

CRYOPRESERVATION OF BISON SEMEN

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By

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ABSTRACT

The cryopreservation of bison semen is not as successful as for dairy and beef bull semen. The overall objective of this research was to develop a cryopreservation procedure to improve the post-thaw survival rate of bison sperm. For this purpose two different studies were conducted. The first study consisted of two experiments. In Experiment 1, damage to bison sperm motility characteristics at different stages of cryopreservation was quantified. In Experiment 2, the effects of extenders and freeze rates on post-thaw motility and structural characteristics were investigated. In Experiment 1, semen was diluted in Triladyl extender and frozen with a freeze rate $-10^{\circ}\text{C}/\text{min}$. Sperm motility characteristics, i.e., total motility, progressive motility, curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) were recorded in fresh, diluted, chilled (4°C) and frozen-thawed semen using computer-assisted sperm analysis (CASA). There was a significant decline ($P < 0.001$) in total and progressive sperm motilities (35% and 42%, respectively) after the freeze-thaw stage compared to pre-freeze stages. All sperm velocities declined ($P < 0.05$) approximately 32% at dilution, and in post-thaw semen, VCL was reduced more ($P < 0.05$) than VAP and VSL. In Experiment 2, semen was diluted in Triladyl or TCA extender and frozen at three different freeze rates: -10 , -25 and $-40^{\circ}\text{C}/\text{min}$. Post-thaw sperm motility characteristics were assessed using CASA, and sperm structural characteristics, i.e., intact plasma membrane (IPM), mitochondrial membrane potential ($\Delta\Psi\text{M}$) and intact acrosomes (IACR) were evaluated using flow cytometry, at 0 and 3 h post-thaw after incubating at 37°C . Triladyl yielded better ($P < 0.05$) post-thaw sperm total and progressive motilities (41% and 34%, respectively) than TCA (36% and 29%, respectively) at 0 h. However, post-thaw percent of decline in sperm motilities and structural

characteristics after 3 h of incubation at 37°C, was less ($P < 0.05$) in TCA than in Triladyl extender. At post-thaw 0 h freeze rate did not affect any sperm characteristics, while a freeze rate of -40°C/min revealed less ($P < 0.05$) percent decline in total and progressive motilities (38% and 45%), than other freeze rates (average 50% and 60%, respectively), after 3 h of post-thaw incubation.

In the second study, three experiments were conducted to determine the effects of zwitterion extender and temperature of glycerol addition (Experiment 1), the addition of reduced glutathione (GSH; Experiment 2) and cholesterol-loaded cyclodextrin (CLC; Experiment 3) on post-thaw quality of bison sperm. In Experiment 1, bison semen was diluted in Triladyl (control) or zwitterion-based (ZI) extenders, i.e. HepesT (Hepes-Tris) and TesT (Tes-Tris). In addition, glycerol in ZI extenders was added either at 37 or 4°C, before cryopreservation. Extenders had no significant effect ($P > 0.05$) on immediate (0 h) post-thaw total and progressive sperm motilities. However, sperm velocities (VCL, VAP, VSL) were better ($P < 0.05$) in ZI extenders than Triladyl. After 3 h of post-thaw incubation at 37°C, sperm motilities were the best ($P < 0.05$) in semen diluted in Triladyl. The percentage of sperm with IPM and IPM-IACR were higher ($P < 0.05$) in Triladyl compared to ZI extenders, while IPM- $\Delta\Psi$ M did not differ due to extenders, at 0 and 3 h post-thaw incubation. Temperature of glycerol addition did not affect sperm motility, or structural characteristics, but sperm VCL and IPM-High $\Delta\Psi$ M at 0 h were higher ($P < 0.05$), when glycerol was added at 4°C than at 37°C. The interaction between ZI extender and temperature of glycerol addition showed significant effect on VCL only. At 0 h, VCL was better ($P < 0.05$) in TesT extender with glycerol addition at 4°C than 37°C and than HepesT with glycerol addition at either 37 or 4°C ($P < 0.05$). Generally, Triladyl was superior than the ZI extenders and addition of glycerol in semen extender at 4°C was not significantly

effective than at 37°C. In Experiment 2, GSH was used as an antioxidant to protect bison sperm against the possible production of reactive oxygen species (ROS) to improve their post-thaw quality. Semen was diluted at 37°C in Triladyl extender containing GSH (0, 0.5, 1.0, 2.0 mM/50 x 10⁶ sperm). Post-thaw analyses at 0 and 3 h did not reveal any significant effect of GSH treatment on sperm motility or structural characteristics ($P > 0.05$). In Experiment 3 fresh bison sperm (100 x 10⁶/ml in Tris-citric acid [TCA] buffer) were pre-treated with different concentrations of CLC (0, 1, 2 or 3 mg) and then diluted in TCA extender for cryopreservation. The CLC-treated sperm showed better ($P < 0.05$) post-thaw (0 h) total and progressive motilities, IPM and IPM-IACR, and also less ($P < 0.05$) percent of decline in these characteristics after 3 h of post-thaw incubation at 37°C compared to control (0 mg CLC) samples.

The overall results of the study showed that freeze-thaw is the most damaging stage to bison sperm. Post-thaw bison sperm quality at 0 h was better in Triladyl than TCA and zwitterion extenders; however, after 3 h sperm survival was better ($P < 0.05$) in TCA extender and in semen frozen at a freeze rate of -40°C/min. The addition of reduced glutathione (GSH) in extender did not improve post-thaw bison sperm quality. The pre-freeze treatment of bison semen with cholesterol-loaded cyclodextrin (CLC) improved ($P < 0.05$) post-thaw bison sperm quality at 0 and 3 h. In conclusion, Triladyl or TCA extender and higher freeze rate -40°C/min can be used successfully for cryopreservation of bison semen, and cholesterol-loaded cyclodextrin should be included in semen cryopreservation procedure for better post-thaw quality of bison sperm.

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LIST OF ABBREVIATIONS

AI:	Artificial insemination
ART:	Assisted reproductive technologies
ATP:	Adenosine triphosphate
BSA:	Bovine serum albumin
CASA:	Computer-assisted sperm analysis
CLC:	Cholesterol-loaded cyclodextrin
cm:	Centimeter
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
FITC-PNA:	Fluorescence isothiocyanate-peanut agglutinin
FSC:	Forward scatter
g:	Gram
GSH:	reduced Glutathione
h:	Hour
$H\Delta\Psi M$:	High mitochondrial membrane potential
IACR:	Intact acrosomes
IPM:	Intact plasma membrane
LN ₂ :	Liquid nitrogen
mg:	Milligram
min:	Minute
ml:	Millilitre
mM:	Millimolar

mOsm:	Milliosmole
MtDR:	Mitotracker deep red
n:	Number
N ₂ :	Nitrogen
PBS:	Phosphate buffered saline
PI:	Propidium iodide
ROS:	Reactive oxygen species
SSC:	Side scatter
TCA:	Tris-citric acid
μm:	Micrometer
μM:	Micromolar
μl:	Microliter
VAP:	Velocity average path
VCL:	Velocity curvilinear
VSL:	Velocity straight-line

CHAPTER 1:

GENERAL INTRODUCCION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

North American bison (*Bison bison*) belong to the family bovidae and has two sub-species; plains bison (*Bison bison bison*) and wood bison (*Bison bison athabascae*) [Prusak et al., 2004]. By the end of 19th century, the bison population declined from millions to a few hundred mainly due to unrestricted commercial hunting [Gates and Aune, 2010]. The Committee On the Status of Endangered Wildlife in Canada (COSEWIC) declared wood bison as “threatened” in the early 1960s [Gates and Aune, 2010]. Due to its threatened status there is a dire need for the conservation of bison genetics. Successful conservation strategies in Canada and United States developed farmers’ interest in bison farming which led to the formation of the commercial bison industry in North America. Furthermore, the bison meat industry is exploring the ways to develop markets in North America and rest of the world.

Being a member of the family bovidae, the cryopreservation procedures available for dairy and beef bull semen are also used for bison semen. However, when compared to dairy and beef semen, cryopreservation of electroejaculated bison semen is not very successful and is characterized by poor post-thaw motility [Lessard et al., 2009] and short longevity [Dorn, 1995]. Due to the unavailability of frozen bison semen, reproductive technologies available for bovines cannot be utilized for the development of bison. So, it is very important to improve bison semen cryopreservation techniques and to bank semen from superior bison bulls to use for the development of the bison industry.

Cryopreservation of cattle semen has already shown great success in previous decades [reviewed by Thurston et al., 2002]. Difficulty freezing bison semen may be attributed to the lack of a bison-specific semen cryopreservation technique and the lack of bison bull selection, for their semen freezing ability. The freeze-thaw stage of cryopreservation is universally accepted as the most damaging stage in almost all species [Watson, 1995]. However, sperm from various mammalian species are sensitive to other stages of cryopreservation as well, e.g., ram sperm to dilution [Purdy, 2006] and boar sperm to initial cooling [Johnson et al., 2000].

For successful semen cryopreservation, the composition of extender and optimal freeze rate are important to minimize extra- and intra-cellular stresses [Hammerstedt et al., 1990; Curry et al., 1994]. A freezing media with good buffering capacity against pH shift is required to sustain the biological activities of sperm and protect them against chemical, dilution, osmotic and thermal stresses during cryopreservation [Rasul et al., 2001]. Tris buffer provides a better buffering system for cryopreservation of bull sperm [Davis et al., 1963]. However, zwitterion buffers such as Tes, Hepes, Pipes and Mes (titrated against Tris) with pKa value close to biological pH 7.0) are more suitable than Tris alone [Good et al., 1966]. Addition of glycerol as a cryoprotectant in freezing media provides protection to cells; possibly due to its salt buffering ability when cells are cooled to subzero temperatures [Lovelock et. al., 1953]. As a cryoprotectant, glycerol may be added in semen as a part of the extender or after cooling [Evans and Maxwell, 1887].

Freeze rate is a crucial factor in semen cryopreservation. The optimum freeze rate required for cryopreservation must be slow enough to minimize the intra-cellular ice formation yet fast enough to reduce cell dehydration and 'solution effect' [Mazur, 1970]. Various freeze rates have been recommended for semen cryopreservation of different species, i.e., -15 to -

60°C/min for ram semen [Byrne et al., 2000; Anel et al., 2003], -30 to -60°C/min for boar semen [Fiser and Fairfull, 1990] and -50 to -100°C/min for bull semen [Woelders et al., 1997].

Physical and chemical stresses on the sperm plasma membrane lead to the excessive production of reactive oxygen species (ROS) [Chatterjee et al., 2001]. Reactive oxygen species are molecules, sometimes known as free radicals, with at least one unpaired electron. They are therefore unstable and highly reactive, often causing cellular damage. Sperm are more vulnerable to ROS due to the polyunsaturated lipid nature of their plasma membranes and lack of cytoplasmic antioxidants, leading to lipid peroxidation and oxidative stress [Sinha et al., 1996]. Free radicals (e.g., O_2^- , H_2O_2) can cause damage to sperm structures by oxidizing lipid membranes, amino acids and nucleic acids [Ochsendort, 1999]. As an antioxidant, glutathione has an important role in intracellular defense mechanism against oxidative stress in the living cells [Irvine, 1996]. The use of thiols, e.g., cystein hydrochloride or glutathione (GSH) as an antioxidant in semen extender effectively reduces losses in post-thaw sperm motility [Bilodeau et al., 2001].

Rapid cooling of sperm from 30 to 4°C in the domestic animals leads to injury known as 'cold shock' [Gilmore et al., 1998; Watson, 2000]. Sperm possessing a high cholesterol to phospholipid ratio in their plasma membranes such as rabbit and human sperm are more resistant to cold shock damage than sperm from boars, stallions, rams and bulls with a low cholesterol:phospholipid ratio [Darin-Bennett and White, 1977; Watson, 1981; Parks and Lynch, 1992; White, 1993]. Cholesterol is an integral part of the sperm plasma membrane and strengthens its structures at temperatures even below the phase transition, by resisting changes in the structural composition of phospholipids hydrocarbon chains [Quinn, 1989]. Cyclodextrins are oligosaccharides with the ability to make soluble inclusion complexes with cholesterol and can

deliver cholesterol into the cell membranes [Cai et al., 2006]. Pre-freeze treatment of bull sperm with cyclodextrin, loaded with cholesterol, results in better post-thaw sperm quality [Purdy and Graham, 2004].

The overall goal of this study was to develop a successful bison semen cryopreservation protocol. The frozen semen can be used for the conservation of North American bison genetics, and for assisted reproductive technologies such as artificial insemination and *in vitro* embryo production and transfer. Cryopreserved semen will also fulfill the needs of a developing North American bison industry for the exploitation of superior bison genetics. In this study we identified the stage(s) causing damage to bison sperm during cryopreservation, and investigated the effects of different factors such as extender, freeze rate, zwitterion buffer, temperatures of glycerol addition, reduced glutathione and cholesterol-loaded cyclodextrin to improve the existing cryopreservation technique for bison semen.

1.2 BISON

1.2.1 Historical perspective and conservation

Bison roamed the grasslands and parklands of central United States and Canada about 5000 years ago [McDonald, 1981]. As the largest grazing land animal in North America, bison populations extended from central Mexico to northern Canada and almost from the east to the west coasts [McDonald, 1981]. Wood bison mainly ranged from the western forests of Canada to eastern Alaska, while plains bison generally inhabited the Great Plains of the United States and Canada to Northern Mexico [Sanderson et al., 2008; Figure 1.1]. Before the arrival of European

settlers in North America in the 19th century, there were about 50 million bison, commonly known as wild buffalo, in the North American continent [Nowak, 1999].

A continuous decline in the bison population of North America started in the 1820s, particularly in southern and eastern parts of their range, due to natural and human factors [Garretson, 1938; Flores, 1991; Isenberg, 2000]. One of the reasons for the decline in bison population was the introduction of non-native animals including horses, cattle and sheep. Horses and repeating rifles were used by the aboriginal people to hunt bison, and competition with sheep and goats for grazing and water sources contributed to the decline in the bison population [Isenberg, 2000].

Fire, predation and severe weather events also served to limit the historical bison population size [Isenberg, 2000]. An extremely harsh and long drought from 1845 to 1860 in southern plains caused widespread losses in the bison herds [Pekka, 2008]. Advancement in the use of firearms expedited hunting and transcontinental rail transportation facilitated the bison trade, leading to a rapid decline in bison by the end of 1800 [Garretson, 1938; Coder, 1975]. In 1820, the sale of hides and other bison products in European commercial markets played a crucial role in the near extinction of bison [Isenberg, 2000]. The number of American bison (both plains and wood bison) declined to less than 1,000 by the end of 19th century [Soper, 1941; Coder, 1975].

Conservation of bison was initiated through the Territories Game Protection Act of 1894 which banned the hunting of wood bison, and the Northwest Game Act of the 1906 which banned the hunting of both wood and plains bison [Environmental Assessment Panel, 1990].



Figure 1.1: Original distribution of plains bison (*Bison bison bison*) and wood bison (*Bison bison athabascae*) in North America based on available zooarchaeological, paleontological, oral and written historical accounts [http://en.wikipedia.org/wiki/American_bison].

The recovery of plains bison in the United States from near extinction was made possible through 4-5 privately owned herds which served as the foundation of present plains bison in America [COSEWIC, 2004]. Meanwhile, from 1840 to 1900, the wood bison population in Canada was also reduced to near extinction from their natural habitat [Raup, 1933]. In 1891, about 300 wood bison were left between Great Slave Lake and the Peace-Athabasca Delta [Ogilvie, 1893] and this number was further reduced to about 250 from 1896 to 1900 [Soper, 1941]. Due to implementation of these acts the wood bison population started to increase slowly

and by 1914 it increased from 250 to about 500 individuals [Banfield and Novakowski, 1960]. In 1922, Wood Buffalo National Park (WBNP) was established under the Forest Reserves and Parks Act to save wood bison from extinction by protecting their habitat [Soper, 1941; Lothian, 1979].

During the 1920's about 6,600 surplus plains bison from Wainright Buffalo Park (WBP) were sent to WBNP [Carbyn et al., 1993, Fuller, 2002, Brower, 2008]. Unfortunately, this resulted in interbreeding of wood and plains bison [Wilson and Strobeck, 1999] and the spread of tuberculosis and brucellosis from plains bison to the native wood bison [Carbyn et al., 1993, Fuller, 2002]. Tuberculosis and brucellosis infections in the North American bison were introduced from the cattle brought by the European settlers, and tuberculosis was identified for the first time in plains bison in Alberta in the 1920's (Banfield, 1974). Bison herds in and around WBNP remain infected with these diseases up the present [Tessaro et al., 1989]. To generate more animals, wood bison were transferred from Wood Buffalo National Park to the Mackenzie Bison Sanctuary and Elk Island National Park (EINP) in 1963 and 1965, respectively [Wilson and Strobeck, 1999]. In Canada, by 1970 there were about 30,000 plains bison, with about half of them located in national parks, wildlife refuges and provincial wildlife areas and on private farms [Shaw and Meagher, 2000]. In Canada, National Parks have played significant role in rescuing the plains bison from extinction [Ogilvie, 1979]. Due to their remoteness from human population centers, wood bison did not get as much attention directed towards conservation efforts as compared to plains bison [Gates et al., 2001].

The recovery of bison from the threshold of extinction in the last quarter of the 19th century is a great conservation success story. At present, there are more than 500,000 bison in North America indicating the secure recovery of this species. However, among these existing

populations, only 5% are maintained as conservation herds by public institutions and private conservation groups, while 95% are maintained by the private sector [Boyd, 2003]. In 2008, the IUCN (International Union for the Conservation of Nature) Red List of Threatened Species declared free ranging North American bison as a “near threatened species” [Gates and Aune, 2010]. At present, Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has specifically declared wood bison in the category of a “threatened species in Canada”.

1.2.2 The bison industry in North America

There were nearly 500,000 bison in North America in 2002. Approximately 169,000 were owned by private farmers in Canada [Nixdorf, 2003], 175, 000 were owned privately in the United States in 2000 [Albrecht, 2000] which in 2002 grew to a number of approximately 231,950 on private farms in the United States [USDA, 2002]. In North America, the bison industry has emerged as a specialized livestock industry and the bison population is expected to grow. According to the Statistic Canada (2006), there was an increase in bison numbers from 145,000 to 195,800 and a small increase in bison farms from 1,887 to 1,898, since 2001. When the number of bison exceeded market demand, the value of bison products decreased. This forced bison producers to improve their farm management and to reduce production costs. Efficient reproductive management practices could have improved the economic efficiency of bison production, but a lack of knowledge about bison reproductive physiology has made this task more difficult.

During the last decade, the bison industry explored ways to develop the bison meat market in the North America and the rest of the world. The number of bison slaughtered in Canada is on the rise, i.e. 11,159 bison were slaughtered in 2001 which reached ~30,000 head in

2005 (AAFC, 2005). The industry has emphasized the unique characteristics of bison meat as a potential competitive advantage over other meat sources. Bison meat is lower in fat and cholesterol than other meats from other species (Table 1.1) and this unique quality appeals to health conscious consumers [Conacher, 2000]. The sale of bison by-products (skull, hide and wool) can further generate farm revenue.

