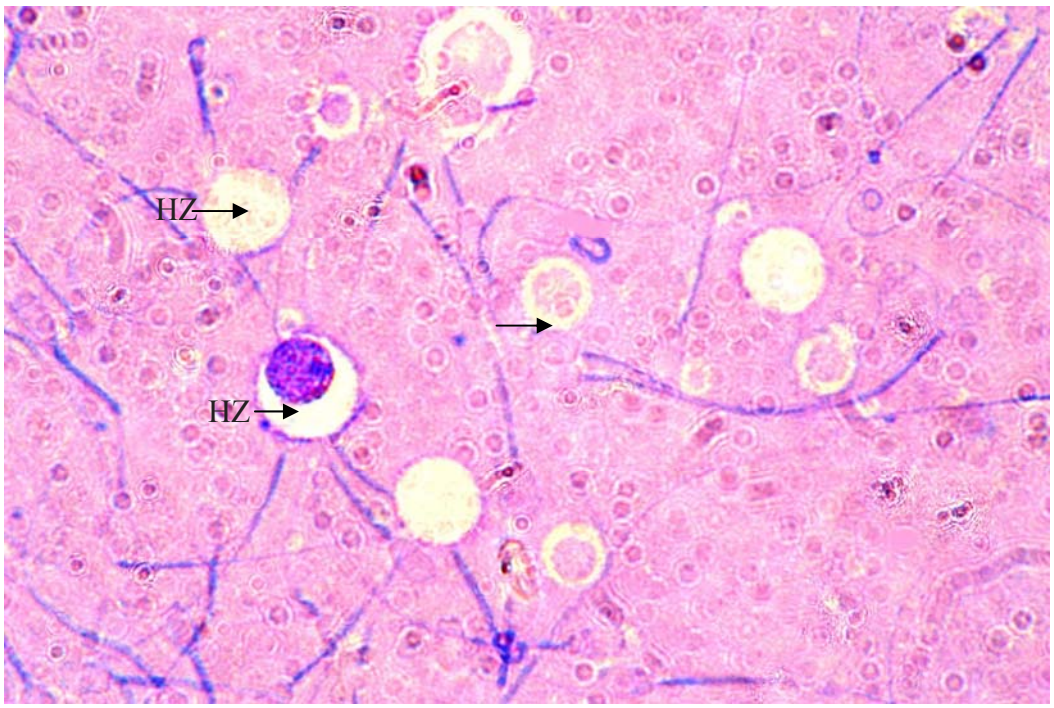


Figure: 3 Photomicrograph of spermatozoa with curve tail (CT) showing intact plasma membrane,i.e HOS +ve. Spermatozoa with straight tail (ST) showing damage plasma membrane, i.e. HOS –ve. After processed by HOS test (X400).



Preparation of Slides

Microscope slides were prepared for the gelatin digestion test. Microscope slides were marked and kept in a moisture chamber at 4°C (refrigerator) for 2 hrs. 2.5% gelatin (BDH) suspension was prepared by dissolving 2.5 gm of gelatin in 100 ml of boiled distilled water. 100 µl of gelatin suspension was placed at the one end of pre cooled slide and smeared evenly towards the other end with another slide and this was air dried. Slides were fixed with 0.05% glutaraldehyde (ICN) and washed thoroughly with phosphate buffer saline and kept again in refrigerator for overnight.

For performing test 50 µl of sperm suspension was placed at the one end of frosted slide and was smeared with cover slip and kept horizontally until dried. Then slides were incubated in a moist chamber at 39°C for 24 hrs. Slides were stained with coomassie blue (0.1 %) for 5 minutes and examined under light microscope at 400 X. Spermatozoa with bright clear halo zone around the head were considered to have ability to digest the gelatin as shown in figure 4. Percentage of sperms with halo zone was counted out of 500 sperm cells.

According to Henkel *et al*., (1995) three parameters were calculated from these results are given below

1. The halo formation rate was calculated as percentage of sperms with halo zone per slide.
2. The halo diameter was measured under phase contrast microscope at 1000 X.
3. Acrosin activity index was calculated by multiplying halo formation rate by halo diameter.

Sperm Glutathione Levels

Sperm glutathione (GSH) levels were evaluated by the method of Sedlak and Lindsay (1986). 0.3 ml sperm suspension belonging to different groups were well mixed with 1.2 ml 20% trichloro acetic acid (TCA, Merck) and placed in an ice bath (0ºC) with occasional shaking for 15 min. The samples were then centrifuged for 15 min at 2500g in order to remove membrane fragments.

Figure: 4 Photomicrograph of spermatozoa with halo zone (HZ) showing acrosin activity. Spermatozoa with no halo zone (⟶) showing no acrosin activity. Processed by geletinolysis test and stained with coomassie blue (X 400).

Glutathione levels were determined spectrophotometrically (optical test system). 1 ml sample, 2 ml of 0.4 M Tris-buffer (pH 8.2) and 0.1 ml of 0.01 M DTNB (5,5'-dithio-bis 2-nitrobenzoic acid, Fluka) were mixed thoroughly and within 5 min optical density of the solution against a reagent blank was read at 412 nm. GSH contents were expressed in µM.

DNA Integrity (Single Cell Gel Eletrophoresis)

Single cell gel electrophoresis or **C**omet assay was performed to determine the cellular DNA status of individual spermatozoa. Cellular DNA damage contains fragments and strand breaks and that separated from intact DNA under an electrophoretic field and yield as a comet tail shape. These comets can be visualized under fluorescence microscope.

The modified alkaline single cell gel electrophoresis was used Mckelvey *et al*., (1993); Hughes *et al*., (1998) and Donnelly *et al*., (2000).

The following steps are involved in processing single cell gel electrophoresis or comet assay.

1. Preparation of slides.
2. Embedding of spermatozoa in agarose gel.
3. Lysing of sperm membrane and breaking down of protein matrices.
4. Separation of DNA fragments by electrophoresis.
5. Neutralization.
6. Staining.
7. Quantification of intact and fragmented DNA by fluorescence microscope.
8. Image analysis.

After removal of cover slip embedded slides with spermatozoa were lowered slowly into the cold freshly prepared lysing solution and refrigerated at least for one hour.

Preparation of Agarose Gel and Slides

For embedding of spermatozoa, microscopic slides (Snail brand 25.4 × 76.2 mm) were marked and coated with agarose (Sigma) gel. The slides were marked with

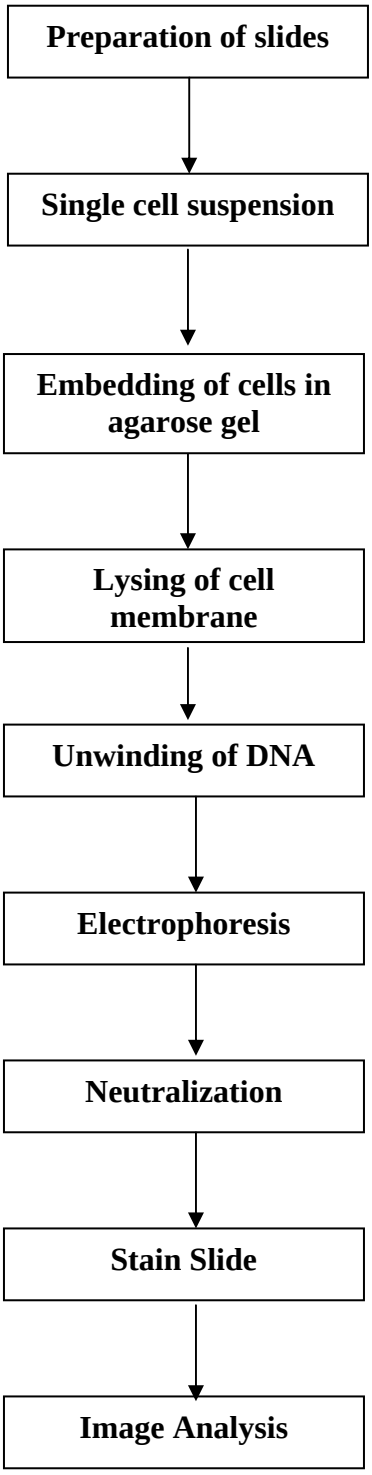


Figure: 5 Schematic diagram of single cell gel electrophoresis process

diamond slide marker for respective groups. Four slides for each group were marked. 1% agarose (normal melting point) in phosphate buffer saline (PBS) containing NaCl (8 g / l), KCl 0.2 g / l, Na_2HPO (1.44 g / l), KH_2PO (0.24 g / l) (BDH) was prepared by heating the solution near to boiling point and then dissolved the agarose in it. Each slide was dipped up to one half in hot agarose solution and removed gently, wiped under side of the slide to remove the agarose gel. The slides were laid on filter paper sheet placed on flat surface of table to dry at room temperature. Slides were stored in a wooden slide box to avoid humidity until used.

Low melting point (0.5%) agarose (250 mg / 50 ml) in phosphate buffer saline was also prepared in the similar manner and 5 ml aliquots were made in falcon tubes that were refrigerated until needed.

Embedding of Spermatozoa in Agarose Gel

An aliquot of 0.5% agarose gel was melted and maintained at 65°C. Large cover slips (Marien field 24 × 50 mm) were placed on hot plate.

20 µl of sperm suspension (~ 10,000 spermatozoa) were mixed with 70 µl of melted agarose gel, placed on coated slides and covered with hot cover slip in order to spread the suspension equally. Slides were placed on ice pack for 3-5 minutes, to harden the gel.

Lysing of Sperm Membrane

Before the embedding of spermatozoa, 100 ml lysing solution was prepared by dissolving NaCl (14 g), Disodium EDTA (3.72 g, Riedel), Tris (0.121 g) in 70 ml dH_2O, pH was adjusted to 10. Distilled H_2O was added in solution up to 89 ml, 10% dimethylsulfoxide (DMSO, Riedel) and 1% Triton X – 100 (Sigma) were mixed and refrigerated in a coplin jar.

Slides were removed from lysing solution and proteinase K (200 µg/100 ml) was dissolved in the same solution again slides were lowered into it and incubated for 4-5 hours at 37˚C to remove any residual protamine that otherwise impede DNA migration through the agarose.

Unwinding of DNA

Slides were carefully removed from proteinase K solution and any remaining liquid was drained off. The slides were placed side by side as close as possible with the agarose end facing the anode in horizontal gel electrophoresis tank. Tank was filled with freshly prepared alkaline electrophoresis buffer leveled at ~ 0.5 cm above the slides, containing NaOH (12 g) and disodium EDTA (0.372 g) dissolved in one litter dH_2O, pH was adjusted to 12.5. The slides were left in alkaline buffer for 30 minutes to allow DNA unwinding in the cells.

Separation of DNA Fragments by Electrophoresis

Power supply was turned on to 25 volt (~ 0.74 V/cm) and the current was adjusted to 300 mA by lowering or raising the buffer level. The slides were electrophoresized for 10 min in order to allow the DNA to migrate. Fresh eletrophoresis buffer was prepared for each run.

Neutralization

Slides were removed from electrophoresis tank and drained off any remaining buffer. Two jars of neutralization solution (containing Tris (0.4 M) pH 7.5) were prepared and slides were placed in each jar for 5 min. This process neutralizes the slides and removes any remaining alkaline buffer or detergent that would interfere with acridine orange (Sigma) stain. At the end for dehydration slides were and dipped in absolute ethanol for 5 min. The slides were then removed from ethanol (Merck), dried and stored in wooden slide box until stained and quantified.

Staining

The slides were stained with acridine orange (20 µg/ml) dissolved in distilled water for 5 minutes and washed thoroughly with distilled water.

Quantification of intact and fragmented DNA by fluorescence microscope

Slides were dried and observed under fluorescence microscope (Leica Microsystems Type = 090-135-002) at 400 X magnification equipped with filters DM510 and green DM 430.

DNA quantification is the measurement of genetic material displacement (comet tail) from nucleus (head). Round & smooth DNA was considered as intact DNA while Scattered DNA as damaged one.

Photographs were captured with canon digital camera attached with fluorescence microscope. For each sample, 200 sperm nuclei were selected randomly and evaluated, percentage of intact and damaged DNA was counted out of 200 cell nuclei.

Image Analysis

For comet image analysis Tritek comet score (software) was used and comet DNA parameters, i.e. comet length, comet height, head diameter, head DNA percentage, tail DNA percentage, tail moment and olive tail moment were analyzed.

Whereas, tail length is used to evaluate DNA damage extent and defined as the distance of DNA migration from the body of nuclear core. Tail moment is defined as a product of the DNA in the tail and mean distance of DNA migration in the tail. Olive tail moment is calculated as product of tail length and percentage of DNA in tail (Olive *et al*., 1990) as shown in Figure 6.

