IMPROVING QUALITY OF CRYOPRESERVED BUFFALO BULL SPERMATOZOA

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A thesis submitted in the partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Physiology

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2017

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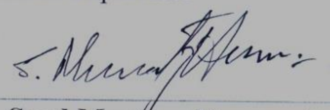

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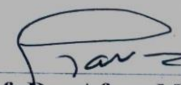

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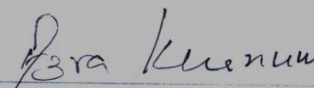

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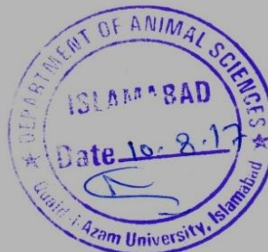

This Thesis is dedicated to my son

Syed Sibtain Ali Shah

as an encouragement to deliver in future.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ALH	Amplitude of lateral displacement of a sperm head
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCF	Beat cross frequency
CASA	Computer assisted semen analyzer
CPAs	Cryoprotectant agents
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-I	DNA integrity
EIF	Extracellular ice formation
EYP	Egg yolk plasma
FAOSTAT	Food and Agriculture Organization Statistics
FR	Frame rate
FRAP	Ferric reducing antioxidant power
GLM	General linear model
GSH/GSSG	Glutathione
GTLS	Gentamicin, tylosin, lincomycin, and spectinomycin
H_2O_2	Hydrogen peroxide
IIF	Intracellular ice formation
JC-1	5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide
LDL	Low density lipoprotein
LIN	Linearity
LN_2	Liquid nitrogen
LPO	Lipid peroxidation
MDA	Malonaldehyde
MMP	Mitochondrial trans-membrane potential
MV	Medium velocity
NAC	N-acetyl-L-cysteine
PM	Progressive motility

ROS	Reactive oxygen species
RTAs	Radical-trapping antioxidants
RV	Rapid velocity
SCN	Surface-catalyzed nucleation
SOD	Superoxide dismutase
STR	Straightness
SV	Slow velocity
SV-PMI	Supra-vital plasma membrane integrity
TALP	Tyrode's albumin lactate pyruvate
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TM	Total motility
Tn	Nucleation temperature
UV–C	Ultra-violet C
VAP	Average path velocity
VCL	Curvilinear velocity
VCN	Volume-catalyzed nucleation
V-IACR	Viable sperm with intact acrosome
VLDL	Very low density lipoprotein
VSL	Straight line velocity
WCEY	Whole chicken egg yolk
ΔΨ MMP	Change in mitochondrial membrane potential

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SUMMARY

Cryopreservation techniques currently in use to preserve buffalo spermatozoa demand improvements in the freezing protocols in order to overcome the cryo-damage that may occur during the process of cryopreservation. Such damage may profoundly affect the *in vitro* quality of spermatozoa in terms of longevity, motility and in vivo fertilization potential. In this connection, the potential of a series of cryodiluents was investigated by applying these into freezing extenders to achieve the desired quality and productivity for water buffalo bull spermatozoa.

Experiment 1; The effects of equilibration times (E1, 2 h; E2, 4 h; E3, 6 h), freezing rates (FR1, manual, 5 cm above liquid nitrogen (LN_2) semen loaded French straws (0.5 ml) for 10 min, plunging into LN_2; and FR2, programmable ultra-fast, holding French straws (0.5 ml) at +4°C for 2 min, from 4 to −10°C at −10°C/min, from −10 to −20°C at −15°C/min, from −20 to −120 °C at −60°C/min, holding at −120°C for 30 sec, plunging into LN_2), and thawing rates (T1, 37 °C for 30 sec; T2, 50°C for 15 sec; T3, 70°C for 7 sec) were evaluated in tris−citric acid extender on the quality of buffalo bull spermatozoa. Progressive motility (PM, %), rapid velocity (RV, %), average path velocity (VAP, µm sec^{-1}), straight line velocity (VSL, µm sec^{-1}), and the mitochondrial transmembrane potential (MMP, %) were found to be greater ($P < 0.05$) with E2, FR2, and T3 as compared to other groups. Sperm curvilinear velocity (VCL, µm sec^{-1}) was greater ($P < 0.05$) with E2 and FR2 compared to other groups. Sperm straightness (STR, %) and linearity (LIN, %) were also greater ($P < 0.05$) with E2 when compared with the other groups. Supravital-plasma membrane integrity (SV-PMI, %), viability and acrosome integrity (V-IACR, %) of spermatozoa were greater ($P < 0.05$) with E2 and FR2 as compared to other groups. Sperm DNA integrity (DNA-I, %) was also greater ($P < 0.05$) with FR2 and T1 as compared to other groups. These experiments concluded that using 4 h equilibration time, programmable ultra-fast freezing rate, and rapid thawing at 70 °C for 7 sec in the cryopreservation protocol improves the post-thaw quality of buffalo bull spermatozoa.

Experiment 2; The antioxidant effect of different concentrations of curcumin at the rate of 0.5, 1.0, 1.5 and 2.0 mM was evaluated in tris−citric acid extender on

freezability of buffalo spermatozoa. At pre-freezing and post-thawing, total antioxidant contents (µM/L) were noticeably higher ($P < 0.05$) with 1.5 and 2.0 mM curcumin as compared to 0.5 and 1.0 mM curcumin and control. At pre-freezing, lipid peroxidation levels (LPO, µM/ml) were lower with 1.5 mM curcumin, while at post-thawing, the LPO levels were lower with 1.5 mM and 2.0 mM curcumin as compared to 0.5 and 1.0 mM curcumin and control. At post-thawing, PM (%), RV (%) and kinematics (VAP, µm sec^{-1}; VSL, µm sec^{-1}; VCL, µm sec^{-1}; STR, %; LIN, %), *in vitro* longevity (%, PM and RV) and DNA-I (%) were significantly higher ($P < 0.05$) with 1.5 mM curcumin compared to control. At post-thawing, SV-PMI (%) and V-IACR (%) were higher with 1.5 curcumin compared to 2.0 mM curcumin and control. These experiments demonstrated that the freezability of water buffalo spermatozoa is appreciably improved with the addition of 1.5 mM curcumin in the extender.

Experiment 3; The effect of different concentrations of UV-C irradiated chicken egg yolk plasma (EYP; v/v; 10%, P1; 15%, P2; 20%, P3) or 20% (v/v) of whole chicken egg yolk (WCEY) in tris−citric acid extender was evaluated on water buffalo sperm quality during cryopreservation (post–dilution, PD; post–equilibration, PE; post–thawing, PT). The effect of best evolved concentration of EYP in extender on *in vivo* fertility capability of buffalo spermatozoa was also evaluated. Overall, at post-thawing, CASA PM (%), RV (%), VAP (µm sec^{-1}) and VSL (µm sec^{-1}), SV-PMI (%), MMP (%), V-IACR (%), and DNA-I (%) were higher in P3 treatment as compared to other treatments and WCEY. The decline percentage (%, longevity) in PM and RV was lower in P3 as compared to WCEY during 2 h incubation under *in vitro* conditions at PT. The *in vivo* fertility rate (%) was significantly higher with P3 as compared to WCEY (76.61 vs. 64.49). This part of the study concluded that WCEY (20%) can be replaced with UV-C irradiated chicken EYP (20%) in tris–citric acid extender for cryopreservation and *in vivo* fertility of water buffalo spermatozoa.

Experiment 4; Another objective of the study was to determine a possible cryoprotection synergism between glycerol and DMSO for water buffalo spermatozoa. The dilution of semen was made with five extenders comprising 7% glycerol at 37 °C, control; 3.5% DMSO at 37°C as well as at 4°C, Group 1; 3.5% glycerol at 37°C and 3.5% DMSO at 4°C, Group 2); 3.5% DMSO at 37°C and 3.5% glycerol at 4°C, Group 3; and 1.75% glycerol and 1.75% DMSO at 37°C as well as at

4°C, Group 4). Moreover, the effect of best concentrations of glycerol and DMSO in extender was assessed on *in vivo* fertility of buffalo spermatozoa. At post thawing (PT), sperm PM (%), RV (%), VAP (µm sec^{-1}), VCL (µm sec^{-1}), *in vitro* longevity (%), SV-PMI (%), MMP (%), V-IACR (%) and DNA-I (% except Group 2) were higher ($P < 0.05$) in extenders having 1.75% glycerol and 1.75% DMSO at 37 as well as at 4°C (Group 4) as compared to other treatment groups and the control. The *in vivo* fertility rate (%) was significantly greater with Group 4 as compared to the control (69.45 vs. 59.81). These experiments concluded that a synergism between glycerol and DMSO (Group 4) potentially improves the quality and *in vivo* fertility of cryopreserved water buffalo spermatozoa.

On the whole, this study concludes that 4 h equilibration time, programmable ultra-fast freezing rate (+4°C for 2 min, from 4 to −10°C at −10°C/min, from −10 to −20°C at −15°C/min, from −20 to −120°C at −60°C/min, holding at −120°C for 30 sec), and rapid thawing at 70°C for 7 sec in the cryopreservation protocol improve the post-thaw quality of buffalo bull spermatozoa. The supplementation of 1.5 mM curcumin as antioxidant in the extender provided better cryoprotection for freezability of water buffalo spermatozoa. The UV-C irradiated chicken EYP (20%) can be effectively used as an alternate to WCEY (20%) in tris–citric acid extender for cryopreservation and *in vivo* fertility of water buffalo spermatozoa. A synergism between glycerol and DMSO (addition of 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C, Group 4) potentially improves the quality as well as *in vivo* fertility of cryopreserved water buffalo spermatozoa. The findings of this study would be helpful for optimization of freezing protocols currently practiced for the cryopreservation of buffalo spermatozoa.

GENERAL INTRODUCTION

Domestic water buffalo *(Bubalus bubalis)* contributes remarkably to cater to the agricultural economy of Asian countries including Pakistan, and few Mediterranean and Latin American countries through milk and meat production (Qureshi and Ahmad, 2008; Andrabi, 2014). The major breeds of dairy buffalo belonging to the river type comprise the Murrah, Surti, Jafarabadi and Nili-Ravi (Perera, 2011). World's population of buffalo is approximately 175.48 million, of which India, Pakistan, China, Nepal and Egypt hold 109.4 million, 33.68 million, 23.25 million, 5.24 million and 3.91 million, respectively. Overall, buffalo population in Asia, Africa, Americas, Europe and Oceania is about 97.1%, 2.1%, 0.7%, 0.2% and 0%, respectively (FAOSTAT, 2013).

The reproductive disorders that have been revealed in buffaloes comprise late onset of puberty, poor estrus expression, longer postpartum ovarian quietness, and most prominently reduced conception rates mostly with artificial insemination (Drost, 2007; Presicce, 2007; Perera, 2011). Although, buffaloes can breed for entire year, seasonal patterns (tropical and temperate) like rainfall and thermal stress, etc, may lead to an elevation of prolactin and melatonin levels, which may drastically influence reproductive efficiency of the buffalo (Perera, 2011).

Despite seasonal effect and other management issues, buffaloes have a remarkable innate adaptation to harsh climates, disease resistance, premium A2 casein milk, and production on minimum provision of food (Singh and Balhara, 2016). Buffaloes contribute about 13% to the world milk production with an annual growth rate of 3.5%, compared with around 2.1% for the cow milk production (Faye and Konuspayeva, 2012). Artificial insemination, a most important reproductive biotechnology, plays a predominate role in the field of animal breeding and genetics (Andrabi, 2014), and provides better quality genetic material from the superior male species. Its role in the progeny testing of male for improving the competence of genetic selection is also well known. It introduces new genetic material through semen at subsidized cost in comparison to cost incurred on import of superior quality animals (Andrabi, 2014; Singh and Balhara, 2016).

1.1. Cryopreservation and its mechanisms

Mammalian cells essentially exist in an ionic environment, which becomes concentrated ice during freezing due to the removal of water from the solution. Increased osmotic pressure in the extracellular fluid produces an osmotic gradient across the plasma membrane that causes water efflux from the cells, as has been described in the conceptual approach introduced by Mazur (1984). The rate of water efflux is controlled by permeability of water via the cell membranes. Thus the possibility of cooling the cells is increased under conditions where the rate of water efflux is insufficient to maintain osmotic equilibrium between the cytoplasm and the unfrozen component of the extracellular solution. Under these conditions, the cytoplasm is supercooled and gets frozen, which is called the intracellular ice formation (IIF) phase. Intracellular ice formation can be easily observed microscopically as there occurs rapid blinking of the cytoplasm due to the array of small ice crystals or microscopic gas bubbles that disperse the light (Steponkus et al., 1983).

Biosynthetic activities of spermatozoa are limited and are dependent normally on catabolic processes for proper execution (Hammerstedt and Andrews, 1997). In order to impede these metabolic changes, the cells are cooled below –130°C (Sathe and Shipley, 2014). Various organelles of spermatozoa also contain water, which is involved in the formation of intracellular and extracellular ice crystals during the cryopreservation. The membranes of spermatozoa possess numerous phospholipids, which are variable within different species and show a specific phase transition temperature. Both temperature and lipid composition of these membranes are responsible for structural damage during the cooling and freezing process (White, 1993). The cholesterol and phospholipid ratio, quantity of nonbilayer- supporting lipids, levels of hydrocarbon chain saturation, and ratio of protein to phospholipids affect sensitivity of the membranes (Medeiros et al., 2002).

During the process of cell freezing, diverse factors have been recognized as detrimental to cell mechanisms (Holt, 2000) including the cooling rate (Rubinsky, 2003), equilibration time, rapid freezing and thawing, nucleation temperature (Tn), class and quantity of cryoprotective agents and post-thaw time interval (Mazur, 1984;

Andrabi, 2014). Moreover, super cooling, formation of extracellular ice crystals, interplay of osmotic effects (Gibson, 2010; Drori et al., 2014) and increase in the quantity of solutes such as sugars, salts and proteins, all cause dehydration of spermatozoa (Adams et al., 2015).

The intracellular and extracellular water is unfrozen in a super cooled form at –5°C. The ice formation occurs extracellularly between –5°C and –10°C while water intracellularly is still in the form of super cooled condition. It is necessary to keep the cooling rate slow to allow cellular dehydration and limiting the intracellular water to be frozen. Moreover, the cells must also be protected against any kind of hyperosmotic exposure after dehydration so that solution-effect injury could be avoided, as which may cause denaturation of the macromolecules. Cold shock to spermatozoa occurs during rapid cooling in the range of 0-30°C, which causes cytoplasmic as well as membrane damage (Gao et al., 1997; Isachenko, 2003). Cold shock also changes the permeability level of the plasma membrane, mitochondria, and acrosome. It is important to mention that in case of global supercooling versus directional solidification, both the supercooling level and freezing rate are responsible to control the number of ice crystals and their size (Kasper and Friess, 2011). Thus, there is a need to standardize both the freezing protocols and the viability assays for evaluating the quality of the cells.

1.2. Intracellular ice formation (IIF)

Intracellular ice formation (IIF) appears to be very critical in cryobiology, since successful freezing considerably depends on it. Although, the cell membrane is thought to be responsible for the formation of IIF, the exact biophysical mechanisms still remain to be explored (Li et al., 2013). Several theories have been put forth to explore the biophysical mechanisms of IIF.

1.2.1. Phenomenological models

Phenomenological models of intracellular ice formation have been proposed which use statistical information to envisage the possibility of intracellular ice formation

throughout the freezing procedures (Mazur, 1977; Pitt and Steponkus, 1989). Mazur's (1966) hypothesis upholds that at a crucial temperature during the cooling, when the smallest radius of ice crystal equals the radius of aqueous pore in the plasma membrane, then intracellular ice formation develops, provided water in the cytoplasm is in supercooled form. Mazur's bifactorial theory of cryoinjury (1965) proposes that there is a need to pair both the freezing as well as thawing rates for obtaining the maximum cell survivability. This theory has experimental evidence in favor of spermatozoa survivability but many studies carried out in bovine could not establish an association between freezing and thawing rates (Watson, 1979).

1.2.2. Mechanistic models

Mechanistic models of IIF have also been suggested on the basis of two general views: that IIF is the cause of cellular injury or that it is a result of damage to the plasma membrane. The former view (Toner et al., 1990), suggests that there are two ways to catalyze the IIF relevant to freezing scenario: the effect of extracellular ice formation (EIF) on the cell membrane (surface catalyzed nucleation, SCN) or intracellular particles (volume catalyzed nucleation, VCN). According to Steponkus et al. (1985), generation of electrical transients at the ice edges in the freezing may be responsible for deterioration of plasma membrane and thus maximizing the damage in the protoplasts. According to Bacic et al. (1990) and Finkelstein (1987) solubility-diffusion mechanism is involved for water passage through lipid bilayers. In case where a concentration gradient exists, depending upon the partition coefficient water penetrates through the lipid bilayer and moves afterward down this gradient to the opposite side (Finkelstein, 1987). There occurs development of a frictional force on the lipid bilayers due to movement of water molecules while passing through them. Since water-water interaction with the lipid bilayer is negligible, the role of water molecules in the membranes is independent (Finkelstein, 1987). Hence, frictional force transmitted by the water molecules would be proportional to velocity of particles while moving through the hydrophobic part of the lipid bilayers (Finkelstein, 1987).

1.2.3. Osmotic rupture hypothesis

The osmotic rupture hypothesis of cryoinjury was introduced to explain the phenomenon of intracellular freezing due to the failure of prevailing theories to account for several experimental challenges (Muldrew and McGann, 1990). Intracellular freezing is almost always associated with fatal injury to the cells. Considerable debate is still continuing about the cellular cryoinjury as a basis or the consequence of IIF (Muldrew and McGann, 1990; Toner et al., 1990). Muldrew and McGann (1990) have hypothesized that rupture in the plasma membrane occurs due to the osmotic stress developed during the cooling process; consequently the passage of extracellular ice into the cell cytoplasm is eased. Tentative mechanism for this rupturing of cell membrane was proposed due to the frictional drag of water driven by the osmotic pressure gradient, which surmounts the ductile strength of the plasma membrane and results into the structural malfunction. Moreover, osmotic pressure is simply a property of a solution; it is not a hydrostatic pressure (Muldrew and McGann, 1990).

1.2.4. Other theories

Wharton and Ferns (1995) have proposed that gap junctions in the plasma membrane are responsible for likely transmission of ice into the cells. A major link between the number of cells forming IIF and the cells loosing the membrane integrity has been found by Acker and McGann (2000). Water and liquefied solutions possess a distinct propensity to cool below their melting point prior to ice formation. In spite of the fact that the freezing point of water is 0°C, in the presence of permeating cryoprotectant agents (CPAs) in excess of 1.0 mol/l an aqueous sample may be cooled to approximately –40°C before ice formation develops. At such low temperatures, conversion of liquid water into ice, once started, occurs very rapidly if the intracellular compartment remains hydrated (Mazur et al., 2005; Mazur, 2010). Because the permeability of cell membrane to permeating CPA is lower in comparison to water, loss of intracellular water is more rapid than gain of CPA. This is of particular relevance to cell viability because excessive volume reductions, presumably above 40%, may represent a source of cell injury (De Santis and Coticchio, 2011).

Cell membranes are considerably damaged after IIF in a condition of high freezing rate (>20°C/min), however their integrity is observed after IIF in the low freezing condition (<20°C/min), (Li et al., 2013). After the successive freezing and thawing, the intact cells again undergo IIF, but the ice within cells forms large crystals and less in growth pace, which indicates a lesser supercooling needed for IIF. Simultaneously, the membrane becomes more brittle due to recurrence of freezing and leads to disruption. These observations uphold Mazur's pore theory and Toner's surface-catalyzed nucleation (SCN) theory (Li et al., 2013). According to Kilbride et al. (2014), both the network solidification and progressive solidification dominate the transition of water into ice phase. The network solidification usually occurs in samples with small cryo-straws or cryo-vials while progressive solidification is mostly monitored in bigger volumes or environmental freezing.

The physical principles of the freezing protocol in either large or small volumes are primarily different (Kilbride et al., 2014), because in cryopreservation, the lower samples (e.g. straws) with typical cooling rates are processed through the smaller temperature regimes while there is uniformity in cooling below the equilibrium melting point for whole volume samples until ice nucleation begins (Pereyra et al., 2011; Morris and Acton, 2013). After the initiation of the ice nucleation by a nucleating agent (Hartmann et al., 2011), there occurs rapid growth of ice in the whole sample (Morris and Acton, 2013). The ice network surges further as a consistent body due to the passage of water from the freeze concentrated matrix which is determined by the nucleation temperature (Ayel et al., 2006) and not the cooling rate (Searles et al., 2001). Biologically, this form of ice solidification is termed as network (or dendritic) solidification (NS), but in materials science it is called cellular growth (Kilbride et al., 2014).

1.3. Freezing methods

Two options are currently available for freezing spermatozoa: 1) conventional vapor freezing or nonprogrammable method, which involves holding of semen loaded straws above liquid nitrogen vapors in a Styrofoam box, for a precise time and distance and, 2) controlled freezing that involves the automated programmable freezers (Andrabi, 2014).

1.3.1. Conventional vapor freezing

In the conventional vapor freezing method as ice crystals grow in terms of velocity and morphology, control on them is not achievable (Watson, 2000). An effective cryopreservation protocol for buffalo spermatozoa can be worked out through orderly examination of different cryobiological aspects (Sukhato et al., 2001). The sensitivity of mammalian sperm to rapid cooling was first recognized by Milovanov (1933) who reported that cellular injury appeared as a loss of selective permeability and integrity of membranes. The most frequently used strategy for the assessment of survivability of spermatozoa during cryopreservation is that their post-thaw outcome should be ≥50% of pre-freezing motility (Wolf and Patton, 1989).

1.3.2. Programmable freezing

Programmable freezers have a greater ease of utility, they permit grouping of cooling ramps without continued operator involvement. Programmable freezers precisely regulate cooling rates over a wide array of temperatures by supplying pressurized vapor-phase liquid nitrogen into a sample chamber controlled by an electronic-solenoid feedback system (Day et al., 2008). Programmable freezers have demonstrated better outcome in reducing variability between freezes (Holt, 2000) as compared to conventional freezing, as this is so because the latter method has issues of repeatability and variation of the samples being studied. It is worth mentioning that faster cooling rates are required for the cryopreservation of sperm of different species, for instance in humans (10°C/min), (McLaughlin et al., 1990), and 20°C/min for dogs (Farstad, 1996), sheep (Fiser et al., 1986), pigs (Fiser et al., 1993) and mice (Stacy et al., 2006). Dias Maziero et al. (2013) could not observe any differences in motility patterns while comparing Styrofoam box or programmable freezing methods.

Amann and Picket (1987) reported that very slow cooling rate causes dehydration of spermatozoa while extremely rapid cooling rate produces intracellular ice formation. Devireddy et al. (2002) reported the suitable cooling rate from 4°C to -80°C equivalent to -60°C/min (range, -20 to -100°C/min) for stallion sperms. The temperature at distance of 2.5–5 cm over the LN_2 level was reported as -160°C

(Heitland et al., 1996) and a freezing rate of -60°C/min was obtained 4 cm over LN_2 (Cochran et al., 1984).

Use of a standardized method and accurate cooling for managing many samples at the targeted freezing rates is essential. It is important to note that if freezers fulfill the first objective (standardized method), they fail to control the cooling. The reason for this uncontrollability is that samples release enough latent heat of fusion, which results into elevation of temperature. Furthermore, the freezers have to dissipate this extra heat quickly; this stops in dropping of temperature of samples although the chamber's temperature falls. The temperature of the sample remains static for 2 to 3 min before the resumption of cooling. This phase between freezing and resumption of cooling is called the freezing point plateau and it is the damaging to viability of spermatozoa (Holt, 2000).

1.4. Pre-requisites of spermatozoa quality during cryopreservation

1.4.1. Initial semen evaluation

There are certain factors that need to be considered when semen samples are to be evaluated. The examination should focus on the overall appearance of the sample, motility, total volume, concentration, live percent, morphologically abnormal sperm percent, and the integrity of the acrosome (Salisbury et al., 1978). The basic aim of semen analysis is to identify and discard subfertile samples precisely in a speedy and economical mode (DeJarnette and Amann, 2010). There is a variable coloration of buffalo semen i.e., milky white to creamy or minor tinge of blue. The volume of the buffalo semen greatly varies with the age and breed of the bulls (Vale, 1994). The pH of buffalo semen ranges from 6.4 to 7.0 (Kumar et al., 1993). The spermatozoa are unable to adapt against the lipid content during low freezing temperatures. This helps in overcoming the phase transitions driven changes (17–36°C) in ionic permeability and enzyme activity which are species dependent (Parks and Lynch, 1992).