Table 1.1

Nutritional comparison of bison meat with other meats (3 oz serving size). From Armstrong et al (1998).

Meat	Calories	Fat	Cholesterol
Bison	93	1.8 g	43 mg
Turkey	125	3.0 g	59 mg
Beef	183	8.7 g	55 mg
Chicken	140	3.0 g	77 mg

In 2012, the bison industry in North American is very active and growing rapidly. Earlier, the bison industry was mostly based on the sale of breeding stock; however, now there is more emphasis on developing meat markets. Although the bison industry has made good progress within a short period, various issues, for example creating awareness and identifying consumers' demand for value-added food trends, quality control from the point of production till distribution and competitive response to the needs of consumers, integrated research for better production, still need to be resolved in this industry. Reproductive breeding technologies (i.e., artificial insemination, *in vitro* fertilization and embryo transfer) developed for dairy and beef cattle can

be used to improve the quality of bison herds. Such improved farming practices are needed to promote the developing bison industry. Successful development of the bison commercial industry will also assist the conservation of the wild bison.

1.2.3 Reproductive biology of male bison

A comprehensive knowledge is required about the reproductive biology of bison. Very few studies are available on reproductive aspects of male bison. The average age for puberty in bison bull is 16.5 months with an average weight of 353 kg; therefore a 2-year old bison bull should be able to pass breeding soundness evaluation [Helbig et al., 2007]. According to the criteria for beef bulls, the onset of puberty occurs when a bull produces ejaculates containing at least 50 million sperm with 10% of individual progressive motility [Wolf et al., 1965]. In an evaluation of breeding soundness of bison bulls between the ages of 18 to 24 month, 72% of the bulls were declared as satisfactory breeders based on given standards of Western Canadian Association of Bovine Practitioners [Haigh et al., 2001]. According to another study conducted on bison, a bison bull should have $\geq 30\%$ progressive motile sperm, $\geq 70\%$ sperm with normal morphology and scrotal circumference ≥ 29 cm, at 28-30 months of age [Keen et. al., 1999].

The dominance of a male bison in the herd is a significant indicator of his reproductive success. Free ranging (wild) male bison of 8-11 years of age are dominant and do not allow younger bulls to breed the females in the herd [Lott, 1971; Berger and Cunningham, 1994]. Male bison start to show active sexual behavior at 1-2 years of age but due to the social dominance of older males, younger bulls cannot fully participate sexually during the rut [Rothstein and Griswold, 1991]. However, if given a chance, a 2 year old bison bull will mount and copulate with estrus females [McHugh, 1958; Berger and Cunningham, 1994]. It has been observed that

bison bulls living under natural conditions can breed successfully in their third breeding season, at the age of about 26 months [McHugh, 1958; Berger and Cunningham, 1994]. It appears from previous studies that the seasonal trend observed in male bison is largely due to seasonal behavior of females, as male bison are physiologically able to produce semen throughout the year [Helbig et al., 2007].

Seasonal reproductive behavior in free ranging bison has been documented in various studies in North America and seasonality in bison reproduction was recorded earlier by the European settlers on the western frontier [Hornaday, 1889]. Bison breed during late summer and females give birth to calves in the spring [Hornaday, 1889; Berger and Cunningham, 1994]. With the exception of Helbig et al (2007), to date no comprehensive scientific literature is available on the seasonal variation of the reproductive ability of male bison. Most of the available information on male bison reproduction is related to social associations [McHugh, 1958] and reproductive behavior [Lott, 1981; Berger and Cunningham, 1994]. A study conducted on free range bison in Yellowstone National Park from 1951 to 1953 revealed that most of the breeding activity takes place from mid June to the end of September [McHugh, 1958] and it has been shown that fecal testosterone in bison bulls was highest in July and August [Mooring et al., 2004]. Bison exhibit more distinct reproductive seasonality than cattle, but electroejaculated and epididymal bison semen collected in different seasons did not show distinct change in sperm quality characteristics [Helbig et al., 2007]. Minimal variation has also been reported in sperm quality of mature dairy bulls which produced higher sperm concentration, volume and total number of normal sperm during the months of April to June [Everett et al., 1978; Barth and Waldner, 2002].

1.3 SEMEN CRYOPRESERVATION

1.3.1 Cryopreservation

Cryopreservation is a process in which cells or tissues are subjected to below 0°C temperatures and preserved in liquid N₂ at –196°C for long term storage. The lowering from body temperature to 4°C reduces cellular metabolic activity and increases the life span of sperm [Barbas and Mascarenhas, 2009]. Cryopreservation effectively stops the biological activity of cells, without affecting their ability to restart normal function on thawing [Mazur, 1984]. Cryopreservation methods include temperature reduction, cellular dehydration, freezing and thawing [Medeiros et al., 2002].

1.3.2 Principles of semen cryopreservation

Sperm like other cells face mechanical, osmotic, chemical, and thermal stresses during the dilution, cooling and freeze-thaw stages of cryopreservation [Rasul et al., 2001]. These stresses are mainly due to the solution effect, the chemical nature of cryoprotectants in the extender, and extra- and intra-cellular ice crystallization [Watson, 2000; Bailey et al., 2003]. Consequently, there is considerable loss of sperm structure and function during different stages of cryopreservation [Garner et al., 1999; Bailey et al., 2003]. The sperm plasma membrane is commonly damaged during the freezing and thawing stages of cryopreservation [Parks and Graham, 1992]. The composition of extender, use of suitable cryoprotectants, and optimal cooling/freezing and thawing rates are important for successful cryopreservation [Hammerstedt et al., 1990; Curry et al., 1994]. Sperm are highly metabolic and extenders used for sperm cryopreservation in domestic animals require optimum pH, good buffering capacity and the capability to protect against the cooling and freeze-thaw stresses of cryopreservation [Salamon

and Maxwell, 2000]. Before freezing, sperm are cooled down from body temperature to 4°C. At certain temperatures during cooling, membrane structural integrity is compromised due to transformation of the membrane from fluid to gel state, known as phase transition [Watson, 2000; Medeiros et al., 2002]. The critical temperature during which phase transition affects plasma membrane integrity ranges from 15 to 5°C [Watson, 2000]. Membrane injury that occurs during rapid cooling of sperm from 30 to 4°C is often referred to as cold shock [Gilmore et al., 1998; Watson, 2000].

Freezing of semen is a crucial phase of cryopreservation. It induces ultra-structural, biochemical, and functional changes to the sperm affecting its ability to transport itself and survive in the female reproductive system, consequently reducing fertility [Salamon and Maxwell, 2000]. Spontaneous ice nucleation usually occurs after a solution is super cooled to a temperature between -5 and -15°C [Woelders, 1997]. As freezing continues to approximately -50°C, osmotic equilibrium is established and the viscosity of the unfrozen fraction becomes very high, causing a solution effect and preventing further crystallization [Holt, 2000]. During freezing, two main factors are injurious for the sperm; a 'solution effect' and 'intracellular ice crystallization' [Marshall, 1984].

A suitable freezing rate is important to minimize damage to sperm from ice formation and solution effect during semen freezing. A slow freezing rate below sub-zero temperature will excessively dehydrate the cells to accommodate the higher extracellular salt concentration resulting from extra-cellular ice formation. This will lead to osmotic shock (solution effect). At a fast freezing rate cells do not find enough time to dehydrate sufficiently, leading to harmful intracellular ice formation called ice crystallization [Mazur et al., 1972]. Below 0°C, the freezing rate should be slow enough to dehydrate the cells and prevent injuries due to intracellular ice

formation, but fast enough to avoid severe cell dehydration and prevent damage to macromolecules and membrane due to a solution effect [Gao et al., 1997].

To minimize freezing stresses, cryoprotectants such as glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol can be used in semen cryopreservation media. These cryoprotectants are able to rearrange the membrane lipid and protein components, increasing membrane fluidity, increasing dehydration at lower temperatures and reducing intracellular ice crystallization for better cell cryosurvival [Holt, 2000]. Since its discovery by Polge et al [1949], glycerol has become the cryoprotectant of choice to spare sperm from ice crystallization injuries during cryopreservation. However, due to its permeating nature, it also exerts some osmotic and toxic stresses on sperm [Purdy, 2006]. The harmful effects of glycerol may be attributed to osmotic stress, changes in membrane structural organization, fluidity, and permeability [Watson, 1995]. However, the protective effects of glycerol are greater than the harmful effects [Garner et al., 1999]. Several experiments have been conducted to find the optimum concentration of glycerol in extender and the best results were obtained with 4-6% glycerol [Byrne et al., 2000; Anel et al., 2003]. Glycerolated semen extenders can be added at the time of initial dilution at body or room temperatures (one-step), or after cooling semen to 5°C as a separate fraction (two-step) [Evans and Maxwell, 1987]. Post-thaw sperm quality in boar improved when glycerol was added at low temperatures [Pursel and Johnson, 1975; Pursel and Parks, 1985].

Like freezing, thawing of semen is also of critical importance as sperm once again traverse through the critical temperature zone from -15 to -50°C [Fisher et al., 1987]. In the case of slow thawing, intra-cellular smaller ice crystals having higher surface energies will be converted to larger crystals [Mazur, 1966], and are harmful to the sperm [Watson, 1995]. Fast thawing not only prevents the re-crystallization of any intracellular ice, but also reduces the

exposure time of concentrated solutes and cryoprotectant to sperm by restoring rapid equilibrium across the sperm plasma membrane [Fisher et al., 1987].

1.3.3 Cryopreservation factors affecting post-thaw quality of mammalian sperm

Generally, it is accepted that approximately 50% of sperm do not survive the process of cryopreservation [Watson, 1995]. Sperm cryosurvival is dependent upon its adaptability to osmotic and thermal stresses while undergoing dilution, cooling, freezing and thawing procedures, the biochemical composition of semen, and species-specific sperm susceptibility to cryoinjury [Holt, 2000].

For successful semen cryopreservation an ideal semen extender should have an optimum pH and osmolarity, good buffering capacity, and cryoprotection ability against thermal shock [Salamon and Maxwell, 2000; Rasul et al., 2001]. Immediately after collection, mammalian semen is usually diluted in an extender containing egg-yolk [Sansone et al., 2000]. Low density lipoproteins (LDLs) in egg yolk are believed to be responsible for sperm protection during cryopreservation [Pace and Graham, 1974; Watson, 1976]. The choice of a suitable buffering system is also an important factor for better sperm cryosurvival [Rasul et al., 2000]. Zwitterion buffers, also known as Good's buffers, resist change in pH of the media even at low temperatures [Good et al., 1966]. Tris, a zwitterion buffer, is a commonly used buffer for the cryopreservation of bull [Davis et al., 1963], goat [Drobnis et al., 1980], boar [Crabo et al., 1972] and buffalo [Rasul et al., 2000] sperm, due to its known buffering capacity.

After dilution in an appropriate extender sperm are subjected to the cooling process. Slow cooling from body temperature to 4°C helps sperm adapt to a reduced metabolic state while rapid cooling leads to cold shock, impairing the functional integrity of membrane proteins [Watson,

2000]. There is reduction in fructose breakdown, oxygen uptake and synthesis of ATP which blocks the energy production and loss of motility [Wales and White, 1959]. On the other hand rapid cooling from 30°C to 4°C causes damage to some proportion of the sperm depending on cooling rate, temperature interval and temperature range [Watson, 1981]. Below 15°C, rapid cooling makes mammalian sperm more vulnerable to hypothermic damage and slow cooling minimizes the detrimental effects on sperm [Parks, 1997].

The rate of semen freezing is important for post-thaw sperm survival. The sperm plasma membrane is the primary structure affected during cryopreservation [Steponkus et al., 1983] and the damage is related to freeze-thaw cycles affecting mainly the plasma and mitochondrial membranes [Blesbois, 2007]. Freezing semen at a faster rate causes irreversible sperm damage due to intracellular ice crystallization reducing their post-thaw sperm survival [Acker and McGann, 2003], while slow freezing leads to solution effect injury or osmotic shock, affecting post-thaw sperm quality [Critser et al., 2002]. The inclusion of cryoprotectant in the extender is essential for cell survival during cryopreservation. Glycerol as a permeable cryoprotectant benefits the sperm for better post-thaw quality by reducing freezing stress [Lovelock, 1953], chelating metallic ions [Lohmann, 1964], dehydrating the cells [Bredderman and Foot, 1969] and preventing freeze fractures of solutions on water solidification [Gao et al., 1995]. Furthermore, glycerol acts directly on the sperm plasma membrane, possibly to reduce the phase transition, and to increase membrane fluidity during cooling [Noiles et al., 1995].

1.3.4 Problems associated with cryopreservation of semen

Semen cryopreservation was made possible more than half a century ago upon the discovery of egg-yolk being protective during cooling [Phillips and Lardy, 1940] and glycerol during freezing [Polge and Rowson, 1952] for the cryopreservation of fowl and bull sperm. Since then research on cryopreservation has introduced and developed several technical modifications of cryopreservation protocols for different species and to improve the existing process [Johnson et al., 2000; Salamon and Maxwell, 2000]. Although several developments have been made, post-thaw quality and fertility of cryopreserved sperm are reduced due to damage during cryopreservation [Medeiros et al., 2002]. Sperm have variable responses to freezing and thawing depending on individual males and species [Holt, 2000; Thurston et al., 2002].

In most mammalian species a substantial number of sperm lose their fertilizing ability at different stages of cryopreservation. For instance, porcine sperm are sensitive to cold temperatures and the fertility of post-thaw pig semen is remarkably low for commercial use [Hofmo and Grevle, 1999]. Similarly, intrauterine insemination of frozen thawed sperm in sheep is not successful and satisfactory conception rates are only achieved when semen is directly deposited into the oviduct using laparoscopy, indicating that cryopreserved ram semen is not able to transit the female reproductive tract efficiently [Salamon and Maxwell, 1995].

Although bovine semen is considered cryoresistant, it still suffers major damage during freezing, and about ten times more frozen-thawed sperm are required than fresh bull sperm to achieve equivalent *in vivo* fertility rates [Shannon and Vishwanath, 1995]. During cryopreservation a significant proportion of sperm which look normal otherwise in terms of motility and morphology, may have suffered sub-lethal damages compromising their fertilizing

potential [Watson, 2000]. A decrease in sperm with IPM-High $\Delta\Psi_M$ has been observed in bull semen shipped overnight (longer equilibration) with a rapid decline in post-thaw longevity, possibly due to sub-lethal damage to sperm mitochondria [Anzar et al., 2011].

1.3.5 Sperm characteristics and cryopreservation

Semen cryopreservation facilitates the expansion of reproductive techniques such as artificial insemination and *in vitro* fertilization [Medeiros et al., 2002]. Sperm are the end product of male spermatogenesis with specific metabolic and anatomical features, having little biosynthetic activity and depend greatly on catabolic function to sustain their life [Barbas and Mascarenhas, 2009]. Sperm receives nutrients from their surrounding environment to maintain the functional integrity of their membranes [Yanagimachi, 1994]. The highly regulated processes in the testes, during which germ cells are transformed into sperm through a series of mitotic divisions, meiosis and spermatid metamorphosis is called spermatogenesis [Senger, 2003]. The metamorphosis of round spermatids into sperm is called spermiogenesis; after which the sperm is released in the seminiferous tubules [Garner and Hafez, 2000; Senger, 2003]. Sperm are covered by a diverse plasma membrane [Garner and Hafez, 2000] made up of a phospholipid bilayer with intercalated proteins and cholesterol [Singer and Nicolson, 1972]. The changes occurring in the sperm plasma membrane in the male reproductive tract prior to ejaculation, and then in female reproductive system, are prerequisite for ovum fertilization [Yanagimachi, 1994].

A mature sperm contains a large nucleus which contains haploid DNA with highly condensed chromosomes. Sperm cells are almost devoid of cytoplasm. Absence of enough endoplasmic reticulum and highly condensed DNA render sperm without transcription ability to make or replace proteins. In the sperm head, the acrosome is a specialized structure involved in

the fertilization process, while mitochondria are present at the anterior region of the sperm flagellum [Eddy and O' Brien, 1994]. Mitochondria are responsible for producing energy in the form of ATP which is mostly consumed to maintain sperm motility [Medeiros et al., 2002].

Generally, sperm motility is believed to be the most important parameter to evaluate semen quality, as *in vitro* fertility rates of oocytes have a definite correlation with sperm motility [Amman, 1989]. Sperm motility is not necessary for zona pellucida binding, but is considered necessary to penetrate through cervical mucous, the female genital tract, and ultimately the oocyte [Saling, 1982].

1.4 FLOW CYTOMETRY TO EVALUATE SPERM QUALITY

Flow cytometry can be used for semen analysis to determine the structural integrity of sperm plasma membrane [Garner et al., 1994], acrosomes [Nagy et al., 2003] and mitochondria [Garner et al., 1997]. The commercial availability of fluorescent dyes for staining different sperm structures allows a fast and accurate assessment of thousands of sperm individually [Garner and Johnson, 1995]. Simultaneous evaluation of different parameters in a sperm can provide a better estimate of its ability to fertilize an oocyte [Graham, 2001].

Intact plasma membrane, an indicator of sperm viability, is usually assessed after staining with membrane impermeable fluorescent dyes, for example yo-pro, propidium iodide, and cells which are capable of excluding these dyes are considered to be live cells with intact plasma membranes. Propidium iodide (PI) binds with the DNA in a cell. An intact plasma membrane will not allow PI to enter the cell. However, PI will permeate the cells possessing a damaged

plasma membrane and binds to the DNA causing the cells to fluoresce red when excited with a 488nm blue laser [Graham et al., 1990; Wilhelm et al., 1996].

Fluoresce isothiocyanate conjugated with peanut agglutinin (FITC-PNA) is commonly used to evaluate the acrosomal integrity [Dalimata and Graham, 1997]. FITC-PNA cannot penetrate the intact acrosomes [Silva and Gadella, 2006]. PNA specifically attaches to galactose moieties on the outer membrane of a damaged acrosome only [Flesch et al., 1998] and gives green fluorescence on excitation with 488 nm blue laser [Nagy et al., 2003].

Mitochondrial membrane potential or mitochondrial integrity can be assessed by using different fluorescent dyes such as Rhodamine 123, JC-1, and mitotracker deep red (MtDR) . Rhodamine washes out if mitochondrial potential is lost and is also less sensitive, while JC-1 does not stain frozen-thawed sperm properly due to its interaction with the extender [reviewed by Hallap et al., 2005]. However, mitotracker deep red 633 dye can be used successfully to evaluate mitochondrial membrane potential. Mitotracker deep red is cell permeant and has the ability to pass through intact plasma and outer mitochondrial membranes to bind selectively with the inner membrane of active mitochondria [Gregory, 2002, Silva and Gadella, 2006]. Sperm having mitochondrial membrane potential will emit red fluorescence on excitation with red diode laser of 633 nm [Anzar et al., 2011].