Statistical Analysis

Data of different sperm characteristics and sperm DNA comet features were taken as percentages and expressed as Mean ± SEM. Student t-test (Graph pad prism version 5) was applied to compare the differences among different experimental groups.

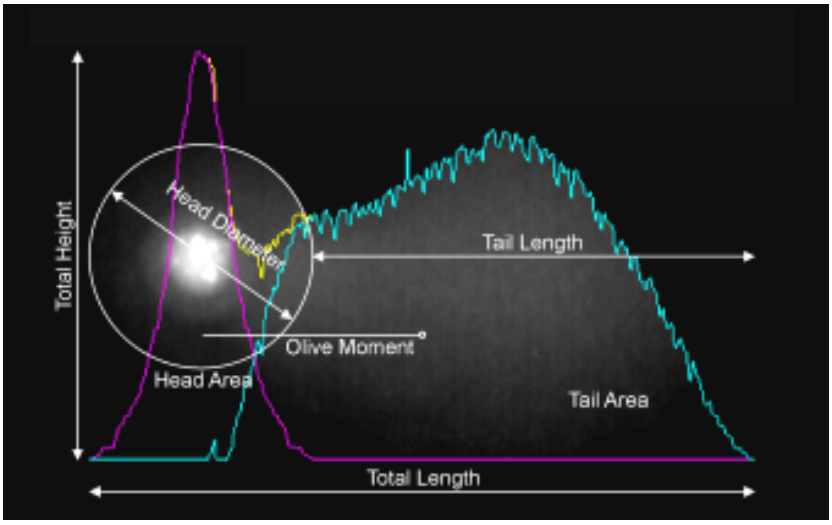


Figure 6. Photograph with diagrammatic explanation of different comet measurements including comet length, comet height, comet head diameter, comet tail length and olive tail moment (comet score).

The sperm characteristic of Nili Ravi buffalo bull i.e. total sperm motility, motility grading, membrane integrity, glutathione levels, acrosomal activity and DNA integrity in fresh semen, cryopreserved semen and cryopreserved semen in the presence of additives glutathione, hydrogen peroxide and combinations of these additives, i.e. glutathione plus hydrogen peroxide in different concentrations were evaluated.

In initial study of undiluted fresh semen sperm mass motility was assessed with phase contrast microscope. Total motile sperms, progressive motile sperm concentration was recorded with the help of semen quality analyzer (SQA-Vb). The informative **s**emen from 5 Nili Ravi Buffalo bulls was based on initial study of semen collected from Semen Production Unit (SPU) Qadirabad, Sahiwal, Pakistan.

Sperm total motility

Mean percentage of sperm total motility in Nili Ravi buffalo bull's fresh semen, cryopreserved semen and semen cryopreserved duly supplemented with Glutathione (GSH), Hydrogen peroxide (H_2O_2) and GSH plus H_2O_2 in different concentrations was studied to find out the effect of these supplements (additives) on sperm motility. The results are shown in Table 5.

Cryopreserved semen (control)

Mean percentage of sperm motility in fresh semen (1-3 hrs after dilution) was recorded. Similarly, mean percentage sperm total motility in cryopreserved semen was recorded. Cryopreservation affected sperm total motility which was significantly decreased compared to that in the fresh semen ($t_{(16)} = 6.504$; $P<0.0001$). The result of cryopreserved group was taken as control for further study.

Glutathione additives

Glutathione additives, i.e. 1 mM GSH and 5 mM GSH were added to semen before cryopreservation to find out their effects on individual sperm total motility.

Additive 1 mM GSH significantly decreased mean percentage of sperm total motility compared to that in control ($t_{(19)}=2.654$; $P=0.0157$). Supplementation of 5 mM GSH highly significantly decreased mean percentage of sperm total motility compared to that of control

($t_{(19)}$=4.931; P<0.0001). Compared to 1 mM GSH additive the decrease in mean motility percentage with 5 mM GSH additive was not significant ($t_{(22)}$=0.9490; P=0.3529).

Hydrogen peroxide Additives

Hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were supplemented prior to semen cryopreservation to see the effect of these two additives on sperm total motility. Additive 100 µM H_2O_2 did not affect mean percentage of sperm total motility which was rather nearly equal to that in control ($t_{(16)}$=0.06607; P=0.9481). (This additive had maintained total sperm motility). Addition of 200 µM H_2O_2 highly significantly decreased mean sperm total motility compared to that in control ($t_{(19)}$=5.045; P<0.001). This decrease in sperm motility is also highly significantly low compared to that with the additive 100 µM H_2O_2 ($t_{(19)}$=5.303; P<0.001).

Combinations of GSH plus H_2O_2

Different combinations of GSH + H_2O_2 additives were separately added before semen cryopreservation to observe the effect of these combinations on sperm total motility. Addition of all these additives highly significantly decreased sperm total motility, 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(19)}$ = 4.801; P<0.0001), 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(19)}$ = 3.953; P=0.0009); 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(19)}$ = 12.33; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(19)}$ = 8.208; P<0.0001).

Comparison was also carried out within these combinations, the additive 1 mM GSH + 100 µM H_2O_2 showed significant decrease in sperm total motility than that with additive 1 mM GSH + 200 µM H_2O_2 ($t_{(22)}$=2.781; P=0.0109). The additive 1 mM GSH + 100 µM H_2O_2 showed no significant difference in sperm total motility compared with 5 mM GSH + 100 µM H_2O_2 additive ($t_{(22)}$=0.1664; P=0.8694). Whereas, highly significant decrease in sperm total motility was observed with the additive 5 mM GSH + 200 µM H_2O_2 than that with additive 1 mM GSH + 200 µM H_2O_2 ($t_{(19)}$=2.689; P=0.0145). Highly significant decrease in sperm total motility was also observed with the additive 5 mM GSH + 100 µM H_2O_2 than that with additive 5 mM GSH + 200 µM H_2O_2 ($t_{(19)}$=3.921; P=0.0009).

Factorial Analysis Of Variance

Two way factorial analysis of variance was carried out using Model 5 × 10 factorial with 5 bulls and 10 treatments (as given in Table 5). The results showed (Table 6 -A) that Model 5 × 10 has highly significant effect on total sperm motility (Table 6 - A). Further compartmentation of 5 × 10 indicated that bulls have no significant effect on rate of motility, but treatments have highly significant inhibitory effect on motility.

Two way factorial analysis of variance was carried out using Model 5 × 3 × 3 factorial with 5 bulls, three concentrations of hydrogen peroxide (0, 100, 200) µM and three concentrations of glutathione (0, 1, 5) mM. Model has significant effect on total sperm motility (Table 7 - A). Compartmentation (Table 7 – B) showed interaction between H_2O_2 + bull and GSH + bull has no significant effect.

Sperm motility gradation

Gradation of sperm motility was done in accordance with their motility pattern. If the sperms were highly motile which move rapidly in a straight line they were included in Progressive 4/4 grade, there were some which were progressive but jerky and a bit slow (3/4 grade); some sperm had circular movement (2/3 grade) and others were in static position (1/4 grade). The percentages of the motility grades are given in Table 8.

Cryopreserved semen

Sperm motility grades were observed in fresh semen, cryopreserved semen and cryopreserved semen in the presence of additives (Glutathione, hydrogen peroxide and GSH + H_2O_2). The result of cryopreserved group in respective grades was taken as control for further study.

In fresh semen and cryopreserved semen, 4/4 and 1/4 grades showed no significant difference in sperm motility grades. There was significant increase in sperm motility grade 3/4 in cryopreserved semen compared to fresh semen ($t_{(16)}$=2.161; P=0.0462), but, in cryopreserved semen sperm motility grade 2/4 decreased highly significantly compared to that of fresh semen ($t_{(16)}$= 6.070; P<0.0001).

Table 6. Two way analysis of variance as a 5 × 10 factorial with 5 bulls and 10 treatments.

Dependent variable : Sperm total motility

Anova table

(A)	Source of variance	Df	SS	MS	F	P
	Model	13	13116.8	1008.98	13 - 17	<0.0001
	Error	36	2758.13	76.61		

Further compartmentation of above results to see there is significant effect of treatment or bull on sperm motility

Anova table

(B)	Source of variance	df	SS	MS	F	P
	Treatment	9	12432.9	1381.42	18.03	<0.0001
	Bull	4	683.95	170.98	2.23	0.0849
	Error	36	2758.13	76.61		

Table 7. Two way analysis as a 5 × 3 × 3 factorial with 5 bulls three concentrations of hydrogen peroxide (0, 100, 200) µM and three concentrations of glutathione (0, 1, 5) mM.

Dependent variable : Sperm total motility

Anova table

(A)	Source of variance	df	SS	MS	F	P
	Model	28	14104.62	503.73	5.98	<0.0001
	Error	21	1770.32	84.3		

Compartmentation of single analysis of variance (A) for the effect of variability, i.e. Hydrogen peroxide (H_2O_2), glutathione (GSH), bull, H_2O_2 + bull, GSH + bull on sperm motility

Anova table

(B)	Source of variance	df	SS	MS	F	P
	H_2O_2	2	4383.62	2151.81	25.53	<0.0001
	GSH	2	5134.28	2567.28	30.45	<0.0001
	H_2O_2 + GSH	4	2202.84	550.71	6.53	0.0014
	Bull	4	683.95	170.98	2.03	0.1271
	H_2O_2 + Bull	8	568.93	71.11	0.84	0.576
	GSH + Bull	8	1210.97	151.37	1.8	0.1348

Glutathione additives

No significant difference in sperm motility grade 4/4 was observed with additive 1 mM GSH as well as 5 mM GSH compared to control. A highly significant decrease in sperm motility grade 4/4 was also observed with 5 mM GSH compared to that with 1 mM GSH ($t_{(22)}$=5.437; P<0.001).

No significant difference in sperm motility grade 3/4 was observed with addition of 5 mM GSH compared to control. However, significant decrease in sperm motility grade 3/4 with 1 mM GSH was observed compared to control ($t_{(19)}$= 2.597; P=0.0177). Highly significant increase in sperm motility grade 3/4 was also observed with 5 mM GSH compared to that with 1 mM GSH ($t_{(22)}$=5.501; P<0.0001).

No significant difference in sperm motility grade 2/4 was observed with additive 1 mM GSH as well as of 5 mM GSH compared to control.

No significant difference in sperm motility grade 1/4 was observed with addition of 1 mM GSH as well as of 5 mM GSH compared to control and with each other.

Hydrogen peroxide additives

No significant difference in sperm motility grade 4/4, 3/4, 2/4 and 1/4 were observed with addition of 100 µM H_2O_2 as well as of 200 µM H_2O_2 when compared to respective control motility grade. Also no significant difference was observed in sperm motility grade 4/4, 3/4, 2/4 and 1/4 with addition of 100 µM H_2O_2 compared to that with 200 µM H_2O_2.

Combinations of GSH + H_2O_2

Cryopreserved semen in the presence of 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 decreased sperm motility grade 4/4 compared to respective control motility grade. But the decrease was not significant. However, there was non-significant increase in sperm motility grade 4/4 with the additive 5 mM GSH + 200 µM H_2O_2 compared with that in the respective control grade.

While making comparison with-in these additives, with additive 5 mM GSH + 200 µM H_2O_2 sperm motility grade (4/4) was higher compared to additives 1 mM GSH +200 µM H_2O_2 and 5 mM GSH +100 µM H_2O_2. But the difference in sperm motility grade 4/4 with-in these combinations was not significant.

No significant difference in sperm motility grade 3/4 was seen with all the four additives, 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 with the respective control motility grade.

While making comparison with-in these additives in sperm motility grade 3/4, the additive 1 mM GSH +100 µM H_2O_2 showed no significant difference with 1 mM GSH + 200 µM H_2O_2, but the same additive showed higher significant difference ($t_{(22)}$= 2.909; P=0.0081) with additive 5 mM GSH +100 µM H_2O_2. Also, no significant difference was seen in sperm motility grade 3/4 with additive 5 mM GSH + 100 µM H_2O_2 vs additive 5 mM GSH +200 µM H_2O_2. However, the latter additive showed significant difference ($t_{(19)}$= 2.184; P=0.0417) in motility grade 3/4 with the additive 1 mM GSH + 200 µM H_2O_2.

In the case of 2/4 sperm motility grade, significant increase ($t_{(19)}$=4.027; P=0.0007) compared to control was seen with additive 1 mM GSH + 200 µM H_2O_2. There was no significant difference in sperm motility grade 2/4 was observed with remaining three additives, 1 mM GSH +100 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and with 5 mM GSH + 200 µM H_2O_2 compared to the respective control.