1.4.2. Freezing protocol

1.4.3. Diluents used in extenders

Extenders or diluents are normally added to the freezing solutions for providing protection to spermatozoa during cryopreservation. Semen extenders have following specific roles:

I. They act as nutrients as an energy source for motility of spermatozoa. Simple sugars contained in extender are lactose, mannose, fructose and arabinose (Sathe and Shipley, 2014).

II. The sugars and polyols (e.g., glycerol) can substitute the water molecules in the generally hydrated polar groups of plasma membrane of spermatozoa, which leads to stability of membrane during the critical temperature zones (−5 to −50°C) of freezing (Woelders et al., 1997; Kumar et al., 2003).

III. They act as buffer against detrimental transformations in pH.

IV. They provide suitable physiologic osmotic pressure and concentration of electrolytes.

V. They thwart harmful bacterial growth. Penicillin (500-1000 IU/ml) and streptomycin (0.5-1.0 mg/ml) being nontoxic to spermatozoa are the most commonly used antibiotics (Akhter et al., 2008). However, gentamicin, tylosin, lincomycin and spectinomycin (GTLS) are also used in the freezing protocols against several bacterial species such as *Mycoplasma*, *Ureaplasma*, *Campylobacter fetus*, *Haemophilus somnus* and *Pseudomonas* (Shin et al., 1988; Lorton et al., 1988).

VI. They provide protection from cold shock during the cooling phase (e.g., glycerol) by enhancing the viscosity of medium.

VII. They hinder the eutectic crystallization of solutes and increase the glass-forming propensity of the media, which is applicable in vitrification process (Nicolajsen and Hvidt, 1994).

VIII. They also reduce the physical and chemical injuries faced by spermatozoa during cooling, freezing and thawing phases of cryopreservation (Gao et al., 1997; Purdy, 2006).

The choice of cryoprotectant in the freezing protocols appears to be a matter of trial and error in almost all the experimental studies. This is partly due to the fact that complete mechanistic explanation about the action of cryoprotectant is still not clear (Holt, 2000). Cryoprotectants are categorized as penetrating (glycerol, DMSO, ethylene glycol, propylene glycol, etc.) or non-penetrating (egg yolk, nonfat skimmed milk, trehalose, amino acids, dextrans, sucrose, etc.). The penetrating cryoprotectant agents (CPAs) rearrange the lipid and protein molecules of the plasma membrane and increase the fluidity and dehydration of membrane and minimize the formation of intracellular ice (IIF), ultimately giving better survivability of spermatozoa (Holt, 2000). The penetrating CPAs also dissolve sugars and salts present in the freezing extender (Purdy, 2006). The role of non-penetrating CPAs is to protect the spermatozoa extracellularly (Aisen et al., 2000). These CPAs may affect, the sperm plasma membrane acting either as a solute or by reducing the temperature during freezing to control the formation of ice in the extracellular surroundings (Amman, 1999; Kundu et al., 2002).

Phosphate-buffered egg yolk (Phillips, 1939) and citrate-buffered egg yolk extenders (Salisbury et al., 1941) were initially used for cryopreservation of bovine spermatozoa. In the later years, glycerol was discovered as the most successful cryoprotectant by Polge et al. (1949) and is still favored in cryobiology. Davis et al. (1963) introduced tris-buffered egg yolk-glycerol extender for cryopreservation of both fresh and frozen-thawed bovine spermatozoa.

1.4.4. Diluents

Different workers have used different diluents with varied success. Dhami and Sahni (1993) found that both tris and milk-based extenders were equally effective for cryopreservation of bovine spermatozoa. Among the soy lecithin (Botu-Bov®-Lecithin (BB-L), egg yolk (Tris, TRIS-R) and Botu-Bov®(BB extenders, lecithin-based medium provided better protection against the lipid peroxidation, resulting in higher pregnancy rate (Crespilho et al., 2014). Akhter et al. (2010) made a comparison of the effect of using Bioxcell® and tris-citric egg yolk as cryodiluent with 4 h equilibration period. Amirat-Briand et al. (2010) found that *in vivo* fertility of bull sperm frozen either with low density lipoproteins (LDL, 59.2%) or tris-egg yolk (20%) provided similar results after artificial insemination. Ansari et al. (2011)

observed that 0.5 mM butylated hydroxytoluene in tris-citric acid extender increased the spermatozoa motility, viability and integrity of plasma membranes in Sahiwal bull. Gloria et al. (2014) demonstrated that the addition of ceftiofur/tylosin and ofloxacin in bull semen extenders offers greater protection against bacteria. According to Celeghini et al. (2008) Botu-Bov® extender is superior to Bioxcell® for cryopreservation of the spermatozoa motility and membrane integrity. Bilodeau et al. (2001) demonstrated that the harmful effects of egg yolk in the extender can be minimized by supplementation of thiols comprising glutathione (GSH/GSSG), cysteine, N-acetyl-L-cysteine (NAC) and 2-mercaptoethanol in low doses. Ansari et al. (2012) showed that glutathione acts in a concentration dependent manner for improving the cryopreservation of buffalo spermatozoa, while *in vivo* fertility was better with 2.0 mM treatment. Akhter et al. (2011) found that the addition of 10% LDL in freezing extender greatly enhances both the *in vitro* quality and *in vivo* fertility of buffalo spermatozoa. Ansari et al. (2014) found that the application of 1.0 mM thioglycol in freezing extender increases the motility, plasma membrane and DNA integrity of buffalo spermatozoa. Taşdemir et al. (2013) also observed that the addition of 6% glycerol in freezing extender is more efficient as compared to the ethylene glycol (EG) or dimethyl sulfoxide (DMSO) for cryopreservation of Eastern Anatolian red bull spermatozoa. Kumar et al. (1992) suggested that lecithin and lipoprotein contents in egg yolk assist in the cryopreservation of the lipoprotein layer of the spermatozoa.

1.4.5. Cooling

Higher cooling rates are considered as essential for successful freezing of spermatozoa, maximal recovery of viable and functional cells using a model sample (Kumar et al., 2003). Slow cooling of semen straws from 30 to 5°C in 2 h in comparison with faster cooling (1 h) or low starting temperature (10°C) and equilibration period of 2 h at 5°C has been establishd to be successful for cryopreservation of buffalo spermatozoa (Dhami et al., 1996). The cooling phase of cryopreservation is linked with the semen quality at post-thawing (Muino et al., 2009).

1.4.6. Equilibration

Equilibration is an essential step during cryopreservation for preserving motility and integrity of sperm cell membranes (Andrabi, 2014). Equilibration for 4 h resulted in the greatest sperm survivability in the case of buffalo (Tuli et al., 1981; Andrabi, 2009; Shahverdi et al., 2014). However, longer equilibration periods with variable success have also been reported in different species; for instance 5 h in goat (Deka and Rao, 1986) and buffalo semen (Fabbrocini et al., 2000), and 4 h in bulls (Leite et al., 2010). Equilibration is an important phenomenon for optimizing the handling conditions during fieldwork (Andrabi, 2014). An equilibration period between 2 and 9 h is appropriate for freezing the epididymal sperm from African buffalo using Triladyl™ extender which appears superior to AndroMed®, (Herold et al., 2006).

1.4.7. Thawing rate

In general, thawing of bovine semen is recommended in a water bath at 33°C or 35°C for 30 sec or 40 sec (DeJarnette and Marshall, 2005). Many studies have evaluated an array of thawing rates for bull sperm and have found that faster thawing rates furnish superior spermatozoa motilities and acrosomal integrity in bull semen: 70°C for 5-6 sec (Rastegarnia et al., 2013; Muino et al., 2008), 75°C for 7 sec (Rodriguez et al., 1975) and 65-70°C for 6-7 sec (Lyashenko, 2015). Thawing rate of 60°C for 15 sec has shown to provide better out-put and longevity at post-thawing and it could improve fertilization potential of buffalo spermatozoa (Dhami et al., 1996).

1.5. Spermatozoa evaluation techniques/assays

A brief account of semen assessment techniques and assays used in different studies is given below.

1.5.1. Computer assisted semen analysis (CASA)

The development of computer assisted semen analysis (CASA; Fig. 1.1) (Amann and Hammerstedt, 1980) on the basis of sperm head movements has facilitated the accuracy and dependability of sperm motility evaluation superior to the conventional

subjective. CASA systems can also analyze sperm concentration, motility, viability (World Health Organization, 2010), and morphology (An et al., 2011). The robust sperm analyzer CASA systems can be used effectively to compare the modifications in spermatozoa motion, velocity and kinematics both prior to freezing and after freeze-thawing of the samples (Liu et al., 2004; Amann and Katz, 2004).

Fig. 1.1. CASA system (CEROS, version 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA) at Animal Sciences Institute, NARC, Islamabad, Pakistan.

The terms used in CASA system are illustrated in Fig. 1.2 (Partyka et al., 2012). Terminologies used in the CASA system include:

Total motility (TM) is the ratio of motile cells to the total concentration of spermatozoa (Mortimer, 2000). Progressive motility (PM) is the sperm number expressed as percentage moving with path velocity (VAP) higher than the medium VAP cut-off and with straightness (STR) higher than the standardized level (Mortimer, 2000). Sperm velocity parameters are rapid velocity, medium velocity, slow velocity and static velocity. Average path velocity (VAP, µm sec^{-1}) is the time averaged velocity of a spermatozoa head along their average path. Straight line velocity (VSL, µm sec^{-1}) is the time averaged velocity of spermatozoa head along the

straight line between their first and last detected positions, respectively. Curvilinear velocity (VCL, μm sec^{-1}) is the time averaged velocity of spermatozoa head along their actual curvilinear path. Amplitude of lateral displacement of a sperm head (ALH, µm) is the magnitude of lateral displacement of a sperm head about its average path, which can be expressed as a maximum or an average of such displacements. Beat cross frequency (BCF, Hz) is the average rate at which curvilinear path crosses the average path. Straightness (STR, VSL⁄VAP) refers to the linearity of the average path. Linearity (LIN, VSL⁄VCL) refers to the linearity of a curvilinear path, (Mortimer and Swan 1999; Lu et al., 2013; Amann and Waberski, 2014).

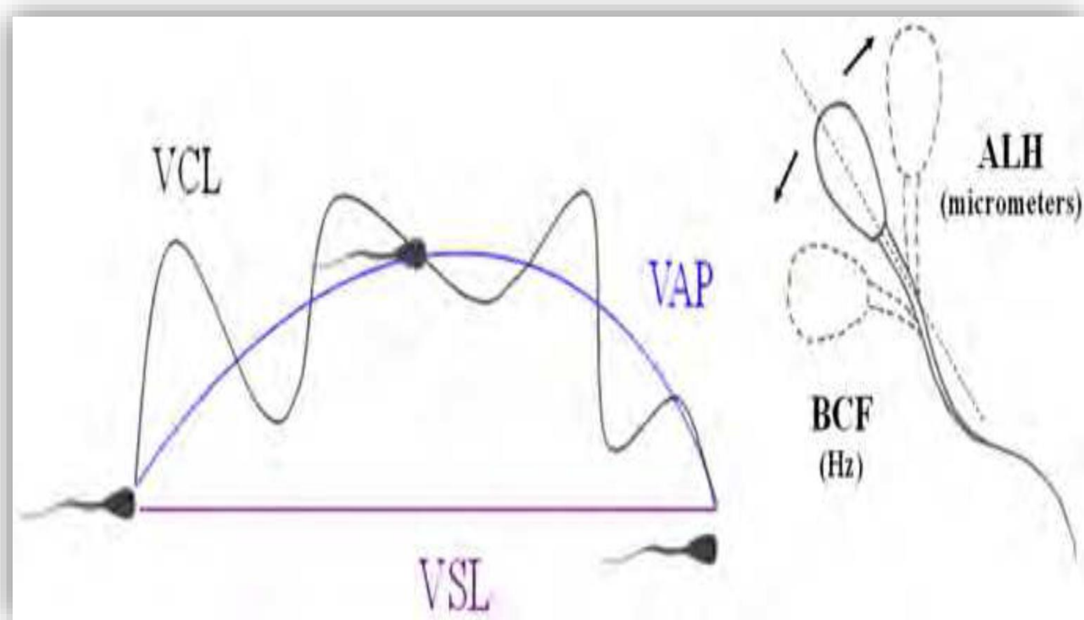

Fig. 1.2. CASA system: Sperm velocities and motion parameters (Partyka et al., 2012).

Several studies have been carried out to compare analyses on manual methods of semen quality with those determined by CASA (Hu et al., 2006; Akashi et al., 2010; Tomlinson et al., 2010; Vested et al., 2011). Liu et al. (1991) and Akashi et al. (2010) have found remarkable correlation between these subjective and objective evaluation methods. Conversely, Spiropoulos (2001) and Vested et al. (2011) reported significant differences among evaluation of sperm parameters in both methods. Some studies have also reported a weaker correlation between ALH and BCF (Ge et al., 2008; Akashi et al., 2010). Different studies revealed correlations of CASA variables (Rapid velocity, VAP, VSL, VCL, ALH, STR, LIN) with *in vitro* fertilization in different

species (Ren et al., 2004). Other studies have however reported a positive correlation among CASA variables particularly the VSL and *in vivo* fertility (Kathiravan et al., 2008).

Many factors can influence the accuracy and precision of CASA output values; hence there is a need to validate all hardware and software settings of the system with the samples and technician(s), and be revalidated on a regular basis. It is also important to determine the appropriate subgroup limits for each measure of sperm motion in typical samples being evaluated, and to use these boundaries in order to visualize and summarize subpopulations of sperms in each sample analyzed, and do not rely on means or medians (Amann and Waberski, 2014). All of the commercial computer assisted sperm analysis (CASA) systems acquire a frame rate (FR) of 30–60 Hz; a higher FR is required to give the estimation output nearer to the ‘‘real path’’ chiefly for fast nonlinear spermatozoa (Mortimer, 2000; Castellini et al., 2011). The depth of the counting chamber is particularly important during evaluation of the motion and kinematic parameters of spermatozoa (Mortimer, 2000; Lu et al., 2014). Other factors that affect CASA motility output include number of fields, concentration and dilution of semen samples (Verstegen et al., 2002; Arruda et al., 2003; Celeghini et al., 2008; Anzar et al., 2010; Partyka et al., 2012; Kumar et al., 2015).

1.5.2. Sperm supra-vital plasma membrane integrity

Following motility, plasma membrane integrity is considered as a vital parameter for the assessment of viability of spermatozoa. Supra vital staining or hypo-osmotic swelling assays are employed for the judgment of sperm plasma membrane structural and functional integrity (Correa and Zavos, 1994). In the supra vital stain, the sperm cells are regarded as viable for the reason that stains cannot pass though the plasma membranes due to their macrcomolecular size. It is because of this reason that the sperm cells in which stains have penetrated are considered as non-viable (Gao et al., 1997).

The composition of phospholipids, water and ion channel proteins and cytoskeletal constituents determine the level and permeability of water (Elmoazzen et al., 2009). The permeability of plasma membrane is also modified by cryoprotective agents

during cryopreservation as these agents increase membrane permeability to water, thereby enhancing cellular dehydration during cryopreservation of cells (Devireddy et al., 2002; Xu et al., 2014). The integrity of plasma membranes is an essential criterion to preserve functions of spermatozoa during storage in the female reproductive tract and until their penetration into the oocytes (Holt, 2000).

1.5.3. Sperm mitochondrial trans-membrane potential

One of the disadvantages of semen cryopreservation is that mitochondrial genes are not preserved, as sperm mitochondria do not contribute to the offspring's mitochondrial population (Woelders et al., 2012). Mitochondrial membrane potential is regarded as the most vital sperm assessment assay (Wang et al., 2003). It varies greatly between animals and groups, and has a significant correlation with the cleavage rate of oocyte (Selvaraju et al., 2008). During cryopreservation, modifications in the ultra structure of mitochondria result in vanishing of the internal mitochondrial structure of spermatozoa after freezing and thawing (Watson, 1995).

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) is a sensitive stain in monitoring sperm mitochondrial function independently (Garner and Thomas, 1999). JC-1 has a changeable fluorescence emission depending upon the change in mitochondrial membrane potential ($\Delta\Psi$). As the membrane polarization occurs, a shift in JC-1 from ~530 nm (monomeric, M- aggregates) to ~590 nm (J-aggregates) takes place at 488 nm excitation. This results in reversible shift of color from green to greenish orange after greater polarization of mitochondrial membranes (Cossarizza and Salvioli, 2001). JC-1 has been used in different studies for the evaluation of mitochondrial membrane potential of spermatozoa among bulls (Garner et al., 1997), ram (Martinez-Pastor et al., 2004), boar (Trzcińska et al., 2008) and stallion (Ortega-Ferrusola et al., 2009).

1.5.4. Sperm viability and acrosome integrity

Stains that have been used for acrosomal visualization of the acrosome include Giemsa (Didion and Graves, 1986), naphthol yellow S/erythrosin B (Bryan and Akruk, 1977), and rose Bengal bismarck brown (Talbot and Chacon, 1981). Giemsa

solutions are well known for their variable nature (Bryan and Akruk, 1977), while variation exists in the intensity of rose Bengal staining of spermatozoa obtained from different bulls (Didion and Graves, 1986).

Spermatozoa can be distinguished into different categories by observing their heads (live or dead), tails (intact, lost or damaged) and acrosomes (Boccia et al., 2007). The vital stain Trypan blue detects live and dead spermatozoa while Giemsa detects the functionality or non-functionality of an acrosome (Kovács and Foote, 1992). This procedure has been effectively used in water buffalo spermatozoa for the evaluation of livability and acrosome status (Presicce et al., 2003). Moreover, Trypan-blue/Giemsa as dual staining can also be used in buffalo for an early selection of the bulls before *in vitro* fertilization (Boccia et al., 2007).

The acrosome develops at the anterior mid of sperm head. The acrosome which is a cap-like structure originates from the Golgi body. The development of acrosome completes at the time of maturation of germ cells during the process of spermiogenesis (Neri et al., 2014). Acrosomal integrity reflects not only the sperm-fertilizing capability but also the cellular damage that might have taken place during cryopreservation (Saacke et al., 1980).

Acrosomal defects are linked with atypical spermiogenesis, sub-fertility and infertility (Meschede et al., 1996). The spermatozoa with damaged acrosome are unable to attach and penetrate the zona pellucida of the oocyte (Thundathil et al., 2000), and are inclined to an early capacitation and casual acrosome reaction (Thundathil et al., 2002). Buffalo sperm acrosome is similar to other species and contains ample hydrolytic enzymes (Kaur et al., 1976). This is mostly substantial for a spermatozoon as it has to sustain an intact acrosome up to binding to oocyte's zona pellucida and undergoing of the acrosome reaction to release acrosome enzymes and thus leading to fertilization (Graham and Moce, 2005).

1.5.5. Acridine orange staining for DNA integrity of sperm

The DNA integrity is another vital parameter for the evaluation of sperms during cryopreservation. The chromatin structure is normally assessed for evaluation of the quality of spermatozoa (Evenson and Jost, 2000).

With respect to the sperm fertility, staining of mammalian spermatozoa with acridine orange has been used to investigate the chromatin structure of the sperm nucleus (Kosower et al., 1992). Martins et al. (2007) have assessed DNA integrity through the acridine orange assay. They reported that spermatozoa with normal DNA emitted a green fluorescence while abnormal DNA emitted red fluorescence. The intercalation of acridine orange takes place into double stranded DNA as a monomer; thereby it binds to single stranded DNA in the form of an aggregate. Monomeric acridine orange excites in the range of 470 nm to 490 nm and binds to double-stranded DNA; thus gives out green fluorescence at 530 nm maximal emission. In contrast, the acridine orange which is aggregated at single-stranded DNA produces red fluorescence at an approximately 640 nm (Peacocke, 1973). It is for this reason that the acridine orange has been employed to distinguish native, double-stranded DNA from denatured, single-stranded DNA in individual cells and to follow the *in situ* vulnerability of the DNA to denaturation by heat (Darzynkiewicz et al., 1975). At low pH, removal of histones by acid enhances acridine orange binding to DNA and vulnerability of the DNA to denaturation (Darzynkiewcz et al., 1975).

There exists possibility that an oocyte will be fertilized with a sperm containing damaged DNA, but epigenetic regulation of the embryo at early stages is perturbed and its further development is blocked (Lewis and Aitken, 2001). Sperm DNA is arranged in an explicit mode that maintains the chromatin in the nucleus compact and steady, nearly in a crystalline position (Fuentes-Mascorro et al., 2000).

1.6. *In vivo* fertility

Artificial breeding is the most established method for the improvement of livestock (Andrabi, 2014). One major advantage of artificial insemination (AI) is that a male can serve for multiple pregnancies throughout the year (Foote, 2002). A successful AI program is however immensely associated with the better storage of semen in straws. Artificial insemination programs have proved very effective in culminating the venereal diseases (Foote, 1999). The fertilization potential is regarded as the most excellent parameter to assess the quality of frozen–thawed spermatozoa (Vale, 1997; Andrabi, 2009). The most common problems concerning AI in buffaloes are however poor conditions of hygiene, difficulty in the detection of estrus and small size of the

uterine body (Drost, 2007; Perera, 2011), method and time of insemination (Andrabi, 2014). A pregnancy outcome greater than 50% is usually considered reliable after AI with frozen thawed buffalo semen (Vale, 1997).

1.7. Curcumin (Diferuloylmethane)

Turmeric, obtained from the rhizomes of a plant *Curcuma longa,* is used as a flavoring and coloring agent as well as a preservative (Cousins et al., 2007). The constituents of turmeric include protein (63%), fat (5.1%), minerals (3.5%), carbohydrates (6.94%), moisture (13.1%) and essential oil (0.8%) (Kapoor, 1990). Curcumin (diferuloylmethane; 2 to 5%), producing yellow color, is the major constituent of turmeric and comprises curcumin (94%), demethoxycurcumin (6%) and bisdemethoxycurcumin (0.3%) (Ruby et al., 1995). Vogel and Pelletier (1815) were the first who isolated the curcumin ($C_{21}H_{20}O_6$). Afterwards, it was crystallized by Daube (1870) and its chemical structure was elucidated by Lampe et al. (1910), who later completed its synthesis (Lampe and I'vlilobedzka, 1913).

Curcumin is although not soluble in water at acidic and neutral pH but it is soluble in polar and non-polar organic solvents and in alkali or in highly acidic solvents such as glacial acetic acid (Tonnesen et al., 2002). The solubility of curcumin therefore appears to be pH dependent for the determination of its concentration (Hung et al., 2008). Curcumin degrades quickly at pH level above 7.0 and adsorbs against the walls of container (Oetari et al., 1996). It is stable up to 70°C temperature when exposed upto 10 min and decomposes at 70°C (Wang et al., 2009).

The fluorescence and absorption spectra signify that curcumin absorbs radiation between 300-400 nm. This permits to absorb UV-radiation and this is how it provides protection to cells and organisms against harmful effects of powerful cancer causing agents, such as ROS (superoxide anions, peroxyl radicals, and hydroxyl radicals) and NOS (nitric oxide and peroxynitrite compounds) (Masek et al., 2013). Curcumin rapidly binds to lipid bilayers and then causes thinning of plasma membrane. It further affects the flexibility of lipid bilayers of plasma membranes (Ingolfsson et al., 2007).

1.7.1. Curcumin as antioxidant

The non-enzymatic antioxidant procedure of the phenol based materials is believed to occue in two steps:

S–OO°+ AH→ SOOH+ A° (Step 1)

A° + X° →Non radical materials (Step 2);

Wherein S is the substance which will be oxidized, AH, a phenolic antioxidant, A°, antioxidant radical, and X°, new or same radical**,** as A°. After dimerization of A° and X°, non-radical product is formed (Frankel, 1998).