1.5 BISON SEMEN CRYOPRESERVATION

1.5.1 Importance of bison semen cryopreservation

Since bison are threatened species bison semen cryopreservation is vital for conservation programs through artificial insemination and *in vitro* embryo production. Cryopreserved semen from healthy bison will reduce the transmission of sexually transmissible diseases. Successful bison semen cryopreservation and its banking from genetically superior bulls will enhance the genetic potential for selection of desired traits. Bison is an animal species unique to North America and semen cryopreservation is important to maintain its genetic diversity. Frozen semen provides insurance for the availability of bison genetics against habitat deterioration, climatic changes, diseases, feeding practices and unforeseen catastrophic events. The optimization of bison-specific semen cryopreservation procedures will not only protect the genetics, but will also contribute to the development of reproductive technologies like AI and *in vitro* embryo production and transfer. The standardization of bison semen cryopreservation and other related reproductive technologies will help to meet the potential demands of emerging bison meat and related industry. The exchange and sale of germplasm from superior bison bulls nationally and internationally will be made possible from the use of cryopreserved semen. The conservation of historically and culturally important North American bison may also serve as a future food security in the North American region.

1.5.2 Current status of bison semen cryopreservation

Unlike their use in various other wild animals, the procedures for reproductive technologies (artificial insemination, embryo transfer) used in domestic cattle can be applied to bison. Bison are wild animals and pose a real danger for the handler as well as for their

companions [Dorn, 1995]. Successful electroejaculated semen collection from bison is possible after sufficient training through a chute system specifically designed for bison [Lessard, et al., 2009]. The extenders and cryoprotectants used for bovines may not be effective for bison semen freezing, as in electroejaculated bison semen there is a greater than 50 % decrease in post-thaw sperm motility [Lessard, et al., 2009] and longevity is very short [Dorn, 1995]. The recovery and cryopreservation of epididymal bison sperm has been reported to be successful with 60% post-thaw progressive motility and normal sperm morphology [Aurini et. al., 2009]. Moreover, the egg-yolk based extender Triladyl is better than non-animal origin based Andromed extender for the cryopreservation of electroejaculated bison semen [Lessard, et al., 2009].

CHAPTER 2:

GENERAL HYPOTHESES AND OBJECTIVES

Hypothesis 1: Maximum damage to bison sperm occurs during freeze-thaw stage than during dilution and initial cooling stages of cryopreservation.

Objective 1: To quantify the damage to bison sperm motility characteristics at fresh, dilution, cooling and freeze-thaw stages of semen cryopreservation by using CASA.

Hypothesis 2: Custom-made Tris-citric acid (TCA) extender can also be used successfully as commercial Triladyl extender, for the cryopreservation of bison semen.

Objective 2: To evaluate the effect of extenders (Triladyl and TCA) on post-thaw quality of bison sperm.

Hypothesis 3: Bison semen cryopreservation at higher freeze rate yields better post-thaw sperm survival than lower freeze rate by reducing solution effect.

Objective 3: To study the effects of freeze rates (-10 , -25 , and $-40^{\circ}\text{C}/\text{min}$) on post-thaw quality of bison sperm.

Hypothesis 4: Zwitterion-based extenders provide better buffering system to bison sperm during cryopreservation procedure and improve post-thaw semen quality.

Objective 4: To investigate the effects of zwitterion-based extenders on cryosurvival of bison sperm during cryopreservation.

Hypothesis 5: Addition of glycerol after cooling semen to 4°C temperature reduces toxic effects on bison sperm and improves post-thaw semen quality.

Objective 5: To assess the effect of glycerol addition in semen at 4°C on post-thaw quality of bison sperm.

Hypothesis 6: Addition of reduced glutathione (GSH) as antioxidant in freezing extender improves cryopreservation of bison semen.

Objective 6: To determine the effect of reduced glutathione (GSH) on post-thaw quality of bison sperm.

Hypothesis 7: Pre-freeze treatment of bison sperm with cholesterol-loaded in cyclodextrin (CLC) improves post-thaw survival rates of bison sperm.

Objective 7: To determine the effect of pre-freeze treatment of bison sperm with cholesterol-loaded cyclodextrin (CLC) on their post-thaw quality.

CHAPTER 3:

QUANTIFICATION OF DAMAGE TO BISON SPERM DURING DIFFERENT STAGES OF CRYOPRESERVATION AND THE EFFECTS OF EXTENDERS AND FREEZE RATES ON POST-THAW BISON SPERM QUALITY

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3.1 ABSTRACT

Semen cryopreservation is an important technique for the banking of animal germplasm from endangered species and exploitation of genetically superior sires through artificial insemination. Despite being a member of the family Bovidae, the freezing ability of semen from bison is poor compared to semen from cattle. This study was designed to quantify the damage to bison sperm at different stages of cryopreservation, and to determine the effects of extender (commercial Triladyl vs. custom made Tris-citric acid [TCA]) and freeze rate (-10 , -25 and $-40^{\circ}\text{C}/\text{min}$) on post-thaw quality of bison semen. Semen was collected from five bison bulls (three wood and two plains) via electroejaculation. In Experiment 1, semen was diluted in Triladyl extender and frozen at a freeze rate of $-10^{\circ}\text{C}/\text{min}$. Sperm motility characteristics were recorded in fresh, diluted, cooled (4°C) and frozen-thawed semen using computer-assisted sperm analysis (CASA). In Experiment 2, semen was diluted in Triladyl or TCA extender, and frozen at three different freeze rates: i.e., -10 , -25 or $-40^{\circ}\text{C}/\text{min}$. Thawing was performed at 37°C for 60 sec. Post-thaw sperm motility characteristics were assessed using CASA, and sperm structural characteristics (plasma membrane, mitochondrial membrane potential and acrosomes) were evaluated at 0 and 3 h after thawing by flow cytometer. In Experiment 1, total and progressive sperm motility did not differ among pre-freeze stages of cryopreservation ($P > 0.05$); however, total and progressive motility declined ($P < 0.001$) in frozen-thawed semen by 35% and 42%, respectively, compared to post-cooled (pre-freeze) semen. In Experiment 2, Triladyl, compared to TCA, yielded greater ($P < 0.05$) post-thaw total sperm motility (41% compared to 36%) and progressive motility (34% compared to 29%) at 0 h, respectively. The percent of decline in post-thaw total and progressive sperm motilities, VCL, VAP, VSL, IPM-High $\Delta\Psi\text{M}$ and IPM-IACR during 3 h incubation at 37°C was less ($P < 0.05$) in TCA than in Triladyl. There was an effect

of freeze rate on post-thaw sperm VAP at 0 h, and total motility, progressive motility, VCL, IPM and IPM-IACR at 3 h were the greatest ($P < 0.05$) when bison semen was frozen at $-40^{\circ}\text{C}/\text{min}$. Likewise, the percent of decline in post-thaw total and progressive sperm motilities, during 3 h of incubation at 37°C , was less ($P < 0.05$) in bison semen frozen at $-40^{\circ}\text{C}/\text{min}$. All post-thaw bison sperm characteristics decreased ($P < 0.05$) from 0 h to 3 h, during incubation at 37°C . The maximum damage to bison sperm occurred during freeze–thaw stage compared to pre-freeze stages of cryopreservation process. Post-thaw total and progressive motilities of bison sperm were greater in Triladyl at 0 h whereas sperm survival was greater in TCA extender after 3 h of post-thaw incubation. Bison sperm had greater survival ($P < 0.05$) when frozen at $-40^{\circ}\text{C}/\text{min}$ freeze rate.

3.2 INTRODUCTION

Bison bison (North American bison) with two existing sub-species *Bison bison bison* (plains bison) and *Bison bison athabascae* (wood bison) belongs to the family Bovidae, genus bison. According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, the bison population in North America declined drastically from millions to a few hundred by the end of 19th Century due to extensive and unrestricted hunting. The Committee on Endangered Species of Wildlife in Canada (COSEWIC) declared the wood bison as ‘threatened’ in the early 1960s [Gates and Aune, 2010]. Therefore, a recovery plan for wood bison was initiated in the 1960’s by the Canadian government [Gates et al., 2001]. Moreover, the occurrence of brucellosis and tuberculosis in wood bison [Robison et al., 1998; Nishi et al., 2006] posed a further threat to Canadian bison and human health. The ideal approach to conserve

the genetic resources of an endangered or threatened species should be *in situ* conservation, but this is a slow and expensive approach. *In vitro* collection and cryopreservation of animal genetic material (semen, oocytes and embryos) and the use of assisted reproductive techniques (ART) offer advantages in the field of conservation biology.

Cryopreservation is a technique used for long-term conservation of genetic material (germplasm) of endangered species [Holt, 1997; Andrabi and Maxwell, 2007]. Semen, due to its abundance, is most commonly used for cryopreservation of genetics. Bull semen can be collected by artificial vagina or electroejaculation. Among these, semen collection with an artificial vagina is a popular method used for bulls located at breeding stations whereas the bulls used for natural mating in field conditions are collected via electroejaculation. Semen collection via electroejaculation yields a greater volume, but lesser sperm concentration, compared to the artificial vagina method [Almquist and Cunningham, 1967; Foster et al., 1970; León et al., 1991]. Unfortunately, there is no comparative study on the effect of extenders and freeze rates on the cryopreservation of semen collected via artificial vagina vs. electroejaculation. Being a member of the family Bovidae, it was anticipated that bison semen can be frozen following cryopreservation procedures available for dairy and beef bull semen. However, cryopreservation of electroejaculated bison semen is not as successful as with dairy and beef bull semen and is characterized by short sperm longevity [Dorn, 1995], and poor total and progressive motilities [Pérez-Garnelo et al., 2006; Lessard et al., 2009]. The freeze-thaw stage of semen cryopreservation is universally accepted as the most damaging stage to the sperm of mammalian species [Watson, 1995]. However, sperm from different species are sensitive to the other stages of cryopreservation process as well: i.e., ram sperm to dilution [Purdy, 2006] and boar sperm to initial cooling [Johnson et al., 2000]. Since sperm experience chemical, osmotic and thermal

shocks during cryopreservation [Rasul et al., 2001] it is important to quantify the damage to bison sperm after dilution, cooling, and freeze-thaw stages of cryopreservation.

For successful semen cryopreservation, the composition of an extender and optimal freeze rate play important roles in minimizing extra- and intra-cellular stresses [Hammerstedt et al., 1990; Curry et al., 1994]. An ideal semen extender should have optimum pH, buffering capacity, suitable osmotic pressure and ability to protect sperm against cold shock [Salamon and Maxwell, 2000]. A few commercial extenders like Triladyl, BioXcell and Andromed have been tried for the cryopreservation of bison epididymal and electroejaculated sperm [Aurini et al., 2009; Lessard et al., 2009; Kozdrowski et al., 2011] with varying success. However, the exact composition of commercial extenders is not known. Moreover, in a typical breeding station, non-commercial (custom-made) extenders are being used for cryopreservation of dairy and beef bull semen. In view of the need to conserve threatened bison genetics and the likelihood of the expanding use of AI for bison in the near future, it is important to determine the efficacy of commercial and custom-made extenders for cryopreservation of electroejaculated bison sperm.

Freeze rate is also a crucial step in semen cryopreservation. Rapid cooling of semen from 30 to 4°C leads to injuries known as “cold shock” [Gilmore et al., 1998; Watson, 2000]. In the 1970, Mazur described the effect of freeze rate on biological systems as: if the freeze rate is slower than optimum, cells keep on dehydrating due to extracellular ice formation between –5 and –10°C. Slower freeze rates lead to an increase in intracellular salt concentration called “solution effect” which denatures the macromolecules and irreversible membrane collapse due to extreme cell shrinkage. If the freeze rate is faster than optimum, there is not enough time for intracellular water to leave the cells and it turns into ice crystals upon freezing which cause irreparable damage to sperm. An optimum freeze rate must be slow enough to allow water to

leave the cells to prevent intracellular ice formation, and fast enough to avoid severe cell dehydration and the solution effect. Different freeze rates have been recommended for cryopreservation of mammalian semen: i.e., -15 to $-60^{\circ}\text{C}/\text{min}$ for ram semen [Byrne et al., 2000; Anel et al., 2003], -30 to $-60^{\circ}\text{C}/\text{min}$ for boar semen [Fiser and Fairfull, 1990], and -50 to $-100^{\circ}\text{C}/\text{min}$ for bull semen [Woelders et al., 1997]. There is no study reported in the literature on the comparison of different freeze rates for cryopreservation of electroejaculated bison semen.

The objectives of present study were to quantify the damage to bison sperm at different stages of cryopreservation (fresh, diluted, cooled and freeze-thawed), and to determine the effects of extenders and freeze rates on post-thaw motility and structural characteristics of bison sperm.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of extenders

A commercially available Triladyl (Minitube Canada, Ingersoll, Ontario) and custom-made Tris-citric acid (TCA; pH 7.2, osmotic pressure $\sim 300\text{mOsm}$) extenders were used. TCA extender containing 2.4% w/v Tris (Sigma, St. Louis, MO; Cat. T1378), 1.4% w/v citric acid monohydrate (Fisher Chemicals, Fairlawn, NJ; Cat. A104), 1.0% w/v fructose (Sigma; Cat. F3510), 7% v/v glycerol (Sigma; Cat G7893), 20% v/v egg yolk, 500 $\mu\text{g}/\text{ml}$ gentamycin (PCCA Houston, TX; Cat. 30-1610), 100 $\mu\text{l}/\text{ml}$ Tylan (Elanco, Guelph, ON) and 300/600 $\mu\text{g}/\text{ml}$ linco-spectin (Pfizer Canada Inc., Kirkland, QC), was prepared in our laboratory. TCA extender was centrifuged at $12,000\times g$ for 15 min at 4°C , supernatant was stored at -20°C and thawed at 37°C before use.

3.3.2 Semen collection and initial evaluation

Five bison bulls (three wood and two plains), 3-6 years of age and weighing between 450-850 Kg, stationed at the Native Hoofstock Centre, University of Saskatchewan, Saskatoon, Saskatchewan (52° 10'N/106° 38'W) were used following Animal Use Protocol # 20090125, approved by the University of Saskatchewan Animal Care Committee,. Initially, bison bulls were allowed time to become familiar with the corrals and the handling system at the centre. Bulls were then trained to move through a hydraulic chute system specifically designed for bison (Berlinic Manufacturing, Quill Lake, SK, Canada). Bulls were gradually adapted to restraint using the hydraulic chute handling system for the semen collection process by electroejaculation (EE). Semen was collected from each bison bull twice weekly during August and September (breeding season) with an electroejaculator (Pulsator IV Auto Adjust™; Lane Manufacturing Inc. Denver, CO, USA). A rectal probe (length 13.5"; diameter 3") containing three electrodes, connected with electroejaculator was gently inserted into the bull's rectum and ejaculation was stimulated by repeated impulses of ~10.2V_{RMS} (root mean squared voltage), 2–3 sec each, for maximum 3 min. Semen was transported to the laboratory in a mobile incubator (37°C) within two hours where it was evaluated and processed within 30 min. Sperm motility characteristics and concentration of each ejaculate were assessed using CASA (Sperm Vision 3.5, Minitube Canada, Ingersoll, Ontario).

3.3.3 Semen processing

Based on initial evaluation, ejaculates possessing sperm motility > 60% and concentration >200 × 10⁶/ml were selected for further processing and diluted to 50 × 10⁶ sperm/ml with the appropriate extender described under each experiment, at 37 °C. The diluted

semen was placed in a 500 ml beaker containing warm water (37°C) and shifted to a cold room (4°C). After cooling to 4°C in 90 to 100 min, the semen was packaged in 0.5 ml French straws. Semen straws were frozen from +4 to –10°C at –3°C/min and from –10 to –80°C at –10, –25 or –40°C/min in a programmable cell freezer (ICE-CUBE 14-S, Sy-Lab Version 1.30, Gerate GmbH, Neupurkdersdorf, Austria) as described under each experiment. After freezing semen straws were plunged in liquid nitrogen (–196°C) and stored for at least 24 h before post-thaw sperm analysis. Three straws per treatment per bull were thawed at 37°C for 60 sec in a water bath, and semen was pooled by bull and treatment.

3.4 EXPERIMENTAL DESIGN

3.4.1 Experiment 1: Motility characteristics of bison sperm during the cryopreservation process

Twenty ejaculates were collected and 18 were qualified for further processing, as discussed in the semen processing section. Each ejaculate was diluted in Triladyl, cooled to 4°C and frozen from +4 to –10°C at –3°C/min and from –10 to –80°C at –10°C/min. After reaching –80°C, semen straws were plunged into liquid nitrogen. Semen was thawed as described in section 3.3.3. An aliquot of fresh, diluted, cooled to 4°C and frozen-thawed semen was removed, and sperm motility characteristics were evaluated with CASA, as described below in semen essays.

3.4.2 Experiment 2: Effects of extenders and freeze rates on post-thaw quality of bison sperm

Twenty two ejaculates were collected and 20 were qualified for further processing, as discussed in the semen processing section. Each ejaculate was diluted in Triladyl or TCA to a final volume of 15 ml in each extender; 5 ml for each freeze rate. The diluted semen was cooled to 4°C as mentioned under semen processing section. After reaching 4°C, semen was packaged in 0.5 ml French straws and held at 4°C until frozen. During freezing, all the semen samples underwent a similar freezing rate of -3°C/min, from 4°C to -10°C, however, from -10 to -80°C, semen straws were frozen using freeze rates cycles of -10, -25 or -40°C/min, in a programmable cell freezer. After reaching -80°C, semen straws were plunged in liquid nitrogen. Semen was thawed as described above in section 3.3.3. Post-thaw sperm motility characteristics were assessed using CASA, whereas sperm intact plasma membrane (IPM), sperm with IPM and high mitochondrial membrane potential (IPM-HΔΨM), and sperm with IPM and intact acrosomes (IPM-IACR) were analyzed with the flow cytometer as described by Anzar et al [2011] at 0 and 3 h, while incubating thawed semen in a water bath at 37°C.

3.5 SEMEN ASSAYS

3.5.1 Computer-assisted semen analysis (CASA)

Sperm motility characteristics and concentration were determined using CASA settings for bison sperm [Lessard et al., 2009]. For fresh semen, each sample was first diluted 1:20 in TCM-199 (37 °C) whereas diluted, cooled, and frozen-thawed semen was evaluated as such in the extender. An aliquot (2.5 µl) of each kind of semen sample was loaded in a pre-warmed (37

°C) chamber slide (Leja Netherlands; 20 µm depth). Approximately 200 sperm in at least five different fields were analyzed for total motility, progressive motility, average path velocity (VAP, µm/sec), curvilinear velocity (VCL, µm/sec) and straight-line velocity (VSL, µm/sec).