While the comparison with-in these additives showed no significant difference in sperm motility grade 2/4. No significant difference in sperm motility grade 1/4 was observed with all the four additives, 1 mM GSH +100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2. While making comparison with-in these additives, no significant difference in sperm motility grade1/4 was seen.

Sperm membrane integrity

Intact membrane

Mean percentages of sperm membrane integrity in fresh semen, cryopreserved semen, cryopreserved semen in the presence of glutathione, hydrogen peroxide and additives in combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) are shown in Table 9.

Cryopreserved semen

The mean sperm membrane integrity was higher in fresh semen than in cryopreserved semen. Mean sperm membrane integrity in fresh semen was, however, not significantly different from that of cryopreserved semen ($t_{(38)}$=1.90; P=0.0648). This indicates that cryopreservation has not affected (damaged) much to sperm membrane integrity. The result of cryopreserved group was taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives.

Glutathione additives

The addition of 1 mM GSH before semen cryopreservation had decreased mean sperm membrane integrity percentage. This decrease was highly significant as compared to that of control ($t_{(40)}$=11.64; P<0.001). The additive of 5 mM GSH decreased sperm membrane integrity which was highly significant as compared to control ($t_{(40)}$=11.66; P<0.0001) but, sperm membrane integrity was seen significantly higher than with additive 1 mM GSH ($t_{(42)}$=2.098; P=0.042). The results showed that glutathione additives cause severe damage in membrane integrity.

Hydrogen peroxide additives

Hydrogen peroxide was added prior to cryopreservation semen in two different concentrations, i.e. 100 µM H_2O_2 and 200 µM H_2O_2. Supplementation of 100 µM H_2O_2 highly significantly lowered sperm membrane integrity than in control ($t_{(37)}$=4.52; P<0.0001). Highly significant decrease in sperm membrane integrity was observed with additives 200 µM H_2O_2 ($t_{(37)}$=11.49; P<0.0001) and 100 µM H_2O_2 ($t_{(36)}$=4.12; P=0.0002) compared to control.

Combinations of GSH + H_2O_2

Different concentrations of this combination, i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5M GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were added prior to semen cryopreservation.

All these additives highly significantly lowered sperm membrane integrity compared to that of control, i.e. 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(33)}$=14.84; P<0.0001), 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(37)}$=15.23; P<0.0001) 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(34)}$=16.05; P<0.0001) and ; 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(34)}$=18.21; P<0.001). Comparisons were carried out among these four additives, no significant difference in sperm membrane integrity was seen within additives 1 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2 ($t_{(32)}$=1.83; P=0.0781). Additive 5 mM GSH + 100 µM H_2O_2 showed significant decrease in sperm membrane integrity ($t_{(29)}$=3.58; P=0.0012) than that of 1 mM GSH + 100 µM H_2O_2. Whereas, highly significant decrease in sperm membrane integrity was observed with additive 5 mM GSH + 200 µM H_2O_2 than with additive 1 mM GSH + 200 µM H_2O_2 ($t_{(33)}$=5.018; P<0.0001) but no significant difference was found with additive 5 mM GSH + 100 µM H_2O_2 ($t_{(30)}$=1.67; P=0.1058).

Damaged membrane

Mean percentages of sperm damaged membrane in fresh semen, cryopreserved semen and semen cryopreserved in the presence of glutathione, hydrogen peroxide and additives in combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) are also shown in Table 9.

Cryopreserved semen

The mean sperm damaged membrane was low in fresh semen. Although in cryopreserved semen membrane damage is more than that in fresh semen, but difference is not significant ($t_{(40)}$=1.90; P=0.0645). This indicates that cryopreservation has not contributed much to sperm membrane damage. The result of cryopreserved group was taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives.

Glutathione additives

The addition of 1 mM GSH before semen cryopreservation increased mean sperm percentage of damaged membrane. This increase was highly significant compared to that of control ($t_{(42)}$=10.83; P<0.0001). The additive 5 mM GSH also increased percentage of sperm damaged membrane, which was highly significantly greater as compared to control

($t_{(42)}$=12.08; P<0.0001), but, it was lower than that with addition of 1 mM GSH ($t_{(44)}$=1.469; P=0.149). The results showed that glutathione additives cause severe damage in sperm membrane.

Hydrogen peroxide additives

Hydrogen peroxide was added prior to cryopreservation of semen in two different concentrations, i.e. 100 µM H_2O_2 and 200 µM H_2O_2. Supplementation of 100 µM H_2O_2 highly significantly increased sperm membrane damage compared to control ($t_{(39)}$=15.54; P<0.0001). The highly significant increase in sperm membrane damage was also observed with additive 200 µM H_2O_2 than that of control ($t_{(39)}$=9.311; P<0.0001) but, sperm membrane damage was lower than that with the addition of 100 µM H_2O_2 ($t_{(38)}$=1.882; P=0.0674).

Combinations of GSH + H_2O_2

Different concentrations of this combination, i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5M GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were added prior to semen cryopreservation. All these additives highly significantly increased sperm membrane damage compared to control, i.e. 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(35)}$=15.27; P<0.0001), 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(39)}$=15.75; P<0.0001); 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(35)}$=16.29; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(35)}$=18.26; P<0.001).

Comparisons were carried out among these four additives, no significant difference in sperm membrane damage was seen within additives 1 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2($t_{(34)}$=2.176; P=0.0366). Additive 5 mM GSH + 100 µM H_2O_2 showed significant increase in sperm membrane damage than that with 1 mM GSH + 100 µM H_2O_2 ($t_{(30)}$=3.412; P=0.0019). Additive 5 mM GSH + 200 µM H_2O_2 showed significant increase in sperm membrane damage than that with additive 1 mM GSH + 200 µM H_2O_2 ($t_{(34)}$=5.145; P<0.0001), however, no significant difference in sperm membrane damage was observed compared to that with additive 5 mM GSH + 100 µM H_2O_2 ($t_{(30)}$=1.67; P=0.1058).

Acrosomal activity

Acrosomal activity parameters i.e. halo formation rate, halo diameter and acrosin activity index are given in Table 10. These parameters were studied in fresh, cryopreserved sperms and sperms cryopreserved in the presence of different additives during the freezing thawing process of bull semen.

Halo formation rate

Halo formation rate was observed in fresh sperms, cryopreserved sperms and sperms cryopreserved in presence of additives of glutathione, hydrogen peroxide and different combinations of GSH + H_2O_2 (Table 10). The test was performed after thawing.

Cryopreserved

Halo formation rate showed highly significant decreased in cryopreserved sperms (control) than that of fresh sperms ($t_{(16)}$=16.90; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Addition of 1 mM GSH highly significantly increased halo formation rate compared to control ($t_{(16)}$=8.69; P<0.0001). Whereas, supplementation of 5 mM GSH showed greater number of halo formation which was highly significantly increased than both the control ($t_{(16)}$=12.90; P<0.0001) as well as that of 1 mM GSH additive ($t_{(16)}$=4.53; P=0.0003).

Hydrogen Peroxide Additives

Two different concentration of hydrogen peroxide additives, i.e. 100 µM H_2O_2 and 200 µM H_2O_2 were added before cryopreservation of semen. The addition of 100 µM H_2O_2 significantly increased halo formation rate compared to control ($t_{(16)}$=2.35; P=0.0315) as well as 200 µM H_2O_2 ($t_{(16)}$=2.28; P=0.0366). But, there was no significant increase in halo formation rate with addition of 200 µM H_2O_2 compared to control ($t_{(16)}$=0.72; P=0.476).

Combination of GSH + H_2O_2

Different combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) were added prior to cryopreservation of semen. The following combinations showed significantly increased halo formation rate compared to control i.e. 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(16)}$=3.18; P=0.0057); and 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(16)}$=2.21; P=0.0416); The additive 5 mM GSH + 200 µM H_2O_2 resulted into highly significantly greater increase in halo formation rate than control ($t_{(16)}$=13.86; P<0.0001). But, there was no significant increase with the additive 5 mM GSH + 100 µM H_2O_2 compared to control ($t_{(16)}$=0.27; P=0.7903). Comparison was also carried out within these combinations, the additive 1 mM GSH + 100 µM showed no significant difference than the additive 1 mM GSH + 200 µM H_2O_2 ($t_{(16)}$=0.464; P=0.6489) and than that of 5 mM GSH + 100 µM additive ($t_{(16)}$=2.07091; P<0.055). Whereas, highly significant increase in halo formation rate was observed with the additive 5 mM GSH + 200 µM compared to that of 1 mM GSH + 200 µM H_2O_2 ($t_{(16)}$=8.125; P<0.0001) and with the additive 5 mM GSH +100 µM ($t_{(428)}$=8.561; P<0.0001).

Halo diameter (µm)

Halo diameter (µm) around the sperm acrosome was measured in the similar groups of sperms, i.e. in fresh sperms, cryopreserved sperms and sperms cryopreserved after addition of glutathione, hydrogen peroxide and different combinations of GSH + H_2O_2 (Table 10).

Cryopreserved

Mean halo diameter (µm) was highly significantly lowered in cryopreserved sperms than that of fresh sperms ($t_{(27)}$=3.65; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

The Glutathione Additives i.e. 1 mM GSH and 5 mM GSH were supplemented during freezing thawing procedure. Additive 1 mM GSH showed highly significant increase in halo diameter measures as compared to that of control ($t_{(35)}$=3.65; P=0.0008). Whereas, no

significant difference was found in halo diameter measurement between the sperms with the additive 5 mM GSH and control ($t_{(33)}$=1.86; P=0243), but, significant decrease in halo diameter measurement was found compared to that with additive 1 mM GSH ($t_{(36)}$=2.35; P=0.3813).

Hydrogen Peroxide Additives

The additives of hydrogen peroxide i.e.100 µM H_2O_2 and 200 µM H_2O_2 were added before cryopreservation of semen. The addition of 100 µM H_2O_2 showed no significant difference in halo diameter measurement compared to control ($t_{(35)}$=0.886; P=0.580) as well as 200 µM H_2O_2 ($t_{(34)}$=0.406; P=0.0687). Also, there was no significant increase in halo formation rate with addition of 200 µM H_2O_2 compared to control ($t_{(31)}$=0.559; P=0.5801).

Combination of GSH + H_2O_2

Different combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) were added prior to cryopreservation of semen. Moreover, all combinations of additives showed no significant difference in halo diameter measurement compared to control.

Moreover no significant difference in halo diameter was observed within these combinations.

Acrosin activity index

Acrosin activity index was calculated by multiplying halo formation rate with halo diameter in fresh sperms, cryopreserved, sperms with the addition of additives prior to cryopreservation, i.e. glutathione, hydrogen peroxide and different combinations of GSH + H_2O_2 (Table 10).

Cryopreserved

Cryopreserved sperms showed highly significant decrease in acrosin activity index than that of fresh sperms ($t_{(16)}$=16.90; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Two concentrations of glutathione additives i.e. 1 mM GSH and 5 mM GSH were added before the freezing and thawing process. Addition of 1 mM GSH and 5 mM GSH highly significantly increased acrosin activity index in sperms compared to that of control ($t_{(16)}$=6.53; P<0.0001) and ($t_{(16)}$=7.18; P<0.0001) respectively. Whereas, no significant difference in acrosin activity index was observed between the additives 1 mM GSH and 5 mM GSH ($t_{(16)}$=0.707; P=0.0003).

Hydrogen Peroxide Additives

Similarly, two concentrations of hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were added before cryopreservation of semen. The addition of 100 µM H_2O_2 significantly increased acrosin activity index than the control ($t_{(16)}$=2.35; P=0.0315). But, no significant difference was observed with additive 200 µM H_2O_2 compared to control. Latter additive showed significant decrease than that of the 100 µM H_2O_2 ($t_{(16)}$=2.53; P=0.022).

Combination of GSH + H_2O_2

Different combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) were added prior to cryopreservation of semen. The combinations 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(16)}$=2.58; P=0.002) and 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(16)}$=1.92; P=0.0077) showed significant increase in sperm acrosin activity index compared to control. But, there was no significant difference with the additive 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(16)}$=1.92; P=0.0723). Whereas, the additive 5 mM GSH + 200 µM H_2O_2 showed highly significant increase in sperm acrosin activity index compared to that of control ($t_{(16)}$=7.30; P<0.0001).