An antioxidant in low amounts, considerably, holdups or stops oxidation of cellular proteins, lipids, carbohydrates and DNA. Curcumin has stronger antioxidant properties than the vitamin E (Zhao et al., 1989). Due to the presence of β-diketone moiety, curcumin exhibits keto–enol tautomerism, which provides curcumin an additional chemical functionality (Baum and Ng, 2004). It is present in enol form in solutions and in solid phases (Pederson et al., 1985). The major enol form makes the midsection of the molecule capable for donation and acceptance of hydrogen bonds. The enol form additionally is responsible for chelating the positively charged metals, which are mostly present at the active sites of target proteins (Baum and Ng, 2004). The phenolic and methoxy groups on phenyl rings and diketones are the major structural traits of curcumiods responsible for provision of antioxidant properties (Masuda et al., 2001; Priyadarsini, 2009). Two phenolic OH occupy a main position in the antioxidant activity for both of the curcumin's enol isomer (CurE) and keto isomer (CurK) tautomers (Barzegar, 2012). Both the CurE and CurK isomers are able to reduce as a minimum two free radicals by two likely labile phenolic O–H sites of reaction. CurK isomer reacts by H-atom transfer mechanism but CurE reacts mainly by electron transfer. As CurK and CurE tautomerism equilibrium exists in neutral conditions in the cells, both H-atom and electron transfer mechanisms assist in loweing the free radicals (Jovanovic et al., 1999; Barzegar, 2012).

Curcumin is an effective reactive oxygen species (ROS) scavenger, a cryoprotective and anti-oxidative (Rashid and Sil, 2015). The role of curcumin as regards its ROS scavenging ability during cryopreservation of buffalo spermatozoa remains to be elucidated.

1.7.2. Curcumin as cryoprotectant

The fluorescence and lipophilic properties of curcumin (Tonnensen et al., 1995) offered the foundation for exploring its cellular localization, local effects on the structure and function of cellular membranes. Curcumin has been applied to a range of cell systems against ROS-induced damage mainly because of its extremely well defined cryoprotective and antioxidative roles against cold shock and oxidative damage (Mathuria and Verma, 2008).

Curcumin has been used as antioxidant both *in vitro* and in animal models (Braga et al., 2003). Curcumin has shown to scavenge the free radicals (peroxides) and phenolic oxidants; it hinders lipid peroxidation stimulated by chemical agents (Reddy and Lokesh, 1994), and also inhibits iron-dependent lipid peroxidation in rat tissues (Sreejayan and Rao, 1993). *In vitro* studies showed that curcumin may thwart oxidative damage to DNA (Subramanian et al., 1994) and that it acts as an effective scavenger of nitric acid (Sreejayan and Rao, 1997).

Addition of curcumin at the concentration of 2.5 mM to Angora goat increased the spermatozoa motility and superoxide dismutase (SOD) activity as compared to the control (Bucak et al., 2010). Addition of both curcumin and dithioerythritol at the concentration of 0.5 mM in the extender offered better membrane protection over non-treated bovine spermatozoa (Bucak et al., 2012). At post thaw, supplementation of 10 μM curcumin provided better protection to plasma membrane from the free radical and lipid peroxidation (LPO) to boar sperm as compared to control (Jeon and Kim, 2013). Both ascorbic acid and curcumin ameliorated the harmful effects of arsenic on libido and semen characteristics of rabbit bucks (El-Seadawy et al., 2014). Furthermore, in Merino rams supplementations of 1 mM and 2 mM curcumin provided better sperm plasma membrane integrity than other experimental groups (Omur and Coyan, 2016).

Treating curcumin along with aflatoxin improved the aflatoxin-induced decrease in sperm count, immobilization, viability, and the morphologic characteristics of the sperm in mice (Mathuria and Verma, 2008). In addition, application of variable curcumin doses (20, 40 and 80 mg kg^{-1}) to mice resulted in better recovery after

inducing the testicular injuries by the scrotal hyperthermia which indicated that curcumin has the potential to prevent male infertility (Lin et al., 2015). According to Ganiger et al. (2007), feeding of Wister rats at curcumin in concentrations of 1500, 3000 and 10,000 ppm showed no toxic effects on fertility or pregnancy and neither any distortion was noticeable in their offsprings. Curcumin provided positive effect at 30 mM and 60 mM supplementations in the blood of male Wistar rats by activating antioxidant enzymes, reducing lipid peroxidation (TBARS) and protein oxidation, (Hsieh et al., 2014). The binding of curcumin to DNA occurs through the hydrogen bonding interactions with the minor groove in AT-rich regions and not by intercalation of the phenyl rings (Nafisi et al., 2009). Curcumin in the presence of Cu (II) caused strand cleavage in DNA through ROS generation, mainly the hydroxyl radical (Ahsan and Hadi, 1998). The structure of curcumin and its derivatives (desmethoxycurcumin and bisdesmethoxycurcumin) seem to protect the DNA against singlet oxygen ($^1O_2$) (Subramanian et al., 1994). Addition of curcumin during cooling phase of the cryopreservation improved rat sperm motility, viability, DNA integrity and TAC level after thawing (Soleimanzadeh and Saberivand, 2013). Rashid and Sil (2015) also found that DNA damage was restored in testes of rats by curcumin and it also protected the cells from apoptosis by its antiapoptotic activity.

In contrast to the positive effects of curcumin as a cryoprotectant or through oral administration, a concentration-dependent decrease in forward motility of both human and mouse sperms has also been reported. Curcumin affects sperm forward motility starting at 100 µM concentration, with a complete block at ≥200 µM concentration within 5-10 min in both human and murine sperms (Naz, 2011; 2014). The exposure of buffalo zygotes to a high dose of curcumin (20 µM) during *in vitro* culture (IVC) resulted into injurious effects on developming embryos (Shang et al., 2013). Moreover, curcumin not only inhibits oocyte maturation and disrupts spindle structure but also promotes injurious effects on *in vitro* fertilization and embryonic development in mouse (Huang et al., 2013). A study in zebrafish embryos treated with curcumin caused the developmental defects (Wu et al., 2007).

Buffalo spermatozoa contain abundant polyunsaturated fatty acids and hence are more prone to oxidative damage as compared to bovine sperms (Nair et al., 2006; Andrabi, 2014). On the basis of both positive and negative effects of curcumin utilization, it is

necessary to confirm its efficacy in the freezing protocol for improving *in vitro* quality of buffalo spermatozoa.

1.7.3. Whole chicken egg yolk (WCEY) and chicken egg yolk plasma (EYP) as cryodiluents

In order to improve on the quality of buffalo semen, various cryopreservation strategies are tried for optimization of the protocols. The central dogma of sperm freezing protocols and to use appropriate extenders is to avert fatal development of intracellular ice crystals and to minimize thermal shock (Andrabi, 2014). Moreover, cellular dehydration, increased solute concentrations and osmotic shock during and after the course of cryopreservation are also considered (Morris et al., 2006).

Hen's egg yolk possesses highly complicated lipid–protein-based emulsifier system (Dickinson, 1998). Egg yolk contains about 50–52% total dry matter. About 80% of the dry matter is present in the water-soluble plasma fraction and 20% is water insoluble granules (Burley and Vadehra, 1989). Low density lipoproteins (LDLs) of yolk provide protection to spermatozoa during cryopreservation (Moussa et al., 2002; Bergeron et al., 2004; Bergeron and Manjunath, 2006). It has been shown previously that the extenders based on 6-10% LDL enhanced the quality of frozen thawed bull spermatozoa as compared to the standard media (tris-citric acid-glucose/fructose) used in egg yolk (Hu et al., 2011).
Hen egg yolk plasma contains 85% LDLs and 15% globular glycoproteins, mostly α-, β and γ-livetins, also referred to as immunoglobulin Y (Anton, 2013; Strixner and Kulozik, 2013). Various studies (Anton et al., 2003; Anton, 2006) have reported that LDL micelles are globular elements with a diameter of about 20–60 nm and with a lipid center stabilization by an outer layer of phospholipids and apoproteins, called lipovitellenins. It is presumed that the predecessor of LDL micelles, the very low density lipoprotein (VLDL) complexes possess a 80 to 350 nm diameter and which exist in the egg yolk plasma fraction (Sirvente et al., 2007). It has been reported that the density of LDL aggregates is in the range of 0.908 g/ml (Li-Chan et al., 1995) and 1.063 g/ml (Hevonoja et al., 2000).

McBee and Cotterill's (1979) method of egg yolk fractionation is exercised at analytical level which includes group-wise centrifugation at high rates (10,000 ×*g*), extended lodging times (45 min) and low product output (Anton et al., 2003; Laca et al., 2015). In contrast, Corlay et al. (1991) described continual centrifugal separation of liquid egg yolk to attain granules and plasma fractions at a maximum force of 5,000 ×*g*.

Egg yolk provides resistance to spermatozoa against cold shock, in connection with the other components (Phillips, 1939). Addition of egg yolk to extenders is a time consuming process mainly due to manual separation at analytical scale. The egg yolk in semen freezing extenders contains granules having a particle size range of 0.8 to 2.0 µm (Chang et al., 1977) under normal environmental states of egg yolk (pH 6.5; 0.15 M NaCl). However, large granule particles with 8 µm size have also been received through laser diffraction measurements (Sirvente et al., 2007). These yolk granules not only interfere with the metabolic changes of the spermatozoa but also lower their mobility (Wall and Foote, 1999) and hence it is necessary to replace whole egg yolk by more suitable cryoprotective agents (Akal et al., 2014). After centrifugation of whole egg yolk into granules and plasma fractions, and observed differences between these specify that there is scope for more efficient or more specific application of these fractions as compared to the whole egg yolk (Laca et al., 2015).

The fractionated egg yolk plasma, due to its clear appearance than the whole egg yolk, can sustain the bovine embryo development to the blastocyst phase in a simple salt solution of NaCl, KCl and $NaHCO_3$ with addition of hemicalcium L-lactate (Elhassan et al., 1999). Yolk plasma has been found to be as capable as whole egg yolk for its use in the freezing protocol of stallion semen (Pillet et al., 2011). Corcini et al. (2016) have reported that in case of dog sperms, addition of 20% egg yolk plasma increased the post thaw quality as compared to whole egg yolk in the freezing extender with regard to sperm motility, integrity of membrane and acrosome. To date, hen egg yolk plasma is one of the vital cryoprotectant, but has not been investigated for the enhancement of semen quality in buffalo bull.

1.8. Cryoprotectant Media

1.8.1. Glycerol

The criteria for the selection of most appropriate cryoprotective compounds as regards the freezing protocols possess lesser toxicity, rapid cell permeability and solubility (Andrabi, 2014). Glycerol is favored as the most effective cryoprotective medium for cryopreservation of spermatozoa (Polge et al., 1949). Lovelock and Bishop (1959) suggested that the protecting effect of glycerol is linked with its colligative properties, i.e., it depresses the freezing point and then finally lowers the electrolyte concentrations in the unfrozen fraction at different temperatures. This may help to neutralize the injurious ‘‘solution effects’’ enforced during the freezing procedure. Glycerol is osmotically active and slowly penetrates through the cell membranes. The cell volume changes due to glycerol and water loss during the freezing and thawing stages (Schneider and Mazur, 1984). The precise mechanism whereby glycerol protects cells from freeze-thawing injury is however not completely understood. Towey et al. (2013) postulated that the glycerol drives water toward its structure at relatively high pressure. This resultantly declines its freezing temperature, thus protecting it from formation of ice. The glycerol reduces membrane fluidity of plasma membrane after binding to phospholipid headgroups (Anchordoguy et al., 1992). Glycerol also interacts with the proteins and glycoproteins of the cell membrane (Armitage, 1986). It also modifies the polymerization and depolymerization function of the microtubules (Keates, 1980), thereby protecting the plasma membrane indirectly. Buffalo semen frozen with 6 or 7% glycerol in the extender provided better outcome (Kumar et al., 1992; Abbas and Andrabi, 2002). Glycerol may be added during one step method, i.e., prior to the semen cooling (Andrabi, 2014) or two steps method, i.e., after cooling at 4°C in a separate fraction, and the second dose (being higher than the first) in a cryopreservation protocol (Fabbrocini et al., 2000). Awad and Graham (2002) reported that there was no negative effect on the motility of bull spermatozoa when the glycerol concentration was reduced to 2–6%. A two-step dilution process for the addition of glycerol has also been used in bull semen to minimize both the toxic and osmotic damage, however some other studies have shown little advantage of these additional precautions (Curry, 1995). Rabbit spermatozoa present an exceptional case in which dimethyl sulfoxide (DMSO)

appears to be the cryoprotectant of choice, instead of glycerol (Wales and O'Shea, 1968).

1.8.2. Dimethyl sulfoxide

Saytzeff (1867) first reported the synthesis of dimethyl sulfoxide (DMSO/ Me_2SO, $(CH_3)_2SO$). DMSO is industrially produced by the oxidation of dimethyl sulfide with oxygen or nitrogen dioxide (Roy, 2002). Lovelock and Bishop (1959) discovered the cryoprotective action of DMSO. It now has extensive applications in many fields as a cryoprotectant (Karlsson et al., 1993), penetrator of biomembranes (Yamashita et al., 2000), scavenger of radicals and fusing the cells (Anchordogny et al., 1992).

DMSO penetrates readily into the sperm cell membrane and enters the cytosol through its interaction with the phospholipids (de Baulny et al., 1996). Its capability to decrease the dehydration of the sperm cell impedes the development of intracellular ice crystals (Kundu et al., 2002). Mandumpal et al. (2011) reported that DMSO averts the formation of ice by inducing a widening of the glass transition of water, which in turn probably decreases the probability of its nucleation and the consequential formation of ice upon fast cooling. DMSO prevents freeze damage of the cell membrane through interaction with the water network and it hydrates the cell surface to decrease water volume change between ice and liquid water (Fahy et al., 2004; Cheng et al., 2015).

DMSO prevents freeze damage of the cell membrane by disturbing the water network hydrating the immediate cell surface to minimize water volume change between ice and liquid water that otherwise cannot easily yield to structural changes and cell death during freeze-thaw (Fahy et al., 2004; Cheng et al., 2015). During cryopreservation, DMSO maintains an osmotic equilibrium between the intracellular and extracellular milieu (Fuller, 2003). DMSO provides protection to the cells by moderately solublizing the membrane to minimize its puncturing, and interrupts the ice lattice, thus allowing the formation of fewer crystals (Lovelock and Bishop, 1959). At lower temperature, growing ice makes the cells dense into smaller and smaller pockets of unfrozen liquid. DMSO prevents the formation of smaller ice pockets of unfrozen liquid during lower temperatures. Additionally, DMSO develops larger unfrozen

pockets at any given temperature than they would be if no cryoprotectants were present. Therefore, larger unfrozen pockets for cells reduce damage from both kinds of freezing injury, that is, mechanical damage from ice and excessive concentration of salt (Wowk, 2007).

Although exact mechanisms of cryoprotectant toxicity are not entirely known yet, a few pragmatic generalizations can be developed. Lipophilicity vigorously correlates with toxicity. Molecules for fat affinity can partition into plasma membranes thus making them unstable. It has also been determineed that strong hydrogen bonding links with the toxicity, probably by disturbing the hydration shell around the macromolecules (Fahy et al., 2004).

Vaisman and Berkowitz (1992) have proposed a hydrogen bonding model wherein interaction of one DMSO molecule takes place with two water molecules (1DMSO:2H_2O). The heat generated after addition of DMSO to water specifies the existence of stronger interactions between DMSO and water (Martin and Hauthal, 1971). This denotes that at comparatively elevated molar proportions of DMSO in water, there occurs a preferable hydrogen bonding of water molecules with DMSO. This stronger association between the molecules of DMSO and water results in a greater lowering of freezing in regions which are enriched with water (Fahy, 1980). In case of 1:2 ratio of DMSO : H_2O, there appears a greater widening of the equilibrium freezing level even under -75°C (Rasmussen and MacKenzie, 1968). This quality signifies the cryoprotective feature of DMSO (Yu and Quinn, 1994). Vibrational spectroscopy has demonstrated that at higher concentrations of DMSO, an interruption in structure of water occurs because of the development of the complexes between DMSO and water (Safford et al., 1969). Vaisman and Berkowitz (1992) while studying the molecular dynamics reported that at 2% (w/w) DMSO concentrations, water structure enhanced and at 15% and 52% (w/w), interruption in water structure occurred. Despite the universal cryoprotective attribute of DMSO, two important aspects should also be considered. First, homogeneous nucleation has comparatively much lower temperature than the heteronucleation, thus deterrence of the latter seems to be a vital aspect in improving cryopreservation. Second, the vitrification system is helpful for attaining the successful cryopreservation of cells without the ice formation at lower freezing points (Yu and Quinn, 1994).

1.8.3. Glycerol and DMSO: differences and synergistic approaches

As regards permeation kinetics, differences exist between glycerol and DMSO. It was reported that owing to its low molecular weight DMSO penetrates the cell membranes faster than the glycerol (Lovelock and Bishop, 1959; Purdy, 2006). Depending on concentration in the extenders and also the species glycerol and DMSO can provoke osmotic strain and toxic outcomes on spermatozoa (Holt, 2000; Rasul et al., 2007). The outcome of freezing media on phase transition behavior of the phospholipid systems has provided useful information about intermolecular interactions. The glycerol, sugars and DMSO are likely to lower the bilayer-non-bilayer transition temperatures (Tn), but have modest control on the liquid-crystal to gel phase transition temperatures (Tin) of non-bilayer forming lipids (Tsvetkova and Quinn, 1994; Yu and Quinn, 1994). High viscosity of glycerol leads to better cryopreservation of cells than the DMSO which due to its low viscosity is unable to adjust the structure of the freeze concentrated material at rapid rates of cooling, thus resulting in poor sperm survival (Morris et al., 2006).

Several studies have revealed combined effect of glycerol and DMSO as regards better turnout of sperm motility in bovine (Snedeker and Gaunya, 1970; El-Harairy et al., 2011), ovine (Jones, 1965) and caprine (Kundu et al., 2002; Farshad et al., 2009). However, Rasul et al. (2007) found the antagonistic effect with different combinations of DMSO and glycerol and lethal results with DMSO alone on post-thaw buffalo bull spermatozoa. The study is warranted to minimize the toxic effect of glycerol and DMSO in freezing protocol for improving the semen quality of buffalo bull by adopting different approaches viz., combined treatment effect, suitable treatment concentrations and temperature of treatment addition.

The dissimilarity between glycero1 and DMSO shows that the mechanisms of freezing and thawing injuries and cryoprotection are much complex than Mazur’s two-factor theory would signify. However, the similarities between DMSO and glycerol for providing cryoprotection strengthen Rapatz and Luyet’s categorization of cryoprotective agents into three forms and favor Mazur’s two factor theory of cryopreservation (Pribor, 1975).

The water buffaloes are major source of milk and meat in Pakistan. Despite the use of AI as modern tool, natural mating is still the predominant method of fertilization in buffalo herds. The use of AI in water buffaloes is still marginal due to several reasons. One of the reasons is lack of truly superior progeny tested bulls and unacceptable low pregnancy rates when using natural or synchronized estrus (Zicarelli et al., 1997). The lackness of long time selection for freezability of semen is another reason which affects AI. Above all, buffalo sperms are prone to oxidative stress due to the higher lipid peroxidation levels probably related to the lower activity of antioxidant enzymes (Andrabi, 2014). It is, therefore, of immense importance to develop different freezing protocols which could overcome the cryo-damage that normally occurs during cryopreservation of water buffalo spermatozoa. The success in developing better freezing protocols would improve not only the *in vitro* quality and longevity of spermatozoa but ultimately the fertilization potential of buffalo. Keeping in view these improvements, a series of cryodiluents were investigated in the freezing extenders to achieve the desired quality and productivity for water buffalo bull spermatozoa.

1.9. Aim and Objectives

The overall aim of the study was to improve the *in vitro* quality and fertility potential of buffalo spermatozoa *in vivo* by using different cryodiluents in the freezing extenders.

The following objectives were planned to conduct this study in four different experimental settings:

1. To study the effect of equilibration times (2, 4 and 6 h), freezing (conventional vs. non-conventional) and thawing rates (T1, 37°C for 30 sec; T2, 50°C for 15 sec; T3, 70°C for 7 sec) on post-thaw quality of buffalo bull (*Bubalus bubalis*) spermatozoa.
2. To study the antioxidant effect of curcumin (diferuoyl methane) in tris-citric acid extender on the freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa.
3. To study the effect of chicken egg yolk plasma (EYP) in tris-citric acid extender on cryopreserved quality and *in vivo* fertility of water buffalo bull (*Bubalus bubalis*) spermatozoa.
4. To study the effect of glycerol and dimethyl sulfoxide on cryopreserved quality and *in vivo* fertility of water buffalo bull (*Bubalus bubalis*) spermatozoa.

MATERIALS AND METHODS

2.1 General experiment settings

Five healthy Nili Ravi buffalo (*Bubalus bubalis*) bulls of about 5-6 years of age were used in all the experiments. The bulls were kept at National Agricultural Research Centre, Islamabad, Pakistan under suitable nutritional and housing conditions. The prevalent ethical standards and guidelines of animal handling were followed during the entrie study. The present research work was carried out at National Agricultural Research Centre (NARC), Animal Science Institute, Islamabad, Pakistan.

2.2. Experiment 1: Determination of the effect of equilibration times, freezing and thawing rates on post-thaw quality of spermatozoa

Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa are illustrated in Figure 2.1.

2.2.1. Preparation of extender

Tris-citric egg yolk extender was prepared by adding tris-(hydroxymethyl)-aminomethane (3.0 g, w/v), citric acid (1.56 g, w/v), fructose (0.2 g, w/v), glycerol (7.0 ml, v/v), streptomycin sulphate (0.01 g, w/v), and hen egg yolk 20 % (v/v) in 73 ml distilled water (Experiment 1 and 2). The pH of the extender was adjusted to 7.0. Finally, the extender was centrifuged at the rate of 1515 ×g for 20 min (Experiment 1). After centrifugation, supernatant was used as diluent and negligibly small sediment portion was left over. The extender was stored at −20°C until use (Andrabi et al., 2008a).

2.2.2. Semen collection and processing

Semen collection from five buffalo bulls was carried out in winter (February-March) for a period of five weeks (5 replicates, n= 25 ejaculates; Experiment1) with artificial

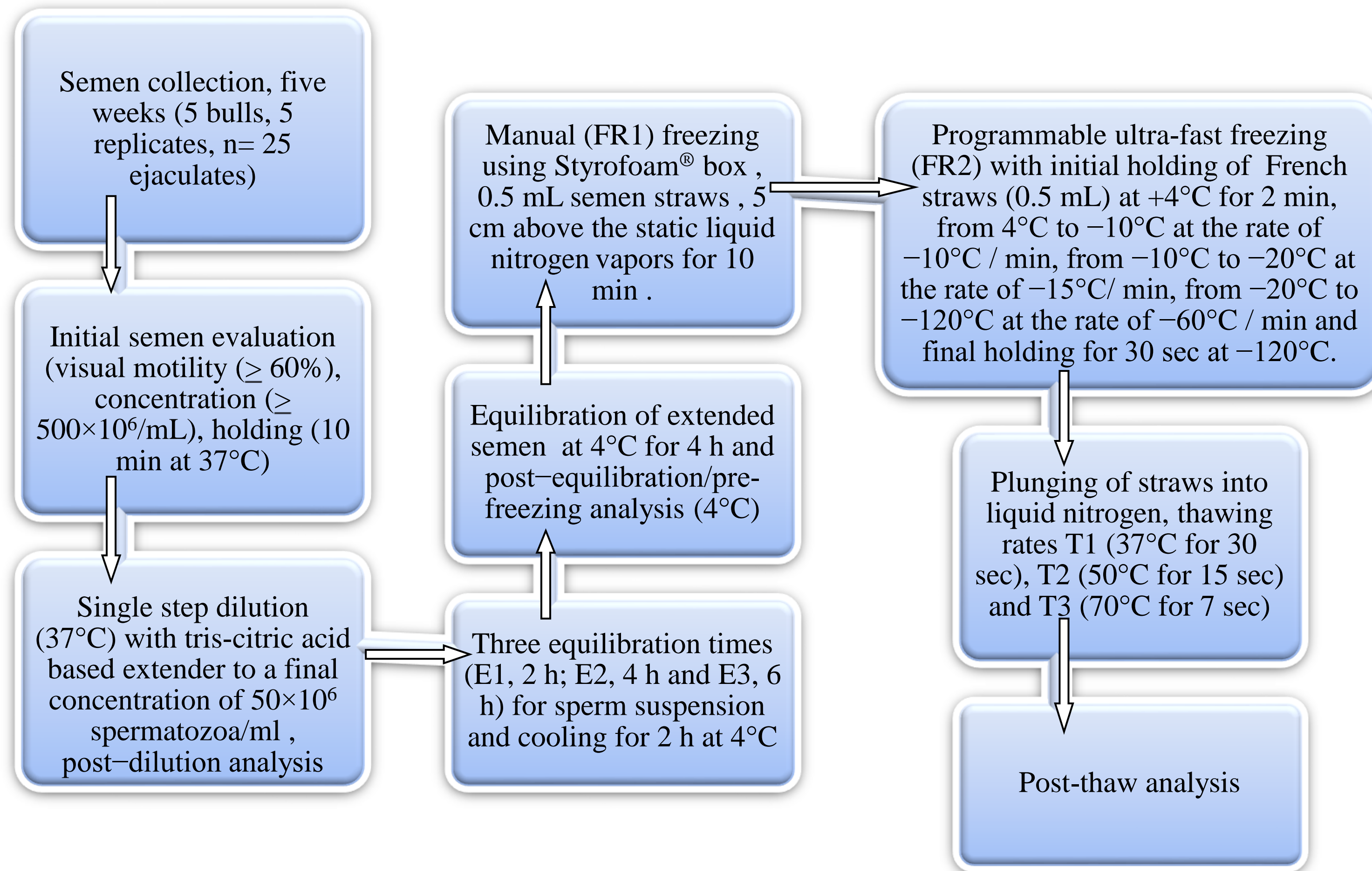

Fig. 2.1. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa in Experiment 1.

vagina (42°C) in a graduated falcon tube. Two consecutive ejaculates with an interval of 10-15 min were collected from each bull once a week. After collection, semen samples were immediately transferred to the laboratory. Sperm progressive motility was evaluated through phase contrast microscope (x 40; BH2, Olympus, Japan) connected with a closed circuit monitor. The concentration of spermatozoa was determined spectrophotometerically (SDM 5, Minitube, GmBH, Germany) at 546 nm. At least one ejaculate from each bull on every collection did qualify (>I ml volume, > 60 % visual progressive motility and > 0.5 x 10^9 sperm/ml concentration) for further processing (Andrabi et al., 2008b; All Experiments). Semen samples from each bull were further sub-divided into three aliquots for one–step dilution (37°C) to a final concentration of 50×10^6 spermatozoa/ml. The sperm suspension was cooled to 4°C in 2 h. The suspension samples of sperms were then subjected to three equilibration times (E1, 2 h; E2, 4 h and E3, 6 h). Cooled sperm suspension samples were frozen either by manual (FR1) or programmable ultra-fast freezing (FR2) methods (Experiment1).