3.5.2 Flow cytometer analysis

A simultaneous assessment of bison sperm plasma membrane, mitochondrial membrane potential and normal acrosomes was conducted following the procedure, as previously described [Anzar et al., 2011]. After thawing, semen was diluted to 1×10^6 sperm/ml in PBS-0.5% BSA solution. The following fluorescent dyes were added per ml sperm suspension: 1 µl fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma chemicals, St. Louis, MO; stock 1mg/ml PBS), 2 µl mito-tracker deep red (MtDR; Invitrogen, Burlington, ON; stock 2 µM in DMSO) and 6.25 µl propidium iodide (PI; Invitrogen; stock 2.4 mM in water). Semen-dye mixture was incubated at room temperature (22°C). Sperm were fixed by adding 10 µl of 10% formaldehyde in each semen sample and evaluated for post-thaw sperm with intact plasma membrane (IPM), IPM and high mitochondrial membrane potential (High $\Delta\Psi$ M) and IPM and intact acrosomes (IACR).

At least 10,000 sperm of each semen sample were analyzed by flow cytometer (Partec Cyflow Space, version 2.4 by Partec GmbH, Münster, Germany) equipped with a 400 mW argon laser. FITC-PNA and PI were excited with 488 nm blue laser and their emission spectra were detected with photo-multiplier detectors at FL-1 and FL-3, respectively. MtDR was excited with 635 nm red diode laser and its emission was detected with photo-multiplier detector FL-6. The data were acquired by FloMax software (version 2.4) for cytometry provided by Partec GmbH. Earlier, the distribution of sperm on forward and side light scatters was identified with Hoechst

33342 stained cells (data not shown). All samples were passed through the flow cytometer with a speed of 1 μ l/sec.

Forward and side light scatters were used to identify the sperm and a region (R1) was gated around sperm-specific events (Figure 3.3, Panel A) for PI, MtDR and FITC-PNA fluorescence. Simultaneous fluorescence data of all three probes were recorded on log scales. Sperm stained with PI had compromised plasma membrane (RN1 region; Figure 3.3, Panel B) whereas the remaining unstained sperm were considered having IPM. Two dimensional MtDR /PI dot plot revealed four sperm subpopulations (Figure 3.3, Panel C): sperm with compromised plasma membrane and low $\Delta\Psi$ M (QA1), sperm with compromised plasma membrane and High $\Delta\Psi$ M (QA2), sperm with IPM and low $\Delta\Psi$ M (QA3), and sperm with IPM and High $\Delta\Psi$ M (QA4). Likewise, four sperm subpopulations were recorded on two dimensional FITC-PNA/PI dot plots (Figure 3.3, Panel D): sperm with compromised plasma membrane and IACR (QB1); sperm with compromised plasma membrane and compromised acrosomes (QB2); sperm with IPM and IACR (QB3); and sperm with IPM and compromised acrosomes (QB4). Compensations were conducted to minimize the overlapping of emission spectra of different fluorophores using FloMax software. For statistical analysis, the data belonging to only IPM, IPM-High $\Delta\Psi$ M (QA4), and IPM-IACR (QB3) were processed.

3.6 STATISTICAL ANALYSIS

The data collected in both experiments were expressed as mean \pm SEM. In Experiment 1, randomized complete block design was used to evaluate motility and velocity characteristics of bison sperm at different stages (fresh, after dilution, after cooling and after freeze-thaw) of

cryopreservation using bull as block due to their random effect. In Experiment 2, the percent of decline in all post-thaw sperm characteristics after 3 h of incubation was calculated as follows:

$$[(\text{Motility at 0 h} - \text{Motility at 3 h}) \div \text{Motility at 0 h}] \times 100$$

Post-thaw sperm motility, velocity and IPM, IPM-High $\Delta\Psi\text{M}$ and IPM-IACR at 0 and 3 h, and percent of decline after 3 h of incubation were analyzed with 2 (extenders) \times 3 (freeze rates) factorial analysis in randomized complete block design using bull as block. If the value of $P < 0.05$, means were separated with Tukey's test. Paired t-tests were used to compare post-thaw sperm characteristics at 0 h and 3 h incubation. All analyses were conducted using SAS MIXED procedure (Version 9.2, SAS Institute Inc. Cary, NC).

3.7 RESULTS

3.7.1 Experiment 1: Motility characteristics of bison sperm during the cryopreservation process

Data on bison sperm motility characteristics at different stages of cryopreservation are presented in Figure 3.1 and 3.2. Bison total and progressive sperm motilities did not change among pre-freeze stages of cryopreservation ($P > 0.05$). However, both total and progressive motilities decreased ($P < 0.0001$) after freeze-thaw by 35% and 42%, respectively (Figure 3.1). Bison sperm VAP and VSL decreased ($P < 0.001$) after dilution in extender by 29% and 34%, respectively and then remained unchanged ($P > 0.05$) after cooling and freeze-thaw stages. VCL of bison sperm first declined ($P < 0.001$) after dilution by 33% and then after freeze-thaw by 22% (Figure 3.2).

3.7.2 Experiment 2: Effects of extenders and freeze rates on post-thaw quality of bison sperm

Statistical analysis revealed a significant effect of extender on post-thaw sperm characteristics. At 0 h, total and progressive sperm motilities were greater ($P < 0.05$) in semen extended in Triladyl than in TCA extender, whereas, the remaining sperm velocities and structural characteristics remained unaffected. All sperm characteristics at 3 h post-thaw were improved ($P < 0.05$) in semen extended in TCA than Triladyl whereas IPM and IPM-IACR did not change due to extender. Likewise, the percent of decline in all sperm characteristics during 3 h of post-thaw incubation was less ($P < 0.05$) in TCA than in Triladyl except that IPM remained similar between the two extenders (Table 3.1 and 3.2).

Freeze rate showed significant effect on post-thaw sperm average path velocity at 0 h, and in total and progressive motilities, VCL, IPM and IPM-IACR at 3 h. These post-thaw sperm characteristics at 3 h differed due to freeze rate and were the greatest at $-40^{\circ}\text{C}/\text{min}$ and the lowest at $-10^{\circ}\text{C}/\text{min}$ freezing rate ($P < 0.05$) whereas the difference between $-25^{\circ}\text{C}/\text{min}$ and $-10^{\circ}\text{C}/\text{min}$ and $-40^{\circ}\text{C}/\text{min}$ was not significant (Table 3.1 and 3.2). A similar trend in freezing rate was observed in the percent of decline in total and progressive motilities during 3 h post-thaw incubation. With both extenders and all three freeze rates, there was a significant difference ($P < 0.05$) in post-thaw sperm motility and structural characteristics between 0 h and 3 h (Table 3.1 and 3.2).

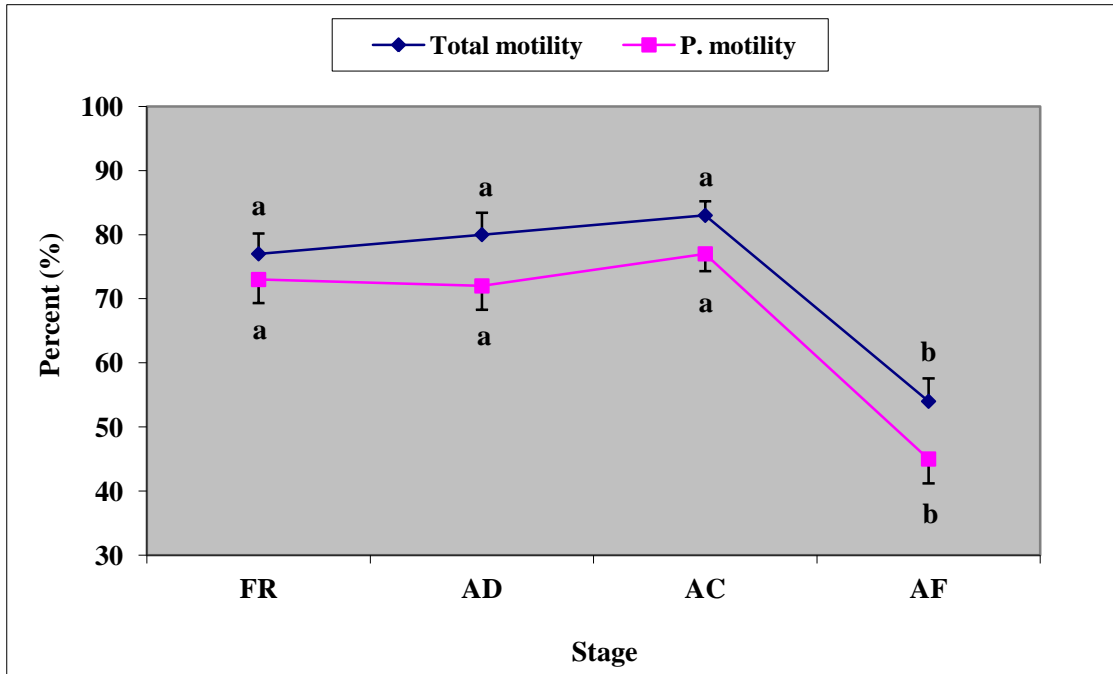


Figure 3.1: Bison sperm motilities at different stages of cryopreservation (Mean \pm SEM; N =18 ejaculates). Within motility, means with different letters (a, b) differed between the stages of cryopreservation ($P < 0.05$).

Abbreviations: P. motility, progressive motility; FR, fresh; AD, after dilution; AC, after cooling; AF, after freezing and thawing.

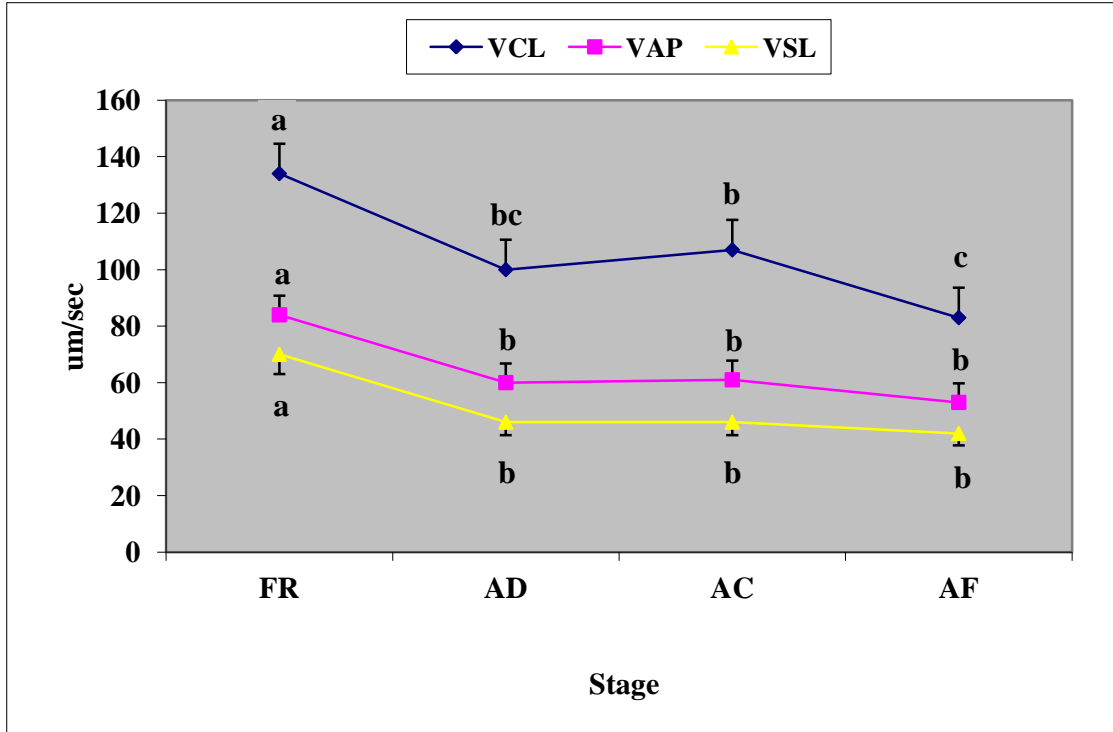


Figure 3.2: Bison sperm velocities at different stages of cryopreservation (Mean \pm SEM; N =18 ejaculates). Within velocity, means with different letters (a-c) differed between the stages of cryopreservation ($P < 0.05$).

Abbreviations: VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; FR, fresh; AD, after dilution; AC, after cooling; AF, after freezing and thawing.

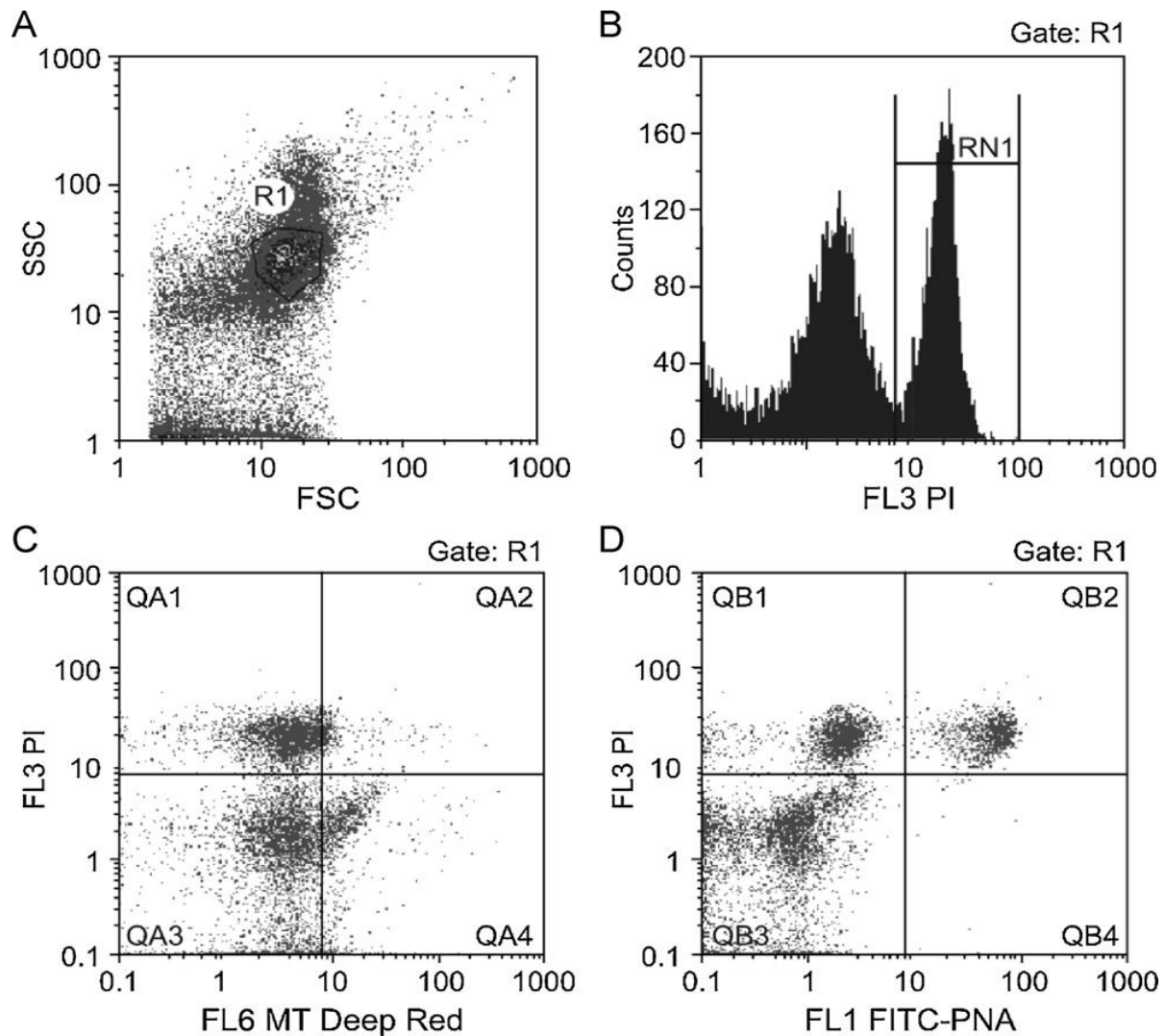


Figure 3.3: Flow cytometry of post-thaw bison bull sperm. Panel A. Forward compared to side scatters. Sperm-specific events were gated as R1. Panel B. PI fluorescence intensity (x-axis) and number of sperm-specific events (y-axis). Sperm with compromised plasma membrane are present in right peak (RN1, PI stained). The remaining subpopulation on the left side represents sperm with intact plasma membrane (PI unstained). Panel C. MtDR compared to PI: QA1 – sperm with compromised plasma membrane and less mitochondrial membrane potential (PI+/MtDR -); QA2 – sperm with compromised plasma membrane and high mitochondrial membrane potential (PI+/MtDR +); QA3 – sperm with intact plasma membrane and less mitochondrial membrane potential (PI-/MtDR -); and QA4 – sperm with intact plasma membrane and high mitochondrial membrane potential (PI-/MtDR +). Panel D. FITC-PNA compared to PI: QB1 – sperm with compromised plasma membrane and intact acrosomes (PI+/FITC-PNA-); QB2 – sperm with compromised plasma membrane and compromised acrosomes (PI+/FITC-PNA+); QB3 – sperm with intact plasma membrane and intact acrosomes (PI-/FITC-PNA-); and QB4 – sperm with intact plasma membrane and compromised acrosomes (PI-/FITC-PNA+)

Table 3.1

Effects of extenders and freeze rates on post-thaw motility characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=20 ejaculates).

Variable	Total motility (%)			Progressive motility (%)			VAP (µm/sec)			VCL (µm/sec)			VSL (µm/sec)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Extender:															
Triladyl	41±1.7	17±1.4*	57±3.2	34±1.7	11±1.3*	67±4.1	51±1.3	34±0.9*	32±1.7	79±2.1	48±4.5*	36±4.5	39±0.7	28±0.6*	27±1.5
TCA	36±1.7	22±1.4*	36±3.3	29±1.8	15±1.4*	43±4.1	48±1.3	38±0.9*	19±3.2	75±2.1	58±1.9*	20±3.2	38±0.7	30±0.6*	19±1.0
<i>P</i> value	0.024	0.022	0.001	0.031	0.036	0.001	NS	0.001	0.001	NS	0.001	0.001	NS	0.016	0.001
Freeze rate (°C/min):															
-10	36±2.1	16±1.7 ^a *	52±1.0 ^a	29±2.1	10±1.6 ^a *	62±1.3 ^a	46±1.5 ^a	34±1.0*	25±1.0	75±2.5	49±2.2 ^a *	31±2.1	37±0.8	28±0.8*	22±1.5
-25	40±2.1	19±1.7 ^{ab} *	49±5.4 ^{ab}	33±2.1	13±1.6 ^{ab} *	58±6.5 ^{ab}	49±1.5 ^{ab}	36±1.0*	26±0.9	80±2.6	55±2.2 ^b *	29±1.4	39±0.8	29±0.8*	25±0.8
-40	40±2.2	23±1.8 ^b *	38±3.2 ^b	33±2.2	17±1.7 ^b *	45±4.5 ^b	53±1.6 ^b	38±1.2*	26±5.4	76±2.7	56±2.4 ^b *	22±8.2	39±0.8	30±0.8*	22±1.4
<i>P</i> value	NS	0.009	0.053	NS	0.009	0.035	0.012	NS	NS	NS	0.058	NS	NS	NS	NS

VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. Each value represents mean±SEM.