Sperm acrosin activity index was also compared within these combinations of additives. The additive 1 mM GSH + 100 µM H_2O_2 showed no significant difference than that of additive 1 mM GSH + 200 µM H_2O_2 ($t_{(16)}$=0.0.17; P=0.8678) and the additive 5 mM GSH + 100 µM ($t_{(16)}$=1.29; P=0.2135). Whereas, a highly significant increase in sperm acrosin

activity index was observed with the additive 5 mM GSH + 200 µM compared to that of 1 mM GSH + 200 µM H_2O_2 ($t_{(16)}$=7.73; P<0.0001) and with the additive 5 mM GSH + 100 µM ($t_{(428)}$=7.77; P<0.0001).

Sperm Glutathione Levels

Sperm glutathione levels in fresh sperms, cryopreserved sperms, cryopreserved sperms in the presence of additives like glutathione, hydrogen peroxide and combinations of GSH + H_2O_2 with different concentrations i.e.1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 are given in Table 11.

Cryopreserved semen

No significant difference sperm in glutathione levels were observed between cryopreserved semen and fresh semen respectively. This shows that cryopreservation has not reduced sperm glutathione levels rather maintained the levels as in fresh semen. The result of cryopreserved group was taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives.

Glutathione additives

Glutathione additives with two different concentrations, i.e. 1 mM GSH and 5 mM GSH were added before semen cryopreservation. Additive1 mM GSH maintained glutathione levels above 4µM as was seen in fresh and cryopreserved (control). However, with this additive there was small significant difference in sperm glutathione compared to control ($t_{(22)}$=4.72; p<0.001). Additive 5 mM GSH caused significantly greater decrease in the glutathione levels compared to control ($t_{(22)}$=10.4; p< 0.001) and that with 1 mM GSH additive ($t_{(22)}$=7.95; p<0.001).

Hydrogen peroxide additives

Hydrogen peroxide additives (100 µM H_2O_2+200 µM H_2O_2) with two different concentrations were supplemented prior to cyopreservation of semen.

A drastic reduction of sperm glutathione level was due to the addition of 100 µM H_2O_2. Obviously, this was highly significantly low compared to that of control ($t_{(28)}$=11.33;

p<0.001). Additive 200 µM H_2O_2 increased the sperm glutathione level significantly compared with 100 µM H_2O_2 additive ($t_{(28)}$ = 5.25; p< 0.001), but compared to that in control, these sperm glutathione levels were highly significantly ($t_{(28)}$=4.73; p<0.001) lesser.

Combination of GSH + H_2O_2

Combination of additives GSH + H_2O_2 with different concentrations were added before semen cryopreservation, they were 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2. Additive1 mM GSH + 100 µM H_2O_2 maintained glutathione levels on higher sides i.e. higher than 4µM. However this higher level of glutathione was significantly less than that of control ($t_{(28)}$=4.0; p<0.001). Additive 1 mM GSH + 200 µM H_2O_2 decreased to very low level and this decrease was highly significantly low compared to that of control ($t_{(25)}$=10.09; p<0.001). Additives of 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 highly significantly decreased sperm glutathione levels compared to control ($t_{(25)}$=10.89; p<0.001); and ($t_{(28)}$ = 18.33; p< 0.001).

Within additives of GSH + H_2O_2 the sperm glutathione levels were significantly higher with the additive 1 mM GSH + 100 µM H_2O_2 compared to additives 1 mM GSH + 200 µM H_2O_2 ($t_{(25)}$=6.42; p<0.001); 5 mM GSH + 100 µM H_2O_2 ($t_{(25)}$=3.63; p<0.001). Similarly the addition of 1 mM GSH + 200 µM H_2O_2 decreased significantly sperm glutathione levels compared to additive 5 mM GSH + 200 µM H_2O_2 ($t_{(25)}$=3.9; p<0.001). There was no significant difference in glutathione levels in comparisons between additives 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2.

DNA Integrity

Comet assay or single cell gel electrophoresis was performed to observe the DNA integrity in different groups of the present study, i.e. fresh sperms, cryopreserved sperms and cryopreserved sperms in the presence of additives glutathione, hydrogen peroxide and different combinations of GSH + H_2O_2.

Intact DNA percentage

Mean percentages of intact DNA in fresh semen, cryopreserved semen, cryopreserved semen in the presence of glutathione, hydrogen peroxide and additives in combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) are shown in Table 12.

Cryopreserved

The mean percentage of intact DNA observed in cryopreserved semen was highly significantly less than that in the fresh semen ($t_{(32)}$=15.89; P<0.0001). This indicated that cryopreservation has damaged greatly the intact DNA of sperm. The result of cryopreserved group was taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives.

Glutathione additives

The addition of 1 mM GSH before semen cryopreservation increased the percentage of intact DNA. This increase was not significant as compared to that of control ($t_{(33)}$=0.9394; P=0.3544). The addition of 5 mM GSH highly significantly increased percentage of DNA intact as compared to control ($t_{(32)}$=14.25; P<0.0001), as well as, to that with addition of 1 mM GSH ($t_{(33)}$=18.20; P<0.0001).

Hydrogen peroxide additives

Hydrogen peroxide was added prior to cryopreservation of semen in two different concentrations, i.e. 100 µM H_2O_2 and 200 µM H_2O_2. Supplementation of 100 µM H_2O_2 and 200 µM H_2O_2 highly significantly lowered percentage of Intact DNA than in control ($t_{(32)}$=7.989; P<0.0001; $t_{(32)}$=6.858; P<0.0001) respectively. There was no significant difference ($t_{(32)}$=0.01994; P=0.9842) in intact DNA percentage when both concentrations of H_2O_2 were compared.

Combinations of GSH + H_2O_2

Different concentrations of this combination, i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5M GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were added prior to semen cryopreservation.

All these additives have increased percentage of Intact DNA compared to that of control. Additives 1 mM GSH + 200 µM H_2O_2, 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 highly significantly increased percentage of intact DNA compared to that of control ($t_{(32)}$=12.04; P<0.0001; $t_{(32)}$= 2.644; P=0.0126; $t_{(32)}$=19.31; P<0.001) respectively. Although percentage of Intact DNA with additives 1 mM GSH + 100 µM H_2O_2 was increase but the same was not significant compared to that of control ($t_{(32)}$=1.055; P=0.2993).

Comparisons were also carried out among these four additives, no significant difference in percentage of Intact DNA was seen within additives 1 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 100 µM H_2O_2 ($t_{(32)}$=27.95; P<0.0001). Additive 1 mM GSH + 200 µM H_2O_2 showed highly significant increase in the percentage Intact DNA compared to 1 mM GSH + 100 µM H_2O_2 ($t_{(32)}$=18.72; P<0.0001). Highly significant increase in percentage of Intact DNA was observed with additive 5 mM GSH + 200 µM H_2O_2 compared with additive 5 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2, ($t_{(32)}$=68.57; P<0.0001; $t_{(32)}$=27.95; P<0.0001) respectively.

Damage DNA percentage

Mean percentages of DNA damage in fresh semen, cryopreserved semen, cryopreserved semen in the presence of glutathione, hydrogen peroxide and additives in combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) are given in Table 12.

Cryopreserved

The mean percentage of DNA damage observed in cryopreserved semen is highly significantly greater than that in the fresh semen ($t_{(32)}$=15.89; P<0.0001). This indicates that cryopreservation has increased sperm DNA damage. The result of cryopreserved group was

taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives (Figure 7).

Glutathione additives

The addition of 1 mM GSH before semen cryopreservation cause no significant change in sperm DNA damage compared to that of the control ($t_{(32)}$=0.7575; P=0.4543). The addition of 5 mM GSH highly significantly decreased percentage of DNA damage compared to control ($t_{(32)}$=14.25; P<0.0001), as well as, to that with the addition of 1 mM GSH ($t_{(32)}$=18.20; P<0.0001) (Figure 8).

Hydrogen peroxide additives

Hydrogen peroxide was added prior to cryopreservation of semen in two different concentrations, i.e. 100 µM H_2O_2 and 200 µM H_2O_2. Supplementation of 100 µM H_2O_2 and 200 µM H_2O_2 highly significantly increased percentage of DNA damage compared to control ($t_{(32)}$=7.989; P<0.0001; $t_{(32)}$=6.858; P<0.0001) respectively. No significant change in percentage of DNA damage was observed with additive 200 µM H_2O_2 than that with additive 100 µM H_2O_2 ($t_{(32)}$=0.01994; P=0.9842) (Figure 8).

Combinations of GSH + H_2O_2

Different concentrations of this combination, i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5M GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were added prior to semen cryopreservation.

All these additives have decreased percentage of DNA damage compared to that of control (Figure 9). Additives 1 mM GSH + 200 µM H_2O_2, 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 highly significantly decreased percentage of DNA damage compared to that of control ($t_{(32)}$=12.04; P<0.0001); ($t_{(32)}$= 2.644; P=0.0126) and ($t_{(32)}$=19.31; P<0.001) respectively. There was no significant ($t_{(32)}$=1.055; P=0.2993) difference in the percentage of DNA damage with additives 1 mM GSH + 100 µM H_2O_2 compared to control.

Comparisons were also carried out among these four additives, significant decrease in percentage of DNA damage was seen with additives 5 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 100 µM H_2O_2 ($t_{(32)}$=27.95; P<0.0001). Additive 1 mM GSH + 200 µM H_2O_2 showed highly significant decrease in percentage DNA damage compared to that with 1 mM GSH + 100 µM H_2O_2 ($t_{(32)}$=2.537; P=0.0163). Highly significant decrease in percentage of DNA damage was observed with additive 5 mM GSH + 200 µM H_2O_2 compared to with additive 5 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2, ($t_{(32)}$=68.57; P<0.0001; $t_{(32)}$=27.95; P<0.0001) respectively.

Comet Length

Mean comet length (µm) is given in Table 13, which was observed in fresh sperms, cryopreserved sperms and cryopreserved sperms with additives glutathione, hydrogen peroxide and different combinations of GSH + H_2O_2.

Cryopreserved

Mean comet length (µm) was significantly low in cryopreserved sperms than the fresh sperms ($t_{(388)}$=21.34; P<0.0001). The result of cryopreserved group was taken as control for further study, i.e. cryopreservation of sperms in the presence of different additives.

Glutathione Additives

Glutathione additives 1 mM GSH and 5 mM GSH were supplemented to extended semen prior to cryopreservation. Addition of 1 mM GSH highly significantly reduced mean comet length compared to that of control ($t_{(393)}$=12.63; P<0.0001). Supplementation of 5 mM GSH led to more reduction in mean comet length which was highly significantly less than that of control ($t_{(377)}$=5.99; P<0.0001) but higher than with 1 mM GSH additive ($t_{(374)}$=7.35; P<0.0001).

Hydrogen Peroxide Additives

Two hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were added to semen before cryopreservation. The addition of 200 µM H_2O_2 reduced mean comet length compared to control ($t_{(477)}$ = 11.80; P<0.0001) and 100 µM H_2O_2 ($t_{(445)}$ = 6.677; P<0.0001). But, no significant reduction from control was seen in mean comet length with the addition of 100 µM H_2O_2 ($t_{(420)}$=1.28; P=0.199).

Combination of GSH + H_2O_2

Different combinations (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) were added before semen cryopreservation. Compared to control there was significantly less mean comet length with the addition of 1 mM GSH + 100 µM H_2O_2 ($t_{(443)}$=7.2; P<0.0001); 1 mM GSH + 200 µM H_2O_2 ($t_{(424)}$=1.9; P=0.048); 5 mM GSH + 100 µM H_2O_2 ($t_{(401)}$=12.13; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 ($t_{(474)}$= 20.21; P<0.0001).

Comparisons were also carried out within these combinations. With the addition of 1 mM GSH + 200 µM H_2O_2, there was highly increased mean comet length compared to that of 1 mM GSH + 100 µM ($t_{(471)}$=5.98; P<0.0001), whereas, the latter additive showed highly significant increase in mean comet length than that of 5 mM GSH + 100 µM H_2O_2 additive ($t_{(448)}$=7.91; P<0.0001). Similarly, the additive 1 mM GSH + 200 µM H_2O_2 highly significantly increased mean comet length compared to that of 5 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=6.51; P<0.0001) and with latter additive highly significant increase in mean comet length was observed than that of 5 mM GSH + 100 µM additive ($t_{(405)}$=4.80; P<0.0001).

Comet height

Comet height (µm) is shown in Table 14, that was observed in sperms obtained from fresh semen and after thawing of cryopreserved semen and semen Supplemented with additives (glutathione, hydrogen peroxide and combination of these additives i.e. GSH + H_2O_2) prior to cryopreservation.