1. Manual (FR1, Experiment 1): 0.5 ml semen straws were kept 5 cm above the static liquid N_2 vapors for 10 min in a Styrofoam® box with a start temperature of approximately 4°C and end temperature of −85°C. The straws were then plunged into liquid nitrogen (LN_2; −196°C).
2. Programmable ultra-fast freezing (FR2, All Experiments): 0.5 ml semen straws were kept in programmable ultra-freezer (Planer Kryo 550−16, Middlesex, UK). The freezing rates were; initial holding of semen straws at 4°C for 2 min, from 4°C to −10°C @−10°C min^{-1}, from −10°C to −20°C @−15°C min^{-1}, and then from −20°C to −120°C @−60°C min^{-1} and final holding time of straws for 30 sec at −120°C. The straws were then plunged into LN_2 (−196°C) after freezing (Shah et al., 2016).

Semen straws were thawed in triplicate from each equilibration and freezing groups at least 24 h after their storage into LN_2 tank. The thawing rates were T1 (37°C for 30 sec; All experiments), T2 (50°C for 15 sec; Experiment 1) and T3 (70°C for 7 sec; Experiment 1).

2.2.3. Semen evaluation

2.2.3.1. Computer-assisted sperm analyzer (CASA; All Experiments)

CASA (CEROS, version 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA) was used for recording the motion, velocity and kinematic variables of spermatozoa (Shah et al. (2016). The standard settings of CASA were: frames acquired, 30; frame rate, 60 Hz; minimum cell size, 5 pixels; minimum static contrast, 30; motile, VAP cutoff, 20 µm sec^{-1}; progressive motile, VAP 80 µm sec^{-1}, STR 80 %; rapid (velocity), VAP > 80 µm sec^{-1}; medium (velocity), VAP > 20 µm sec^{-1} < 80 µm sec^{-1}; slow (velocity), VAP < 20 µm sec^{-1} > 10 µm sec^{-1}; magnification, 1.89; and temperature, set, 37°C. CASA motion, velocity and kinematic variables recorded were total motility (TM, %), progressive motility (PM, %); rapid velocity (RV, %), medium velocity (MV, %), slow velocity (SV, %); VAP (average path velocity, µm sec^{-1}), VCL (curvilinear velocity, µm sec^{-1}), VSL (straight line velocity, µm sec^{-1}), ALH (amplitude of lateral displacement of sperm head, µm*),* BCF (beat cross frequency, Hz), STR (straightness – VSL: VAP, %) and LIN (linearity – VSL: VCL, %). A drop (5µL) of sperm suspension (freshly diluted, cooled/equilibrated (Experiment 1, 2 and 3) and frozen-thawed (all experiments) was loaded into a pre-warmed (37°C) glass slide, cover-slipped and positioned in a portable MiniTherm stage (37°C) of microscope. About 200 sperms in 2-5 fields were analyzed (x 100; CX41, Olympus, Japan).

2.2.3.2. Sperm supra-vital plasma membrane integrity (SV-PMI, %; All Experiments)

Sperm supra vital plasma membrane integrity was evaluated according to Chan et al. (1991), Tartaglione and Ritta (2004) and Shah et al. (2016). An aliquot of sperm suspension (50 µL) for each experimental group was mixed with hypo-osmotic solution [(500 µL; 0.735 g tri sodium citrate, CAS 1545801, Merck, Darmstadt, Germany and 1.351 g fructose in 100 ml distilled water; osmolarity 190 mOsmol/kg)] in falcon tubes, and was then incubated at 37°C for 30−40 min. Equal drops (5 µL) of incubated sperm suspension and eosin solution (0.5%, w/v eosin; CAS 45380, Merck, Darmstadt, Germany) in 2.92% (w/v) tri-sodium citrate dihydrate were then mixed for 10 sec on a slide, and finally cover–slipped. Two hundred spermatozoa per evaluation

period (PD, PE and PT) were counted under phase contrast microscope (x 400; Leica, Leitz Wetzlar GmbH, Germany). The spermatozoa with unstained heads and swollen tails were regarded as structurally and functionally intact, while structurally and functionally non-viable spermatozoa were identified by their pink heads and un-swollen tails.

2.2.3.3. Viable sperm with intact acrosome (%; All Experiments)

Viability and acrosome integrity of spermatozoa was evaluated with Trypan blue (CAS 23850 Merck, Darmstadt, Germany and Giemsa (CAS 106-22-9, Merck, Darmstadt, Germany) using the staining protocol as described by Kovács and Foote (1992). Equal drops (5 µL) of Trypan-blue solution (0.2%) and sperm suspension were placed on a slide at room temperature and mixed well by using edge of the cover slip. After vertical air-drying of the smears, fixing in formaldehyde-neutral red solution [(86 ml 1N HCl, CAS 1001645-58-4; Merck, Darmstadt, Germany), 14 ml 37% formaldehyde; CAS 51-21-8, Merck, Darmstadt, Germany) and 0.2 g neutral red; CAS 553-24-2, Merck, Darmstadt, Germany)] was undertaken for 2–3 min. After rinsing with distilled water and air–drying, the slides were placed in Giemsa stain (7.5%, w/v) for 2.5 h at 37°C. Later, slides were washed with distilled water, air–dried and mounted with Balsam of Canada (CAS 8007-47-4, Merck, Darmstadt, Germany). Two hundred sperms per period (PD, PE; Experiments 2, 3 and 4 and PT; all Experiments) were evaluated under a phase contrast microscope (x 400; Leica, Leitz Wetzlar GmbH, Germany). Sperms having white head region (Trypan blue negative) and purple acrosomal region (Giemsa positive) were classified as viable with intact acrosome, whereas sperms having blue head region (Trypan blue positive) and pale lavender acrosomal region (Giemsa negative) were considered as nonviable with damaged acrosome.

2.2.3.4. Sperm mitochondrial trans-membrane potential

Mitochondrial trans–membrane potential (MMP, %) was evaluated with JC−1 (CAS 47729-63-5, Merck, Darmstadt, Germany; a lipophilic cationic probe 5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) as described by Ahmed et al. (2016a). JC-1 (20 µL) was added to 100 µL of sperm suspension (5×10^6

total cells) of each experimental group, and then incubated at 37°C for 10 min. Evaluation was performed under an epifluorescence microscope (x 40; 480/550 nm excitation/barrier filter; Nikon Optiphot, Japan) in two hundred spermatozoa per evaluation period (PD, PE and PT). Spermatozoa with orange mid−piece were considered having high mitochondrial transmembrane potential and those with green mid-piece were considered having low mitochondrial transmembrane potential. MMP was evaluated in experiment 1, 3, and 4, respectively.

2.2.3.5. Sperm DNA integrity

DNA integrity (DNA-I, %) was evaluated thourgh acridine orange test as described by Martins et al. (2007). The smears from each experimental group were prepared on glass slides and air–dried. Smears were fixed in Carnoy's solution [methanol (CAS 67-56-1, Merck Darmstadt, Germany) and glacial acetic acid (CAS 64-19-7, Merck, Darmstadt, Germany) in a 3:1 proportion] for 2 h at room temperature instead of overnight as was done by Martins et al. (2007). After air-drying of slides, incubation in tampon solution [(80 mmol/L citric acid and 15 mmol/L $Na_2HPO_4.7H_2O$ (CAS 7782-85-6 Merck, Darmstadt, Germany), pH 2.5)] was carried out at 75°C for 5 min to evaluate if the DNA was intact. Finally, the slides were stainined for 2-3 min with acridine orange (CAS, 65-61-2, Merck, Darmstadt, Germany; 0.2 mg/ml in distilled water), and washed with water to remove background staining. Slides were cover slipped and evaluated in wet condition. Two hundred spermatozoa per period (PD, PE; Experiments 2, 3 and 4 and PT; all Experiments) were evaluated under epifluorescence microscope (x 400; 480/550 nm excitation/barrier filter; Nikon Optiphot, Japan). Spermatozoa with intact DNA emitted green fluorescence while spermatozoa with damaged DNA emitted yellow to red fluorescence.

2.3. **Experiment 2:** To study antioxidant effect of curcumin (diferuoyl methane) in tris-citric acid extender on spermatozoa freezability. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa are illustrated in Figure 2.2.

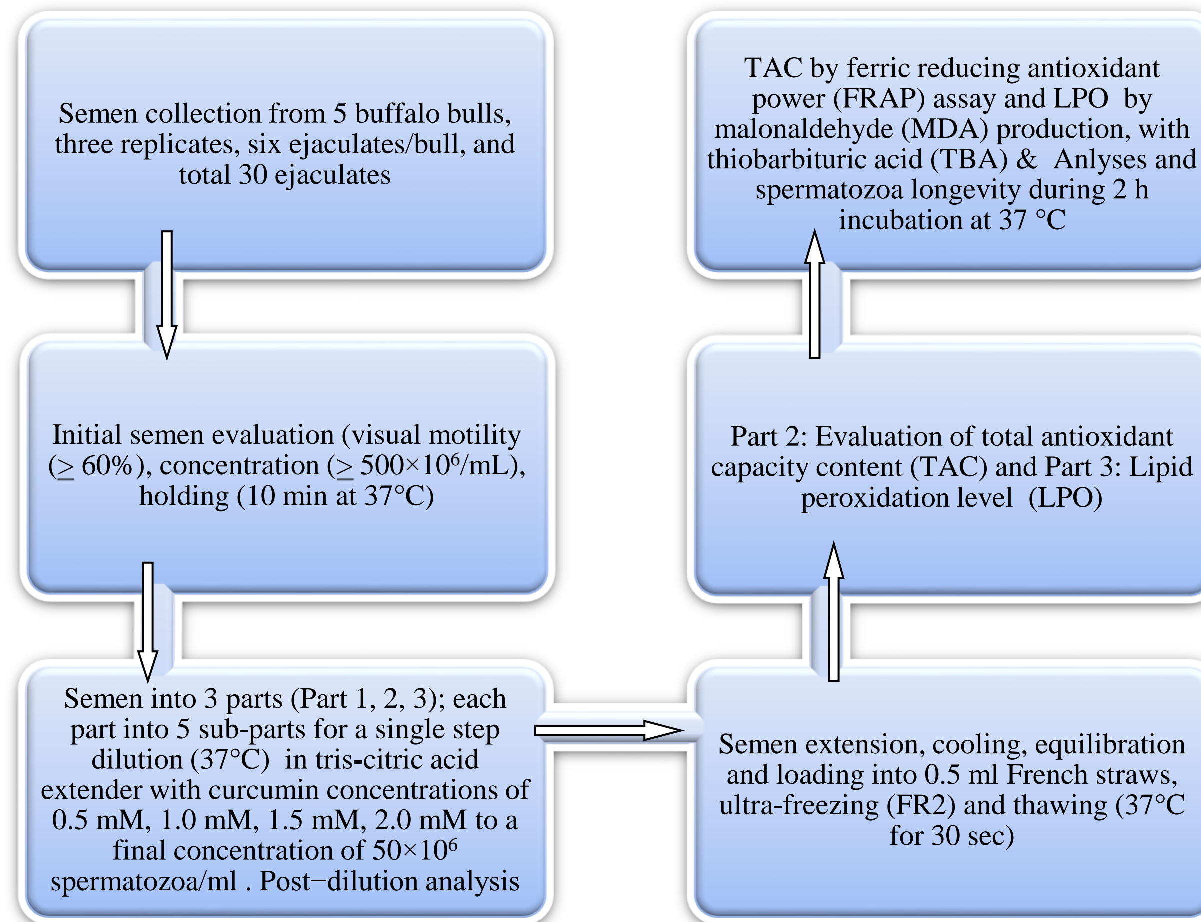

Fig. 2.2. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa in Experiment 2.

2.3.1. Semen processing

Semen collected (Section 2.2.2) from five buffalo bulls with artificial vagina (42°C) during the summer season. Semen collection was undertaken as two consecutive ejaculates per week from each bull (three replicates, six ejaculates/bull, and total 30 ejaculates). Initial evaluation and holding of semen was carried out according to Andrabi et al. (2008b). Semen samples from each bull were aliquoted into three equal parts. Part 1 of the semen samples from each bull were again equally distributed into five aliquots for dilution in tris-citric acid extender containing curcumin (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, CAS 458-37-7, Sigma-Aldrich®, Merck) and control (without curcumin). The semen extension, cooling, equilibration and loading into 0.5 ml French straws, freezing and thawing were conducted as described in Experiment 1 (Section 2.2.2) (Shah et al., 2016). Part 2 and part 3 of the semen samples from each bull were used for the evaluation of total antioxidant capacity (TAC) and lipid peroxidation (LPO) level, respectively. Employing centrifugation (1515 ×g for 10 min), the sperm and seminal plasma of post-diluted (Iqbal et al., 2016a), equilibrated and frozen– thawed semen were separated to obtain the pellets and seminal plasma from each experimental group. The spermatozoa pellets were cleansed with TALP (100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1.0 mM pyruvate (Parrish et al., 1988) by centrifugation at 1515 ×g for 10 min. The cleansed spermatozoa pellets from each experimental group were re-suspended in incubation TALP (sperm TALP with 6 mg ml^{-1} bovine serum albumin) for LPO assay (Garg et al., 2009). The extended seminal plasma was used for TAC content by the ferric reducing antioxidant power (FRAP) protocol (Benzie and Strain, 1996; Kumar et al., 2011; Soleimanzadeh and Saberivand, 2013).

2.3.2. Semen evaluation

All semen quality assays were performed at post−dilution, pre−freezing/post-equilibration and post−thaw stages. CASA, SV-PMI, V-IACR and DNA-I (%) were conducted as described in Experiment 1 (Shah et al., 2016; Sections 2.2.3-2.2.3.5).

2.3.3. Sperm total antioxidant capacity assessment (FRAP Assay)

In order to formulate a FRAP reagent, 300 mM/L acetate buffer, pH 3.6, was combined with 10 mM/L 2,4,6-tri-(2-pyridyl)-1,3,5-triazine, 98.00% and 20 mM/L $FeCl_3 \cdot 6H_2O$ in ratio of 10:1:1. Aliquots of 50 µL spermatozoa of each experimental group were added separately to FRAP reagent (1 ml) in a plastic cuvette. Absorbances were recorded after 10 min at 593 nm on a spectrophotometer after mixing-up against a reagent blank. The change in absorbance was translated into FRAP value (µM) by relating ΔA_{593} of test sample with that of standard solution of known FRAP value using following formula: (0-5min ΔA_{593} test sample/0-5min ΔA_{593} standard)× 2000 (FRAP value of 1 M ascorbic used as standard). The FRAP value of freezing extender utilized in cryopreservation was also evaluated and subtracted from the FRAP value of extended seminal plasma to finally measure the TAC levels of seminal plasma.

2.3.4. Lipid peroxidation (LPO) assay

LPO levels of spermatozoa were evaluated by production of malonaldehyde (MDA), with thiobarbituric acid (TBA) as described by Garg et al. (2009). The spermatozoa pellet attained by centrifugation (1515 ×g; 10 min) was re–suspended in PBS (2 ml; pH 7.2). LPO level was measured after adding 2 ml of TBA– trichloroacetic acid (TCA)–reagent (15% w⁄ v TCA, 0.375% w⁄ v TBA and 0.25 N HCl) to 1 ml of sperm suspension. Boiling water bath was used to process the contents for 15 min. The sperm suspension was centrifuged at 1515 ×g for 10 min soon after cooling. Afterward, the supernatant was separated. The absorbance was measured at 535 nm. The concentration of malonaldehyde was determined by the specific absorbance coefficient (1.56×10^5 mol^{-1} cm $cube^{-1}$) as described by Garg et al. (2009).

$$\text{MDA produced } (\mu mol/ml) = \frac{\text{Optical density (OD)} \times 106 \times \text{total volume (3 ml)}}{1.56 \times 10^5 \times \text{test volume (1 ml)}}$$

$$= \frac{\text{Optical density (OD)} \times 30}{1.56}$$

2.3.5. Longevity of spermatozoa during *in vitro* incubation

The sperm longevity was studied from the output of two CASA variables (progressive motility and rapid velocity) after thawing the treatments at 37°C for 2 h in a water bath.

2.4. **Experiment 3:** To study the effect of chicken egg yolk plasma (EYP) in tris-citric acid extender on cryopreserved quality and *in vivo* fertility of spermatozoa. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa are illustrated in Figure 2.3.

2.4.1. Collection of whole chicken egg yolk (WCEY)

Freshly laid eggs (n=12) from *Nick Chick*-White (commercial hybrid) chicken were collected in a single batch from a commercial farm (Islamabad, Pakistan) and used within 36 h. Before separation of the yolk, eggs were washed in lukewarm water and wiped with disposable cloth towels soaked in 70% ethanol (CAS 64-17-5, Merck, Darmstadt, Germany) for disinfection. The eggs were then placed in a refrigerator at 4°C for 3 h for cooling and compaction of yolk. After cooling, separation of egg yolk was done manually by breaking the shell, placing the yolk on filter paper (Whatman No. 1, Maidstone, UK) and rolling the yolk sac until it was dried. The egg yolk was held between folded filter paper, and yolk sac was squeezed from both sides by applying gentle pressure. As a result, the egg yolk sac was ruptured and the yolk started to flow into the pre-sterilized glass beaker. Some of the egg yolk was left on the filter paper so that the albumen may not contaminate the collected yolk (WCEY). The final volume of the pooled WCEY was 144 ml, which was used immediately for both fractionation (EYP preparation) and direct addition into in the extender.

2.4.2. Preparation of chicken egg yolk plasma (EYP) by fractionation of WCEY

Whole chicken egg yolk (40 ml) was fractionated into plasma by slightly modifying the earlier procedures (McBee and Cotterill, 1979; Pillet et al., 2011). An equal volume (40 ml) of whole egg yolk and 0.17 M NaCl (CAS 7647-14-5, Merck,

Darmstadt, Germany) was stirred by using a magnetic stirrer (Wisestir®, MSH-20A, witeg Labortechnik GmbH, Germany) for 75 min at 4°C in cold cabinet unit (Minitǜb GmbH, Germany). After that, suspension (80 ml) of whole egg yolk and 0.17 M NaCl solution was packed in 50 ml ultra-centrifuge tubes (40 ml/tube) and transferred into a refrigerated centrifuge (Heraeus, Biofuge Primo R, Thermo Scientific GmbH, Germany). The centrifugation was performed at 10,015 ×*g* for 45 min at 4°C. The supernatant (72 ml) was collected and transferred to new 50 ml centrifuge tubes (36 ml/tube) for the second centrifugation at 10,015 ×*g* for 30 min at 4°C. The time-lapse between the first and second centrifugation was about 3 min. The supernatant (70 ml, EYP) was poured into a pre-sterilized 100 ml glass beaker (Pyrex Iwaki, Japan) with 5 cm diameter.

2.4.3. Dynamic UV–C treatment of chicken EYP

The EYP extracted in a glass beaker (5 cm diameter) was treated with dynamic UV–C irradiation in a horizontal laminar flow cabinet (Streamline® Maxwell, Whitehouse, Singapore) which was turned on 60 min in advance. The sample was exposed for 40 min at 25°C to a UV lamp (TUV 30W, Philips, G30T8, Holland) at a distance of 10 cm. For dynamic irradiation treatment (de Souza and Fernandez, 2011), the sample was continuously stirred by using a magnetic stirrer (Wisestir®, MSH-20A). As per manufacturer's instructions, the wavelength of the UV light produced by the lamp was 253.7 nm, with an energy output of UV light 83 µW/cm^2 at a distance of 1 m. Density of UV-C irradiation (E) was calculated as 8300 µW/cm^2, using the formula:

$x = 100\sqrt{N/E}$, where

x = distance in cm

E = effective energy output µW/cm^2 according to the manufacturer's declaration

N = total energy output µW/cm^2 at 100 cm distance according to the manufacturer's declaration. Finally, the dynamic UV-C irradiation dose given to EYP was calculated as 19920000 µW s/cm^2 by applying the following formula:

UV-C dose = UV-C density x time (s)

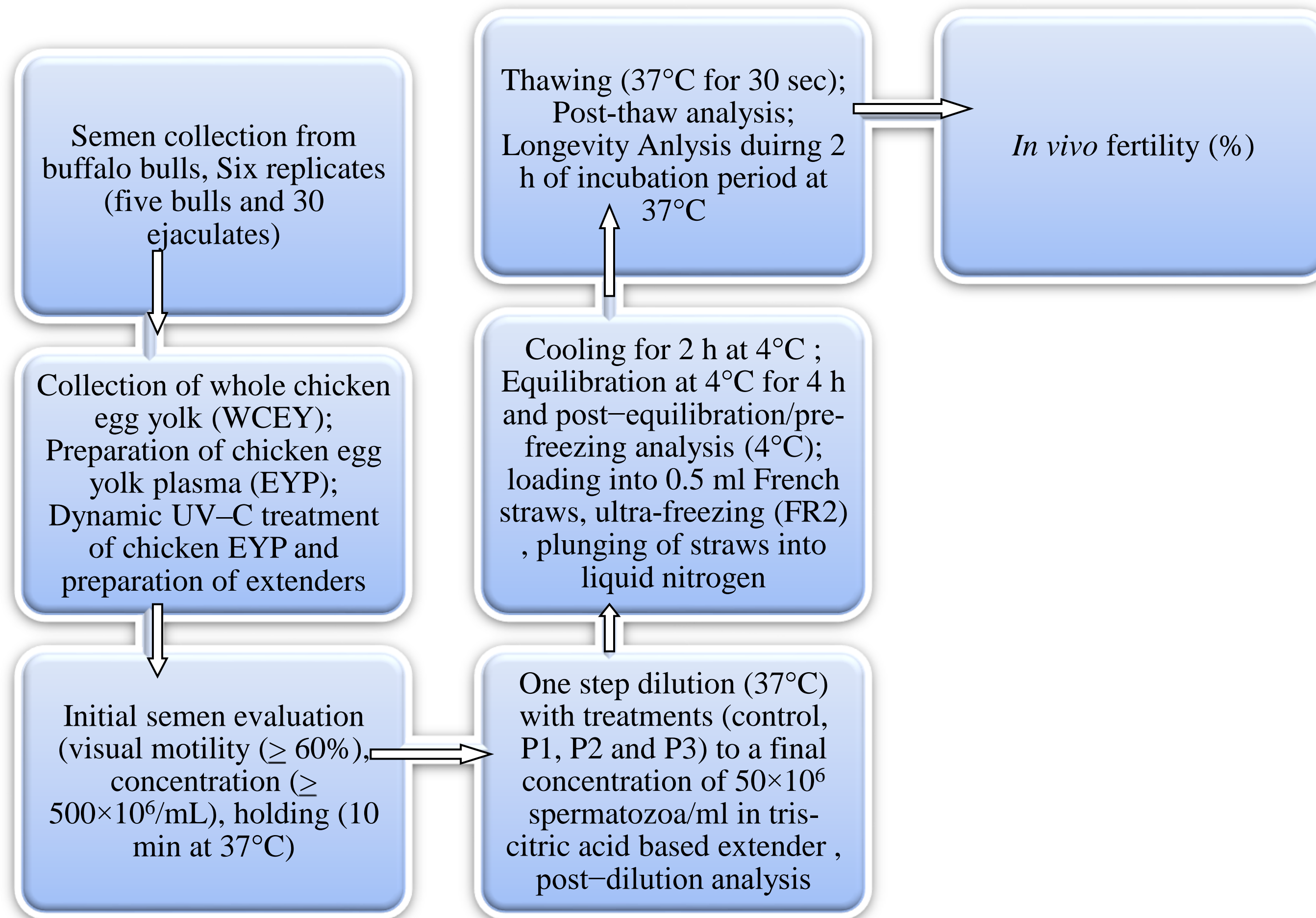

Fig. 2.3. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa in Experiment 3.