P-value, within a column, indicates the difference in sperm motility characteristics due to extender and freeze rate.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 3.2

Effects of extenders and freeze rates on post-thaw structural characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=20 ejaculates).

Variable	IPM (%)			IPM-High $\Delta\Psi$ M (%)			IPM-IACR (%)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Extender:									
Triladyl	53±2.8	41±2.7*	24±1.4	16±0.9	9.0±0.9*	41±3.3	50±2.5	36±3.4*	31±3.8
TCA	51±2.1	42±1.1*	17±1.4	15±1.1	11±1.1*	25±2.7	48±1.7	38±0.8*	21±1.2
P value	NS	NS	NS	NS	0.024	0.037	NS	NS	0.023
Freeze rate (°C/min):									
-10	49±2.6	37±1.6 ^{a,*}	26±2.1	15±1.4	9.0±1.2*	37±3.1	46±2.3	32±1.7 ^{a,*}	31±2.0
-25	54±2.5	44±1.9 ^{b,*}	17±2.2	15±1.4	10±0.9*	29±1.0	51±2.3	39±2.2 ^{b,*}	22±2.3
-40	55±2.3	45±2.1 ^{b,*}	19±2.2	16±1.4	10±0.9*	34±0.8	52±1.8	40±2.4 ^{b,*}	24±2.1
P value	NS	0.037	NS	NS	NS	NS	NS	0.038	NS

IPM, intact plasma membrane; IPM-High $\Delta\Psi$ M, intact plasma membrane and high mitochondrial membrane potential; IPM-IACR, intact plasma membrane and intact acrosomes. Each value represents mean±SEM.

P-value, within a column, indicates the difference in sperm structural characteristics due to extender and freeze rate.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

3.8 DISCUSSION

This is the first published report on the quantitative assessment of damage to electroejaculated bison sperm, as they undergo cryopreservation (Experiment 1) and on the detailed evaluation of post-thaw bison sperm plasma membrane, mitochondrial membrane potential and normal acrosomes simultaneously, using flow cytometry (Experiment 2).

It is generally accepted that approximately 50% sperm are killed during cryopreservation [Watson, 1995]. According to the published reports to date there has been very little success in cryopreservation of electroejaculated bison semen [Dorn, 1995; Pérez-Garnelo et al., 2006; Lessard et al., 2009], when compared to post-thaw motilities of cattle semen [Anzar et al., 2011]. In Experiment 1, an attempt was made to investigate if bison sperm are sensitive to dilution and cooling stages prior to undergoing the freeze–thaw cycle which is known to be the most damaging stage in cryopreservation [Watson, 1995]. Data from the present study revealed bison sperm total and progressive motilities remained unaffected after dilution and initial cooling, reflecting their ability to withstand dilution and cooling stresses efficiently. These findings are in contrast to ram and boar sperm which are sensitive to dilution [Purdy, 2006] and initial cooling [Johnson et al., 2000], respectively. In the present study, bison sperm total and progressive motilities decreased by 35% and 42%, respectively, after freeze–thaw stage. A similar major decrease in sperm quality due to freeze–thaw occurs in water buffalo [Matharoo and Singh, 1980; Tuli et al., 1981; Rasul et al., 2001] and cattle [Budworth et al., 1987; Perumal et al., 2011] sperm. The damage to sperm characteristics during freeze–thaw is mainly attributed to ice crystallization and intracellular solute concentration [Mazur, 1984]. Sperm, like other cells, pass

through the lethal temperature zone (-10 to -50°C) and solution effect once during freezing and once more during thawing [Mazur, 1970].

Sperm velocity is associated with bull fertility [Budworth et al., 1988; Kjaestad et al., 1993; Perumal et al., 2011]. In the present study, bison sperm velocities at each stage of cryopreservation were slower (Figure 3.2) than reported in water buffalo [Rasul et al., 2001]. This difference in sperm characteristics between domestic (water buffalo) and wild buffalo (bison) bulls may be due to species, domestication, bulls' age and health, season, semen processing, and evaluation method. Moreover, in the present study, bison sperm average path, curvilinear and straight-line velocities in frozen-thawed semen were 51, 79 and 39 $\mu\text{m}/\text{sec}$ as compared to dairy bull sperm, at 61, 107 and 49 $\mu\text{m}/\text{sec}$ [Anzar et al., 2011], respectively. In the present study, it was revealed that dilution has an adverse effect on the fine and sophisticated parameters, like sperm velocities, of bison sperm. It is speculated that the decrease in sperm velocities immediately after dilution, which also remained low after subsequent stages, may be due to the difference in viscosities of extender and bison seminal plasma. Further, the decrease in VCL after the freeze–thaw stage is mainly attributed to cryoinjuries to sperm mitochondrial apparatus [Jones and Stewart, 1979] and the axoneme [Courstens et al., 1989].

The success of semen cryopreservation largely depends upon an extender with optimum pH, desirable buffering potential, and cryoprotectants to protect sperm against cold shock and freeze–thaw stresses [Salamon and Maxwell, 2000]. Recently, the effect of various commercial extenders such as Triladyl, Andromed, BioXcell have been studied for cryopreservation of epididymal sperm collected from slaughtered North American [Aurini et al., 2009; Lessard et al., 2009; Krishnakumar et al., 2011] and European [Kozdrowski et al., 2011] bison bulls. Post-thaw motility, intact acrosomes and *in vitro* fertilization rate of frozen-thawed epididymal sperm did

not differ due to extenders, i.e., Triladyl and Andromed. The plant origin Andromed may be a more desirable extender for the cryopreservation of epididymal semen [Aurini et al., 2009; Krishnakumar et al., 2011]. In a pilot study on European bison epididymal sperm, Tris-citric acid–egg yolk–glycerol extender was superior to a commercial extender, BioXcell [Kozdrowski et al., 2011]. These attempts on cryopreservation of epididymal sperm are valuable for the conservation of bison genetics. Unfortunately, detailed information about the reproductive potential (semen quality or fertility rates) of bison bulls slaughtered for food is usually not available. Moreover, the cryopreservation of ejaculated semen from a live animal is more desirable for maximum exploitation of superior male genetics to achieve the objectives of artificial insemination. Semen collection via artificial vagina is a popular approach for cattle housed at breeding stations whereas semen from cattle on pasture is usually collected via electroejaculation. Bison are still considered wild animals. Due to their aggressive behavior it would be very difficult to collect semen from bison bulls with an artificial vagina. Therefore, semen collection via electroejaculation was the only choice due to safety reasons. Presently, species-specific extenders are available in the market for the cryopreservation of semen but the exact composition of these extenders is proprietary information. Previously, Triladyl was assessed in our laboratory for cryopreservation of electroejaculated bison semen. It yielded post-thaw total and progressive motilities of 35% and 15% respectively [Lessard et al., 2009]. In semen cryopreservation research focusing on species with poor freezing ability, it is desirable to use an extender that can be modified easily to study the effect of different additives in the extender on post-thaw semen quality. TCA is a commonly used extender for research and production of dairy and beef bull semen in our laboratory and bull breeding stations [Anzar et al., 2002, 2011]. Therefore, in the second experiment, two extenders (commercial Triladyl and

custom-made TCA) were compared for use with bison semen collected via electroejaculation. Results from our study indicated that Triladyl, as compared to TCA, yielded slightly greater, but statistically significant higher post-thaw total and progressive motilities immediately after thawing (0 h). Post-thaw total and progressive sperm motilities in Triladyl extender were better in the present study, (41% and 34%) than reported earlier (35% and 15%, respectively) [Lessard et al., 2009] in electroejaculated bison semen. Surprisingly, the percent decline in total and progressive sperm motilities and structural characteristics (except IPM) during 3 h post-thaw incubation at 37 °C was greater in Triladyl than in TCA indicating that custom-made TCA provides a more desirable buffering environment to bison sperm after thawing. It further suggests longer survival rate of bison sperm cryopreserved in TCA than in Triladyl, and possibly better survival in the female reproductive tract following artificial insemination. Both Triladyl and TCA are Tris-based extenders and ideally should have similar effect. The different sources of main ingredients in both extenders might be reflected in the difference in post-thaw quality of bison sperm. In our opinion, in addition to Triladyl, TCA extender can also be used for the cryopreservation of bison semen on commercial and/or research basis, because it can be modified easily to study the effects of different additives in the extender and is less expensive.

Freeze rate is also very important to the successful cryopreservation of any cell or tissue. An optimum freeze rate should be fast enough to minimize the solution effect and slow enough to avoid intracellular ice crystal formation [Mazur, 1970]. In an earlier study, bison semen was frozen at -10 °C/min and post-thaw total and progressive motilities were only 35% and 15%, respectively [Lessard et al., 2009]. Therefore, three different freeze rates were assessed in the present study to determine any beneficial effect of a greater freeze rate for bison semen cryopreservation. Among three freeze rates, there was no significant difference in sperm

motilities and sperm structural characteristics, except VAP, at 0 h. Sperm from dairy and water buffalo bulls can tolerate a wide range of freeze rates without any significant adverse effect on post-thaw survival [Gilbert and Almquist, 1978; Hultnaes, 1982]. Freeze rate had a significant effect on bison sperm motility, progressive motility, VCL, IPM and IPM-IACR at 3 h post-thaw and lesser decline in total and progressive motilities after 3 h of incubation. Therefore, higher freeze rate of $-40^{\circ}\text{C}/\text{min}$, using programmable cell freezer, is recommended for the cryopreservation of bison semen.

Post-thaw quality of bison sperm found in the present and previous studies [Dorn, 1995; Lessard et al., 2009] is not as high as dairy and beef bull semen. In dairy bull semen cryopreservation, we have reported high post-thaw motility ($>70\%$) and structural characteristics and very little decline (1–6%) in these characteristics during post-thaw incubation [Anzar et al., 2011]. The lesser post-thaw bison sperm characteristics might lead to a failure to fertilize oocytes after artificial insemination with frozen-thawed bison semen. Therefore, attempts should be made to sustain the post-thaw quality of bison sperm to ensure the availability of viable sperm for fertilization process and to inseminate the females close to ovulation time. The difference in post-thaw quality in bison and dairy bull semen is mainly due to the intensive selection pressure in dairy bulls over decades. Bison bulls used in the present study were purchased from the field without any selection whereas dairy and beef breeding bulls undergo rigorous selection in terms of productive and reproductive traits. Few studies have been reported in literature on the development of breeding soundness evaluation of bison bulls [Keen et al., 1999; Helbig et al., 2007], but more comprehensive work is required to develop the selection parameters of bison breeding sires. The present study also indicates a tremendous potential to improve semen cryopreservation technology for bison semen.

In conclusion, the maximum damage to bison sperm occurred during freeze–thaw stage. For cryopreservation of electroejaculated bison semen, Triladyl yielded slightly greater but significantly better motilities immediately after thawing, whereas, TCA enhanced the ability of bison sperm to sustain motility and structural characteristics during post-thaw incubation to a greater extent than Triladyl. Both Triladyl and TCA can be used for cryopreservation of bison semen. The cryodamage to bison sperm can be reduced using a greater freezing rate ($-40^{\circ}\text{C}/\text{min}$). Bison semen collected via electroejaculation can be frozen in liquid nitrogen but more research is required to further improve its post-thaw quality.

CHAPTER 4:

STRATEGIES TO IMPROVE POST-THAW QUALITY OF BISON SPERM

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4.1 ABSTRACT

Bison are known for their poor semen quality following cryopreservation. This study was conducted to improve post-thaw quality of bison semen using zwitterion (ZI) extenders, glycerol addition at lower temperature (4°C), and treatment of sperm with reduced glutathione (GSH), or cholesterol loaded-cyclodextrin (CLC) before freezing. Electroejaculated semen having sperm motility >60% and concentration >200 x10⁶/ml was processed for cryopreservation. Post-thaw sperm motility characteristics were analyzed using computer-assisted sperm analysis (CASA) and the sperm structural characteristics of intact plasma membrane, mitochondrial membrane potential and intact acrosomes were evaluated using a flow cytometer, at 0 and 3 h of post-thaw incubation at 37°C. In Experiment 1, semen was diluted in Triladyl extender (control) or ZI extenders (TesT [Tes-Tris] and HepesT [Hepes-Tris]). In addition, glycerol in ZI extender was added either at 37 or 4°C before cryopreservation. The extenders evaluated did not show any significant effect ($P > 0.05$) on post-thaw total and progressive sperm motilities at 0 h. However, curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL) were better ($P < 0.05$) in ZI extenders than Triladyl. After 3 h of post-thaw incubation at 37°C, sperm motilities were superior ($P < 0.05$) in semen diluted in Triladyl. Sperm had a higher intact plasma membrane and more intact acrosomes in Triladyl compared to ZI extenders ($P < 0.05$). Effect of ZI extenders and temperature of glycerol addition was significant ($P < 0.05$) only on sperm with IPM-High $\Delta\Psi_M$ at 0 h, when glycerol was added at 4°C. The only interaction between ZI extenders and temperature of glycerol addition revealed better ($P < 0.05$) post-thaw VCL at 0 h in TesT extender with glycerol addition at 4°C. In Experiment 2, GSH was used as antioxidant against the production of reactive oxygen species (ROS) in bison sperm for better post-thaw semen quality. Semen was diluted in Triladyl extender containing 0, 0.5, 1.0 or 2.0

mM GSH/50 x 10⁶sperm/ml, at 37°C. Post-thaw analyses at 0 and 3 h incubation did not reveal any significant effect of GSH treatment on post-thaw sperm motility or structural characteristics compared to control samples (0 mM GSH). There was comparatively less but statistically non significant percent of decline in all motility characteristics due to GSH treatment during 3 h post-thaw incubation, at 37 °C. In Experiment 3, fresh bison sperm (100 x 10⁶ in 1 ml) were pre-treated with different concentrations of CLC (0, 1, 2 or 3 mg) at 22 °C for 15 min and then diluted to 50 x 10⁶ sperm/ml in Tris-citric acid (TCA)-egg yolk-glycerol extender before cryopreservation. The CLC-treated sperm, compared with control, showed better (*P* < 0.05) post-thaw total and progressive motilities, IPM and IACR at 0 and 3 h post-thaw incubation at 37 °C and less percent of decline in these characteristics after 3 h of post-thaw incubation.

In conclusion, zwitterion extenders, various temperatures of glycerol addition and pre-treatment of bison semen with GSH did not improve post-thaw quality of bison sperm. However, pre-treatment of bison semen with cholesterol-loaded cyclodextrin significantly improved post-thaw sperm quality and thus can be used as an integral step for bison semen cryopreservation.

4.2 INTRODUCTION

Cryopreservation is a basic technique that can be used for the conservation of endangered and threatened species and exploitation of genetically superior sires [Holt, 1997; Nishi et al., 2002; Halbert et al., 2005; Andrabi and Maxwell, 2007]. Semen cryopreservation is very successful in cattle compared to other mammalian species [reviewed by Thurston et al., 2002]. However, bison semen cryopreservation is not as successful as in cattle [Dorn, 1995; Moreno et al., 1994]. After cryopreservation, electroejaculated bison sperm showed more than 50% decline

in total and progressive motilities [Dorn, 1995; Lessard et al., 2009] and short term post-thaw longevity [Dorn, 1995]. Due to unavailability of frozen bison semen, the usage of assisted reproductive technologies is limited for the development of the bison industry. Therefore, it is important to optimize the cryopreservation procedures to bank bison semen for genetic conservation and to use frozen-thawed semen in a rapidly expanding bison industry.

Sperm are metabolically active cells and require freezing media with good buffering capacity to provide substantial protection against pH changes during and after cryopreservation. Our preliminary experiment on cryopreservation of bison semen using Triladyl extender demonstrated acceptable post-thaw sperm quality at 0 hr [Hussain et al., 2011]. However, there was a significant decline in bison sperm motility and structural characteristics after 3 h of post-thaw incubation, when compared to bovine sperm [Anzar et al., 2011]. Moreover, post-thaw bison sperm motility varies with extenders [Lessard et al., 2009].

For successful semen cryopreservation, an ideal semen extender should have an optimum pH and osmolarity, good buffering capacity, and cryoprotection ability against thermal shocks [Salamon and Maxwell, 2000, Rasul et al., 2001]. Zwitterion (ZI) buffers have the ability to resist change in pH of media even at low temperatures [Good et al., 1966]. Tris is an efficient zwitterion buffer commonly used for the cryopreservation of bovine bull [Davis et al., 1963], goat [Drobnis et al., 1980], boar [Crabo et al., 1972] and water buffalo [Rasul et al., 2000] sperm. Other zwitterion buffers (Tes and Bes) were demonstrated to maintain a significant level of sperm with intact acrosomes after cryopreservation [Graham et al., 1984]. Tes-Tris (TesT) at pH 7.0 provided a good buffering system for the cryopreservation of cattle sperm [Graham et al., 1972]. In contrast, Tris-citrate buffering system was better than Tris-Tes and Tris-Hepes for cryopreservation of water buffalo semen [Rasul et al., 2000].

Since the discovery of glycerol as a cryoprotectant [Polge et al., 1949], it has been extensively used for the cryopreservation of mammalian semen. Due to its permeating nature, glycerol also causes some osmotic and toxic stresses on sperm [Purdy, 2006]. The osmotic and toxic effects of glycerol mostly affect the sperm plasma membrane [Hammerstedt et al., 1990]. However, glycerol addition at low temperature yields better post-thaw sperm quality in boar [Pursel and Johnson, 1975; Pursel and Parks, 1985] and bovine [Erickson et al., 1954] semen. The glycerolated semen extenders can be added at the time of initial dilution at body or room temperatures, or after cooling semen to 5°C as a separate fraction [Evans and Maxwell, 1987].

The cryopreservation of bovine bull semen is associated with a decrease in the antioxidant contents of glutathione (GSH) and superoxide dismutase (SOD) within sperm cells [Bilodeau et al., 2000]. During the freezing process there is a considerable decline in GSH content in porcine [Gadea et al., 2004] and bovine sperm [Bilodeau et al., 2000]. Addition of antioxidant(s) helps to control ROS production and preserves the motility and survival of bull sperm in unfrozen semen [Foote, 1967]. Use of thiols such as cysteine hydrochloride or glutathione (GSH) for semen cryopreservation effectively reduces loss in post-thaw sperm motility of bull semen [Bilodeau et al., 2001]. In cattle, the addition of GSH to the thawing extender results in a significant increase in sperm fertilizing ability [Gadea et al., 2008]. Addition of GSH in the freezing extender improves boar sperm cryosurvival and with an increase in post-thaw sperm motility and recover of a high number of viable sperm [Gadea et al., 2004].