Cryopreserved

Mean comet height was significantly decreased in cryopreserved sperms compared to that of fresh sperms ($t_{(388)}$=16.32; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Comet height was observed in cryopreserved sperms with 1 mM GSH and 5 mM GSH additives. Addition of 1 mM GSH highly significantly reduced mean comet height compared to that of control ($t_{(393)}$=17.02; P<0.0001) and with 5 mM GSH additive ($t_{(374)}$=7.99; P<0.0001). Mean comet height was also significantly lowered with the addition of 5 mM GSH compared to control ($t_{(377)}$=10.60; P<0.0001).

Hydrogen Peroxide Additives

Hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were supplemented prior to sperm cryopreservation. There was significant decrease in mean comet height with both the additives compared to control ($t_{(420)}$=7.43; P<0.0001; $t_{(399)}$=8.93; P<0.0001) respectively. There was no significant difference in mean comet height when both concentrations of H_2O_2 were compared ($t_{(423)}$=2.164; P=0.031).

Combination of GSH + H_2O_2 Additives

Additives of this combination with different concentrations, i.e. (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) were supplemented before cryopreservation of sperms. Highly significant decrease in mean comet height with all of these additions was observed compared to that of control, i.e. 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(443)}$=15.87; P<0.0001); 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(424)}$ = 9.44; P<0.0001); 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(401)}$=17.14; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=11.19; P<0.0001).

When comparison was carried out within these additives, the additive 1 mM GSH + 200 µM H_2O_2 showed significantly higher mean comet height compared to that of 1 mM GSH + 100 µM H_2O_2 ($t_{(471)}$=6.01; P<0.0001) and the latter additive increased mean comet height

compared to that of 5 mM GSH + 100 µM H_2O_2 ($t_{(448)}$=3.59; P=0.0004). Similarly, additive 1 mM GSH + 200 µM H_2O_2 increased significantly mean comet height than additive 5 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=2.38; P<0.0001). Also, there was higher mean comet height with the additive 5 mM GSH + 200 µM H_2O_2 compared to that of 5 mM GSH + 100 µM H_2O_2 ($t_{(405)}$=5.78; P<0.0001).

Head Diameter (µm)

Mean comet head diameters was measured in fresh sperms, cryopreserved sperms (control) and cryopreserved sperms supplemented with glutathione, hydrogen peroxide different combination of GSH + H_2O_2 are given in Table 15.

Cryopreserved

A highly significant increase in head diameter was observed in cryopreserved sperms (control) compared to that of fresh sperms ($t_{(388)}$=18.68; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Cryopreservation of sperms after supplementation of glutathione additive 5 mM GSH significantly increased mean head diameter compared to control and to that of 1 mM GSH ($t_{(377)}$=4.41; P<0.0001; $t_{(374)}$=7.84; P<0.0001) respectively. There was no significant increase in mean head diameter with the addition of 1 mM GSH compared to that of control ($t_{(393)}$=0.47; P=047).

Hydrogen Peroxide Additives

Supplementation of hydrogen peroxide additive 200 µM H_2O_2 decreased significantly mean head diameter on cryopreservation compared to that of control ($t_{(399)}$=8.54; P<0.0001). Addition of additive 100 µM H_2O_2 highly significantly increased mean head diameter compared to that of control ($t_{(420)}$=6.58; P<0.0001) as well as to that of 200 µM H_2O_2 additive ($t_{(423)}$=16.99; P<0.0001).

Combination of GSH + H_2O_2 Additives

Different concentrations of this combination, i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were supplemented prior to cryopreservation of sperms.

The addition of all these combinations increased mean head diameter compared to that of control. Additive 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(443)}$=2.53; P=0.118), 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(424)}$=4.57; P = P<0.0001), 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=3.67; P<0.0001). The lowest mean head diameter of these combinations, was observed with the addition of 5 mM GSH + 100 µM H_2O_2, that showed non significant increase compared to that of control ($t_{(401)}$=0.57; P=0.56).

The comparison within all these combinations was also noted. The highest mean head diameter was observed with the addition of additive 1 mM GSH + 200 µM H_2O_2 that was significantly higher than that of additive 1 mM GSH + 100 µM H_2O_2 ($t_{(471)}$=4.12; P<0.0001). Whereas, this latter additive significantly increased mean head diameter compared to the addition of 5 mM GSH + 100 µM H_2O_2. ($t_{(448)}$=3.59; P=0.0004). Similarly, The above mentioned additive1 mM GSH + 200 µM H_2O_2 showed non significant increased than that of additive 5 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=1.25; P<0.0001). Whereas, the latter additive significantly increased mean head diameter than that of the additive 5 mM GSH + 100 µM H_2O_2 ($t_{(405)}$=5.51; P<0.0004).

Head DNA Percentage

Mean head DNA percentage of fresh sperms cryopreserved sperm, supplementation of different concentrations of glutathione, hydrogen peroxide and there combinations to cryopreserved sperms are given in Table 16.

Cryopreserved

Mean head DNA percentage in cryopreserved sperms was significantly low compared to fresh sperms ($t_{(388)}$=5.45; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Two Glutathione additives i.e. 1 mM GSH and 5 mM GSH were added to before cryopreservation of sperms. There was significantly higher mean head DNA percentage with the addition of 1 mM GSH additive compared to control ($t_{(393)}$=7.18; P<0.0001). With the addition of 5 mM GSH mean head DNA percentage was highly significantly lower compared to control ($t_{(377)}$= 11.87; P<0.0001) and also with the additive 1 mM GSH ($t_{(374)}$=30.52; P<0.0001).

Hydrogen Peroxide Additives

Hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were added to prior to cryopreservation of sperms. With the addition of both these additives mean head DNA percentage was significantly lowered compared to control i.e. 100 µM H_2O_2 vs control ($t_{(420)}$=7.62; P<0.0001) and 200 µM H_2O_2 vs control ($t_{(399)}$=11.21; P<0.0001). While, mean head DNA percentage with the supplementation of 200 µM H_2O_2 was significantly lower compared to that of 100 µM H_2O_2 ($t_{(423)}$=3.26; P=0.0012).

Combination of GSH + H_2O_2 Additives

Mean head DNA percentage was noted after cryopreservation with different additives like 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2.

Mean head DNA percentage was significantly lowered with the additives compared to control i.e. 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(443)}$=3.14; P=0.0018); 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(424)}$=15.65; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=12.34; P<0.0001). The addition of 1 mM GSH + 200 µM H_2O_2 showed no significant decrease compared to control.

The addition of 1 mM GSH + 200 µM H_2O_2 showed highly significant increase in head DNA Percentage compared to that with additive 1 mM GSH + 100 µM H_2O_2 ($t_{(471)}$=22.61; P<0.0001). Additive 5 mM GSH + 100 µM H_2O_2 showed significant increase in head DNA Percentage than that of 1 mM GSH + 200 µM H_2O_2 ($t_{(448)}$=3.43; P=0.0006).

The additive 5 mM GSH + 200 µM H_2O_2 significantly increased head DNA Percentage compared to sperms with the additive 1 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=4.97; P<0.0001)

and significantly decreased head DNA Percentage compared to sperms with additive 5 mM GSH + 100 µM H_2O_2 ($t_{(405)}$=20.01; P<0.0001).

Tail DNA Percentage

Mean tail DNA percentage was observed in fresh sperms, cryopreserved sperms and supplemented cryopreserved sperms with the additives (Table 17).

Cryopreserved

There was highly significant increase in mean tail DNA percentage in cryopreserved sperms compared to that of fresh sperms ($t_{(385)}$=5.17; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Glutathione additives 1 mM GSH and 5 mM GSH were added prior to cryopreservation of sperms. The addition of 1 mM GSH additive showed highly significant lower mean tail DNA percentage compared to control ($t_{(393)}$= 7.18; P<0.0001) as well as to that of the additive 5 mM GSH ($t_{(374)}$=30.52; P<0.0001). Also, the latter additive increased significantly the mean tail DNA percentage compared to control ($t_{(377)}$=11.87; P<0.0001)

Hydrogen Peroxide Additives

Two hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were supplemented before cryopreservation of sperms. The additive 100 µM H_2O_2 highly significantly increased mean tail DNA percentage as compared to control. Also, the supplementation of 200 µM H_2O_2 very highly significantly increased mean tail DNA percentage compared to control ($t_{(399)}$=11.21; P<0.0001) and with the additive of 100 µM H_2O_2 ($t_{(423)}$=3.26; P<0.0001).

Combination of GSH + H_2O_2 Additives

The tail DNA percentage was observed with the combination of GSH + H_2O_2 additives of different concentrations supplemented prior to cryopreservation of sperms i.e. 1 mM GSH

+ 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 and the comparison was made with control and also made within these additives.

The sperms with additive 1 mM GSH + 100 µM H_2O_2 significantly increased tail DNA percentage than control ($t_{(443)}$=3.14; P=0.0018) and no significant increase was seen with 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(4o1)}$=1.2; P=0.226). Whereas, 1 mM GSH + 200 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 highly significantly increased tail DNA percentage compared to control ($t_{(424)}$=15.65; P<0.0001; $t_{(400)}$=12.34; P<0.0001) respectively.

When these combinations were compared with each other, the additive 1 mM GSH + 200 µM H_2O_2 highly significantly increased tail DNA percentage compared to additive 1 mM GSH + 100 µM H_2O_2 ($t_{(471)}$=22.61; P<0.0001). And 1 mM GSH + 100 µM H_2O_2 showed significantly higher tail DNA percentage than that of 5 mM GSH + 100 µM H_2O_2 ($t_{(448)}$=3.4; P<0.0001). The addition of 5 mM GSH + 200 µM H_2O_2 very highly significantly increased tail DNA percentage compared to additive 5 mM GSH + 100 µM H_2O_2 ($t_{(405)}$=20.01; P<0.0001). Whereas, the same additive showed significantly low mean tail DNA percentage compared to that of 1 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=4.9; P<0.0001).

Tail Length

Mean tail length (µm) in fresh sperms, cryopreserved sperms and supplemented cryopreserved sperms with the additives is given in Table 18.

Cryopreserved

Mean tail length (µm) in cryopreserved sperms (control) was significantly higher than in fresh sperms ($t_{(388)}$ = 5.48; P<0.0001) (Figure 7). The result of cryopreserved group was taken as control for further study

Glutathione Additives

Two glutathione additives with different concentrations, i.e. 1 mM GSH and 5 mM GSH were added prior to cryopreservation of sperms. Addition of the both additives i.e. 1 mM GSH and 5 mM GSH highly significantly decrease the mean tail length compared to the control ($t_{(393)}$=10.95; P<0.0001; $t_{(377)}$=10.53; P<0.0001) respectively. Whereas within both of these additives no significant difference was noted ($t_{(374)}$=0.15; P=0.876) (Figure 8).

Hydrogen Peroxide Additive

Hydrogen peroxide additives 100 µM H_2O_2 and 200 µM H_2O_2 were supplemented before cryopreservation of sperms. The addition of 100 µM H_2O_2 additive highly significantly decreased the mean tail length than control ($t_{(420)}$=9.49; P<0.0001). The additive 200 µM H_2O_2 has significantly increased mean tail length compared to control ($t_{(399)}$=2.31; P=0.0212) and of 100 µM H_2O_2 ($t_{(423)}$=12.07; P<0.0001) (Figure 8).

Combinations of GSH + H_2O_2

Four additives of this combination i.e. 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were supplemented prior to cryopreservation of sperms. With all these supplementations highly significant decrease was observed in mean tail length compared to that of control i.e. 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(431)}$ = 8.79; P<0.0001); 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(424)}$=7.24; P<0.0001); 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(401)}$=11.09; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=11.62; P<0.0001).

Mean tail length was observed within the GSH + H_2O_2 combinations, there was no significant difference between 1 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2 but, 5 mM GSH + 100 µM H_2O_2 significantly lowered mean tail length compared to that of 1 mM GSH + 100 µM H_2O_2 ($t_{(448)}$=3.94; P<0.0001).

Similarly, mean tail length showed no significant difference between 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 ($t_{(405)}$=0.94; P=0.343) while, 5 mM GSH + 200 µM H_2O_2 significantly reduced the mean tail length compared to 1 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=5.20; P<0.0001) (Figure 9).

Comet tail moment

Observation of mean comet tail moment in fresh sperms, cryopreserved and in supplemented cryopreserved sperms with different additives is given in Table 19.

Cryopreserved

Mean comet tail moment measured in cryopreserved sperms was significantly high compared to that in fresh sperms ($t_{(385)}$ = 4.94; P<0.0001). The result of cryopreserved group was taken as control for further study.