2.4.4. Preparation of extenders

A base extender comprising of 15.0 g tris−(hydroxymethyl)−aminomethane (CAS 77-86-1, Merck, Darmstadt, Germany), 7.8 g citric acid (CAS 77-98-9, Merck, Darmstadt, Germany), 1.0 g fructose (CAS 26177-86-6, Merck, Darmstadt, Germany), and 0.5 g streptomycin sulphate (CAS 3810-74-0, Merck, Darmstadt, Germany) was prepared in 500 ml of distilled water. The base extender was then divided into four equal parts for addition of glycerol (v/v; 7%; CAS 56-81-5, Merck, Darmstadt, Germany) and UV-C irradiated chicken EYP (v/v; 10%, P1 or 15%, P2 or 20%, P3) or WCEY (v/v; 20%, control). The final pH of all the extenders was adjusted to 7.00 and stored at −20°C until use (Andrabi et al., 2008a).

2.4.5. Semen collection and processing

Semen collection was carried out as above (Section 2.2.2) for a period of six weeks (five bulls, six replicates, and thirty ejaculates) with an artificial vagina (42°C). Frequency of semen collection, initial evaluation, i.e., visual motility, concentration of sperm, holding, dilution (control, P1, P2 and P3) to a final concentration of 50×10^{6} spermatozoa/ml, cooling, equilibration, loading into 0.5 ml French straws, freezing (FR2), storage and thawing were conducted as described in Experiment 1 (Section 2.2.2; Shah et al., 2016).

2.4.6. Evaluation of sperm quality

Sperm quality was evaluated at PD immediately after dilution at 37°C, PE after 2 h of cooling and 4 h of equilibration at 4°C and PT after 24 h of freezing/storage at −196°C and then thawing at 37°C for 30 sec stages of cryopreservation. CASA, SV-PMI, MMP, V-IACR and DNA-I (%) were conducted as described in Experiment 1 (Sections 2.2.3-2.2.3.5).

2.4.7. *In vitro* sperm longevity based on CASA (% recovery; Experiment 2, 3 and 4)

The sperm longevity in terms of percent decline (Δ) and recovery in post–thaw sperm characteristics i.e., progressive motility and rapid velocity was evaluated during 2 h of incubation at 37°C. The post–thaw sperm characteristics were assessed through CASA.

The following formula used by Anzar et al. (2011) was applied:

$$\frac{\text{Variable at 2 h} - \text{Variable at 0 h}}{\text{Variable at 0 h}} \times 100$$

2.4.8. *In vivo* fertility estimation (%)

The best post–thaw *in vitro* sperm quality variables were found in P3 (20% chicken EYP) treatment. Moreover, no interaction was found between bull (n = 5) and treatment (EYP and WCEY) with GLM procedure, in any of the sperm quality variables. Therefore, AI doses were prepared separately from semen of two bulls (2 ejaculates/bull) for *in vivo* fertility trial using WCEY and P3. Semen collection, extension and cryopreservation protocols for the preparation of AI doses were the same as described above (Sections 2.2.2 and 2.2.3-2.2.3.5).

2.4.9. Animal selection and artificial insemination

A total of two hundred and thirty one buffaloes in their 2nd to 3rd lactation and good body condition were selected for the *in vivo* fertility trial during the October and November (breeding season). The buffaloes were maintained and managed in a group of 2-5 buffaloes by farmers in Islamabad (Pakistan). The common feeding practice among these farmers was the use of seasonal green fodder (oats, maize, or wheat), hay (maize and millet), wheat straw, wheat bran and cotton seed cake. The animals had free access to clean drinking water *ad libitum*.

The number of inseminations performed with frozen–thawed semen was 124 and 107 for P3 and WCEY, respectively. All the artificially bred buffaloes showed clinically normal reproductive tract with true indications of estrus. The release of mucus and

decline in milk production were the major criteria for estrus detection. The inseminations under trial were executed about 24 h following the inception of heat. Confirmation of pregnancy was done through rectal palpation at day 60 after AI.

2.5. **Experiment 4:** To study the effect of glycerol and dimethyl sulfoxide on cryopreserved quality and *in vivo* fertility of spermatozoa. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa during Experiment 4 are illustrated in Figure 2.4.

2.5.1. Experiment I

2.5.2. Preparation of extender

The base extender comprising of tris−(hydroxymethyl)−aminomethane (15.0 g), citric acid (7.8 g), fructose (1.0 g), streptomycin sulphate (0.5 g), hen egg yolk (100 ml) and 400 ml of distilled water (Andrabi et al., 2008a) was prepared. The base extender was then distributed into five equal parts for addition of glycerol and DMSO at specific concentrations. The first extender had 7% glycerol (v/v); the second extender had 3.5% DMSO (v/v); the third extender had 3.5% glycerol (v/v); the fourth extender had 1.75% (v/v) DMSO, and the fifth extender contained 1.75% glycerol (v/v). The pH of all the extenders was adjusted to 7.0 and these were stored at −20°C until use.

2.5.3. Semen collection and processing

Semen collection was carried out for a period of six weeks (five bulls, six replicates, and thirty ejaculates) with an artificial vagina (42°C) in a graduated falcon tube. Semen from each bull was collected twice consecutively. Initial evaluation, i.e., motility (> 60%), spermatozoa concentration (> 500×106 /ml), and handling of semen was carried out according to Andrabi et al. (2008b). Semen samples from each bull were distributed into five aliquots for dilution with the modified base extenders. The first aliquot was diluted at 37°C in extender containing 7% glycerol (control, C). The second aliquot was diluted at 37°C and as well as at 4°C in the extender containing 3.5% DMSO (Group 1, G1). The third aliquot was diluted at 37°C in the extender containing 3.5% glycerol and then at 4°C in the extender containing 3.5% DMSO (Group 2, G2). The fourth aliquot was diluted at 37°C in the extender containing 3.5%

DMSO and then at 4°C in the extender containing 3.5% glycerol (Group 3, G3). The fifth aliquot was diluted in extenders having 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C (Group 4, G4). The final concentration of viable spermatozoa in all the experimental groups was 50×10^6/ml. Sperm suspension was cooled from 37°C to 4°C in 2 h and equilibrated at 4°C for 4 h. The loading, freezing, storage and thawing of sperm suspension was carried out as described in Experiment 1 (Section 2.2.2).

2.5.4. Evaluation of sperm quality

Experiment I

All semen quality assays were performed at post−dilution, pre−freezing/post-equilibration and post−thawing stages of cryopreservation. CASA, SV-PMI, MMP, V-IACR and DNA-I (%) were conducted as described in Experiment 1 (Sections 2.2.3-2.2.3.5).

2.5.5. Experiment II

2.5.5.1. *In vivo* fertility (%)

As per statistical analysis of data, more promising results were found in G4 treatment on post–thaw *in vitro* sperm quality variables. Additionally, no interaction was found between bull (n = 5) and treatment (DMSO and/or glycerol) with GLM procedure, in any of the sperm quality variables. Therefore, artificial insemination (AI) doses were prepared individually from semen of two bulls for *in vivo* fertility trial by using the extender having 7% glycerol (C, 37°C) or the extenders having 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C (G4). Semen collection, extension and cryopreservation protocols for the preparation of AI doses were the same as illustrated in Experiment 1 (Section 2.2.2).

Post–thaw sperm quality was evaluated after 24 h of storage in LN_2 prior to use for *in vivo* fertility trial (Andrabi et al. 2016). A total of four hundred and seventeen buffaloes (C, n = 226 214; G4, n = 203) were inseminated with frozen–thawed semen in their 2nd to 3rd lactation. The buffaloes showed clinically normal reproductive tract

with true indications of estrus. The release of mucus and decline in milk production were the major criteria for estrus detection. The inseminations under trial were executed about 24 h following the inception of heat. The rectal palpation at day 60 post–AI was undertaken to assess the pregnancy.

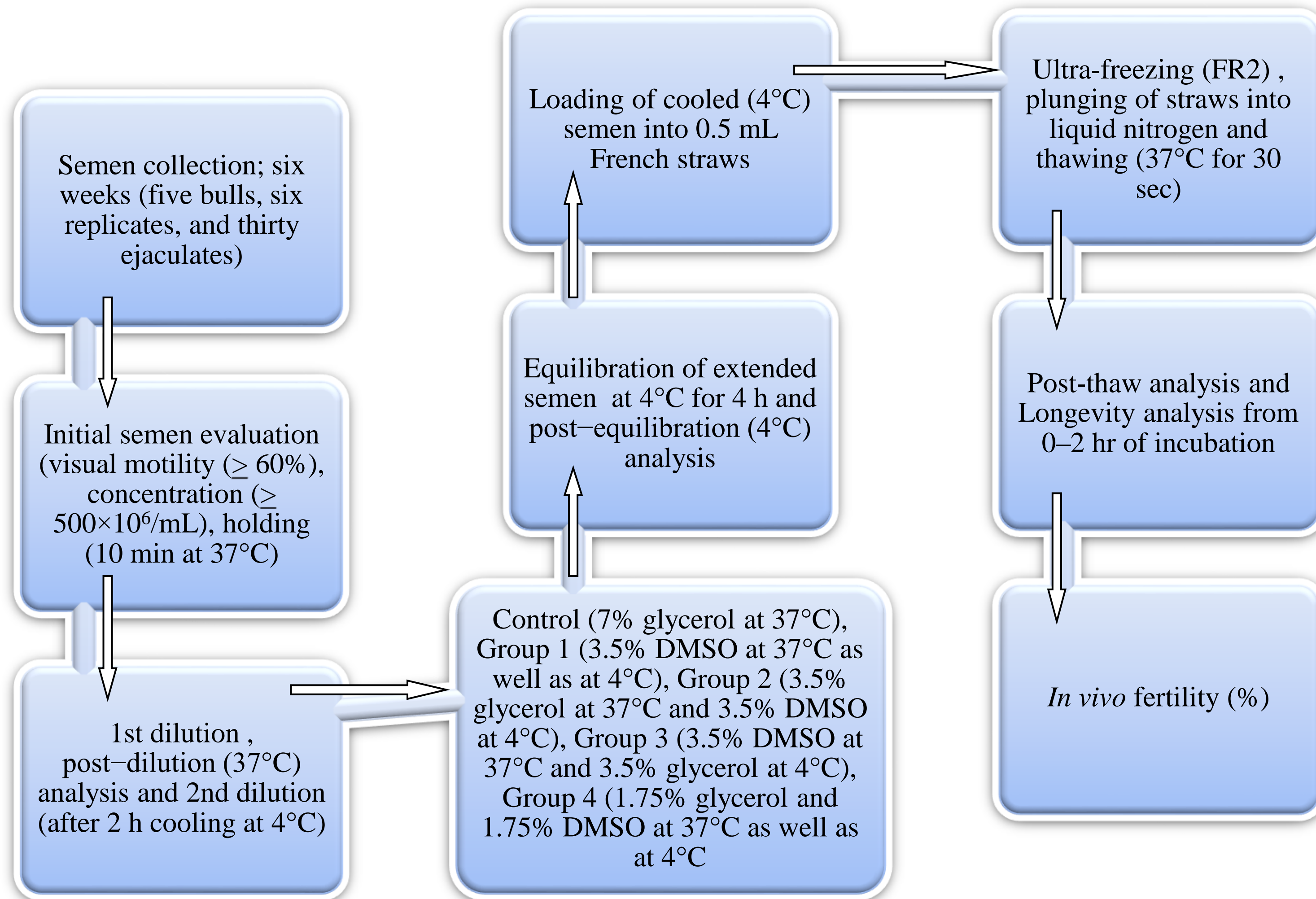

Fig. 2.4. Flow diagram showing series of steps undertaken during cryopreservation and evaluation of buffalo bull spermatozoa in Experiment 4

2.6. STATISTICAL ANALYSES

2.6.1. Experiment 1

The data were analyzed by using a 3-factor, factorial randomized block design (3 × 2 × 3). GLM procedure within the ANOVA module in Minitab (Release 17.3.1®, Minitab, Inc., United States) was used with three equilibration times, two freezing rates and three thawing rates as fixed factors and response variables were CASA motility, velocity distribution and kinematics, and SV-PMI, V-IACR, MMP, and DNA-I. When F-ratio was significant ($P < 0.05$), Tukey's pair-wise comparison test was used to further differentiate between the mean values (Minitab). The results are presented as mean±SEM of the mean.

2.6.2. Experiment 2

The experiment was replicated three times (six ejaculates/bull, and total 30 ejaculates). Minitab (Release 17.3.1R, Minitab, Inc., United States) was used for all the data analyses. Grubbs' test was applied for outlier detection and data normalization. As no interaction was observed between bull and treatment (curcumin) with GLM procedure, so bull data were pooled and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Posthoc test for pairwise comparisons between the mean. Pearson's correlation was performed among CASA parameters and supra vital plasma membrane integrity, viable sperm with intact acrosome, DNA integrity, total antioxidant capacity and lipid peroxidation levels. The differences were considered significant at $P < 0.05$ and results are presented as mean±SEM of the mean.

2.6.3. Experiment 3

The experiment was repeated six times (five bulls, six replicates, and thirty ejaculates). All the statistical analyses were conducted using the Minitab (Release 17.3.1®, Minitab, Inc., Pine Hall Road, State College, PA, United States). Grubbs' test was applied for outlier detection and normalization of data. No interaction was observed between bull ($n = 5$) and treatment (EYP and WCEY) with GLM procedure,

so bull data were pooled and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Posthoc test for pairwise comparisons between the treatment means. The *in vivo* fertility data were analyzed by Chi-square test. The differences were considered significant at $P < 0.05$ and results are presented as mean±SEM of the mean.

2.6.4. Experiment 4

Experiment I was repeated six times for each group (five bulls, six replicates, and thirty ejaculates). The statistical analyses were conducted using Minitab (Release 17.3.1®, Minitab, Inc., Pine Hall Road, State College, PA, United States). Grubbs' test was applied for outlier detection and to normalize the data. No interaction was observed between bull ($n = 5$) and treatment (DMSO and/or glycerol) with GLM procedure, so bull data were pooled and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Posthoc test for pairwise comparisons between the treatment means. The *in vivo* fertility in experiment II was analyzed by Chi-square test. The differences were considered significant at $P < 0.05$ and results are presented as mean±SEM.

EFFECT OF EQUILIBRATION TIMES, FREEZING, AND THAWING RATES ON POST-THAW QUALITY OF BUFFALO (*Bubalus bubalis*) BULL SPERMATOZOA

3.1. SUMMARY

The effects of equilibration times (E1, 2 h; E2, 4 h; E3, 6 h), freezing rates (FR1, manual, 5 cm over liquid nitrogen (LN_2) for 10 min, plunging in LN_2; FR2, programmable ultra-fast, holding at +4°C for 2 min, from 4 to −10°C at −10°C/min, from −10 to −20°C at −15°C/min, from −20 to −120°C at −60°C/min, holding at −120°C for 30 sec, plunging in LN_2), and thawing rates (T1, 37°C for 30 sec; T2, 50°C for 15 sec; T3, 70°C for 7 sec) were evaluated on quality of buffalo bull spermatozoa. Progressive motility (%), rapid velocity (%), average path velocity (VAP, µm sec^{-1}), straight line velocity (VSL, µm sec^{-1}), and mitochondrial transmembrane potential (%) were greater ($P < 0.05$) with E2, FR2, and T3 as compared to other groups. Sperm curvilinear velocity (VCL, µm sec^{-1}) was higher ($P < 0.05$) with E2 and FR2 as compared to other groups. Sperm straightness (%) and linearity (LIN, %) were greater ($P < 0.05$) with E2 as compared to other groups. Sperm LIN was affected ($P < 0.05$) with T3 as compared to other groups. Supravital-plasma membrane integrity (%), viability and acrosome integrity (%) of spermatozoa were greater ($P < 0.05$) with E2 and FR2 than the other groups. Sperm DNA integrity (%) was higher ($P < 0.05$) with FR2 and T1 compared to other groups. It is concluded that using 4 h equilibration time, programmable ultra-fast freezing rate (FR2), and rapid thawing at 70°C for 7 sec in cryopreservation protocol improves the post-thaw quality of buffalo bull spermatozoa.

3.2. INTRODUCTION

During cryopreservation, the spermatozoa are subjected to osmotic, thermal, and mechanical stresses, which are detectable at dilution, cooling, equilibration, freezing, and thawing stages (Andrabi, 2009). As regards, equilibration, it is traditionally called the 'glycerol equilibration' period, during which spermatozoa interact with glycerol before they freeze. During this stage, glycerol rapidly penetrates into the spermatozoa to establish equilibrium between its intracellular and extracellular concentration and other osmotically active extender constituents (Salamon and Maxwell, 2000). Therefore, this phenomenon interacts with the type of extender (buffer and cryoprotectant) used and could interact with other cryogenic procedures (Marshall, 1984). Equilibration time suggested for buffalo spermatozoa diluted intris-citric acid-based extender is 2–6 h at 4–5°C (Tuli et al., 1981; Dhami et al., 1996; Swelum et al., 2011). However, these studies have evaluated only subjective sperm quality parameters with varying success.

Biological materials can be frozen either with the help of rather simple non-programmable freezers or with more highly developed programmable freezers. Non-programmable freezers depend mostly on the volume to surface ratio of the straw or vial and the rate of ventilation. The cooling curve in non-programmable systems can be theoretically envisaged to be optimal for slow freezing (Woelders and Chaveiro, 2004), with comparativey low cooling rates directly after beginning of ice formation and higher cooling rates later on. While in programmable freezers, the temperature inside the cooling chamber can be accurately controlled and the time track of that temperature can be programmed as per experimental set-up (Andrabi, 2007). Freezing rates using programmable freezers suggested for cryopreservation of buffalo spermatozoa are: (i) 20 or 30°C/min from +4 to −120°C (Sukhato et al., 2001), (ii) 17.32°C/min between +4 and −40°C (Bhosrekaret al., 1994), and (iii) from −10 to −80°C at a rate of −30°C/min (Anzar et al., 2010). It is important to mention at this point that studies conducted by Sukhato et al. (2001) and Anzar et al. (2010) are limited in investigating the freezing rate higher than 30°C/min during the critical temperature zone.

In the freeze–thaw procedure, the thawing phase is as imperative for the survival of spermatozoa as the freezing stage (Andrabi, 2009). It is in general thought that fast rewarming rates are essential for the recovery of spermatozoa with higher quality. This is attributed to the possibility that intracellular ice crystals formed during freezing might grow in a slow re-warming process and cause injuries to spermatozoa (Holt, 2000; Arav and Natan, 2009). Many investigators have reported a wider range of thawing rates with improved quality of buffalo bull spermatozoa viz., 37°C for 30 sec (Rao et al*.,* 1986), 60°C for 15 sec (Dhami et al*.,*1992, 1996), (rapid) 1000°C, or (slow) 200°C/min (Sukhato et al*.,* 2001). However, there is a need to find out the most appropriate thawing rate for buffalo bull spermatozoa in conjunction with optimum equilibration time and freezing rate(s).

On the whole, the objectives of this part of the study were to evaluate the effects of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo bull spermatozoa.

3.3. MATERIALS AND METHODS

The details regarding Materials and Methods have been provided in Chapter 2 (Sections 2.1, 2.2.1-2.2.3.5 and 2.6.1).

3.4. RESULTS

3.4.1. Effects of equilibration times on post-thaw quality parameters

The effects of equilibration times on post-thaw quality parameters of buffalo bull spermatozoa are presented in Table 3.1. Mean values of sperm progressive motility (%), rapid velocity (%), average path velocity (VAP, $\mu m\ sec^{-1}$), curvilinear velocity (VCL, $\mu m\ sec^{-1}$), straight line velocity (VSL, $\mu m\ sec^{-1}$), straightness (STR– VSL: VAP, %), linearity (LIN – VSL: VCL, %), supra vital plasma membrane integrity, mitochondrial trans–membrane potential, and viability and acrosome integrity were significantly greater ($P < 0.05$) with E2 than the E1 or E3. DNA integrity did not differ significantly in relation to the equilibration times.

3.4.2. Effects of freezing rates on post-thaw quality parameters

The effects of freezing rates on post-thaw quality parameters of buffalo bull spermatozoa are presented in Table 3.2. Mean values of sperm progressive motility (%), rapid velocity (%), medium velocity (%), VAP (µm sec^{-1}), VSL (µm sec^{-1}), VCL (µm sec^{-1}), supra vital plasma membrane integrity, mitochondrial trans–membrane potential, viability and acrosome integrity and DNA integrity were significantly higher ($P < 0.05$) with FR2 than FR1.

3.4.3. Effects of thawing rates on post-thaw quality parameters

The effects of thawing rates on post-thaw quality parameters of buffalo bull spermatozoa are presented in Table 3.3. Mean values of sperm progressive motility (%), VAP, VSL, LIN and MMP were higher ($P < 0.05$) at T3 compared to T1 and T2. Sperm DNA integrity was higher ($P < 0.05$) at T1 as compared to T2 and T3.

Table 3.1 Effect of equilibration times (E1, 2h; E2, 4h; E3, 6h) on post-thaw quality parameters of buffalo (*Bubalus bubalis*) spermatozoa (five bulls; five replicates, 25 ejaculates)

Parameters	**Equilibration times (h)**			
	2	**4**	**6**	**S.E.M.**
Progressive motility (%)	09.73^{b}	15.25^{a}	8.33^{b}	0.51
Rapid velocity (%)	17.95^{b}	22.63^{a}	13.80^{c}	0.60
Medium velocity (%)	76.10^{b}	70.01^{c}	80.00^{a}	0.89
Slow velocity (%)	4.00^{a}	3.21^{a}	03.96^{a}	0.49
Average path velocity (µm sec^{-1})	56.81^{b}	62.36^{a}	53.70^{c}	0.53
Straight line velocity (µm sec^{-1})	44.16^{b}	51.58^{a}	42.78^{b}	0.63
Curvilinear velocity (µm sec^{-1})	94.40^{a}	96.67^{a}	88.24^{b}	1.00
Amplitude of lateral head displacement (µm)	06.49^{a}	06.19^{a}	06.09^{a}	0.12
Beat cross frequency (Hz)	25.52^{a}	25.04^{a}	25.70^{a}	0.21
Straightness (VSL: VAP %)	78.38^{b}	82.70^{a}	79.98^{b}	0.63
Linearity (VSL: VCL %)	48.41^{b}	54.87^{a}	49.80^{b}	0.93
Supra-vital plasma membrane integrity (%)	26.00^{b}	30.70^{a}	23.93^{c}	0.39
Mitochondrial trans-membrane potential (%)	41.20^{b}	45.90^{a}	35.33^{c}	0.65
Viability and intact acrosome (%)	58.33^{b}	60.26^{a}	53.50^{c}	0.52
DNA integrity (%)	94.06^{a}	94.53^{a}	94.73^{a}	0.27

Means with different superscripts in a row differed significantly ($P < 0.05$).