During cryopreservation, initial cooling of sperm to 4°C leads to membrane alterations due to phase transition [Watson, 2000; Medeiros et al., 2002] while the freeze-thaw cycle affects the integrity of plasma and mitochondrial membranes [Blesbois, 2007]. At certain temperatures

during cooling, membrane integrity is compromised due to the formation of lipid aggregates into micro-domains, forming gaps in the membrane leading to altered membrane function [Amman, 1999]. Membrane functions are compromised at lower temperatures, between 5-15°C, [Drobnis et al., 1980] due to transition from liquid to gel phase and the lipids present in egg yolk help in reducing these membrane damages [Hammerstedt et al., 1990]. Beside proteins and phospholipids, cholesterol is an integral part of the sperm plasma membrane and controls membrane structure by interacting with the phospholipid hydrocarbon chains [Darin-Bennett and White, 1977]. Cholesterol strengthens membrane structures at temperatures, even below the phase transition, by resisting change in the structural composition of phospholipid hydrocarbon chains [Quinn, 1989]. Sperm possessing a high cholesterol:phospholipid ratio in their plasma membranes such as rabbit and human sperm, are more resistant to cold shock damage than sperm from boars, stallions, rams and bulls having low cholesterol:phospholipid ratio [Darin-Bennett and White, 1977; Watson, 1981; Parks and Lynch, 1992; White, 1993]. Cholesterol is insoluble in water and can be removed or incorporated in sperm membranes using cyclodextrins [Choi and Toyoda, 1998; Cross, 1999; Iborra et al., 2000]. Cyclodextrins (oligosaccharides) make soluble inclusion complexes with cholesterol and can deliver it to the cell membranes [Cai et al., 2006]. An addition of methyl- β -cyclodextrin pre-loaded with cholesterol to the semen before cryopreservation has improved sperm survival in cattle [Purdy and Graham, 2004], stallion [Combes et al., 2000; Moore et al., 2005], donkey [Alvarez et al., 2006], pig [Galantino-Homer et al., 2006], and ram [Morrier et al., 2004] semen.

The main aim of this study was to improve the current bison semen cryopreservation procedures, which would be useful to pool semen from genetically superior bulls for advancing the bison industry, conservation of genetic diversity and for future research. In this study,

several attempts were made to improve the post-thaw quality of bison semen using ZI extenders for good buffering system, glycerol addition at lower temperature (4°C), and pre-freeze treatment of bison sperm with reduced glutathione (GSH) antioxidant and cholesterol pre-loaded in cyclodextrin (CLC).

4.3 MATERIALS AND METHODS

4.3.1 Semen collection and initial evaluation

Five bison bulls (3 wood and 2 plains), 3-6 years of age and weighing between 450-850 Kg, located at the Native Hoofstock Centre, University of Saskatchewan, Saskatoon, Saskatchewan (52° 10'N/106° 38'W) were used following Animal Use Protocol # 20090125, approved by the Animal Use and Ethics Committee at the University of Saskatchewan. Initially, bison bulls were allowed time to become familiar with the corrals and the handling system at the centre. They were then trained to move through a hydraulic chute system specifically designed for bison (Berlinic Manufacturing, Quill Lake, SK, Canada) and were gradually adapted to restraint for the semen collection process by electroejaculation (EE). Semen was collected from each bison bull twice weekly during July to September (breeding season) with an electroejaculator (Pulsator IV Auto Adjust™; Lane Manufacturing Inc. Denver, CO, USA). A rectal probe (length 13.5"; diameter 3") containing three electrodes, connected to the electroejaculator unit, was gently inserted into the bull's rectum and ejaculation was stimulated by repeated impulses of ~10.2V_{RMS} (root mean squared voltage), 2–3 sec each, for maximum 3 min. Semen was transported to the laboratory in a mobile incubator (37°C) within two hours where it was evaluated and processed within 30 min. Sperm motility characteristics and

concentration of each ejaculate were assessed using CASA (Sperm Vision 3.5, Minitube Canada, Ingersoll, Ontario).

4.3.2 Semen processing

Based on initial evaluation, ejaculates possessing sperm motility > 60% and concentration > 200×10^6 /ml were selected for further processing and diluted to 50×10^6 sperm/ml with the extender, mentioned under each experiment, at 37 °C. The diluted semen was placed in a 500 ml beaker containing warm water (37°C) and shifted to a cold room (4°C). After cooling to 4°C in 90 to 100 min, the semen was packaged in 0.5 ml French straws. Semen straws were frozen from +4 to -10°C at -3°C/min and from -10 to -80°C at -10, -25 or -40°C/min in a programmable cell freezer (ICE-CUBE 14-S, Sy-Lab Version 1.30, Gerate GmbH, Neupurkdersdorf, Austria) as mentioned under each experiment. After freezing, semen straws were plunged in liquid nitrogen (-196°C) and stored for at least 24 h before post-thaw sperm analysis. Three straws (per treatment per bull) were thawed at 37°C for 60 sec in a water bath, and semen was pooled by bull and treatment.

4.4 EXPERIMENTAL DESIGN

4.4.1 Experiment 1: Effects of zwitterion (ZI) extenders and temperatures of glycerol addition on cryopreservation of bison semen

4.4.1.1 Preparation of zwitterion extenders

A commercial extender, Triladyl, composed of Tris and citric acid, was prepared according to manufacturer's instructions (Minitube Canada, Ingersoll, Ontario) and considered as

the control extender. Two ZI buffers, TesT (Tes-Tris) and HepesT (Hepes-Tris), were prepared by the procedure described earlier [Rasul et al., 2000] with slight modifications. Briefly, TesT and HepesT buffers were made by titrating Tes and Hepes (~ 300 mOsm kg^{-1} each) with Tris (~ 300 mOsm kg^{-1}) to pH 7.0. Three different kinds of each ZI extender (TesT and HepesT) were prepared containing final glycerol concentration of 0, 7 or 14% (v/v). Each extender contained egg yolk (20%; v/v), gentamycin sulfate (500 $\mu\text{g}/\text{ml}$), tylan (100 $\mu\text{l}/\text{ml}$) and linco-spectin (300/600 $\mu\text{g}/\text{ml}$). All the extenders were centrifuged at 12,000xg for 15 min at 4°C. The supernatant was aliquoted, stored at -20°C , and thawed at 37°C before use.

4.4.1.2 Experimental procedure

Thirteen ejaculates were collected and eleven were qualified for further processing, as discussed in semen processing section. Each ejaculate was divided in five aliquots and diluted to 50×10^6 sperm/ml with Triladyl (aliquot 1), TesT (aliquot 2) and HepesT (aliquot 3) containing 7% glycerol at 37°C and cooled to 4°C. Two aliquots were diluted to 100×10^6 sperm/ml in half of the required volume of TesT (aliquot 4) and HepesT (aliquot 5) containing 0% glycerol and cooled to 4°C. The remaining half volume of chilled (4°C) TesT and HepesT extenders containing 14% glycerol was added in aliquots 4 and 5 respectively, to achieve final sperm concentration $50 \times 10^6/\text{ml}$ and glycerol concentration 7% (v/v) and were equilibrated at 4°C for additional 30 min. After cooling to 4°C, semen samples were packaged in 0.5 ml French straws (IMV Canada, Woodstock, Ontario) for freezing, as described in section 4.3.2.

4.4.2 Experiment 2: Effect of addition of reduced glutathione (GSH) in freezing extender on post-thaw quality of bison semen

4.4.2.1 Preparation of Triladyl extender containing GSH

In this experiment, commercial Triladyl (Minitube Canada, Ingersoll, Ontario) extender was prepared according to the manufacturers' instructions and was divided into four treatment fractions containing 0 (control), 0.5, 1.0 or 2.0 mM of GSH (L-Glutathione reduced; Sigma-Aldrich, Cat.G6529-5G). The prepared extender was stored at -20°C and thawed at 37°C before use.

4.4.2.2 Experimental procedure

Eleven ejaculates were collected and nine were qualified for further processing, as discussed in semen processing section. Each ejaculate was diluted to 50×10^6 sperm/ml in Triladyl extender containing 0 (control), 0.5, 1.0 or 2.0 mM /ml of GSH, at 37°C . Semen was cooled to 4°C and packaged in 0.5 ml French straws for freezing, as mentioned under section 4.3.2.

4.4.3 Experiment 3: Effect of cholesterol-loaded cyclodextrin (CLC) on cryopreservation of bison semen

4.4.3.1 Preparation of Tris-citric acid buffer and extender

Tris-citric acid (TCA, control) buffer was prepared as described earlier [Purdy and Graham, 2004] with minor modifications. Briefly, Tris was dissolved at 3.03% (w/v) with citric acid monohydrate 1.74% (w/v) and fructose 1.2% (w/v) in Milli-Q distilled water. TCA-egg yolk-glycerol (TCA-EYG) extender was prepared by adding glycerol 14 % (v/v), egg yolk 40% (v/v), gentamycin sulfate (500 $\mu\text{g}/\text{ml}$), tylan (100 $\mu\text{l}/\text{ml}$) and linco-spectin (300/600 $\mu\text{g}/\text{ml}$). The

extender was centrifuged at 12000xg for 15 min at 4°C and the supernatant was aliquoted, stored at -20°C, and thawed at 37°C before use.

4.4.3.2 Preparation of cholesterol-loaded cyclodextrin (CLC)

Methyl β -cyclodextrin (Sigma-Aldrich; Cat. C4555-5G) was loaded with cholesterol (Sigma-Aldrich; Cat. C8667-5G) according to the procedure described earlier [Purdy and Graham, 2004]. Briefly, the 'Solution A' was prepared by dissolving 200 mg cholesterol in 1 ml chloroform in a glass tube. In a separate glass tube, the 'Solution B' was prepared by dissolving 1 g of methyl β -cyclodextrin in 2 ml of methanol. Then 0.45 ml from Solution A was added to Solution B and the mixture was stirred well until the solution became homogenous (90 mg of cholesterol/g of cyclodextrin). The mixture was then dried in a glass petri-dish by blowing with nitrogen (N₂) gas slowly. The solute crystals achieved by drying were kept overnight in a desiccator for further drying and were stored at 22°C. On the day of experiment, fresh working solution was prepared by dissolving 50 mg CLC/ml of Tris-citric acid buffer, at 37°C.

4.4.3.3 Experimental procedure

Ten ejaculates were collected and nine were qualified for further processing, as discussed in the semen processing section above. Each ejaculate was diluted to 100 x10⁶ sperm/ml with TCA buffer at 22°C. Then, either 0 (control), or a concentration of CLC (1, 2 or 3 mg/ml) was added and incubated at 22°C for 15 min. After incubation, TCA-egg yolk-glycerol (TCA-EYG) extender was added to final sperm concentration 50x10⁶/ml. Semen samples were cooled to 4°C and packaged in 0.5 ml French straws for freezing, as mentioned under section 4.3.2.

4.5 SEMEN ASSAYS

4.5.1 Computer-assisted semen analysis (CASA)

Sperm motility characteristics and concentration were determined using CASA settings for bison sperm [Lessard et al., 2009]. For fresh semen, each sample was first diluted 1:20 in TCM-199 (37 °C) whereas diluted, cooled, and frozen-thawed semen was evaluated as such in the extender. An aliquot (2.5 µl) of each kind of semen sample was loaded in a pre-warmed (37 °C) chamber slide (Leja Netherlands; 20 µm depth). Approximately 200 sperm in at least five different fields were analyzed for total motility, progressive motility, average path velocity (VAP, µm/sec), curvilinear velocity (VCL, µm/sec) and straight-line velocity (VSL, µm/sec).

4.5.2 Flow cytometer analysis

A simultaneous assessment of bison sperm plasma membrane, mitochondrial membrane potential, and normal acrosomes was conducted following the procedure, as previously described [Anzar et al., 2011]. After thawing, semen was diluted to 1×10^6 sperm/ml in PBS-0.5% BSA solution. The following fluorescent dyes were added per ml sperm suspension: 1 µl fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma chemicals, St. Louis, MO; stock 1mg/ml PBS), 2 µl mito-tracker deep red (MtDR; Invitrogen, Burlington, ON; stock 2 µM in DMSO) and 6.25 µl propidium iodide (PI; Invitrogen; stock 2.4 mM in water). Semen-dye mixtures were incubated at room temperature (22°C). Sperm were fixed by adding 10 µl of 10% formaldehyde in each semen sample and evaluated for the post-thaw presence of intact plasma membrane (IPM), IPM and high mitochondrial membrane potential (High $\Delta\Psi$ M) and IPM and intact acrosomes (IACR).

At least 10,000 sperm of each semen sample were analyzed by flow cytometer (Partec Cyflow Space, version 2.4 by Partec GmbH, Münster, Germany) equipped with a 400 mW argon laser. FITC-PNA and PI were excited with 488 nm blue laser and their emission spectra were detected with photo-multiplier detectors at FL-1 and FL-3, respectively. MtDR was excited with 635 nm red diode laser and its emission was detected with photo-multiplier detector FL-6. The data were acquired by FloMax software (version 2.4) for cytometry provided by Partec GmbH. Earlier, the distribution of sperm on forward and side light scatters was identified with Hoechst 33342 stained cells (data not shown). All samples were passed through the flow cytometer with a speed of 1 μ l/sec.

Forward and side light scatters were used to identify the sperm and a region (R1) was gated around sperm-specific events (Figure 3.3, Panel A) for PI, MtDR and FITC-PNA fluorescence. Simultaneous fluorescence data of all three probes were recorded on log scales. Any sperm stained with PI has a compromised plasma membrane (RN1 region; Figure 3.3, Panel B) whereas the remaining unstained sperm were considered to have IPM. Two dimensional MtDR /PI dot plot revealed four sperm subpopulations (Figure 3.3, Panel C): sperm with compromised plasma membrane and low $\Delta\Psi$ M (QA1), sperm with compromised plasma membrane and High $\Delta\Psi$ M (QA2), sperm with IPM and low $\Delta\Psi$ M (QA3), and sperm with IPM and High $\Delta\Psi$ M (QA4). Likewise, four sperm subpopulations were recorded on two dimensional FITC-PNA/PI dot plots (Figure 3.3, Panel D): sperm with compromised plasma membrane and IACR (QB1); sperm with compromised plasma membrane and compromised acrosomes (QB2); sperm with IPM and IACR (QB3); and sperm with IPM and compromised acrosomes (QB4). Compensations were conducted to minimize the overlapping of emission spectra of different

fluorophores using FloMax software. For statistical analysis, the data belonging to only IPM, IPM-High $\Delta\Psi\text{M}$ (QA4), and IPM-IACR (QB3) were processed.

4.6 STATISTICAL ANALYSIS

Data collected at 0 and 3 h post-thaw incubation at 37°C for sperm motility (total and progressive motilities, VCL, VSL, VAP) and structural (IPM, IPM-High $\Delta\Psi\text{M}$, IPM-IACR) characteristics were used for statistical analyses. Data for post-thaw percent of decline in all sperm characteristics after 3 h of incubation was calculated as follow (e.g.):

$$[(\text{Motility at 0 h} - \text{Motility at 3 h}) \div \text{Motility at 0 h}] \times 100$$

In Experiment 1, the effects of ZI extenders on sperm motility and structural characteristics were analyzed using randomized complete block design using bulls as blocks due to their random effect. Factorial analysis (2x2) was used to assess the effect of ZI extenders (TesT and HepesT), temperatures (37 and 4°C) of glycerol addition and their interaction on sperm characteristics. In Experiment 2 and 3, randomized complete block design with bulls as a random effect, was used to assess effects of pre-freeze GSH and CLC treatments of bison semen on post-thaw sperm motility and structural characteristics. Data were expressed as the means \pm SEM. If the value of $P < 0.05$, means were separated with Tukey's test. All analyses were conducted using SAS MIXED procedure (version 9.2, SAS institute Inc. Carry, NC).

4.7 RESULTS

4.7.1 Experiment 1: Effects of zwitterion (ZI) extenders and temperatures of glycerol addition on cryopreservation of bison semen

Glycerol addition in different extenders at 37°C did not show any significant effect on immediate (0 h) post-thaw sperm total and progressive motilities. However, among velocities, sperm VCL and VAP were higher ($P < 0.05$) in TesT extender and VSL was higher in both ZI extenders, compared to Triladyl (control; Table 4.1). During 3 h of post-thaw incubation, sperm total and progressive motilities were better preserved ($P < 0.05$) in Triladyl than ZI extenders, while sperm velocities were not affected ($P > 0.05$) due to extenders. Post-thaw percent of decline after 3 h of incubation in all sperm motility characteristics was less ($P < 0.05$) in semen diluted in Triladyl than in ZI extenders (Table 4.1). Among sperm structural characteristics, IPM and IPM-IACR were higher ($P < 0.05$) in Triladyl than in ZI extenders, at both 0 and 3 h of post-thaw incubation (Table 4.2). The percent of decline in sperm structural characteristics after 3 h of post-thaw incubation did not vary due to extenders with glycerol addition at 37°C (Table 4.2).

ZI extenders (TesT & HepesT) and the temperature (37 or 4°C) of glycerol addition did not generally reveal significant effect on sperm motility or structural characteristics. Except, sperm VCL at 0 h was better ($P < 0.05$) in TesT extender and in samples in which glycerol was added at 4°C, while percent of decline after 3 h was less ($P < 0.05$) in HepesT extender (Table 4.3). Likewise, sperm with higher mitochondrial membrane potential at 0 h were better ($P < 0.05$) in semen samples in which glycerol was added at 4°C than at 37°C (Table 4.4). The interaction of ZI extenders and temperature of glycerol addition only revealed better ($P < 0.05$) sperm VCL at 0 h in TesT extender in which glycerol was added at 4°C than HepesT with glycerol addition at either 37 °C or 4°C (Table 4.3). All sperm motility and structural characteristics declined significantly during 3 h of post-thaw incubation at 37°C ($P < 0.05$).

Table 4.1

Effects of extenders (with glycerol addition at 37°C) on post-thaw motility characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=11 ejaculates).

Extender	Total motility (%)			Progressive motility (%)			VCL (µm/sec)			VAP (µm/sec)			VSL (µm/sec)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Triladyl	43± 4.3	18± 4.5 ^{a,*}	60± 7.9 ^a	35± 4.3	11± 4.3 ^{a,*}	71± 9.1 ^a	78± 4.2 ^a	46± 5.2*	39± 6.5 ^a	47± 2.1 ^a	33± 2.4*	29± 4.2 ^a	37± 1.6 ^a	28± 1.6*	23± 3.1 ^a
HepesT	32± 3.4	6± 0.5 ^{b,*}	80± 2.5 ^b	26± 3.2	2± 0.3 ^{b,*}	92± 1.3 ^b	86± 3.3 ^{ab}	40± 2.7*	53± 3.3 ^{ab}	51± 1.7 ^{ab}	32± 1.1*	37± 2.3 ^{ab}	41± 1.4 ^b	28± 1.0*	31± 2.4 ^b
Test	36± 3.4	7± 0.6 ^{b,*}	77± 4.7 ^{ab}	29± 3.4	3± 0.4 ^{b,*}	85± 5.1 ^{ab}	88± 3.8 ^b	35± 1.4*	59± 2.6 ^b	52± 1.9 ^b	29± 1.1*	43± 2.7 ^b	41± 1.6 ^b	25± 1.0*	37± 2.8 ^b
P-Value	NS	0.006	0.040	NS	0.025	0.050	0.050	NS	0.011	0.034	NS	0.005	0.032	NS	0.001

VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity. Each value represents mean ± SEM.