Glutathione additives

Cryopreservation of sperms was carried out after supplementation of glutathione additives, 1 mM GSH and 5 mM GSH. The addition of 1 mM GSH highly significantly reduced comet tail moment (DNA damage) compared to control ($t_{(393)}$=9.34; P<0.0001). Mean comet tail moment significantly increased by the addition of 5 mM GSH additive compared to that of 1 mM GSH additive ($t_{(374)}$=9.51; P<0.0001), but this additive significantly lowered the mean comet tail moment compared to control ($t_{(377)}$=3.44; P=0.0007).

Hydrogen Peroxide Additives

Hydrogen peroxide additives (i.e. 100 µM H_2O_2 and 200 µM H_2O_2) were added prior to cryopreservation of sperms. The addition of 100 µM H_2O_2 additive resulted in significantly decreased comet tail moment compared to control ($t_{(420)}$=3.35; P=0.0009). Mean comet tail moment with the addition of 200 µM H_2O_2 showed highly significant increase compared to control ($t_{(399)}$ = 6.82; P<0.0001) and with the 100 µM H_2O_2 additive ($t_{(423)}$=11.18; P<0.0001).

Combinations of GSH + H_2O_2 Additives

Different combinations of GSH + H_2O_2 additives, i.e. 1 mM GSH +100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH +100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 were added before cryopreservation of sperms. The lowest mean comet tail moment was observed with additive 5 mM GSH + 100 µM H_2O_2 (2.918±0.12 µm) and the highest mean

comet tail moment was observed with additive 1 mM GSH + 200 µM H_2O_2 (6.534±0.31µm).The highly significant decrease in comet tail moment was observed in comparison with control, i.e. 1 mM GSH +100 µM H_2O_2 vs control ($t_{(443)}$=5.65; P<0.0001); 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(401)}$=7.26; P<0.0001) and 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=4.09; P<0.0001), where as 1 mM GSH + 200 µM H_2O_2 vs control showed no significant difference ($t_{(424)}$=0.225; P=0.822).

Comparison between the combinations of GSH + H_2O_2 additives was also made. The additive 1 mM GSH + 100 µM H_2O_2 highly significantly decreased mean comet tail moment compared to 1 mM GSH + 200 µM H_2O_2 ($t_{(471)}$=3.43; P=0.0006). Whereas, the additive 5 mM GSH + 100 µM H_2O_2 showed highly significant decrease in mean comet tail moment compared to that of 1 mM GSH + 100 µM H_2O_2 ($t_{(448)}$=4.95; P<0.0001). Also, highly significant low mean comet tail moment was observed with the additive 5 mM GSH + 100 µM H_2O_2 in comparison with 5 mM GSH + 200 µM H_2O_2 ($t_{(405)}$=5.9; P<0.0001) and with 1 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=5.81; P<0.0001).

Olive tail moment

The Olive tail moment was observed in fresh sperms, cryopreserved and sperms with different additives like that of Glutathione, Hydrogen peroxide, and combination of GSH + H_2O_2 supplemented to cryopreserved sperms (Table 20).

Cryopreserved

Mean olive tail moment (µm) in cryopreserved sperms was significantly higher than in fresh sperms ($t_{(385)}$=2.70; P=0.007). The result of cryopreserved group was taken as control for further study.

Glutathione Additives

Additives 1 mM GSH and 5 mM GSH were added prior to cryopreservation of sperms. The addition of 1 mM GSH highly significantly lowered the mean olive tail moment compared to control ($t_{(393)}$=6.65; P<0.0001). Whereas, supplementation of 5 mM GSH highly significantly increased mean olive tail moment compared to control ($t_{(377)}$=16.03;

P<0.0001) as well as to that of the sperms with the additive 1 mM GSH ($t_{(374)}$=28.00; P<0.0001).

Hydrogen Peroxide Additives

Hydrogen peroxide additives, 100 µM H_2O_2 and 200 µM H_2O_2 were added before cryopreservation of sperms. Supplementation of 100 µM H_2O_2 showed highly significant increase in mean olive tail moment (µm) than that of control ($t_{(420)}$=13.54; P<0.0001) and the sperms with addition of 200 µM H_2O_2 ($t_{(423)}$=5.03; P<0.0001). The additive 200 µM H_2O_2 also caused significantly higher mean olive tail moment than that of control ($t_{(399)}$=10.23; P<0.0001).

Combination of GSH + H_2O_2 Additives

Cryopreservation of sperms was carried out after supplementation of additives of GSH + H_2O_2 with different concentrations i.e. (1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2).

All the above added combinations, significantly increased mean olive tail moment (µm) than that of control, such as 1 mM GSH + 100 µM H_2O_2 vs control ($t_{(443)}$=7.07; P<0.0001); 1 mM GSH + 200 µM H_2O_2 vs control ($t_{(424)}$=17.44; P<0.0001); 5 mM GSH + 200 µM H_2O_2 vs control ($t_{(400)}$=16.42; P<0.0001) and 5 mM GSH + 100 µM H_2O_2 vs control ($t_{(401)}$=2.42; P=0.016).

When the combinations were compared with each other, the addition of 1 mM GSH + 100 µM H_2O_2 highly significantly increased mean olive tail moment as compared to that with 5 mM GSH + 100 µM H_2O_2 ($t_{(448)}$=6.72; P<0.0001). While, the additive 1 mM GSH + 200 µM H_2O_2 further increased mean olive tail moment than with the additive 1 mM GSH + 100 µM H_2O_2 ($t_{(471)}$=16.54; P<0.0001).

The higher mean olive tail moment was observed with the addition of 5 mM GSH + 100 µM H_2O_2 in comparison with that of 5 mM GSH + 200 µM H_2O_2 ($t_{(405)}$=19.58; P<0.0001). But, no significant difference was found with the additives of 5 mM GSH + 200 µM H_2O_2 and 1 mM GSH + 200 µM H_2O_2 ($t_{(428)}$=1.009; P=0.31).

Semen cryopreservation is an important part of assisted reproduction (Keshavarz, 2007) and is a potentially useful way of sperm banking until needed for experimentation or insemination. Long-term preservation of spermatozoa in liquid nitrogen is a subject of paramount interest because of extensive use of frozen semen for artificial insemination (AI) in cattle breeding. (Mossad, 1994) in human (Donnelly *et al.,* 2001) and other animals (Li *et al.,* 2007; Anel, *et al.,* 2005).

In dairy cattle, the majority of routine inseminations are done with frozen–thawed semen (Vermes *et al.,* 1995). Cryopreserved bull semen has been used commercially in dairy cattle for decades. Conception results are now comparable or better than those with natural mating (Watson, 2000). The response of sperm to cryopreservation and the fertility of frozen–thawed semen vary between species (Waterhouse *et al.,* 2006). In the majority of mammalian species fertility is clearly reduced by the cryopreservation protocol (Watson, 2000).

There are many features of spermatozoon which are essential for fertilization and must be preserved after cryopreservation which include DNA content, acrosomal integrity, motility and viability (Ozkavukcu *et al.,* 2008). Chatherjee *et al.,* (2001) observed that cooling and freezing/thawing exert physical and chemical stresses on the sperm membrane. Salvodor *et al.,* (2006) reported that cryopreservation is associated with an oxidative stress induced by free radicals. The freezing process produces physical and chemical stress on the sperm membrane which reduces sperm viability and fertilizing ability (Bailey *et al.,* 2000).

Bilodeau *et al.,* (2000) and Ball *et al.,* (2001) reported that two important processes during cryopreservation take place firstly, production of ROS that can bring about changes in the membrane system. Secondly, due to freezing changes in antioxidant defense systems including decrease in intracellular GSH contents (Gadea *et al.,* 2004).

Baumber et al., (2000) examined that in recent years antioxidants have been used to protect spermatozoa from deleterious effects of cryopreservation and free radicals are eliminated by antioxidants systems. Antioxidants commonly used according to Bilodeau *et al.,* (2001) are thiols such as glutathione and cystein that prevents the loss of sperm motility in frozen thawed bull semen. Szczesniak-Fabianczyk *et al.,* (2006) investigated

that a semen extender with cystine improved the viability, chromatin structure and membrane integrity of bore sperm during liquid preservation.

Sperm motility evaluation is the most common and major determinant of male fertility and sperm quality assessment (Pena, 2007; Kjaestad *et al.,* 1993; Hartman, 1965), whereas, poor sperm motility causes male infertility (Hong *et al.,* 1991). It is an important parameter for sperm viability and fertilizing ability (Kjaestad *et al.,* 1993). However, motility should be evaluated together with other parameters when estimating the fertilizing potential of spermatozoa (Kenney *et al.,,* 1893).

Uysal and Bucak (2007) observed the effects of oxidized glutathione, cysteine and lycopene as the quality of frozen-thawed ram semen. They used different concentrations of these antioxidants. They reported that antioxidants GSH at 5 mM had significant ($P<0.001$) effect in maintaining post thaw sperm mobility, sperm morphology, acrosome integrity, viability and membrane integrity compared to other concentration groups. Similarly BSA at 20 mg/ml, cysteine at 10 mM and lycopene at 200 µg concentration had significantly ($P<0.001$) improved features of cryopreserved sperm of ram. They are of the opinion that particular concentrations of antioxidants during semen cryopreservation may exert beneficial effects on the quality of the freezing-thawing of ram semen. They indicated that this is new approach to the cryopreservation of sperm from ram of different breeds.

Gadea *et al.,* (2005) observed that reduced glutathione improved functions and in vitro fertilizing ability of boar spermatozoa after cryopreservation. They are of the opinion that contact time of GSH with the sperm cells is important. The contact time in their study was 30, 60, and 90 minutes. They observed that 30 minutes was an insufficient contact time to produce a significant effect on the motility pattern. Ninety minutes contact time of GSH produced significant effects on sperm motility. They observed that the motility percentage was better with 5 mM GSH concentrations. They suggested that addition of GSH to the thawing extender could be of significant benefit in improving the function and freezing capacity of frozen bore spermatozoa.

Uysal *et al.,* (2007) used nine different additives and observed that additives had cryoprotective influence on improving post-thawed sperm motility, sperm morphology, acrosomal and membrane integrity and sperm viability. They found that the highest post-

thawing sperm motility and membrane integrity were obtained with 5 mM concentrations of GSH. They also observed that Taurine at 50 mM concentration showed positive effect in protecting sperm morphology, Cysteine at 5 mM had significant effect on viability. They indicated that different additives with different concentrations had significantly positive effect on different sperm features.

Whitaker *et al.,* (2008) supplemented 5 mM glutathione to the media during semen thawing. They examined supplementation of 5 mM glutathione had significant effect on forward progressive motility, viability or DNA fragmentation at 0.5 hour post-thawing compared to control. Forward progressive motility was significantly less at 6.0 hour after thawing in the 5 mM GSH supplemented group. DNA fragmentation was significantly higher at 6.0 hour after thawing in the 5 mM GSH supplemented group compared to the control.

Nili Ravi buffalo bull, studied here, is one of the most important breed in dairy industry as well as for semen production. In this study sperm characteristics of Nili Ravi buffalo bull were evaluated in fresh and cryopreserved semen, i.e. total sperm motility, motility grading, membrane integrity, glutathione levels, acrosomal activity and DNA integrity. The same characteristics were investigated in the presence of additives glutathione (antioxidant), hydrogen peroxide (oxidant) and combinations of these additives, i.e. glutathione plus hydrogen peroxide in different concentrations. Egg yolk tris glycerol (EYTG) extender was used as cryopreservation media.

In this study GSH as well as H_2O_2 with different concentrations were used to see their influence on different parameters of semen in fresh and cryopreserved condition. In addition, these two additives, in combination, with different concentrations were also supplemented to semen before cryopreservation. The intention was to find out their influence in combined form on parameters of semen. In present study cryopreservation significantly reduced ($P<0.0001$) total sperm motility in Nili Ravi buffalo bull. This reduction is comparable with frozen-thawed sperm total motility in human (Donnelly *et al.,* 2001), in boar (Gadea *et al.,* 2005), in holstein bull (Uysal *et al.,* 2007, Chatterjee *et al.,* 2001). Initial sperm motility of Swedish red and white dairy bulls was 58.0±7.2% and after thawing it was 49.9±7.3% (Januskauska *et al.,* 2000).