Table 3.2 Effect of freezing rates on post-thaw quality parameters of buffalo (*Bubalus bubalis*) spermatozoa (five bulls; five replicates, 25 ejaculates)

Parameters	**Freezing rates**		**S.E.M.**
	FR1	**FR2**	
Progressive motility (%)	9.83^{b}	12.37^{a}	0.42
Rapid velocity (%)	16.51^{b}	19.74^{a}	0.49
Medium velocity (%)	74.23^{b}	76.51^{a}	0.73
Slow velocity (%)	05.04^{a}	02.41^{b}	0.40
Average path velocity (µm sec^{-1})	56.22^{b}	59.02^{a}	0.44
Straight line velocity (µm sec^{-1})	45.32^{b}	47.03^{a}	0.51
Curvilinear velocity (µm sec^{-1})	91.08^{b}	95.11^{a}	0.82
Amplitude of lateral head displacement (µm)	6.22^{a}	06.28^{a}	0.10
Beat cross frequency (Hz)	25.28^{a}	25.57^{a}	0.17
Straightness (VSL: VAP %)	81.06^{a}	79.64^{a}	0.51
Linearity (VSL: VCL %)	51.58^{a}	50.47^{a}	0.75
Supra-vital plasma membrane integrity (%)	25.00^{b}	28.75^{a}	0.32
Mitochondrial trans-membrane potential (%)	38.17^{b}	43.44^{a}	0.53
Viability and intact acrosome (%)	56.51^{b}	58.22^{a}	0.42
DNA integrity (%)	94.02^{b}	94.86^{a}	0.22

Means with different superscripts in a row differed significantly ($P < 0.05$).

Freezing rates: Manual, FR1, from 4°C to −85°C, plunging in LN_2; programmable ultra-fast, FR2, holding at 4°C for 2 min, from 4°C to −10°C @−10°C min^{-1}, from −10°C to −20°C @−15°C min^{-1}, from −20°C to −120°C @−60°C min^{-1}, holding at −120°C for 30 sec, and plunging in LN_2).

Table 3.3 Effect of thawing rates (T1, at 37°C for 30 sec; T2, at 50°C for 15 sec; T3, at 70°C for 7 sec) on post-thaw quality parameters of buffalo (*Bubalus bubalis*) spermatozoa (five bulls; five replicates, 25 ejaculates)

Parameters	**Thawing rates**			**S.E.M.**
	T1	**T2**	**T3**	
Progressive motility (%)	09.75^{b}	10.85^{b}	12.71^{a}	0.51
Rapid velocity (%)	16.80^{b}	18.93^{a}	18.64^{ab}	0.60
Medium velocity (%)	75.85^{a}	75.15^{a}	75.11^{a}	0.89
Slow velocity (%)	03.91^{a}	03.81^{a}	03.45^{a}	0.49
Average path velocity (µm sec^{-1})	56.34^{b}	57.34^{b}	59.19^{a}	0.53
Straight line velocity (µm sec^{-1})	44.77^{b}	45.45^{b}	48.30^{a}	0.63
Curvilinear velocity (µm sec^{-1})	92.62^{a}	94.20^{a}	92.48^{a}	1.00
Amplitude of lateral head displacement (µm)	06.24^{ab}	06.61^{a}	05.92^{b}	0.12
Beat cross frequency (Hz)	25.37^{a}	25.50^{a}	25.39^{a}	0.21
Straightness (VSL: VAP %)	79.81^{a}	80.05^{a}	81.20^{a}	0.63
Linearity (VSL: VCL %)	49.93^{b}	49.94^{b}	53.21^{a}	0.93
Supra-vital plasma membrane integrity (%)	26.33^{a}	26.83^{a}	27.46^{a}	0.39
Mitochondrial trans-membrane potential (%)	39.66^{b}	40.73^{ab}	42.03^{a}	0.65
Viability and intact acrosome (%)	58.20^{a}	56.46^{a}	57.43^{a}	0.52
DNA integrity (%)	95.56^{a}	93.83^{b}	93.93^{b}	0.27

Means with different superscripts in a row differed significantly ($P < 0.05$).

3.5. DISCUSSION

This study was designed to evaluate the effect of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo bull spermatozoa.

Traditionally, equilibration is the total time during which spermatozoa remain in contact with glycerol prior to freezing. This is the stage at which glycerol penetrates into the spermatozoa to establish a balance between the intracellular and extracellular concentration (Andrabi, 2014). In the present study, 4h compared to other equilibration times (2h or 6h) resulted in a significantly higher post-thaw sperm PM, RV, VAP, VSL, VCL, STR, LIN, SV-PMI, MMP, and V-IACR. Similar to the present study, a few studies have also reported improved post-thaw sperm STR and LIN with 4 h equilibration time in buffalo (Kumar et al., 2015) and cattle (Celeghini et al., 2008; Leite et al., 2010). Tuli et al. (1981) showed higher sperm motility in buffalo with 4 h equilibration as compared to 2 h and 6 h equilibration times. In contrast, equilibration times viz-a-viz 2, 4, 8, and 16 h did not affect sperm PMI and motion attributes (VAP, VSL, VCL, LIN) in buffalo bull (Shahverdi et al.,2014). This discrepancy is perhaps due to the CASA settings used by Shahverdi et al. (2014), for instance in their case, minimum velocity limit for forward motile spermatozoa was 10 µm sec^{-1}. This CASA appears to be too low to explain any difference due to the equilibration times. Dhami et al. (1996) have demonstrated that only 2 h equilibration time was required for successful cryopreservation of buffalo spermatozoa in zwitterion buffers. As regards post-thaw sperm DNA integrity, no significant effect was observed in the current study among the equilibration times. This study hypothesized that extender composition may have protected the sperm DNA during equilibration. Moreover, the cryo-protocol used in the present study may have helped to curtail the over generation of reactive oxygen species. Similar to results of this study, Shahverdiet al. (2014) have not observed the ‘equilibration time effect’ on post-thaw sperm DNA integrity in buffalo.

In principle, it is the freezing rate during the critical temperature zone (−5 to −50°C), that establishes whether the spermatozoa will remain in equilibrium with their extracellular environment or will become progressively supercooled, with the increasing likelihood of intracellular ice nucleation (Kumar et al., 2003). At optimum

freezing rates, the spermatozoa remain exposed to the adverse conditions for a shorter moment (Woelders, 1997). Programmable freezers are efficient in controlling the freezing rates during critical temperature range (Holt, 2000). The current study reports, for the first time, that there occurs successful freezing of buffalo bull spermatozoa using FR2 (−60°C/min) during the critical temperature range. This freezing rate resulted in significantly higher sperm PM, RV, VAP, VSL and VCL, SV-PMI, MMP, V-IACR and DNA-I than FR1. In contrast, Anzar et al. (2010) demonstrated that programmable freezing at the rate of −30°C/min as compared to freezing at the rate of −10 and −20°C/min during the critical temperature zone resulted in improved sperm PM, VSL, PMI, and acrosomal integrity in buffalo. Similarly, Sukhato et al. (2001) reported that automated freezing at the rate of −20 or −30°C/min yielded better sperm PM in buffalo as compared to automated freezing at the rate of −10°C/min. It is, therefore, suggested that if the freezing rate is within the required values (50–100 °C/min), this culminates in less excessive intracellular dehydration, less excessive intracellular solute concentrations, and less shrinkage of the cells (Mazur, 1984; Woelders, 1997).

The thawing protocol depends on the specific rate of freezing used in the different studies. If it has been suitably higher to induce the intracellular freezing, or lower enough to produce cell dehydration, then in the first case, fast thawing is suitable to prevent recrystallization of any intracellular ice which may be present in the spermatozoa (Andrabi, 2009). Currently, significantly higher sperm PM, RV, VAP, VSL and VCL, LIN and MMP were found at T3. Similar to the current study, higher PM, kinematics, and viability of bull spermatozoa have been reported using fast thawing rate at 65–70°C for 6–7 sec (Lyashenko, 2015) and 60°C for 15 sec (Dhami et al., 1992). Conversely, Rao et al. (1986) found that thawing rate of 37°C for 30 sec was comparatively better than 30°C for 30 sec and 75°C for 9 sec for buffalo spermatozoa. The difference in spermatozoa quality outcome in the present study could be due to the use of programmable ultrafast freezing (FR2) against the conventional static vapor freezing method that was used by Rao et al. (1986). Therefore, it appears that spermatozoa thawed at a faster rate may have also been exposed for a shorter time to the concentrated solute and cryoprotectant-glycerol. Moreover, the reinstatement of the intracellular and extracellular equilibrium is more rapid in fast thawing as compared to slow thawing (Salamon and Maxwell, 2000).

Higher sperm DNA-I was reported with T1 (95.56%) compared to T2 (93.83%) and T3 (93.93%) in this study. According to Kumar et al. (2012), buffalo bulls are classified as being of high-fertility had about 90% cryopreserved spermatozoa with intact DNA. Therefore, this statistical difference is unlikely to be of biological relevance.

3.5.1. CONCLUSION

The study concluded that inclusion of 4 h equilibration time, programmable ultra-fast freezing rate (FR2), and rapid thawing at 70°C for 7 sec in the cryopreservation protocol improves the post-thaw quality of buffalo bull spermatozoa.

FREEZABILITY OF WATER BUFFALO BULL (*Bubalus bubalis*) SPERMATOZOA IS IMPROVED WITH THE ADDITION OF CURCUMIN (DIFERUOYL METHANE) IN SEMEN EXTENDER

4.1. SUMMARY

Effects of curcumin as an antioxidant in the extender were evaluated on freezability of buffalo spermatozoa. Semen from each bull (five bulls, three replicates, six ejaculates/bull, a total of 30 ejaculates) was diluted in tris-citric acid extender containing curcumin (0.5, 1.0, 1.5 or 2.0 mM) or control. At pre-freezing and post-thawing, total antioxidant contents (µM/L) were higher ($P < 0.05$), while the lipid peroxidation levels (µM/ml) were lower ($P < 0.05$) with 1.5 and 2.0 mM curcumin compared to 0.5 and 1.0 mM curcumin and control. At post-thawing, progressive motility (PM, %) and rapid velocity (RV, %) were higher ($P < 0.05$) with 1.5 mM as compared to other doses of curcumin and control (except in case of RV, 1.5 was similar with 1.0 mM curcumin). Kinematics (average path velocity, µm sec^{-1}; straight-line velocity, µm sec^{-1}; curvilinear velocity, µm sec^{-1}; straightness, %; linearity, %), *in vitro* longevity (%, PM and RV) and DNA integrity (%) at post-thawing were higher ($P < 0.05$) with 1.5 mM curcumin as compared to the control. At post-thawing, supravital plasma membrane integrity (%) and viable spermatozoa with intact acrosome (%) were higher with 1.5 compared to 2.0 mM curcumin and control. It is concluded that freezability of buffalo spermatozoa is improved with the addition of 1.5 mM curcumin in extender.

4.2. INTRODUCTION

Although, redox biology implies that a small increase in level of reactive oxygen species (ROS) is sufficient to activate the signaling pathways, but the oxidative stress normally involves elevated ROS levels This results in an impairment of cellular nucleic acids, proteins or lipids (Schieber and Chandel, 2014). It is well documented that ROS generated as a result of univalent reduction of oxygen (Bilodeau et al.,2000) is responsible for the maintenance of the physiological processes in spermatozoa, like the capacitation and acrosome reaction (O'Flaherty et al.,1997). Therefore, the balance between ROS generation and antioxidants should be appropriate for the protection of spermatozoa (Iqbal et al., 2016b).

Antioxidants are generally defined as compounds with slow autoxidation (Li and Pratt, 2015). Based on their mechanism, antioxidants are categorized mainly into two types that is preventive antioxidants (PO; e.g. glutathione peroxidase and catalase) and radical-trapping antioxidants (RTAs; e.g. phenols; Valgimigli and Pratt, 2012). Many RTAs are electron-rich phenolic compounds that undergo spontaneous reactions with O_2 at competitive rates (Griesser et al., 2011). Curcumin (diferuoyl methane), a natural RTA, is a yellow colored compound isolated from the dried ground rhizomes of the perennial herb *Curcuma longa* L. (family Zingiberaceae). From cyclic voltammetric studies, it is reveald that H-atom transfer from CH_2 group at the centre of the heptadione link has an antioxidant function in curcumin together with that of its phenolic –OH group. The conversion of 1, 3-dicarbonyl moiety of curcumin to an isosteric heterocycle as in pyrazole curcumin, which decreases its rotational freedom, enhances its redox properties as well as antioxidant activity (Jha et al., 2015).

During cryopreservation, a disparity between the ROS and the natural antioxidants results into oxidative stress. To reduce the effects of oxidative stress on spermatozoa during cryopreservation, it is necessary that antioxidants are added into semen extenders (Andrabi et al., 2008a). Outcomes of spermatozoa cryopreservation with curcumin in the extender have been reported in Angora buck (Bucak et al., 2010), Holstein bull (Bucak et al., 2012), miniature boar (Jeon and Kim, 2013) and albino Wistar rats (Soleimanzadeh and Saberivand, 2013). Moreover, oral administration of

curcumin have been reported to improve the morphologic features of spermatozoa in New Zealand White rabbit buck (Seadawy et al., 2014) and mice (Mathuria and Verma, 2008; Głombik et al., 2014; Lin et al., 2015). To date, no study is available on the use of curcumin as antioxidant in semen extender for cryopreservation of water buffalo spermatozoa. This study was, therefore, aimed to evaluate the effects of curcumin (diferuoyl methane) as antioxidant in extender on freezability of buffalo bull spermatozoa.

4.3. MATERIALS AND METHODS

The details with respect to this experimental section have been provided in Chapter 2 (Sections 2.1, 2.2-2.2.3.5, 2.3.-2.3.5 and 2.6.2).

4.4. RESULTS

4.4.1. Effect of curcumin (diferuoyl methane) on total antioxidant capacity and lipid peroxidation

Data as regards the effect of curcumin (diferuoyl methane) on total antioxidant capacity assessment (FRAP assay) and lipid peroxidation assay (MDA determination) at post-dilution, pre-freezing and post-thawing in buffalo bull semen are presented in Table 4.1. At post-dilution, total antioxidant content was greater ($P < 0.05$) with all doses of curcumin as compared to control. At pre-freezing and post-thawing, total antioxidant contents were higher ($P < 0.05$) with 1.5 mM and 2.0 mM curcumin when compared with the other concentrations and control.

At post-dilution, lipid peroxidation level decreased ($P < 0.05$) with 1.5 mM than 0.5 mM curcumin and control. At pre-freezing and post-thawing, lipid peroxidation levels were lower ($P < 0.05$) with 1.5 mM and 2.0 mM curcumin as compared to other concentrations and control.

4.4.2. Effect of curcumin (diferuoyl methane) on CASA progressive motility, velocity distribution and kinematics

Data on the effect of curcumin (diferuoyl methane) in the extender on CASA progressive motility, velocity distribution and kinematics at post-dilution, pre-freezing and post-thawing in buffalo bull are presented in Table 4.2. At pre-freezing, mean value of progressive motility was higher ($P < 0.05$) with 1.5 mM compared to 2.0 mM curcumin supplementation and control. At post-thawing, mean value of progressive motility was higher ($P < 0.05$) with 1.5 mM curcumin compared to other concentrations and control. At post-thawing, mean value of rapid velocity was higher ($P < 0.05$) with 1.5 mM compared to 0.5, and 2.0 mM curcumin concentrations and control.

At post-thawing, mean value of average path velocity was higher ($P < 0.05$) with 1.5 mM compared to 2.0 mM curcumin supplementation and control. At post-thawing, mean value of straight-line velocity was higher ($P < 0.05$) with 1.5 mM compared to 0.5 mM curcumin and control. At post-dilution, mean value of curvilinear velocity was higher ($P < 0.05$) with 1.5 mM than 2.0 mM curcumin. At post-thawing, mean value of curvilinear velocity was higher ($P < 0.05$) with 1.5 mM compared to 2.0 mM curcumin and control. At post-dilution and post-thawing, mean values of sperm straightness were higher ($P < 0.05$) with 1.5 mM curcumin than control. At post-dilution, mean value of sperm linearity was higher ($P < 0.05$) with 1.5 mM curcumin compared to other concentrations and control. At post-thawing, mean value of sperm linearity was higher ($P < 0.05$) with 1.5 mM compared to control.

4.4.3. Effect of curcumin (diferuoyl methane) on longevity of spermatozoa during *in vitro* incubation

Data on the effect of (diferuoyl methane) on post-thawing CASA variables (progressive motility and rapid velocity) assessed during 2-hr incubation at 37°C are presented in Table 4.3. Progressive motility was higher ($P < 0.05$) with 1.5 mM curcumin at 1 h of incubation compared to other curcumin concentrations and control. Rapid velocity was higher ($P < 0.05$) with 1.5 mM curcumin at 1 h of incubation compared to 0.5 and 2.0 mM curcumin and control. At 2 h incubation, the progressive

motility and rapid velocity were higher (P < 0.05) with 1.5 mM curcumin than the control.

4.4.4. Effect of curcumin (diferuoyl methane) on supravital plasma membrane integrity, viable spermatozoa with intact acrosome and DNA integrity

Data on the effect of curcumin (diferuoyl methane) in extender on sperm supravital plasma membrane integrity, viable spermatozoa with intact acrosome and DNA integrity at post-dilution, pre-freezing and post-thawing in buffalo bull are presented in Table 4.4. At post-dilution and post-thawing, mean value of supravital plasma membrane integrity was higher (P < 0.05) with 1.5 mM compared to 2.0 mM curcumin and control. At post-dilution, mean value of viable spermatozoa with intact acrosome was higher (P < 0.05) with 1.5 mM curcumin than control. At post-thawing, mean value of viable spermatozoa with intact acrosome was higher (P < 0.05) with 1.5 compared to 2.0 mM curcumin and control. At pre-freezing, mean value of DNA integrity was higher (P < 0.05) with 1.5 mM curcumin than control. At post-thawing, DNA integrity was higher (P < 0.05) among all the curcumin concentrations compared to control.

4.4.5. Correlation analysis among CASA variables and other seminal assays

Pearson's correlation analysis among CASA variables and other semen quality assays at post-dilution, pre-freezing and post-thawing are presented in Table 4.5. Progressive motility was positively correlated (P < 0.05) with supravital plasma membrane integrity at all stages, viable spermatozoa with intact acrosome, DNA integrity and total antioxidant capacity at post-dilution and post-thawing. Rapid velocity was positively correlated (P < 0.05) with viable spermatozoa with intact acrosome at all stages, supravital plasma membrane integrity, total antioxidant capacity at pre-freezing and post-thawing and DNA integrity at pre-freezing. Average path velocity was positively correlated (P < 0.05) with viable spermatozoa with intact acrosome at all stages; supravital plasma membrane integrity at pre-freezing and post-thawing; DNA integrity at pre-freezing; total antioxidant capacity at post-thawing; and DNA integrity at pre-freezing and post-thawing. Curvilinear velocity was positively correlated (P < 0.05) with viable spermatozoa with intact acrosome at pre-freezing

and post-thawing, supravital plasma membrane integrity, DNA integrity at pre-freezing and total antioxidant capacity at post-thawing. Amplitude of lateral head displacement was positively correlated ($P < 0.05$) with supravital plasma membrane integrity, viable spermatozoa with intact acrosome, DNA integrity at pre-freezing and lipid peroxidation at post-thawing.

Progressive motility, average path velocity, straight-line velocity, curvilinear velocity, straightness and linearity were negatively correlated ($P < 0.05$) with lipid peroxidation at post-thawing, except rapid velocity, which was negatively correlated ($P < 0.05$) with lipid peroxidation at pre-freezing and post-thawing. Medium velocity was negatively correlated ($P < 0.05$) with supravital plasma membrane integrity at pre-freezing and post-thawing, viable spermatozoa with intact acrosome, DNA integrity at post-dilution and pre-freezing and total antioxidant capacity at post-thawing. Amplitude of lateral head displacement was negatively correlated ($P < 0.05$) with DNA integrity at post-thawing.

Table 4.1 Effect of curcumin (diferuoyl methane) in extender on total antioxidant capacity and lipid per oxidation (malonaldehyde, MDA levels) of buffalo bull semen during cryopreservation (post–dilution, PD; pre–freezing, PF; post–thawing, PT; three replicates, five bulls, six ejaculates/bull, and a total of 30 ejaculates)

Stage	**Curcumin concentration**	**Total antioxidant capacity ($\mu M\ l^{-1}$)**	**Lipid per oxidation (MDA levels, $\mu M\ ml^{-1}$)**
PD	0.5 mM	74.67±1.25[a]	493.00±1.20[ac]
	1.0 mM	76.93±1.04[a]	487.00±2.60[bc]
	1.5 mM	78.80±1.51[a]	483.20±2.07[b]
	2.0 mM	75.73±1.26[a]	490.00±1.47[abc]
	Control	67.80±1.37[b]	496.40±1.34[a]
PF	0.5 mM	66.00±0.56[b]	520.80±2.25[a]
	1.0 mM	69.33±1.35[b]	517.30±4.14[a]
	1.5 mM	84.33±0.82[a]	499.50±2.47[b]
	2.0 mM	80.93±1.51[a]	522.70±4.41[a]
	Control	56.60±2.77[c]	528.90±2.04[a]
PT	0.5 mM	48.67±2.08[b]	543.30±4.50[b]
	1.0 mM	51.93±1.27[b]	540.30±4.16[b]
	1.5 mM	71.20±1.28[a]	517.90±2.52[d]
	2.0 mM	69.93±1.46[a]	558.20±3.06[c]
	Control	30.40±1.17[c]	614.10±3.76[a]

Values are expressed as mean ± S.E.M. Values lacking a common superscript in a column indicate significant ($P < 0.05$) difference among the experimental groups at a particular stage.

Table 4.2 Effect of curcumin (diferuoyl methane) on CASA parameters of buffalo bull during cryopreservation (post–dilution, PD; pre–freezing, PF; post–thawing, PT; three replicates, five bulls, six ejaculates/bull, and a total of 30 ejaculates)

Variable	Stage	Curcumin concentration				Control
		0.5 mM	1.0 mM	1.5 mM	2.0 mM	
PM	PD	31.47±2.24	28.19±1.93	38.36±3.57	23.93±2.53	28.73±2.75
	PF	19.37±1.40^{ab}	17.68±1.38^{ab}	21.90±2.09^{a}	14.97±1.17^{b}	15.10±0.94^{b}
	PT	15.49±1.12^{b}	17.07±1.37^{b}	23.27±1.75^{a}	14.87±1.36^{bc}	9.83±0.79^{c}
RV	PD	59.80±4.23	56.59±4.48	60.04±3.99	44.96±2.80	56.70±3.78
	PF	37.20±3.46	34.22±2.93	39.36±2.94	34.00±2.89	33.60±2.55
	PT	24.36±1.87^{b}	26.07±1.32^{ab}	31.53±1.92^{a}	22.13±1.36^{b}	22.30±1.07^{b}
MV	PD	36.67±4.21	38.51±4.49	36.61±3.76	49.80±2.97	36.47±3.92
	PF	58.03±3.29	60.90±3.40	56.78±2.11	61.13±2.99	61.93±2.22
	PT	64.91±2.24	64.27±1.89	58.44±2.70	66.93±1.78	65.43±1.99
VAP	PD	95.11±3.97	90.87±3.88	91.51±3.10	82.33±2.07	84.74±4.10
	PF	75.92±2.50	74.29±2.19	77.22±2.27	74.28±2.47	73.84±2.24
	PT	65.68±1.72^{ab}	67.67±1.11^{ab}	71.55±2.12^{a}	64.68±1.74^{b}	63.06±1.42^{b}
VSL	PD	72.06±2.67	70.32±3.02	72.30±2.50	62.34±1.86	72.30±2.42
	PF	57.44±1.27	55.48±1.27	58.58±2.00	53.95±1.47	53.86±1.27
	PT	52.37±1.35^{b}	53.25±1.19^{ab}	59.44±2.17^{a}	53.10±2.00^{ab}	47.34±1.39^{b}
VCL	PD	143.7±5.97^{ab}	141.9±6.01^{ab}	153.0±4.61^{a}	128.2±4.21^{b}	131.2±7.79^{ab}
	PF	126.97±5.08	123.87±4.25	128.24±4.35	124.71±5.30	126.06±4.32
	PT	106.6±3.35^{ab}	110.4±2.62^{ab}	117.2±2.32^{a}	101.5±1.97^{b}	99.17±3.40^{b}
ALH	PD	7.55±0.22	7.16±0.26	6.78±0.19	6.89±0.29	7.53±0.20
	PF	6.66±0.17	6.67±0.22	6.69±0.20	6.48±0.27	6.86±0.22
	PT	6.00±0.20	6.22±0.18	5.58±0.22	5.65±0.14	6.28±0.17
BCF	PD	25.06±0.38	25.85±1.12	25.58±1.52	24.66±0.55	25.76±0.28
	PF	24.88±0.58	24.28±0.76	24.69±1.03	24.40±0.79	25.48±0.79
	PT	24.93±0.54	25.00±0.61	24.90±0.91	24.81±1.33	25.06±0.16
STR	PD	81.17±1.21^{ab}	80.65±0.92^{ab}	81.88±1.47^{a}	79.82±1.55^{ab}	76.90±0.76^{b}
	PF	80.30±1.42	77.53±1.24	79.03±0.77	77.84±1.27	76.45±1.40
	PT	75.14±1.18^{ab}	75.71±1.40^{ab}	78.63±1.71^{a}	75.67±0.84^{ab}	73.00±0.57^{b}
LIN	PD	48.90±1.23^{b}	49.38±1.97^{b}	54.98±1.43^{a}	49.15±1.99^{b}	47.95±1.08^{b}
	PF	47.35±1.68	47.63±1.49	51.05±1.45	46.73±1.70	45.60±1.31
	PT	45.86±1.03^{ab}	43.43±0.71^{ab}	47.00±1.70^{a}	45.80±1.39^{ab}	42.00±0.57^{b}

Values are expressed as Mean±S.E.M. Values lacking a common superscript in a row indicate significant ($P < 0.05$) difference among the experimental groups at a particular stage. Progressive motility (PM, %), velocity distribution (rapid velocity and RV, medium velocity, MV) and kinematics (μm sec^{-1}; average path velocity, VAP, straight line velocity, VSL, curvilinear velocity, VCL; amplitude of lateral head displacement, ALH, μm; beat cross frequency, BCF, Hz; straightness, STR, VSL/VAP, % and linearity, LIN, VSL/VCL, %)

Table 4.3 Effect of curcumin (diferuoyl methane) on CASA progressive motility (PM, %) and rapid velocity (RV, %) of buffalo bull spermatozoa during 1–2 h incubation (37°C) at post–thawing (three replicates, five bulls, six ejaculates/bull, and a total of 30 ejaculates)

Variable	**Incubation Time (h)**	**Curcumin concentration**				**Control**
		0.5 mM	**1.0 mM**	**1.5 mM**	**2.0 mM**	
PM	1	10.21 ± 0.51^{b}	10.00 ± 0.42^{b}	17.30 ± 0.34^{a}	9.43 ± 0.51^{b}	6.29 ± 0.49^{c}
	2	8.00 ± 0.26^{a}	7.25 ± 0.68^{ab}	8.40 ± 0.50^{a}	5.50 ± 0.44^{ab}	4.75 ± 0.28^{b}
RV	1	20.17 ± 1.07^{b}	22.54 ± 0.69^{a}	25.93 ± 0.41^{a}	15.14 ± 0.35^{c}	16.86 ± 0.99^{bc}
	2	14.14 ± 0.77^{ab}	15.63 ± 0.36^{ab}	16.67 ± 0.60^{a}	13.07 ± 0.57^{ab}	10.86 ± 0.48^{b}

Values are expressed as mean ± S.E.M. Values lacking a common superscript in a row indicate significant ($P < 0.05$) difference among the experimental groups at a particular incubation time.