P-value, within a column, indicates the difference in sperm motility characteristics due to extenders.

^{ab}Means in column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 4.2

Effects of extenders (with glycerol addition at 37°C) on post-thaw sperm structural characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=11 ejaculates).

Extender	IPM (%)			IPM-High $\Delta\Psi$ M (%)			IPM-IACR (%)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Triladyl	49± 2.8 ^a	40± 3.3 ^{a,*}	18± 5.6	21± 2.2	14± 1.9*	32± 7.4	47± 3.1 ^a	36± 3.8 ^{a,*}	25± 4.7
HepesT	39± 2.4 ^b	30± 1.7 ^{b,*}	23± 3.6	26± 1.1	14± 1.7*	47± 6.5	35± 2.5 ^b	25± 2.0 ^{b,*}	27± 5.9
TesT	38± 2.4 ^b	29± 1.6 ^{b,*}	22± 5.2	26± 1.8	14± 1.4*	43± 5.8	35± 2.2 ^b	22± 1.6 ^{b,*}	36± 3.5
P-Value	0.001	0.002	NS	NS	NS	NS	0.001	0.001	NS

IPM, intact plasma membrane; IPM-High $\Delta\Psi$ M, intact plasma membrane and high mitochondrial membrane potential; IPM-IACR, intact plasma membrane and intact acrosomes. Each value represents mean \pm SEM.

P-value within a column indicates the difference in sperm structural characteristics due to extenders.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 4.3

Effects of zwitterion extenders and temperatures of glycerol addition on post-thaw motility characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=11 ejaculates).

Variable	Total motility (%)			Progressive motility (%)			VCL (µm/sec)			VAP (µm/sec)			VSL (µm/sec)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Extender:															
HepesT	32± 2.3	6± 0.5*	80± 1.4	27± 2.2	2± 0.4*	92± 0.8	87± 2.7	37± 1.4*	55± 2.2	51± 1.3	29± 0.7*	40± 2.1	41± 1.1	25± 0.6*	34± 2.7
TesT	35± 2.5	6± 0.4*	78± 3.1	29± 2.4	2± 0.3*	87± 2.9	94± 3.0	38± 1.5*	60± 1.8	54± 1.3	30± 0.8*	45± 1.9	43± 1.0	27± 0.7*	40± 2.1
P-values	NS	NS	NS	NS	NS	NS	0.017	NS	0.025	NS	NS	NS	NS	NS	NS
Temperature of glycerol addition:															
37°C	34± 2.4	7± 0.5*	78± 2.6	28± 2.3	3± 0.4*	89± 2.7	87± 2.5	36± 1.3*	56± 2.1	51± 1.2	29± 0.8*	40± 1.9	41± 1.1	25± 0.6*	34± 1.9
4°C	33± 2.4	6± 0.4*	80± 2.3	28± 2.3	2± 0.2*	81± 1.6	94± 3.2	39± 1.6*	59± 2	54± 1.3	30± 0.7*	45± 2.1	43± 1.0	26± 0.7*	40± 2.8
P-Value	NS	NS	NS	NS	NS	NS	0.026	NS	NS	NS	NS	NS	NS	NS	NS

VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity. Each value represents mean ± SEM.

P-value, within a column, indicates the difference in sperm motility characteristics due to extender & temperature of glycerol addition.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.01$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 4.4

Effects of zwitterion extenders and temperatures of glycerol addition on post-thaw structural characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=11 ejaculates).

Variable	IPM (%)			IPM-High $\Delta\Psi$ M (%)			IPM-IACR (%)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
Extender:									
HepesT	41±1.8	31±1.5*	23±3.5	28±1.1	16±1.4*	46±4.4	37±1.8	26±1.6*	30±4.0
TesT	39±1.9	29±1.2*	23±3.6	29±1.2	15±1.0*	47±3.7	36±1.7	22±1.2*	37±2.7
P-values	NS	NS	NS	NS	NS	NS	NS	NS	NS
Temperature of glycerol addition:									
37°C	39±1.7	29±1.1*	22±3.1	26±1.0	14±1.1*	45±4.3	35±1.6	24±1.3*	32±3.5
4°C	41±2.0	30±1.5*	24±4.1	31±1.0	16±1.4*	48±3.7	38±1.8	24±1.6*	36±3.4
P-Value	NS	NS	NS	0.001	NS	NS	NS	NS	NS

IPM; intact plasma membrane, IPM-High $\Delta\Psi$ M; intact plasma membrane and high mitochondrial membrane potential, IPM-IACR; intact plasma membrane and intact acrosomes. Each value represents mean \pm SEM.

P-value, within a column, indicates difference in sperm structural characteristics due to extender & temperature of glycerol addition.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.01$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

4.7.2 Experiment 2: Effect of addition of reduced glutathione (GSH) in freezing extender on post-thaw quality of bison semen

Data analysis showed that bison semen diluted in GSH-containing extender has no significant effect on immediate (0 h) post-thaw total and progressive sperm motilities and structural characteristics (Table 4.5 and 4.6). Among velocities, VCL was significantly lower ($P < 0.05$) in semen samples diluted in extender containing 2 mM GSH. The percent of decline in post-thaw sperm motility characteristics was comparatively less but statistically non-significant ($P > 0.05$) in GSH treated samples than untreated control, during 3 h of incubation at 37°C (Table 4.5). Similarly, there was no significant effect of GSH treatments on any of post-thaw sperm structural characteristics during 3 h of incubation at 37°C (Table 4.6). All sperm motility and structural characteristics declined significantly during 3 h of post-thaw incubation at 37°C ($P < 0.05$).

Table 4.5

Effect of reduced glutathione (GSH) on post-thaw sperm motility characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=9 ejaculates).

GSH	Motility (%)			Progressive motility (%)			VCL (µm/s)			VAP (µm/s)			VSL (µm/s)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
0.0 mM	50± 3.0	26± 4.8*	51± 6.9	43± 3.0	18± 4.6*	62± 8.0	82± 2.0 ^a	48± 3.3*	37± 5.1	48± 0.7	34± 1.6*	30± 3.7	38± 0.7	29± 1.4*	24± 3.9
0.5 mM	46± 3.6	30± 5.3*	35± 9.6	37± 3.5	22± 5.1*	41± 11.0	81± 2.2 ^{ab}	64± 5.2*	25± 7.4	48± 0.8	40± 2.3*	19± 5.2	37± 0.8	32± 1.4*	17± 3.6
1.0 mM	46± 3.5	31± 5.1*	36± 8.3	38± 3.2	23± 5.0*	42± 10.4	81± 2.0 ^{ab}	56± 5.6*	25± 6.6	48± 0.6	39± 2.2*	20± 4.4	37± 0.5	32± 1.0*	15± 2.6
2.0 mM	48± 2.6	31± 4.6*	36± 8.5	39± 2.9	22± 4.4*	44± 9.5	77± 2.2 ^b	64± 4.7*	22± 7.0	46± 0.8	37± 3.1*	16± 5.8	36± 0.8	30± 1.8*	14± 4.4
P-Value	NS	NS	NS	NS	NS	NS	0.035	NS	NS	NS	NS	NS	NS	NS	NS

VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity. Each value represents mean ± SEM.

P-value, within a column, indicates the difference in sperm motility characteristics due to GSH treatment.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 4.6

Effect of reduced glutathione (GSH) on post-thaw sperm structural characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=9 ejaculates).

GSH	IPM (%)			IPM-High $\Delta\Psi$ M (%)			IPM-IACR (%)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
0.0 mM	40± 3.3	37± 3.4*	8± 3.1	18± 1.9	9± 2.1*	44± 11.8	40± 3.6	34± 3.7*	16± 4.5
0.5 mM	42± 3.8	37± 3.7*	10± 2.5	21± 1.5	11± 2.6*	43± 13.8	41± 4.0	33± 3.7*	17± 3.3
1.0 mM	42± 3.3	37± 3.7*	12± 4.3	20± 1.6	9± 1.4*	50± 8.2	41± 3.5	34± 3.7*	18± 4.6
2.0 mM	39± 4.0	36± 3.7*	8± 2.4	20± 1.0	9± 1.7*	53± 8.8	39± 4.1	33± 3.8*	15± 3.1
P-Value	NS	NS	NS	NS	NS	NS	NS	NS	NS

IPM, intact plasma membrane; IPM-High $\Delta\Psi$ M, intact plasma membrane and high mitochondrial membrane potential; IPM-IACR, intact plasma membrane and intact acrosomes. Each value represents mean \pm SEM.

P-value within a column indicates the difference in sperm structural characteristics due to GSH treatment.

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

4.7.3 Experiment 3: Effect of cholesterol-loaded cyclodextrin (CLC) on cryopreservation of bison semen

The treatment of bison sperm with cholesterol-loaded cyclodextrin (CLC) significantly improved post-thaw quality of bison sperm (Tables 4.7 and 4.8). At 0 and 3 h of post-thaw incubation, total and progressive sperm motilities were better for every sample treated with CLC ($P < 0.05$), and post-thaw percent of decline in motility and velocity characteristics after 3 h was less ($P < 0.05$) in CLC treated samples than in control (0 mg CLC). Sperm structural characteristics (IPM and IPM-IACR at 0 and 3 h) were higher ($P < 0.05$) in 2 & 3 mg CLC treated samples than in control samples. The post-thaw percent of decline in IPM and IPM-IACR after 3 h was significantly less ($P < 0.05$) in samples treated with 3 mg CLC than control and 1 mg CLC treated sperm. There was no significant difference ($P > 0.05$) within CLC treatments (1, 2 and 3 mg) on sperm motility and structural characteristics at 0 and 3 h. Post-thaw sperm velocities and sperm with IPM-High $\Delta\Psi_M$ were not affected ($P > 0.05$) by CLC treatments. All sperm motility & structural characteristics declined significantly during 3 h of post-thaw incubation at 37°C ($P < 0.05$).

Table 4.7

Effect of cholesterol-loaded cyclodextrin (CLC) on post-thaw motility characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=9 ejaculates).

CLC	Total motility (%)			Progressive motility (%)			VCL (µm/sec)			VAP (µm/sec)			VSL (µm/sec)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
0 mg	33± 2.8 ^a	11± 2.5 ^{a,*}	69± 4.8 ^a	28± 2.7 ^a	6± 1.9 ^{a,*}	81± 4.2 ^a	91± 2.2	55± 4.5 [*]	40± 4.7	53± 1.1	33± 7.6 [*]	37± 4.8	41± 2.0	25± 3.1 [*]	39± 6.8
1 mg	42± 5.9 ^{ab}	19± 6.1 ^{ab,*}	61± 7.3 ^{ab}	37± 5.9 ^{ab}	15± 5.7 ^{ab,*}	68± 7.9 ^{ab}	95± 3.7	58± 6.0 [*]	39± 4.9	55± 1.8	36± 10.1 [*]	35± 4.8	44± 1.6	30± 8.0 [*]	33± 5.0
2 mg	48± 5.4 ^b	29± 6.3 ^{b,*}	44± 7.2 ^b	43± 5.4 ^b	24± 6.1 ^{b,*}	50± 8.0 ^b	96± 5.1	66± 4.6 [*]	31± 3.3	56± 2.5	41± 8.7 [*]	27± 3.8	43± 1.9	33± 7.2 [*]	23± 4.4
3 mg	46± 4.9 ^{ab}	26± 5.4 ^{ab,*}	49± 7.1 ^{ab}	41± 4.6 ^{ab}	20± 5.2 ^{ab,*}	57± 8.5 ^{ab}	93± 3.2	64± 5.7 [*]	32± 5.2	53± 1.2	39± 11.0 [*]	26± 6.4	40± 0.8	32± 3.1 [*]	22± 7.0
P-Value	0.040	0.035	0.032	0.039	0.028	0.013	NS	NS	NS	NS	NS	NS	NS	NS	NS

VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. Each value represents mean ± SEM.

P-value, within a column, indicates the difference in sperm motility characteristics due to CLC treatment.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

*Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

Table 4.8

Effect of cholesterol-loaded cyclodextrin (CLC) on post-thaw structural characteristics of bison sperm at 0 and 3 h, and on percent decline after 3 h of incubation at 37°C (n=9 ejaculates).

CLC	IPM (%)			IPM-High $\Delta\Psi$ M (%)			IPM-IACR (%)		
	0 h	3 h	% decline	0 h	3 h	% decline	0 h	3 h	% decline
0 mg	33± 2.8 ^a	23± 3.2 ^{a,*}	32± 5.0 ^a	16± 3.6	6± 2.0*	66± 6.5	32± 2.7 ^a	21± 3.0 ^{a,*}	36± 4.8 ^a
1 mg	44± 4.6 ^{ab}	31± 4.2 ^{ab,*}	30± 5.2 ^a	19± 2.9	8± 2.6*	64± 8.1	43± 4.6 ^{ab}	29± 4.0 ^{ab,*}	32± 5.3 ^a
2 mg	47± 4.8 ^b	37± 4.6 ^{b,*}	21± 3.8 ^{ab}	16± 3.3	9± 3.5*	56± 13.4	46± 4.8 ^b	36± 4.5 ^{b,*}	21± 3.8 ^{ab}
3 mg	46± 4.3 ^b	39± 3.5 ^{b,*}	14± 2.3 ^b	14± 3.0	6± 2.2*	62± 6.9	45± 4.2 ^b	38± 3.5 ^{b,*}	15± 2.8 ^b
P-Values	0.021	0.007	0.021	NS	NS	NS	0.020	0.003	0.006

IPM, intact plasma membrane; IPM-High $\Delta\Psi$ M, intact plasma membrane and high mitochondrial membrane potential; IPM-IACR, intact plasma membrane and intact acrosomes. Each value represents mean \pm SEM.

P-value, within a column, indicates the difference in sperm structural characteristics due to CLC treatment.

^{ab}Means in a column with different superscripts are different ($P < 0.05$).

* Asterisk denotes a significant decline ($P < 0.05$) in a sperm characteristic after 3 h of post-thaw incubation at 37°C.

4.8 DISCUSSION

To our knowledge, this is the first study in which four approaches were used to improve post-thaw quality of electroejaculated bison semen. These approaches were: 1) use of zwitterion extenders to provide suitable buffering system against change in pH during and after cryopreservation, 2) glycerol addition at lower temperature to reduce its possible toxic effect at body temperature, 3) the addition of reduced glutathione (GSH) in semen extender as an antioxidant and 4) the pre-treatment of bison sperm with cholesterol-loaded cyclodextrin (CLC) to stabilize their plasma membrane against thermal stress.

Zwitterion (ZI) buffers are superior to other buffers due to their optimum buffering capacity and they provide better sperm motility in goat and cattle semen compared to citrate, phosphate or Tris buffers [Good et al., 1966; Graham et al., 1972]. In experiment 1, contrary to our expectations, there was no added beneficial effect of zwitterion extenders on immediate (0 h) post-thaw sperm motilities of bison sperm compared to Triladyl (control; Table 4.1). Triladyl extender is composed of Tris (a zwitterion buffer) and citric acid, whereas TesT and HepesT extenders have Tes and Hepes containing Tris (all zwitterion buffers). These results suggest Tris buffer, a common ingredient in all three extenders, can provide a suitable buffering system for bison sperm when it is titrated with weak citric acid or with Tes or Hepes (zwitterions). Similar findings were reported earlier for post-thaw sperm motility in buffalo and Holstein bulls [Anzar and Graham, 1995; Rasul et al., 2000]. Mitochondria are the basic source of energy required for sperm motility; therefore, sperm velocity indicates the functional integrity of the sperm mitochondria [Graham et al., 1984] and is also related to bovine bull fertility [Budworth et al., 1988]. During *in vitro* fertilization sperm with higher VCL, VAP and VSL fertilize more

oocytes, and VCL alone is a good indicator of fertility [Marshburn et al., 1992]. In this study, post-thaw VCL, VAP and VSL at 0 h were higher in TesT extender than control Triladyl (Table 4.1). Interestingly, similar findings of higher post-thaw velocities with TesT buffer were observed in buffalo sperm [Rasul et al., 2000]. Post-thaw sperm structural analysis (0 h) with flow cytometer revealed better IPM and IPM-IACR with Triladyl. Oocyte fertilization basically depends on the integrity of the acrosome, an organelle directly involved in fertilization [Saacke and White, 1972]. Post-thaw IPM-IACR of bison sperm were affected adversely in TesT buffer, as found in bovine bull sperm [Anzar and Graham, 1995]. In another study, TesT buffer yielded more damaged acrosomes compared to TCA due to early sperm hyperactivation and capacitation resulting in damage to the acrosomes [Ijaz et al., 1989].

There is controversy in published literature about the temperature of glycerol addition in various species. The addition of glycerol at low temperature is better for sperm cryopreservation [Pursel and Jhonson, 1975; Pursel and Parks, 1985]. In previous research, the post-thaw quality of buffalo sperm was better when glycerol was added at 37°C than at 4°C [Rasul et al., 2007]. Also, ram sperm are more sensitive to glycerol addition at 30°C than at 5°C [Fiser and Fairful, 1989] but boar sperm were not affected by temperature of glycerol addition [Maxwell and Salamon, 1979]. In this study, the effect of temperature of glycerol addition was also evaluated on post-thaw sperm motility and structural characteristics. Glycerol as a cryoprotectant, rearranges membrane lipids and proteins resulting in increased fluidity, causes more cellular dehydration at lower temperatures, and less intracellular ice formation leading to better sperm cryosurvival [Holt, 2000]. There was no significant interaction of zwitterion buffers (HepesT, TesT) and temperatures of glycerol addition on bison sperm motility and structural characteristics. Data analysis of ZI extenders and temperature of glycerol addition revealed better

curvilinear velocity at 0 h in TesT extender than in HepesT and for glycerol addition at 4°C than at 37°C. Likewise, mitochondrial membrane potential was better when glycerol was added at 4°C than at 37°C, while extender effect was non significant. In general, Triladyl was a better extender than zwitterion extenders (TesT & HepesT) and addition of glycerol at 4°C did not significantly improve post-thaw bison sperm quality. This indicates that bison sperm can tolerate the possible toxic effects of glycerol very well at higher temperature (37°C). Consequently, for the sake of procedural convenience, bison semen can be extended confidently in glycerolated extender at the time of initial dilution at 37°C.