This study showed that cryopreserved semen significantly reduced the total sperm motility compared to fresh semen. Addition of 1 mM GSH showed increase in mean motility compared to supplementation of 5 mM GSH additive but this was not significantly different from the latter additive. It was observed that supplementation of 1 mM GSH and 5 mM GSH to freezing extender significantly reduced mean percentage of total sperm motility ($P<0.01$; $P<0.0001$ respectively) than in cryopreserved semen without supplements. Both these additives maintain motility more than 50 percent. The decrease in motility may be attributed to deficiency of the glutathione redox-cycle in the extender (Bilodeau *et al.,* 2001) as sperm thiol oxidation is important in acquisition of sperm motility (Seligman *et al.,* 2005). Chattergee *et al.,* (2001) observed freezing/thawing caused a decrease in the percentage of sperm motility. Addition of GSSG but not of GSH to EYTG before freezing partially prevented the loss of motility. And measured the changes in sulfhydryl groups by fluorescence, electrophoresis and spin labeling that could be the reflection of various degrees of damages due to cooling and freezing/thawing. The exposure of sulfhydryl groups could lead to the formation of loosely packed membrane structures more prone to further physical damages (Calvin *et al.,* 1973; Seligman *et al.,* 1992).

Changes in redox status of spermatozoa that could interfere with signal transduction mechanisms, which control sperm functions. Inhibitors of phosphoproteins phosphatases (calyculin A and okadaic acid) increased velocity and capacitation in human spermatozoa (Leclerc et al., 1996).

In this study supplementation of 100 µM H_2O_2 helped in increasing motility equivalent to cryopreserved (control) 71% while higher concentration (200 µM H_2O_2) to freezing media reduced total motility less than 50%. low and controlled concentrations of the ROS play an important role in sperm physiology. Reactive oxygen species, such as the superoxide anion, hydrogen peroxide and nitric oxide, induce sperm hyperactivation, capacitation or the acrosome reaction in vitro. The ROS involved in these processes may vary depending on experimental conditions (Lamirande *et al.,* 1997). Griveau *et al.,* (1994) reported a low concentration of H_2O_2 (50 µmol l-1) accelerates the development of sperm hyperactivation (by 37%) and capacitation (by 43%) after incubation for 3 hours. The concentration of H_2O_2 (50 µmol l^{-1}) needed to induce sperm capacitation

(Griveau *et al.,* 1994). Freshly ejaculated sperm cannot fertilize until they have spent some time in a suitable environment in order to capacitate. Capacitated sperm acquire the ability to exhibit hyperactivated motility and to undergo a physiological acrosome reaction (Yanagimachi, 1994). Capacitation has been assessed by the chlortetracycline (CTC) staining pattern, by the ability of the sperm to acrosome-react spontaneously or after stimulation with lysophosphatidyl choline, A23187 or progesterone, by the zona-free hamster oocyte test, by the prevalence of hyperactivation and by IVF (Ford, 2004). Capacitation is associated with a number of biochemical events, most notably an increase in protein tyrosine phosphorylation (Visconti and Kopf, 1998; Visconti *et al.,* 1998; Baldi *et al.,* 2000, 2002; Guraya, 2000; Breitbart, 2003). Adding ROS increased capacitation whereas removing them decreased it whatever methods were used. Some of the effects of ROS could be mimicked by other oxidizing agents, notably thiol oxidants (Ford, 2004).

The lowest mean percentage motility was observed with the supplementation of additives 5 mM GSH + 100 µM H_2O_2 and 1 mM GSH + 100 µM H_2O_2. The remaining combinations (1 mM GSH + 200 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2) also showed highly significant reduction in mean motility compared to cryopreserved condition. Motility was maintained by 100 µM H_2O_2 additive, but this additive with different concentrations did not improve sperm motility in Nili Ravi buffalo bull. Unlike Gadea *et al.,* (2005); Uysal and Bucak (2007).

Bilodeau *et al.,* (2000) strongly suggested that freeze-thaw cycle generates the oxidative stress and during this freezing and thawing process reactive oxygen species (ROS) are produced (Chatterjee and Gangnon, 2001) that resulted in detrimental effect on sperm function, for example, sperm viability, motility, membrane integrity, permeability, fluidity, calcium fluxes (Hammerstedt *et al.,* 1990; Bailey and Buhr, 1994; Zhao and Buhr, 1995). ROS alters cytoskeleton and effects on sperm axoneme that lead to loss of sperm motility, consequently fertility potential is reduced (de Lamirande and Gangnon, 2001). Involvement of antioxidant defense system in the maintenance of frozen thawed sperm quality against reactive oxygen species elimination and their changes during the semen cryopreservation process has been investigated in different species such as, in ram (Marti *et al.,* 2008; Bucak and Tekin, 2007), bovine (Uysal *et al.,* 2007; Bilodeau *et*

al., 2001, Chatterjee *et al.,* 2001), boar (Gadea *et al.,* 2005; 2003) and human (Donnelly *et al.,* 2000).

As far as progressive motility is concerned (gradation 4/4) there was no appreciable difference in fresh and cryopreserved semen. An improved and better progressive motility was observed with 1 mM GSH (66.52±2.15%). Donnelly *et al.,* (2001) showed that cryopreservation has reduced sperm progressive motility in fertile human from 44% to 21% and in infertile human from 33% to 12 %. In human freeze-thawed semen progressively motile sperm decreased significantly ($P<0.0001$) (Connell *et al.,* 2002). Gadea *et al.,* (2005) also observed decrease in boar sperm forward progressive motility (FPM) in fresh and after freezing-thawing in freezing media (lactose egg yolk) respectively.

All the groups i.e. cryopreserved semen with and with out supplementations including fresh semen showed no significant difference in 4/4 grade except 5 mM GSH. Sperm 4/4 grade are those which were highly motile and move rapidly in a straight line. Because of this characteristic of these type of sperms can be expected that they will be highly motile compared to other sperm-grades. This is what has been observed in this study.

Supplementations of 1 mM GSH, 100 μM H_2O_2, 1 mM GSH + 200 μM H_2O_2 increased sperm progressive motility more than 60% (66.52±2.15%; 63.49±2.63%; 69.14±4.57%) respectively which was not significant increase. While, supplementation of 5 mM GSH significantly ($P<0.0001$) reduced sperm progressive motility than that with 1 mM GSH. Gadea *et al.,* (2005) concluded that addition of GSH to freezing media improved boar sperm motility and motion parameters of thawed spermatozoa. Boar sperm FPM was increased significantly ($P<0.05$) with the supplementation of 1 mM GSH and 5 mM GSH respectively.

Sperm membrane integrity

Sperm membrane integrity is of importance for metabolism, sperm capacitation, the acrosome reaction and the binding of spermatozoa to the egg surface. Injury of sperm membrane may cause loss of normal sperm function such as motility, viability and fertilizing capacity. One property of the sperm membrane is its ability to permit the selective transport of molecules. In hypo osmotic condition water can flow across the

membrane resulting into an increase in sperm volume and swelling of plasma membrane (Song *et al.,* 1991). Mammalian spermatozoa undergo capacitation involving membrane alterations that occur in vivo during their transit in the female reproductive tract (Chang, 1951). Cryopreservation of semen damages spermatozoal plasma membrane (Park and Graham, 1992), which indicates swelling and breakage (Pace *et al.,* 1981), loss of permeability and changes in membrane fluidity (Canvin and Buhr, 1989). This also leads to leakage and aggregation of phospholipids and protein, reduction of motility, viability and enzyme activity (Gordon 1994; Parrish *et al.,* 1986,). After thawing, bovine spermatozoa demonstrate alteration in capacitation and acrosome activity (Cormier *et al.,* 1997; Fraser and McDermott, 1992; Florman and Babcock, 1991) showed that frozen-thawed spermatozoa capacitate faster than fresh spermatozoa in vitro. They concluded that fertilizing life–span of capacitated spermatozoa is limited that the fertilizing capacity of cryopreserved semen in vivo is poorer compared to fresh semen. Premature capacitation may reduce fertility. In this study cryopreserved semen (75.32 %) did not show much difference in mean percentage of intact plasma membrane compared to fresh semen (79.75%). The damage of sperm plasma membrane was not of the order as mentioned by different authors. It appears that the cryopreserved semen of Nili Ravi buffalo bull may not be behaving like that of male animals of other animal species that have been studied by other scientists. Although researchers cryopreserved sperms with different additives did show very high damage to sperm plasma membrane compared to cryopreserved semen, but in this study below 50% damage was seen with additive 1 mM GSH, 200 µM H_2O_2; 1 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; 5 mM GSH + 100 µM H_2O_2; 1 mM GSH + 200 µM H_2O_2; and the highest damage to plasma membrane was with the supplementation of 5 mM GSH + 100 µM H_2O_2 additive (25.18%). Miester and Tale (1976) indicated that thiol compounds such as cysteine protects sperm cells from toxic oxygen metabolites causing lipid peroxidation of sperm plasma membrane under in vitro condition. Fuvabasko and Sano (2005) reported that a semen extender with 5 mM cystein improve viability and membrane integrity of boar sperm cells during liquid storage. In this study glutathione additives 1 mM GSH (48.57%) and 5 mM GSH (52.39%) did not improve sperm membrane rather these two thiol additives damaged sperm membrane compared to cryopreserved semen.

Compared to cryopreserved semen significant damage to sperm membrane was done with the supplementation of hydrogen peroxide additives 100 µM H_2O_2 (36.52%) and 200 µM H_2O_2 (42.17%). Uysal and Bucak (2007) investigated that best post-thawed spermatological indicators were obtained from goat semen frozen with 5 mM cystein. It appears from different studies and from this study that maintenance of sperm membrane integrity varies from animal to animal and also from addititive to additive with different concentrations.

Acrosin activity index.

Clinical cause for male infertility in human may be due to absence or reduced activity of acrosin in spermatozoa, also in patients with unexplained infertility (Goodpasture *et al.,* 1982; Mohsenian *et al.,* 1982). Henkel *et al.,* (1995) investigated that this can be well defined halo around spermatozoa which indicates sufficient acrosin activity; with a poorly defined halo showing insufficient acrosin activity and with no halo formation indicating no acrosin activity. They have identified good fertilization whose fertilization rate is greater than 50% and poor fertilization if the rate of fertilization is less than 50%. They found that probability of successful fertilization decreased at halo formation rate < 60%. In their study incidence of low acrosin activity was related to 6 - 4 µm of halo diameter, 14 - 50 % halo formation rate and 7 – 3 acrosin activity index. They suggested that subfertility could be detected because of low acrosin activity.

Looking at the results from this study the highest halo formation rate and halo diameter was observed in fresh semen resulting in the highest acrosin activity index which indicates sign of good fertilization. Cryopreserved semen showed mirror image of fresh semen. There was lowest halo formation rate and halo diameter and very low acrosin activity index indicating poor fertilization rate. Addition of 5 mM GSH improves significantly halo formation rate, halo diameter and acrosin activity compared to cryopreserved semen. Some improvement in these parameters was also seen with the addition of 1 mM GSH showing the effect is dose dependent. Similar significant improvement compared to cryopreserved semen was also seen with the addition of 5 mM GSH +200 µM H_2O_2 to cryopreserved semen. The lowest acrosin activity index was 5.0 with supplementation of 200 µM H_2O_2 (5.0) suggesting poor fertilization.

Supplementation of remaining additives showed acrosin activity index which ranged from 6.1 to 6.4 which shows poor fertilization based on Henkle *et al.,* (1995) investigation. Zalata *et al.,* (2004) studied relationship between acrosin activity of human spermatozoa and oxidative stress. They concluded that presence of oxidative stress in an individual with leukocytospermia and / or abnormal semen parameters is associated with impaired sperm function measured by its acrosin activity. Oxidative stress is defined as increased rate of cellular damage induced by oxygen derived oxidants called reactive oxygen species (ROS). Oxidative stress occurs when there is an imbalance between the production of ROS and the scavenging ability of the antioxidants (Sharma and Agarwal, 1996; Garrido *et al.,* 2004 and Fujii *et al.,* 2003). This study also shows oxidative stress caused due to hydrogen peroxide additives in two concentrations 100 µM H_2O_2 (acrosin activity index 6.4) and 200 µM H_2O_2 (acrosin activity index 5.0) must have impaired sperm function as acrosin activity measured is very low. We do see that combination of an oxidant and antioxidant (5 mM GSH+200 µM H_2O_2) with higher concentration results in higher halo formation rate and halo diameter and gives improved acrosin activity index. It is suggested that presence of antioxidant (GSH) reduces the cellular damage and also lessens the imbalance between ROS production and scavenging ability of antioxidant as given by Sharma and Agarwal (1996) and Garrido *et al.,* (2004). Perhaps this way acrosin activity improved with this combination.