Table 4.4 Effect of curcumin (diferuoyl methane) on SV–PMI, V–IACR and DNA–I of buffalo bull spermatozoa during cryopreservation (three replicates, five bulls, six ejaculates/bull, and a total of 30 ejaculates)

Variable	**Stage**	**Curcumin concentration**				**Control**
		0.5 mM	**1.0 mM**	**1.5 mM**	**2.0 mM**	
SV–PMI	PD	72.47±0.92[ab]	72.47±0.89[ab]	75.87±1.2[a]	71.27±1.24[b]	70.40±0.76[b]
	PF	66.73±1.02	66.73±1.02	70.20±1.65	67.20±1.40	65.53±1.01
	PT	28.27±0.97[ab]	28.27±0.97[ab]	30.47±1.11[a]	26.00±0.99[b]	24.53±0.48[b]
V–IACR	PD	68.80±0.95[ab]	69.00±1.02[ab]	71.13±1.21[a]	68.47±0.93[ab]	66.93±0.88[b]
	PF	65.07±1.10	66.27±1.24	67.80±1.56	64.07±1.27	63.93±1.00
	PT	58.13±1.02[ab]	59.47±1.04[ab]	61.87±1.40[a]	56.33±0.88[b]	56.40±0.75[b]
DNA–I	PD	94.20±0.29	94.27±0.28	94.87±0.34	94.47±0.26	93.73±0.28
	PF	93.20±0.35[ab]	94.07±0.37[ab]	94.47±0.39[a]	93.60±0.34[ab]	92.67±0.40[b]
	PT	93.27±0.27[b]	93.60±0.29[a]	94.53±0.26[a]	94.20±0.20[a]	91.33±0.39[c]

Values are expressed as mean ± S.E.M. Values lacking a common superscript in a row indicate significant ($P < 0.05$) difference among the experimental groups at a particular stage.

Supra–vital plasma membrane integrity (SV–PMI, %), viable and intact acrosome (V–IACR, %) and DNA integrity (DNA–I, %); post–dilution, PD; pre–freezing, PF; and post–thawing, PT.

Table 4.5 Pearson's correlation among CASA parameters; other semen quality variables, total antioxidant capacity, TAC, µM l^{-1}; and lipid per oxidation levels, LPO, malonaldehyde levels, µM ml^{-1}) during cryopreservation of buffalo bull semen (three replicates, five bulls, six ejaculates/bull, and a total of 30 ejaculates)

Stage	Other variable	CASA variable						
		PM	**RV**	**MV**	**VAP**	**VSL**	**VCL**	**ALH**
PD	SV-PMI	0.51***	0.17	-0.17	0.13	0.29*	0.05	-0.19
	V-IACR	0.48***	0.32*	-0.37***	0.28*	0.37***	0.21	-0.02
	DNA-I	0.07	0.22	-0.24*	0.23	0.13	0.20	0.06
	TAC	0.05	-0.05	0.12	-0.04	-0.04	-0.07	-0.17
	LPO	0.04	-0.01	-0.03	-0.06	0.11	-0.21	0.05
PF	SV-PMI	0.33***	0.52***	-0.46***	0.55***	0.46***	0.48***	0.37***
	V-IACR	0.17	0.37***	-0.30*	0.43***	0.28*	0.41***	0.36***
	DNA-I	0.31*	0.33***	-0.28*	0.36***	0.39***	0.33***	0.27*
	TAC	0.28*	0.10	-0.10	0.06	0.17	0.02	-0.07
	LPO	-0.16	-0.24*	0.22	-0.17	-0.03	-0.16	-0.10
PT	SV-PMI	0.39***	0.25*	-0.27*	0.27*	0.39***	0.14	-0.06
	V-IACR	0.32***	0.27*	-0.10	0.30*	0.27*	0.31*	0.00
	DNA-I	0.36***	0.11	-0.07	0.16	0.31*	0.06	-0.23*
	TAC	0.62***	0.42***	-0.24*	0.36***	0.47***	0.32*	-0.25
	LPO	-0.49***	-0.30*	0.14	-0.27*	-0.38***	-0.24*	0.23*

*P < 0.05 and ***P < 0.001

Progressive motility, PM, %; rapid velocity, RV, %; medium velocity, MV, %; average path velocity, VAP, µm sec^{-1}; straight line velocity, VSL, µm sec^{-1}; curvilinear velocity, VCL, µm sec^{-1}; amplitude of lateral head displacement, ALH, µm); and other semen quality variables (supra vital plasma membrane integrity, SV–PMI, %; viable and intact acrosome, V–IACR, %; DNA integrity, DNA–I, %; post–dilution, PD; pre-freezing, PF; and post-thawing, PT.

4.5. DISCUSSION

It is well known that there occurs an overproduction of ROS because of oxidative stress during semen processing and cryopreservation (Andrabi, 2007). Addition of antioxidants in semen extender controls the degree of ROS generation during cryopreservation (Iqbal et al., 2016 a,b). Presently, at post-dilution, a higher seminal total antioxidant content was found with all concentrations of curcumin added into the extender as compared to control. While at pre-freezing and post-thawing, a higher seminal total antioxidant content was found with 1.5 mM and 2.0 mM concentrations of curcumin in the extender compared to 0.5 mM and 1.0 mM curcumin and control. This demonstrated that curcumin added at the concentrations of 1.5 mM and 2.0 mM in the cryodiluents were able to alleviate the total seminal antioxidant contents during the processes of cooling and freezing.

The susceptibility of mammalian spermatozoa to oxidative stress is due to their higher concentrations of unsaturated fatty acids and of limited repair mechanisms (Van Loon et al., 1991; Andrabi, 2009). The lipid peroxidation surge begins with the attack of ROS on polyunsaturated fatty acids of the sperm plasmalemma (Baumber et al., 2003). Moreover, it has been reported that the seminal antioxidant content is inadequate for the prevention of lipid peroxidation during freezing and thawing processes (Storey, 1997). Consequently, fortification of cryodiluent with the antioxidant is required (Andrabi et al., 2008a). At post-dilution, this study found lower lipid peroxidation level with 1.5mM curcumin than the 0.5mM curcumin and control. At pre-freezing and post-thawing, lower lipid peroxidation levels were found with 1.5 mM and 2.0 mM compared to other concentrations of curcumin in the extender and control. This highlights that 1.5 mM and 2.0 mM dose level of curcumin not only sustained the seminal total antioxidant capacity but also restricted the lipid peroxidation during cryopreservation. Studies on bull and goat spermatozoa however report contrasting findings that curcumin did not significantly affect the lipid peroxidation and antioxidant potential levels at post-thawing (Bucak et al., 2010; 2012). This difference can be attributed to the species differences (water buffalo versus buck and Taurus bull) in response to the doses of curcumin in cryodiluents. It is relevant to mention that cooling and freezing rates themselves affect the levels of ROS generation (Wang et al., 1997), which can be countered with the addition of

antioxidants in the extender. It is therefore suggested the ability of curcumin to assemble in the plasma membrane (Jaruga et al., 1998), indicates that curcumin at a certain dose level can protect the plasma membrane against lipid peroxidation during cryopreservation.

Computer assisted sperm analysis allows an objective assessment of motility parameters, velocity distribution and kinematics in buffalo (Andrabi, 2014; Shah et al., 2016). The prognostic values of sperm motility and motion characteristics significantly predicted the *in vitro* and *in vivo* fertility in buffalo (Sohail et al., 2013; Ahmed et al., 2016 a,b). At post-thawing, the current study found higher sperm progressive motility with 1.5 mM curcumin compared to other concentrations of curcumin and control. Similarly, Bucak et al. (2012) reported that addition of 0.5 and 2.0 mM curcumin in the extender did not improve the post-thawing progressive motility in Holstein bull. This highlights that 1.5 mM curcumin was efficacious enough to keep a balance between ROS generation and antioxidant capacity to counteract the oxidative stress and ultimately increasing the progressive motility (Sharma and Agarwal, 1996). Interestingly, at post-thawing, the correlation matrix in this study revealed a significantly higher association between sperm progressive motility and seminal total antioxidant capacity. Similarly, a highly significant negative correlation was found between sperm progressive motility and lipid per oxidation. It is worth mentioning that dose level of 2.0 mM curcumin in extender may have cytotoxic effects on buffalo bull spermatozoa (Naz, 2011). The mechanism by which curcumin impedes the progressive motility appears to be related to acidification of spermatozoa intracellularly and hyperpolarization of its membrane due to concentration-dependent decrease in intracellular pH from 7.3 to 6.81 in human and mouse (Naz, 2014).

High rapid velocity of spermatozoa is indicative of superior prognostic value in terms of *in vivo* fertility in water buffalo (Ahmed et al., 2016 a,b). At post-thawing, the current study found higher sperm rapid velocity with 1.5 concentration of curcumin compared to 0.5 and 2.0 mM curcumin and control. This indicates that 1.5mM curcumin has played a role in maintaining the seminal antioxidant levels, which may have enhanced the rapid sperm velocity after the freeze-thawing cycle. It is noteworthy that the present data of rapid velocity are very much supported with that

of progressive motility, that is, a similar pattern was observed in progressive motility of buffalo bull spermatozoa with the addition of curcumin in cryodiluents. Moreover, at post-thawing, the correlation matrix in the present study revealed a highly significant association between sperm rapid velocity and seminal total antioxidant capacity. Similarly at post-thawing, a highly significant negative correlation was found between sperm rapid velocity and lipid per oxidation.

The sperm kinematics reflects the physical and biochemical conditions imposed on spermatozoa during cryopreservation (Andrabi, 2009). In the present study, average path velocity, straight-line velocity, curvilinear velocity, straightness and linearity of spermatozoa were significantly increased with 1.5 mM curcumin concentration than the control. Conversely, Bucak et al. (2010; 2012) did not report any significant improvement with curcumin in the kinematics of buck and bull spermatozoa except the linearity in bovine. The overall discrepancy may be due to species differences and strict criteria for CASA settings as used in the current study which provided rational conclusions in terms of CASA output (Shah et al., 2016). Besides the above, this study found that most of the objective CASA variables were significantly correlated with supravital plasma membrane integrity, percentage of viable sperm with intact acrosome, DNA integrity and biochemical assays particularly at the post-thawing stage of cryopreservation. This signifies the efficacy of CASA for objective evaluation of buffalo bull spermatozoa in the current study.

Evaluation of *in vitro* longevity predicts the viability of spermatozoa in female reproductive tract (Akhter et al., 2008). In present study, at 1 h incubation, the progressive motility was significantly greater with 1.5 mM curcumin as compared to other concentrations and control. Regarding the rapid velocity, at 1 h of incubation, it was found to be significantly greater with 1.5 mM curcumin as compared to 0.5 mM and 2.0 mM curcumin and control. While at 2 h of incubation, both the progressive motility and rapid velocity were significantly greater with 1.5 mM curcumin than the control. This shows an improved survivability of buffalo bull spermatozoa during 2 h incubation under *in vitro* conditions. This improvement can be due the maintenance of seminal total antioxidant capacity and prevention of lipid peroxidation by curcumin at 1.5 mM dose level. However in the present study, the total antioxidative profiles were

similar in extenders containing 1.5 mM and 2.0 mM curcumin; this discrepancy could be due to the toxic effects of curcumin at dose level of 2.0 mM (Naz, 2011).

The integrity of sperm plasma membrane and acrosome is an essential criterion to sustain spermatozoal functions in the female's reproductive tract and oocyte penetration (Holt, 2000; Esteves et al., 2007). At post-thawing, this study found higher supravital plasma membrane integrity and percentage of viable spermatozoa with intact acrosome with the addition of 1.5 mM curcumin in the extender as compared to 2.0 mM dose and control. These findings signify that 1.5 mM curcumin in cryodiluent acted as a membrane stabilizing antioxidant by preventing the elevation of lipid peroxidation level. However in the present study, the lipid peroxidation levels were similar in extenders containing 1.5 and 2.0 mM curcumin; as narrated above, this discrepancy could be due to the toxic effects of curcumin at dose level of 2.0 mM (Naz, 2011). It is worth mentioning that in this study at post-thawing, a significant association between supravital plasma membrane integrity and sperm progressive motility/rapid velocity was found. Regarding the acrosome integrity, Omur and Coyan (2016) reported that it was higher with 1, 2 and 4 mM curcumin in Merino ram. However, Bucak et al. (2012) reported no significant differences in bovine sperm acrosome abnormalities with 0.5 mM and 2 mM curcumin in the extender. Interestingly, both of these studies had limitations in evaluating only the acrosomal integrity and ignoring the percentage of viable/nonviable spermatozoa. It is relevant to mention that this study at post-thawing, percentage of viable spermatozoa with intact acrosome was significantly correlated with sperm progressive motility and rapid velocity.

DNA integrity is considered as an essential factor for the determination of spermatozoa to endure the cryopreservation process (Evenson et al., 2002). At post-thawing, the current study found higher DNA integrity with all the concentrations of curcumin in extender compared to control. It is an established fact that during cryopreservation, sperm DNA damage is induced by the generation of ROS (Gorczyza et al., 1993). Moreover, the two factors that may provide protection to sperm DNA from oxidative injury include the characteristic tight packaging of the DNA and the antioxidants present in the seminal plasma (Twigg et al., 1998). This indicates that curcumin as an antioxidant in the extender may have a stronger link for

providing protection to sperm DNA against the oxidative stress during the freeze-thawing process. This was evident in this study by quantifying the seminal total antioxidant capacity and sperm lipid peroxidation at different stages of cryopreservation. Similarly, Soleimanzadeh and Saberivand (2013) found higher post-thawing sperm DNA integrity with curcumin in albino Wistar rat. However, Kelley et al. (2001) have reported that curcumin not only failed to avert the single-strand DNA breaks by H_2O_2, but also generated DNA damage in Jurkat T-lymphocyte cells. These differences in the effect of curcumin may be due to different types of cells and their physiological responses upon its supplementation. Therefore, it can be put forward that curcumin at certain dose level in extender protects the sperm DNA of buffalo bull during the freeze-thawing cycle.

Overall, a dose-dependent response was not found in this study. Curcumin at 0.5, 1.0 and 2.0 mM in extender was inferior in protecting the sperm structures and functions compared to 1.5 mM. The possible reason for low efficacy of curcumin at 0.5 and 1.0 mM could be its insufficient concentrations in creating redox homoeostasis as it was observed during the seminal total antioxidant capacity and lipid peroxidation evaluation. Moreover, there is a possibility that 2.0 mM curcumin in extender may have cytotoxic effects (Naz, 2011) on buffalo bull spermatozoa resulting in compromised sperm structures and functions. It is, therefore, proposed that addition of 1.5 mM curcumin in the extender has been well tolerated by buffalo spermatozoa and seems to be rather beneficial in terms of its antioxidative role during cryopreservation.

4.5.1. CONCLUSION

It is concluded that freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of 1.5 mM curcumin (diferuoyl methane) as antioxidant in semen extender.

CHICKEN EGG YOLK PLASMA IN TRIS-CITRIC ACID EXTENDER IMPROVES THE QUALITY AND FERTILITY OF CRYOPRESERVED WATER BUFFALO (*Bubalus bubalis*) SPERMATOZOA

5.1. SUMMARY

This study was primarily designed to evaluate the effect of different concentrations of UV-C irradiated chicken egg yolk plasma (EYP; v/v; 10%, P1; 15%, P2; 20%, P3) or 20% (v/v) of whole chicken egg yolk (WCEY) in tris−citric acid extender on quality of water buffalo sperm during cryopreservation (post–dilution, PD; post–equilibration, PE; post–thawing, PT). The effect of best experimental concentration of EYP in extender on *in vivo* fertility of buffalo spermatozoa was also evaluated. At PE and PT, CASA progressive motility (PM, %) was significantly higher in P3 as compared to P1 and WCEY. Rapid velocity (RV, %) was higher in P3 compared to P1 and WCEY during cryopreservation (PD, PE and PT). Average path velocity (µm sec^{-1}) and straight line velocity (µm sec^{-1}) were higher in P2 and P3 than WCEY at PE and PT. The decline percentage (%, longevity) in PM and RV was lower in P3 when compared with WCEY during the 2 h incubation period under *in vitro* conditions at PT. Supra–vital plasma membrane integrity (%) was higher in P2 and P3 as compared to control at different stages (PE and PT). Mitochondrial transmembrane potential (%) was higher in P2 and P3 than the P1 and WCEY at different stages (PD and PT). Percentage of viable sperm with intact acrosome, and sperm DNA integrity (%) were higher in P2 and P3 as compared to WCEY at PT. The *in vivo* fertility rate (%) was significantly higher with P3 as compared to WCEY (76.61 vs. 64.49). In conclusion, WCEY (20%) can be replaced with UV-C irradiated chicken EYP (20%) in tris–citric acid extender for cryopreservation of water buffalo spermatozoa.

5.2. INTRODUCTION

Whole chicken egg yolk (WCEY) has been conventionally used as a general constituent of semen freezing extenders for majoirty of the livestock species including buffalo possibly due to its easy availability (Andrabi, 2009).

Being a non-membrane permeable cryoprotectant, major role of WCEY in extender is to provide protection against sperm damages during the cooling and freezing procedures (Andrabi, 2014). However, the use of WCEY in cryodiluents is also associated with some disadvantages. Earlier studies have revealed that WCEY contains substances like high density lipoproteins (HDLs) and minerals, which have the potential to affect the sperm functionality (Manjunath et al., 2002). It has been demonstrated that low density fraction of WCEY, which is mainly composed of low density lipoproteins (LDLs) is responsible for the cryoprotective properties (Bergeron et al., 2004). The mechanisms that have been suggested to elucidate these cryoprotective properties range from reversible binding of exogenous lipid (Ricker et al., 2006) to unification of liposomes with plasma membrane of sperms (De Leeuw et al., 1993). Several recent studies have tested replacing WCEY with purified LDLs for cryopreservation of bovine (Manjunath et al., 2002), canine (Bencharif et al., 2010), equine (Pillet et al., 2012) and bubaline (Akhter et al., 2011) spermatozoa with the aim to develop extenders that are chemically better defined than those containing WCEY. However, LDLs cannot be routinely used in freezing extenders, because they are not produced at an industrial scale (Pillet et al., 2011). Although, soy lecithin extenders are available at commercial levels; they are not meant for cryopreservation of water buffalo spermatozoa, therefore they are still under examination and are not generally acknowledged (Layek et al., 2016).

Alternatively, through ultracentrifugation, WCEY can be isolated into two of its major fractions, the plasma and granules (Pillet et al., 2011). Chicken egg yolk plasma (EYP) is composed of 85% LDLs and 15% livetin fraction and it also presents the edge to be easily produced in the laboratory (Anton, 2013; Strixner and Kulozik, 2013; Laca et al., 2015). In recent experiments, it was found that chicken EYP was better than WCEY in improving the post thaw quality and fertility of spermatozoa in dogs (Corcini et al., 2016) and horses (Pillet et al., 2011), respectively. Additionally,

keeping in view the disinfected requirements in semen processing (Andrabi et al., 2016), sterilization of chicken EYP is an essential step before it is included in the extender. Although, gamma–irradiation of chicken EYP has been used for equine cryodiluent, yet it is not easily available, requires specialized handling and may cause denaturation of the product (Pillet et al., 2011). Alternatively, ultraviolet light (UV–C) having an electromagnetic spectrum of 100 to 280 nm, has been referred to as 'germicidal UV', as it effectively inactivates bacteria and viruses (Caillet-Fauquet et al., 2004; Lytle and Sagripanti, 2005). Therefore, dynamic UV–C as an alternative and economical non-thermal/non-ionizing treatment can be used to eradicate the microorganisms present in chicken EYP. Importantly the compactness and higher viscosity involved in this technique does not work effectively to sterilize the WCEY (Unluturk et al., 2008).

The experiments presented in this chapter were primarily designed to explore the result of different concentrations of UV-C irradiated chicken EYP (v/v; 10% or 15% or 20%) in tris−citric acid based extender on water buffalo sperm quality during cryopreservation (post–dilution, PD; post–equilibration, PE; post–thawing, PT). In addition, the effect of best developed concentration of UV-C irradiated EYP in extender on *in vivo* fertility of buffalo spermatozoa was evaluated.

5.3. MATERIALS AND METHODS

The details of this section have been described in Chapter 2 (Sections 2.1, 2.2.1-2.2.3.5 and 2.6.3).

5.4. RESULTS

5.4.1. Effect of whole chicken egg yolk (WCEY) and chicken egg yolk plasma (EYP) in extender on sperm motility parameters and velocity distribution

Data on the effect of WCEY and chicken EYP in extender on sperm motility parameters and velocity distribution in buffalo bull at PD, PE and PT stages of cryopreservation are presented in Table 5.1. At PD, mean value of PM was higher (P

< 0.05) in P3 as compared to other experimental groups and WCEY. At PE and PT, mean values of PM were greater ($P < 0.05$) in P3 as compared to P1 and WCEY. Mean values of RV were greater ($P < 0.05$) in P3 as compared to P1 and WCEY during cryopreservation (PD, PE and PT). At PD and PE, mean values of MV were greater ($P < 0.05$) in WCEY as compared to P2 and P3. At PT, mean value of MV was greater ($P < 0.05$) in WCEY than P3.