Cryodamage to sperm cells affects various functions and processes important for successful fertilization [Bailey et al, 2000]. A significant level of ROS is produced during cryopreservation procedures, which could damage the membranes and sperm functions, and also destabilize the antioxidant defense system including a reduction in intracellular GSH contents. This reduction in glutathione in seminal plasma can be compensated by the exogenous addition of reduced glutathione (GSH) in the media, to improve post-thaw semen quality [Bilodeau et. al., 2000]. Addition of GSH improves semen quality by increasing lactate production and fructose consumption, and pre-freeze addition of 5 mM GSH in the media renders better post-thaw motility during 3 h of incubation at 37°C [Wiechetek and Slaweta, 1987]. For this study, addition of GSH to freezing media did not reveal any significant improvement in immediate (0 h) post-thaw quality of the bison sperm. However, the percent of decline in motility characteristics after 3 h of incubation at 37°C was (non-significantly, $P > 0.05$) less in GSH treated samples than in control. Similar findings were observed in boar sperm in that the addition of GSH in the extender did not improve post-thaw viability characteristics or *in vitro* fertilizing ability [Gadea et al, 2004]. GSH treatment also did not reveal any significant affect on the percent of decline in

sperm structural characteristics during 3 h of post-thaw incubation. This study indicated that GSH was obviously not effective during freezing as the immediate post-thaw standard sperm characteristics were not different in GSH treated sperm than control sperm. In our opinion, a long period between semen collection and semen arrival in the lab may have caused some sub-lethal damage to sperm due to ROS production in fresh semen and this was reflected in depressed longevity after thawing. Post-thaw incubation of semen revealed a beneficial effect of GSH treatments indicating the ROS production in frozen-thawed sperm. Interestingly, frozen-thawed cattle sperm are more adversely affected by ROS production than fresh sperm [Trincherro et al., 1990].

Cholesterol modulates membrane fluidity by interacting with the fatty acyl chains of the phospholipids and cholesterol:phospholipid ratio in the sperm plasma membrane is an important determinant of membrane fluidity and resistance of sperm against cold shock damage [Watson, 1981]. Binder sperm proteins (BSPs) are secreted by the seminal vesicles and bind to sperm membranes at ejaculation [Manjunath et al., 2007]. They play an important role in sperm function and fertility [Manjunath et al., 2007]. These BSPs are involved in cholesterol efflux to initiate sperm capacitation [Therien et al., 1998]. Cryopreserved sperm undergo capacitation-like changes [Bailey et al., 2000; Watson, 2000] including loss of cholesterol from the plasma membrane leading to acrosome reaction and reduced life span [Medeiros et al. 2002]. Cyclodextrin (cyclic oligosaccharides) can be used to alter the cholesterol contents of cell membranes [Christian et al., 1997; Combes et al., 2000; Purdy and Graham, 2004; Moore et al., 2005]. Compared to natural cyclodextrins, the use of its derivatives has proven better for incorporating cholesterol into cellular membranes [Pitha et al., 1988]. Among cyclodextrin derivatives, methyl- β -cyclodextrin is the most efficient to accept cholesterol from mouse L-cell

fibroblasts [Yancey et al., 1996]. In our study, pre-treatment of bison semen with cholesterol-loaded-methyl- β -cyclodextrin (CLC) before freezing, resulted in better sperm cryosurvival than in untreated control samples. The optimum concentration of CLC for bison sperm cryosurvival lies between 2 and 3 mg/100 $\times 10^6$ sperm, which is about 0.020–0.030 mg CLC/10 6 sperm compared to 0.0125–0.025 mg/10 6 for bull sperm [Purdy and Graham, 2004] and 0.0125–0.026 mg/10 6 for stallion sperm [Combes et al., 2000; Moore et al., 2005]. Thus the optimum CLC requirement appears to differ among species. An improvement in post-thaw sperm quality by adding CLC indicates that poor cryosurvival of bison semen is either due to a lower cholesterol:phospholipid ratio in bison sperm membranes or presence of BSP factors in seminal plasma might be causing cholesterol efflux after ejaculation from sperm membranes, as identified in bovine seminal plasma initiating capacitation [Therien et al., 1998]. Cholesterol efflux could reduce sperm resistance to cold shock during cryopreservation [Darin-Bennett and White, 1977; White, 1993]. Total and progressive motilities of bison sperm were up to 15% higher and IPM and IPM-IACR up to 14% higher in CLC treated semen than control semen. In comparison, CLC treatment increased total motility up to 18% and sperm with intact plasma membrane up to 14% in bull sperm [Purdy and Graham, 2004]. Similarly, stallion sperm treated with CLC showed an increase in total sperm motility up to 19% and sperm with intact plasma membrane up to 21% [Combes, et al., 2000]. Fresh bison semen quality is not as good as that of bovine semen, but post-thaw improvement of bison sperm motility and structural characteristics due to cholesterol addition is likely as good as that of bovine semen.

The pre-treatment of bison sperm with CLC not only improved their cryosurvival (at 0 h) but also significantly reduced the percent of decline in sperm motility and structural characteristics during 3 h of post-thaw incubation at 37°C. The percent of decline in total and

progressive motility, IPM and IPM-IACR was significantly less in CLC treated sperm than untreated control by 25%, 31%, 18% and 21% respectively. Our results suggested that maintaining higher cholesterol concentrations in the sperm plasma membrane via CLC treatment minimized cryodamage and improved post-thaw survival rates. The co-incubation of bison sperm with CLC in Tris buffer alone (without glycerol and egg-yolk) successfully transfers cholesterol to the sperm membranes [Purdy and Graham, 2004]. The addition of cholesterol in CLC probably decreases the lipid phase transition temperature and saves the sperm plasma membranes from the damaging effect of cold shock [Ladbroke et al., 1968].

In conclusion, 1) Triladyl extender can be used for better post-thaw quality of bison semen. 2) Glycerol addition at 37°C along with whole extender is more practical and easy procedure. 3) Pre-freeze addition of GSH is not significantly effective to improve post-thaw bison semen quality. Therefore, a controlled study is required to investigate the effect of GSH addition in the extender on post-thaw quality (longevity) of the bison sperm. Moreover, ROS production and presence of an antioxidant defense mechanism in bison sperm needs to be determined for future research to improve techniques for the cryopreservation of bison sperm. 4) Bison semen must be pre-treated with a minimum of 2 mg of CLC to incorporate exogenous cholesterol into sperm plasma membranes to minimize damage induced by the cryopreservation procedures. Overall, these recommendations will improve the survival rate of frozen-thawed bison semen and allow a successful usage of frozen semen in assisted reproductive technologies like artificial insemination, embryo transfer or *in vitro* production of embryos.

CHAPTER 5:

GENERAL DISCUSSION AND RECOMENDATIONS

The main goal of this thesis study was development of a bison specific semen cryopreservation technique to improve the poor freezing ability of bison sperm. For this purpose, different factors were studied, including various extenders, freeze rates, temperatures of glycerol addition, addition of reduced glutathione (GSH) addition to extenders and pre-treatment of sperm with cholesterol-loaded cyclodextrin (CLC).

The first series of the experiment were designed to measure the degree of damage to bison sperm motility characteristics during the cryopreservation process. Total and progressive sperm motilities were well maintained during the dilution and cooling phases (Figure 3.1). This is in contrast to ram and boar sperm which are sensitive to dilution [Purdy, 2006] or initial cooling [Johnson et al., 2000]. However, sperm motilities declined significantly after the freeze-thaw stage (Figure 3.2). Similar results were observed in water buffalo and cattle sperm [Matharoo and Singh, 1980; Tuli et al., 1981; Budworth et al., 1987; Rasul et al., 2001]. This experiment concluded that most of the damage to bison sperm occurs during the freeze-thaw stage of cryopreservation, probably due to intracellular ice formation or solution effect [Mazur, 1970]. During the freezing process formation of extra- and intra-cellular ice damages several structures and components of the sperm including the plasma membrane, intra-cellular macromolecules, and the mitochondrial apparatus [Watson, 1995]. Similarly, solution effect can lead to irreversible damages to plasma membranes and osmotic shock due to severe dehydration and an increase of intra cellular salt concentration [Gao et al., 1997]. To minimize the damage to

bison sperm during freeze-thaw stage, an extender with good buffering capacity and optimum freeze rate could significantly improve the post-thaw quality of bison sperm.

The second series of experiments were to compare the quality of the sperm frozen with homemade or commercial extenders (custom-made Tris-citric acid (TCA) and commercial Triladyl), and different freeze rates (-10 , -25 or $-40^{\circ}\text{C}/\text{min}$). Triladyl was a better extender than TCA for immediate post-thaw sperm quality, while after 3 h of post-thaw incubation TCA extender proved to be better for preservation of sperm viability characteristics. Commercial Triladyl as well as TCA provided an equivalent buffering system during cryopreservation. Thus, custom-made TCA extender could be used confidently as a lower cost alternative to study the effect of different additives for cryopreservation of bison semen. For successful cryopreservation of cells, an optimum freeze rate is required to minimize the solution effect and the formation of deleterious intracellular ice crystals during freezing and thawing [Mazur, 1970]. In this experiment different freeze rates (i.e. -10 , -25 or $-40^{\circ}\text{C}/\text{min}$) were used to assess a suitable freeze rate for cryopreservation of bison semen. Immediate (0 h) post-thaw sperm motility and structural characteristics were not affected by freeze rate because the results did not differ (Table 3.1 and 3.2). It can be speculated that a sperm subpopulation with stable membranes can resist cooling phase damage and can survive equally within the freeze rate range used in this experiment. In other words, it is possible that cryo-sensitive sperm with sub-lethal damages did not survive before freezing and a homogenous sperm population was able to survive within freezing rate range from -10 to $-40^{\circ}\text{C}/\text{min}$. The decline of post-thaw sperm total and progressive motilities after 3 h were significantly less for the sperm samples frozen at -40°C than those at $-10^{\circ}\text{C}/\text{min}$, suggesting less cryo-damage to the sperm when it is frozen at a high freeze rate. It has been demonstrated that a higher freeze rate used on water buffalo semen yielded better post-

thaw sperm quality than a lower freeze rate [Anzar et al., 2010]. This suggests that a rapid freeze rate is more desirable for improved post-thaw cryosurvival of bison semen. Also, it is less time consuming for the freezing process.

Sperm are very metabolically active cells and may cause a change in pH of the media during the cryopreservation procedure. An extender with good buffering capacity is required to maintain appropriate pH during and after cryopreservation. Zwitterion (ZI) buffers are known for their optimum buffering capacity [Good et al. 1966] and they provide better sperm motility in goat semen compared to citrate, phosphate or Tris buffers [Graham et al. 1972]. Glycerol as a cryo-protectant is an important component included in all extenders. For some species, it is considered toxic to sperm survival due to its permeating nature [Purdy, 2006]. Generally, glycerol is added in the extender before dilution of the semen. However, glycerol can be added after semen cooling at a lower temperature to minimize its toxic effects [Evan and Maxwell, 1987]. So, zwitterion buffers were expected to maintain a better buffering system through temperature changes during cryopreservation [Good et al., 1966]. In the present study, zwitterion buffers did not show any improvement in the post-thaw quality of bison sperm (Table 4.1 and 4.2). These results are in agreement with previous studies in water buffalo and Holstein bull sperm [Anzar and Graham, 1995; Rasul et al., 2000]. It can be concluded that the different buffers used in this study have a similar effect on bison sperm and are useful in cryopreservation protocols. Also, the temperature at which glycerol was added did not significantly affect post-thaw sperm motility and structural characteristics (4.3 and 4.4). It can be speculated that bison sperm are able to tolerate and equilibrate the glycerol added either at ambient or lower temperatures. It is suggested that the use of glycerol addition at ambient temperature is a practical and convenient approach for semen cryopreservation.

In the previous experiments, there was no significant improvement in post-thaw bison semen quality with different buffering systems, different freeze rates, or glycerol addition at different temperatures. So, it was hypothesized that the poor freezing ability of bison sperm was due to excessive production of reactive oxygen species (ROS; free radicals) during cryopreservation. The polyunsaturated lipid nature of sperm membranes [Agarwal et al, 1994; Kobayashi et al, 2001] and deficient cytoplasmic antioxidant defense mechanism [Donnelly et al, 1999; Saleh and Agarwal, 2002] of sperm could make them vulnerable to damage by ROS produced during cryopreservation. Moreover, cryopreservation also reduces the antioxidant contents present in sperm [Bilodeau et al., 2000]. An experiment was designed to counter the possible production of ROS in bison sperm by supplementing the extender with reduced glutathione (GSH; an antioxidant). In our experiment, addition of GSH in freezing media did not improve immediate (0 h) post-thaw quality of bison sperm (Table 4.5 and 4.6), suggesting that bison semen has sufficient GSH to protect it against ROS. It has been demonstrated that bovine semen samples treated with GSH maintains better motility [Bilodeau et al, 2001; Foote et al, 2002] and provides better protection against oxidative damage to mammalian sperm [Alvarez and Storey, 1989]. Reduced glutathione (GSH) is the main antioxidant in cells, but various other antioxidants are also present in the seminal plasma and sperm, which might have been protecting bison sperm against ROS production during cryopreservation. So, more controlled studies on the identification of ROS and the role of various antioxidants in bison semen will be of significant importance to determine the effect of exogenous addition of antioxidant to achieve better post-thaw quality of bison sperm.

For the last series of the experiment, it was hypothesized that pre-freeze treatment with cholesterol can improve freezing ability of bison sperm by strengthening the sperm plasma

membrane against cold shock. It was demonstrated that pre-freeze treatment of bison semen with cholesterol loaded in cyclodextrin (CLC) resulted in better sperm cryosurvival than untreated control samples (Table 4.7 and 4.8). Bison sperm has been reported to have short post-thaw survival [Dorn, 1995]. However, bison sperm survived for a longer time as the decline in sperm viability during 3 h of post-thaw incubation at 37°C was less in CLC treated semen (Table 4.7 and 4.8). Proper cholesterol to phospholipids ratio is important in maintaining sperm membrane functions during cryopreservation process [Watson, 1995]. Sperm from rabbits and humans with higher cholesterol:phospholipid ratio in the membranes are more resistant to cold shock damage than sperm from boar, stallion, rams and bulls which have a lower ratio [Watson, 1981, Parks and Lynch, 1992, White, 1993]. Sperm binder proteins (BSPs) in bovine seminal plasma are responsible for the first cholesterol efflux after ejaculation [Therien et al., 1998] and initiate capacitation, normally occurring in female reproductive tract [Manjunath et al., 2007]. So, it can be speculated that there is a reduction of cholesterol in the plasma membrane of electroejaculated bison sperm due to the BSP proteins present in the seminal plasma. During cryopreservation, cholesterol efflux from the sperm plasma membrane reduces post-thaw sperm viability due to early capacitation [Medeiros, 2002]. Cholesterol pre-loaded in cyclodextrin has been used with varying success to improve the post-thaw quality of semen in bovine bulls [Purdy and Graham, 2004], horses [Moore et al., 2005] and rams [Morrier et al., 2004]. In our study, the concentrations found suitable for bison sperm cryosurvival were 2 and 3 mg of CLC/100×10⁶ sperm. Improvement in bison sperm quality after cholesterol addition confirmed that the poor freezing ability of bison was due to lower cholesterol to phospholipids ratio in bison sperm membranes. Also, increased time interval from collection to processing (> 2 h) might have led to more cholesterol efflux due to the presence of seminal plasma protein factors (BSPs), resulting in

poor freezing ability of semen. In conclusion, addition of cholesterol seems an appropriate approach in bison semen cryopreservation technique for better post-thaw viability of bison sperm.

In summary, the main goal of this research study was the development of a bison semen specific cryopreservation technique to improve freezing ability of the sperm. In our opinion, electroejaculated bison semen can be frozen and preserved in liquid nitrogen successfully using these recommendations: 1) Triladyl is a better extender and glycerol addition at 37°C as part of extender is an easy and practical approach for the cryopreservation of bison semen, 2) Use of cholesterol (2-3 mg / 1×10^9 sperm) must be included in the semen cryopreservation technique for better post-thaw quality of bison sperm. Improvement in bison semen cryopreservation technique is vital to exploit semen from elite bison bulls for the conservation and development of bison genetic potential. Available frozen semen can be used through AI and *in vitro* embryo production to furnish the developing needs of current bison industry.

Successful bison semen cryopreservation is essential for the development of the commercial bison industry in North America. Abundantly available frozen bison semen will provide the basis for assisted reproductive technologies such as artificial insemination, *in vitro* embryo production and embryo transfer to exploit the desired traits of any particular bison, as it is done in beef and dairy cattle industries. There is great potential for the sale of frozen bison semen in the North American market. In the cattle industry, a single semen dose is worth an average of about \$ 20. In our current study, the bison bulls produced an average volume of 5 ml semen/ejaculate with a concentration of about 0.5 to 1.0×10^9 sperm/ml. So, a single bull can produce 50-100 semen doses of 50×10^6 sperm /ml, representing a market value of \$1000-2000 per ejaculate, and semen with a potential value of \$20,000/month (2 ejaculates per week). If a

market for frozen bison semen can be developed, the industry will be able to generate millions of dollars in revenue. Finally, industry can conserve the genetic diversity of the iconic North American bison species using the methods developed in this study.

Based on these research findings and the study of related literature, several factors should be considered to improve freezing ability of bison semen including:

1. Bison bull selection criteria

Presently, bison bulls are acquired without considering any selection criteria for their reproductive potential. For successful bison semen cryopreservation, it is significant to establish parameters for the selection of bull with greater reproductive potential and good freezing ability.

2. Knowledge of the reproductive potential of bison

There is a paucity of bison knowledge about reproductive potential in comparison with beef and dairy cattle. Literature available on breeding soundness of bison bulls for breeding purposes or semen collection for AI is insufficient. The published studies available on breeding soundness evaluation by Keen et al [1999], onset of puberty in bison [Helbig et al., 2007] and bison semen quality in different seasons [Helbig et al.,2007] have provided the basis to understand the reproductive biology of male bison. Further studies will be considerably important to develop potential selection indices for breeding soundness evaluation of bison sires.

3. Biochemical composition of bison semen/sperm

Availability of knowledge on these aspects will be helpful to select a suitable media for collection and cryopreservation of bison semen. In this regard, more focus is required to identify bison seminal plasma proteins and their role during cryopreservation. BSPs identified in bison seminal plasma, [Gratton and Lessard, personal communication] may have been involved in cholesterol depletion from sperm plasma membranes, leading to poor post-thaw semen quality. Also, characterization of the bison sperm plasma membrane in terms of its protein, lipid and sterol components is very important to identify the changes occurring in sperm during the cryopreservation process.

4. Bison semen dilution at ejaculation

Dilution of semen in the required extender at the time of collection can also play an important role to provide support against pH change, thermal and osmotic shocks, leading to a better cryopreservation for the bison sperm.

5. Handling stress on bison

Stress associated with captivity and difficulties handling bison bulls before and during semen collection may affect the quality of bison semen and its freezing ability.

Success in developing suitable bison semen cryopreservation technology is of significantly important to exploit the genetic potential of this unique species in North America. Availability of cryopreserved bison semen will create new avenues to apply existing reproductive technologies for the conservation, sustainable use, and development of North American bison species.

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