Glutathione Levels

GSH is a tripeptide in living cells which plays an important role in the intracellular protective mechanism against oxidative stress as it can react both with many ROS and as cofactor for glutathione peroxidase that catalyze the reduction of toxic H_2O_2 and lipoperoxide to alkyl alcohols (Bilodeau *et al.,* 2001). The resulting oxidized glutathione (GSSG) is reduced to glutathione by glutathione reductase using NADPH as the cofactor. The GSSG / GSH pair plays important role as redox sensor and as protective agents against ROS-induced damage, in many cell types (Halliwell and Gutteridge, 1998). GSH is present in both bull spermatozoa and seminal plasma at the mM and µM range respectively (Bilodeau *et al.,* 2000; Jain and Arora, 1988). According to Bilodeau *et al*., (2000) cryopreservation of bull spermatozoa in egg yolk tris-glycerol extender

reduced GSH level by 5 fold without increasing the GSSG level, which suggests that GSH leak out from the sperm cells. The addition of several thiols to the extender improves sperm motility (Bilodeau *et al.,* 2001).

In studying Nilli Ravi Buffalo bull, fresh semen and cryopreserved semen showed no difference in GSH levels rather in cryopreserved semen GSH levels (4.39 μM/10^6) were slightly higher than in the fresh semen (4.38 μM/10^6). cryopreservation of Nilli Ravi Buffalo bull semen with 1 mM GSH (4.22 μM/10^6) and 1 mM GSH + 100 μM H_2O_2 (4.11μM/10^6) additives did show significant reduction of GSH levels compared to cryopreserved semen but compared to other additives used here the reduction in GSH levels were significantly very low. The highest reduction of GSH levels was seen due to supplementation of 100 μM H_2O_2 with cryopreserved semen (3.03 μM /10^6). Stradaioli *et al.,* (2007) also observed that cryopreservation reduces the spermatozoal GSH content. They collected six semen samples from five bulls which were frozen and thawed in two extenders i.e. egg yolk tris citrate extender and commercial extenders (Bioxcell). They found that Bioxcell extender was superior in preserving GSH content than egg yolk tris citrate. They are of the view that high GSH level contained in the commercial extender was able to alleviate oxidative damage to spermatozoa surviving freezing thawing procedures. This study also shows that reduction in GSH levels vary from additive to additive as better results have been observed with additives 1 mM GSH and 1 mM GSH + 100 μM H_2O_2 than other additives. A higher concentration of glutathione additive, 5 mM GSH (3.87 μM /10^6) and that of hydrogen peroxide, 200 μM H_2O_2 (3.87μM /10^6) gave better results that GSH levels improved with these additives. In other additives supplemented to cryopreserved semen GSH levels ranged from 3.03 μM /10^6 to 3.73 μM /10^6. This has also been reported by Ochsendorf *et al.,* (1998) that sperm GSH levels vary between species, from 0.1nM/10^9 spermatozoa in rabbit to 90 nM/10^8 spermatozoa in mouse. The method used to assay and the bull species from which spermatozoa are isolated also influenced the values which makes comparison difficult (Jain and Arora, 1988). Different authors have calculated percentage GSH reduction due to freezing and thawing in different animal species. Decrease in spermatozoal glutathione levels upon cryopreservation has been reported 80% in bull (Bilodeau *et al.,* 2000), 63% in human (Molla *et al.,* 2004), 32% in boar (Gadea *et al*,

2004) and 58% in bovine spermatozoa (Stradaioli *et al.,* 2007). In this study percentage GSH reductuion ranges from 3.79% to 44.55%. According to Stradaioli *et al.,* (2007) the decrease in GSH level can be ascribed both to a leakage from cell due to cell membranes rupture and to an oxidative stress.

DNA Damage

In this study DNA damage in cryopreserved semen was significantly higher (40.1±1.55%) than in fresh semen (14.37± 0.65%). The best additive was 5 mM GSH + 200 µM H_2O_2 of whose presence showed the least DNA damage (10.88 ±0.2%) compared to fresh semen. Other better additive was 5 mM GSH which maintained intact DNA 83.43 ±0.74% which was significantly higher than in cryopreserved semen. Fraser *et al.,* (2006) indicated that in cryopreserved bull semen the sperm genome is well preserved. They also observed that percentage of head DNA of fresh semen (81.6%) was not significantly decreased after cryopreservation (80.7%) when they modified the protocol for comet assay. This study shows highly significant decrease in head DNA in cryopreserved semen (69.69±1.32%) compared to fresh semen (79.06 ±1.09%). However, supplementation of 1 mM GSH additive before cry preservation of semen protected head DNA percentage slightly higher (79.54 ±0.32%) than in fresh semen. The lowest head DNA was observed with the addition of 5 mM GSH+200 µM H_2O_2 additive (47.03 ±0.70%), This may be due to higher concentration of oxidant H_2O_2 for which evidence we get when in higher concentration oxidant 200 µM H_2O_2 (49.94 ±1.12) additive is supplemented to semen cryopreservation and this gives near lowest mean head DNA.

Yildiz *et al.,* (2007) have used variable combinations and concentrations of cryoprotectants on sperm assessment parameters of frozen-thawed mean sperm. They used raffinose alone (Raffinose 0.3M) and raffinose combined with fructose (Raffinose 0.3M+ Fructose 0.1 M) and glycerol (Raffinose 0.3 M+ Glycerol 0.1 M) and evaluated DNA fragmentation in different strains of mice. They observed compared to raffinose alone the combinations of raffinose with fructose and glycerol showed significant decrease in DNA fragmentation. In this study also different combinations of additives were used. Of which 1 mM GSH additive indicated the highest protection to head DNA.

Above 50% head DNA was protected with this supplementation of 5 mM GSH; 100 µM H_2O_2; 1 mM GSH+100 µM H_2O_2; 5 mM GSH+100 µM H_2O_2 and 5 mM GSH + 200 µM H_2O_2 additives. The beneficial effects and improved sperm-assessment parameters produced as a result of varying sugar types and cryoprotectant agents combination (i.e. glycerol) have also been reported for ram (Molinia *et al.,* 1994), bovine (Garcia and Graham, 1989), dog (Yildiz *et al.,*2000) and human sperm (Critser *et al.,*1988; McGonagle *et al.,* 2000).

In this study tail parameters of comet features like tail moment (µm), olive tail moment, tail DNA % and tail length (µm) were studied. Tail moment (µm) evaluation showed that there was highly significant DNA damage due to cryopreservation compared to that of fresh semen. The lowest DNA damage in tail moment feature was observed with the supplementation of 1 mM GSH additive before cryopreservation (1.886 µm) the other additive which protected DNA was with the addition of 5 mM GSH + 100 µM H_2O_2 additive before cryopreservation which protected DNA (2.918µm) better than in fresh diluted semen. Near equivalent to fresh semen DNA damage was due to the addition of 1 mM GSH + 100 µM H_2O_2 additive before cryopreservation (3.85 µm). Compared to cryopreserved semen DNA damage the other cryoprotectants like 5 mM GSH (4.49µm), 1 mM GSH + 100 µM H_2O_2 (4.64 µm), and 5 mM GSH + 200 µM H_2O_2 (4.31µm) showed better protection to DNA damage. The highest DNA damage was with the supplementation of 200 µM H_2O_2 (11.48 µm) additive which may be expected because this being oxidant in characteristic. In the case of olive tail moment as well cryopreserved semen showed highly significant DNA damage compared to fresh semen. Supplementation of 1 mM GSH (4.45 µm) before cryopreservation showed as the best protectant against DNA damage than in cryopreserved semen and other additives. The highest DNA damage was seen in these combinations where H_2O_2 was present with higher concentration, i.e. 1 mM GSH + 200 µM H_2O_2 (12.11 µm); 5 mM GSH + 200 µM H_2O_2 (11.82%); 100 µM H_2O_2 (11.96 µm) and 200 µM H_2O_2 (9.989µm). Surprisingly supplementation of 5 mM GSH (12.69 µm) showed the highest DNA damage (12.69 µm).

Tail DNA feature also showed significantly higher DNA damage in cryopreserved semen than in fresh semen. Addition of 1 mM GSH (20.46 µm) proved to be the best

cryoprotectant against DNA damage compared to cryopreserved semen and fresh semen. Here also DNA damage was the highest with the addition of those additives which had higher concentration of H_2O_2. This is seen in the case of the additives 1 mMGSH+200µMH_2O_2 (52.97 µm); 5 mM GSH+200 µM H_2O_2 (48.25 µm); and alone 200 µM H_2O_2 (50.09 µm).

In the case of comet tail length there was highly significant DNA damage was found in cryopreserved semen compared to fresh semen. Glutathione additives 1 mM GSH and 5 mM GSH well protected DNA damage (reduced comet tail length (8.0 µm)). Similarly, DNA was protected with the supplementation of 5 mM GSH+100 µM H_2O_2 (8.2 µm) and 5 mM GSH+200 µM H_2O_2 (7.8 µm) prior to cryopreservation of semen.

In this study all the four tail parameters showed significant DNA damage in cryopreserved semen. Different additives were used to see if they reduce the possibility of DNA damage when supplemented to semen before cryopreservation. Among these additives 1 mM GSH additive proved most favorable in the four tail parameters in protecting DNA better than in fresh semen too. Some additives of glutathione and hydrogen peroxide combinations were also useful (as mentioned above) giving protection to DNA against damage.

Lopes *et al.,* (1998) while investigating potential cause for DNA fragmentation in human spermatozoa observed that when samples were incubated in the presence of ROS, DNA damage was evident. They also observed that addition of antioxidant to samples significantly decreased the amount of DNA damage induced by ROS. They suggested that ROS can cause an increase in DNA fragmentation and pretreatment with antioxidant can reduce DNA damage. This is what has been observed in this study oxidants supplemented (alone or in combinations) before cryopreservation had significantly damaged DNA than with the supplementation of antioxidants (glutathione additive).

The information regarding DNA integrity reported includes general information about DNA fragmentation in sperm under cryopreserved condition. In this study the effect of different additives (supplemented alone or in combination) on sperm DNA under cryopreserved condition indicated that supplementation of 1 mM GSH was beneficial in protecting DNA from fragmentation.

CONCLUSION

In conclusion, this investigation showed that addition of additives 1 mM GSH and 5 mM GSH did not show improvement in sperm motility rather rate of motility and sperm membrane integrity reduced significantly compared to that due to cryopreservation. The effect of these two on the rate of motility is not significantly different from each other. Additive 5 mM GSH protected sperm membrane integrity in a better way than with 1 mM GSH concentration. Better motility of 4/4 grade sperm motility was observed with 1 mM GSH than with 5 mM GSH concentration. The reason for this is not clear. Similarly, halo formation rate with 5 mM concentration was very high (80.50%) than with 1 mM GSH concentration. There was no appreciable difference in halo diameter and acrosine activity index with 1 mM GSH and 5 mM GSH concentrations. DNA integrity with 5 mM GSH increased compared to that with 1 mM GSH. Mechanisms have been discussed in this discussion. While regarding comet features supplementation of 1 mM GSH additive showed highest percentage of comet head DNA; tail DNA damage was the least compared to fresh semen, cryopreserved semen and other additives used here, tail moment DNA damage and olive tail moment DNA damage was the least with the addition of 1 mM GSH additive when compared with the supplementation of other additives to semen before cryopreservation, fresh semen and cryopreserved semen. However, tail length DNA damage was slightly higher than with additive 5 mM GSH + 200 μM H_2O_2.

These variations among different cryoprotectants (alone or in combination) were also observed by Uysal and Bucak (2007) that particular concentrations of antioxidants during semen preservations may exert beneficial effects on the quality of the freezing/thawing of ram semen. Similar situation may be in the case of Nili Ravi bull where different concentrations of different additives may have beneficial effects on the quality of preserved semen of Nili Ravi bull.

FUTURE SUGGESTIONS

- Various additives (antioxidants) have been investigated to date in terms of their effects on the sperm characteristics including motility during cryopreservation. These include GSH, GSSH, cysteine, taurine, hypotaurin, trehalose, hyaluronan, etc. The effects of oxidants and antioxidants on lipid peroxidation are also well known. The effect of H_2O_2 as an additive with these antioxidants needs more careful investigations and the mechanism of its action can be elaborated by using a combination of above mentioned antioxidants along with various concentrations of H_2O_2.
- The concentrations of the additives used here can also be varied over a wide range to get better understanding for future studies.
- Some other more advanced characterization techniques such as immuno-localization of antioxidant/protein by indirect immune-fluorescence can be employed to get a better insight into the detailed mechanism of action of these additives on Nili Ravi Buffalo sperms motility.
- Similar studies can be applied to other mammalian species.

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