5.4.2. Effect of WCEY and chicken EYP in extender on sperm kinematics

Data on the effect of WCEY and chicken EYP in extender on sperm kinematics in buffalo bull at PD, PE and PT stages of cryopreservation are presented in Table 5.2. At PD, mean value of VAP was greater ($P < 0.05$) in all the experimental groups as compared to control. At PE, mean value of VAP was greater ($P < 0.05$) in P2 and P3 than WCEY. At PT, mean value of VAP was greater ($P < 0.05$) in P3 than WCEY. At PE, mean values of VSL were greater ($P < 0.05$) in P2 and P3 than WCEY. At PT, mean value of VSL was greater ($P < 0.05$) in P3 as compared to P1 and WCEY. At PD, mean value of VCL was greater ($P < 0.05$) in P1 and P2 as compared to P3 and WCEY. At PD, mean value of ALH was lower ($P < 0.05$) in P3 as compared to P1 and P2. At PD, mean value of BCF was lower ($P < 0.05$) in P3 as compared to other experimental groups and WCEY. At PE, mean value of BCF was lower ($P < 0.05$) in WCEY than P2. At PE, mean value of BCF was greater ($P < 0.05$) in P2 as compared to WCEY.

5.4.3. Effect of WCEY and chicken EYP on *in vitro* longevity (%)

Data on the effect of WCEY and chicken EYP on percent decline of PM and RV from 0–2 h of incubation period (37°C) at PT is presented in Table 5.3. The percent decline in PM was lower ($P < 0.05$) with P3 as compared to P1 and WCEY during 2 h of incubation. The percent decline in RV was lower ($P < 0.05$) with P2 and P3 as compared to WCEY during 2 h of incubation period.

5.4.4. Effect of WCEY and chicken EYP in extender on sperm SV–PMI, MMP, V–IACR and DNA–I

Data on the effect of WCEY and chicken EYP in extender on sperm SV–PMI, MMP, V–IACR and DNA–I in buffalo bull at PD, PE and PT stages of cryopreservation are presented in Table 5.4. At PD, mean value of SV–PMI was greater ($P < 0.05$) in P2 and P3 as compared to P1 and WCEY. At PE and PT, mean values of SV–PMI were greater ($P < 0.05$) in P2 and P3 as compared to WCEY. At PD and PT, mean values of MMP were greater ($P < 0.05$) in P2 and P3 as compared to P1 and WCEY. At PE, mean value of MMP was greater ($P < 0.05$) in P3 as compared to P1 and WCEY. At PD and PE, mean values of V–IACR were greater ($P < 0.05$) in P3 as compared to WCEY. At PT, mean values of V–IACR were greater ($P < 0.05$) in P2 and P3 as compared to WCEY. At PT, mean value of DNA–I was greater ($P < 0.05$) in P2 and P3 as compared to WCEY.

5.4.5. Effect of WCEY and chicken EYP (P3) in extender on *in vivo* fertility (%)

Data on the effect of WCEY and chicken EYP (P3) in extender *in vivo* fertility (%) of buffalo spermatozoa are presented in Table 5.5. *In vivo* fertility (%) of buffalo spermatozoa was significantly greater ($P < 0.05$) in P3 as compared to WCEY.

Table 5.1 Effect of differential chicken egg yolk plasma (EYP) and whole chicken egg yolk (WCEY) in tris-citric acid extender on sperm motility parameters and velocity distribution in buffalo bull during cryopreservation (5 bulls, 6 replicates, 30 ejaculates)

Variable	Stage	Experimental groups			
			EYP		
		WCEY	**10%**	**15%**	**20%**
PM	PD	44.5 ± 3.58^{b}	46.33 ± 4.65^{b}	46.5 ± 1.09^{b}	62.78 ± 0.99^{a}
	PE	20.83 ± 1.42^{c}	28.25 ± 2.53^{bc}	41.75 ± 4.19^{ab}	52.20 ± 4.34^{a}
	PT	19.25 ± 3.99^{c}	26.83 ± 3.55^{bc}	36.25 ± 4.07^{ab}	49.83 ± 2.28^{a}
RV	PD	57.33 ± 3.22^{b}	70.17 ± 3.09^{b}	80.33 ± 2.53^{a}	79.97 ± 2.27^{a}
	PE	37.83 ± 3.43^{c}	51.33 ± 3.02^{bc}	66.25 ± 3.67^{ab}	68.83 ± 1.87^{a}
	PT	28.17 ± 3.41^{c}	38.20 ± 3.15^{bc}	45.75 ± 4.70^{ab}	60.75 ± 2.29^{a}
MV	PD	39.33 ± 3.38^{a}	28.17 ± 3.45^{ab}	16.17 ± 2.81^{b}	17.22 ± 1.76^{b}
	PE	53.33 ± 5.06^{a}	42.83 ± 3.29^{ab}	30.08 ± 4.68^{b}	26.31 ± 2.49^{b}
	PT	60.83 ± 4.88^{a}	55.40 ± 2.42^{a}	47.75 ± 3.79^{ab}	33.75 ± 2.37^{b}
SV	PD	1.67±0.21	1.17±0.48	1.00±0.00	0.14±0.09
	PE	3.33±0.56	1.58±0.90	2.25±0.85	1.28±0.41
	PT	6.67±2.55	4.25±1.26	2.17±0.87	2.42±0.52

Values are shown as Mean±S.E.M. Values without a common superscript in a row indicate significant difference ($P < 0.05$) among the experimental groups at a particular stage. Post–dilution, PD; post–equilibration, PE; and post–thawing, PT;; WCEY (20%, v/v); EYP (v/v); P1, 10%; P2, 15%; P3, 20%; progressive motility (PM, %), rapid velocity (RV, %), medium velocity (MV, %) and slow velocity (SV, %)

Table 5.2 Effect of differential chicken egg yolk plasma (EYP) and whole chicken egg yolk (WCEY) in tris-citric acid extender on sperm kinematics in buffalo bull during cryopreservation (5 bulls, 6 replicates, 30 ejaculates)

Variable	Stage	Experimental groups			
			EYP		
		WCEY	**10%**	**15%**	**20%**
VAP	PD	94.8±2.38[b]	113.6±5.58[a]	120.1±2.48[a]	105.9±3.15[ab]
	PE	78.78±4.07[b]	89.09± 3.78[ab]	98.16± 3.02[a]	97.92±1.08[a]
	PT	68.23±2.45[b]	78.67±3.60[ab]	82.53±3.50[ab]	91.53±1.40[a]
VSL	PD	80.97±3.17	91.82±6.20	90.9± 1.57	89.32±2.09
	PE	59.98±2.43[b]	67.91±2.48[ab]	77.7± 3.76[a]	81.66±1.88[a]
	PT	55.73±2.91[b]	62.79±3.34[b]	69.63±3.65[ab]	78.39±1.54[a]
VCL	PD	137.6±1.68[b]	179.5±6.84[a]	196.9±4.89[a]	146.8±6.15[b]
	PE	134.3±7.03	145.8±8.30	158.5±3.52	149.6±8.21
	PT	108.9±2.36	123.7±2.71	122.5±4.26	131.9±3.65
ALH	PD	6.43±0.36[ab]	7.43±0.25[a]	7.73±0.32[a]	5.65±0.27[b]
	PE	7.28±0.25	6.51± 0.37	6.70± 0.08	6.20± 0.60
	PT	6.38±0.26	5.85±0.26	5.66±0.35	5.55±0.22
BCF	PD	31.23±1.76[a]	32.83±1.93[a]	31.08±0.43[a]	23.55±0.29[b]
	PE	23.90±0.45[b]	27.71± 0.90[ab]	29.38±0.87[a]	27.93± 0.42[ab]
	PT	27.90±0.53	31.40±0.98	30.33±1.11	29.24±0.40

Values are shown as Mean±S.E.M. Values without a common superscript in a row indicate significant difference ($P < 0.05$) among the experimental groups at a particular stage. Post–dilution, PD; post–equilibration, PE; and post–thawing, PT; WCEY (20%, v/v); EYP (v/v); P1, 10%; P2, 15%; P3, 20%; average path velocity (VAP, µm sec^{-1}), straight line velocity (VSL, µm sec^{-1}), curvilinear velocity (VCL, µm sec^{-1}), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz)

Table 5.3 Effect of differential chicken egg yolk plasma (EYP) and whole chicken egg yolk (WCEY) in tris-citric acid extender on percent decline in progressive motility (%) and rapid velocity (%) of buffalo bull spermatozoa from 0–2 h of incubation period (37°C) at post–thawing (5 bulls, 6 replicates, 30 ejaculates)

Variable	Experimental groups	Percent decline
Progressive motility	WCEY	30.90±6.17^{a}
	10% EYP	36.11±3.09^{a}
	15% EYP	19.42±4.91^{ab}
	20% EYP	12.01±2.36^{b}
Rapid velocity	WCEY	41.81±6.57^{a}
	10% EYP	27.59±1.68^{ab}
	15% EYP	15.21±3.97^{b}
	20% EYP	10.75±2.24^{b}

Values are shown as Mean±S.E.M. Values without a common superscript in a column indicate significant difference ($P < 0.05$) among the experimental groups. WCEY (20%, v/v); EYP (v/v); P1, 10%; P2, 15%; and P3, 20%

Table 5.4 Effect of differential chicken egg yolk plasma (EYP) and whole chicken egg yolk (WCEY) in tris-citric acid extender on semen quality parameters of buffalo bull spermatozoa during cryopreservation (5 bulls, 6 replicates, 30 ejaculates)

Variable	**Stage**	**Experimental groups**			
			EYP		
		WCEY	**10%**	**15%**	**20%**
SV–PMI	PD	72.17±0.70^{b}	74.17±0.95^{b}	80.50±0.43^{a}	84.00±0.77^{a}
	PE	61.83±0.75^{c}	65.17±0.65^{bc}	67.00±0.82^{ab}	69.67±1.28^{a}
	PT	25.17±0.79^{c}	35.17±0.95^{b}	38.67±0.84^{ab}	40.50±0.62^{a}
MMP	PD	77.33±0.88^{b}	81.17±1.17^{b}	83.33±0.84^{a}	87.00±0.73^{a}
	PE	69.67±0.71^{b}	71.67±0.67^{b}	76.33±0.67^{ab}	79.33±0.95^{a}
	PT	41.00±0.97^{b}	50.33±1.17^{c}	56.50±0.96^{a}	59.50±1.69^{a}
V–IACR	PD	67.17±0.48^{b}	69.17±1.62^{ab}	69.83±2.36^{ab}	75.00±1.59^{a}
	PE	60.00±1.69^{b}	63.50±1.06^{ab}	66.17±0.95^{ab}	68.00±1.39^{a}
	PT	55.67±1.61^{b}	61.83±1.11^{ab}	62.67±1.02^{a}	65.00±0.52^{a}
DNA–I	PD	94.00±0.37	94.50±0.22	94.00±0.58	95.67±0.33
	PE	93.33±0.49	94.00±0.52	94.50±0.43	94.83±0.60
	PT	92.67±0.33^{b}	94.33±0.33^{ab}	94.83±0.31^{a}	95.33±0.33^{a}

Values are shown as Mean±S.E.M. Values without a common superscript in a row indicate significant difference ($P < 0.05$) among the experimental groups at a particular stage. Post–dilution, PD; post–equilibration, PE; and post–thawing, PT; WCEY (20%, v/v); EYP (v/v); P1, 10%; P2, 15%; and P3, 20%; supra–vital plasma membrane integrity (SV–PMI, %), mitochondrial transmembrane potential (MMP, %), viable and intact acrosome (V–IACR, %) and DNA integrity (DNA–I, %)

Table 5.5 Effect of chicken egg yolk plasma (EYP, P3, 20%, v/v) and whole chicken egg yolk (WCEY, 20%, v/v) in tris-citric acid extender on *in vivo* fertility of buffalo bull spermatozoa

Experimental groups	**Buffaloes inseminated (n)**	**Buffaloes pregnant (n)**	**Pregnancy rate (%)**	**Chi-square value**	**P value**
WCEY	107	69	64.49[b]	4.102	0.043
20% EYP	124	95	76.61[a]		

Values without a common superscript in a column indicate significant difference ($P < 0.05$).

5.5. DISCUSSION

This study was primarily designed to assess the effect of different concentrations of UV-C irradiated chicken EYP (v/v; 10% or 15% or 20%) in tris−citric acid based extender on water buffalo sperm quality during cryopreservation (post–dilution, PD; post–equilibration, PE; post–thawing, PT). The effect of best evolved concentration of UV-C irradiated EYP in extender on *in vivo* fertility of buffalo spermatozoa was also evaluated.

The motility plays an important role during sperm transport in the female reproductive tract. The results of this study demonstrated that PM of buffalo bull spermatozoa at PE and PT was significantly increased in extender containing P3 (EYP, 20%) as compared to P1 (EYP, 10%) and (WCEY). Similarly, increased post-thaw sperm motility has been reported with 20% EYP as compared to 20% WCEY in canine (Corcini et al., 2016). In another study in dogs, the post-thaw percentage of motile spermatozoa was increased (55.3% vs. 27.7%) with purified 6% LDL extender compared to WCEY extender (Bencharif et al., 2008). Earlier studies have demonstrated that WCEY has cryoprotectant antagonists, variable composition, HDLs and egg yolk granules that hamper the motility of sperm (Akhter et al., 2011). Therefore, it is suggested that these disadvantages can be minimized by using the chicken EYP.

Computer assisted sperm analysis acquires detailed digital data of sperm movement in many microscopic fields and presents an objective assessment of velocity distribution and kinematics, which are not possible to measure and/or observe manually (Verstegen et al., 2002). The results of current study demonstrated that RV was greater ($P < 0.05$) with P3 as compared to P1 and WCEY during cryopreservation (PD, PE and PT). Medium velocity was increased in control as compared to other experimental groups at all the stages of cryopreservation. At PE, increased VAP was observed with P2 and P3 compared to WCEY. Other sperm kinematics (VAP, VSL) were increased in extender containing P2 and P3 at PE and PT as compared with the WCEY. Similarly, CASA parameters were reported to be better in bovine with the addition of LDLs in extender compared to WCEY (Amirat et al., 2004; 2005). This improvement in CASA motion characteristics could be due to increased free

movement of spermatozoa with EYP (Pillet et al., 2011).

In vitro evaluation of longevity is performed to foresee the viability of spermatozoa in the female reproductive tract (Akhter et al., 2008). In this study, the decline percentage (Δ, longevity) of PM and RV was lower in P3 as compared to WCEY. This shows a better survivability of buffalo bull spermatozoa during 2 h incubation under *in vitro* condition at PT. It is suggested that WCEY could contain some unwanted components that potentially affect the longevity and survivability of spermatozoa (Pillet et al., 2011; Layek et al., 2016).

Supra–vital HOST gives an assessment of both functional and structural PMI of spermatozoa simultaneously (Chan et al., 1991). A better SV–PMI of buffalo bull spermatozoa in extenders with P2 and P3 compared to P1 and WCEY at PD was observed, while it was significantly greater in P2 and P3 as compared to WCEY at PE and PT. The present results are comparable with the findings of a previous study in buffalo (Akhter et al., 2011) in which PMI was significantly higher in TCA extender containing commercially available LDLs as compared to the WCEY. It is thought that in the present study the concentration of LDLs available in chicken EYP (P2 and P3) was sufficient enough to interact with the buffalo spermatozoa and thus provided better cryoprotection. Similarly, a highly significant increment in terms of PMI has been shown in extender containing 20% chicken EYP (Corcini et al., 2016) and commercially available 6% LDLs (Bencharif et al., 2010) as compared to the extender containing WCEY in canine. In contrast, PMI of stallion spermatozoa was found to be higher when freezing extender was supplemented with WCEY (2%) as compared to chicken EYP (2%), (Pillet et al., 2011). This is supposed to be due to a difference in the composition of sperm plasma membranes of the two species (stallion vs. buffalo bull). In another study, PMI of frozen thawed cattle bull spermatozoa was similar in the extenders containing 8% LDLs or 20% WCEY (Amirat et al., 2004). It is pertinent to mention that the required concentrations of LDLs in semen extenders could be due to species related differences of sperm plasma membrane (Labbe et al., 2001). Moreover, spermatozoa from different species also differ in their membrane elasticity, swelling volume, water permeability and cell geometry (Courtens and Rety, 2001).

Mitochondria are the energy centers of spermatozoa (Graham and Moce, 2005), and low MMP is considered as a sign of early sperm apoptosis (Barroso et al., 2006). The present study found significantly higher MMP with P2 and P3 compared to P1 and WCEY during PD and PT, while it was higher with P3 compared to P1 and WCEY at PE. This significant finding appears to be due to an adequate availability of LDLs and glycoproteins (Anton, 2013) with P3, which protected the mitochondrial membrane during cryopreservation. It has been reported that sperm motility is maintained by intact mitochondria (Lindemann et al., 1991), which is indicated in the present study by higher MMP with P3 during cryopreservation.

The assessment of acrosomal integrity provides an estimate of sperm fertility (Graham and Moce, 2005). The present study described significantly better V–IACR protection to buffalo spermatozoa in P3 compared to control during PD and PE and in P2 and P3 compared to WCEY at PT. Similarly, higher acrosomal integrity in dog spermatozoa was reported with 20% EYP than 20% WCEY (Corcini et al., 2016). Presently, significant increase in acrosome integrity may be attributed to the availability of sufficient LDLs in P2 and P3. It is reported that during cryopreservation, HDLs in WCEY interact with bovine seminal plasma proteins and accelerate the sperm capacitation (Manjunath et al., 2002). The LDLs interact with seminal plasma proteins by lowering the efflux of cholesterol and phospholipids from the plasma membrane and prevent premature capacitation and subsequent acrosome reaction (Manjunath et al., 2002; Bergeron et al., 2004). It is noteworthy to mention that plasma extracted from egg yolk is composed of 85% LDLs and 15% livetin fraction (Pillet et al., 2011; Anton, 2013; Strixner and Kulozik, 2013), and thus offers an advantage over WCEY. It is, therefore, hypothesized that LDL enriched chicken EYP may have maintained the acrosome integrity of buffalo spermatozoa either directly through the exchange or restoring of phospholipids of acrosomal membrane (Bencharif et al., 2008).

Sperm DNA integrity is a vital evaluation parameter and is associated with fertilization potential in buffalo (Ahmed et al., 2016a). This study found significantly higher DNA–Iin P2 and P3 compared to control at post–thawing. It is relevant to mention that WCEY provides substrates (aromatic amino acids such as L–phenylalanine) for H_2O_2 production by an aromatic amino acid oxidase released from

dead spermatozoa, thus resulting in oxidative stress (Shannon and Curson, 1982). It is therefore, hypothesized that due to WCEY, there may be an over production of reactive oxygen species (ROS) during freezing and thawing, which may have compromised the sperm DNA–I compared to chicken EYP.

The production of livestock can be improved by using one of the modern reproductive techniques, that is, AI (Andrabi, 2014). A significantly higher *in vivo* fertility with P3 compared to control (76.61% vs. 64.49%, $P < 0.05$) was observed in this study. Similarly, favorable AI results were found in mares with semen cryopreserved in extender containing EYP (Pillet et al., 2011). In bovine, significantly higher cleavage rate with semen frozen in LDLs extracted from chicken egg yolk was found (Amirat et al., 2004). It is noteworthy that a fertility rate higher than 50% is regarded as a good result after AI with frozen–thawed spermatozoa in water buffalo (Vale, 1997; Andrabi, 2009). Thus, present results as regards the *in vivo* fertility are promising and suggest that EYP (20%) can replace WCEY for cryopreservation of water buffalo spermatozoa.

Lastly, it is relevant to discuss that the dynamic UV–C irradiation is a promising technology to lower the microbial loads without damaging the relevant quality attributes of chicken egg products (de Souza and Fernandez, 2011). It is reported that UV-C is mutagenic to microorganisms through its capability to break the molecular bonds within microorganism nucleic acid (DNA or RNA), thereby destroying them, rendering them harmless or prohibiting their growth and multiplication (Caillet-Fauquet et al., 2004; Lytle and Sagripanti, 2005). The UV–C irradiation dose ranging from 2,000 to 8,000 $\mu W \cdot s/cm^2$ is reported to be lethal for 90% of bacteria and viruses (Kowalski et al., 2000). In the current study, the dynamic UV–C irradiation dose given to chicken EYP was calculated to be 19920000 $\mu W\ s/cm^2$. It is therefore, suggested that the UV–C irradiation dose was high enough to irradiate the microorganisms from chicken EYP in this study. Thus, the chicken EYP being enriched with LDLs (Anton, 2013; Strixner and Kulozik, 2013), may also have met the sanitary requirements (Andrabi et al., 2016) for addition in extender as a non-membrane permeable cryoprotectant.

5.5.1. CONCLUSION

In conclusion, WCEY (20%, v/v) can be replaced with UV-C irradiated chicken EYP (20%, v/v) in tris citric acid extender to improve the post-thaw *in vitro* quality and *in vivo* fertility of water buffalo spermatozoa The method of EYP extraction is simple and can be used in routine buffalo semen cryopreservation process. Moreover, the protocol of dynamic UV-C irradiation used for sterilization of EYP may be used to meet the sanitary requirements.

SYNERGISM BETWEEN GLYCEROL AND DIMETHYL SULFOXIDE AND ITS EFFECT ON *IN VITRO* QUALITY AND IN VIVO FERTILITY OF WATER BUFFALO (*Bubalus bubalis*) SPERMATOZOA

6.1. SUMMARY

The objective of this part of the study was to evaluate a cryoprotection synergism between glycerol and DMSO for water buffalo spermatozoa. In addition, the effect of best developed concentrations of glycerol and DMSO in extender on *in vivo* fertility of buffalo spermatozoa was assessed. Ejaculates (n=30) were equally distributed into five aliquots; first aliquot was diluted at 37°C in extender having 7% glycerol (control); second aliquot was diluted at 37°C as well as at 4°C in extender having 3.5% DMSO (Group 1); third aliquot was diluted at 37°C in extender having 3.5% glycerol and then at 4°C in extender having 3.5% DMSO (Group 2); fourth aliquot was diluted at 37°C in extender having 3.5% DMSO and then at 4°C in extender having 3.5% glycerol (Group 3); fifth aliquot was diluted in extenders having 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C (Group 4). At post thawing (PT), sperm progressive motility (%), rapid velocity (%), average path velocity (µm sec^{-1}), curvilinear velocity (µm sec^{-1}), *in vitro* longevity (%), structural and functional integrity of plasmalemma (%), mitochondrial transmembrane potential (%) and viable sperm with intact acrosome (%) were greater ($P < 0.05$) in Group 4 compared to other treatment groups and control. Regarding sperm DNA integrity (%) at PT; it was greater ($P < 0.05$) in Group 4 compared to Group 1, 3 and control. The *in vivo* fertility rate (%) was significantly greater with Group 4 compared to control (69.45 vs. 59.81). In conclusion, synergism exists between glycerol and DMSO (Group 4) in improving the quality and *in vivo* fertility of cryopreserved water buffalo spermatozoa.

6.2. INTRODUCTION

Cryopreservation is a non–physiological process. It involves the adaptability of spermatozoa to the osmotic and temperature shocks (Holt, 2000). The damages to spermatozoa during cryopreservation affect primarily the cellular membranes (plasma and mitochondrial) and in the worst circumstances, the DNA (Blesbois, 2007). These injuries to sperm membranes result into compromised viability and metabolic factors. Consequently, such alterations in the structural and functional integrity of spermatozoa affect their fertilizing potential. Studies have indicated that composition of extender, suitable cryoprotectant, cooling and thawing rates are involved in the retrieval of cryopreserved buffalo spermatozoa from such injuries (Andrabi, 2014).

Suitable cryoprotectant is added in semen extender to minimize the deleterious effects of freeze-thaw cycle. Many cryoprotectants like glycerol, dimethyl sulfoxide (DMSO) and sugar have been used with contrasting results in cryopreservation protocols for buffalo spermatozoa (Andrabi, 2009). Among these, glycerol is the cryoprotectant of choice and is added at the concentration of 6–7% in tris citric acid extender at 37°C for buffalo spermatozoa (Andrabi 2009). Glycerol performs its role by causing a lowering of the freezing point of water, binding with metallic ions, replacing intracellular water, and by reducing the electrolyte profile in the unfrozen portion (Medeiros et al., 2002). Besides this, glycerol has been demonstrated to have contraceptive effect on the sperm of some species (Jeyendran et al., 1985; Hammerstedt and Graham, 1992; Buhr et al., 2001; Wundrich et al., 2006). This effect is most likely due to the osmotic shock following rapid loss of glycerol from frozen-thawed spermatozoa in female's reproductive tract (Long, 2014).

As an alternative, DMSO is a rapidly penetrating cryoprotectant possessing lower molecular weight than glycerol. Also DMSO may reduce the deleterious effect of hydroxyl radicals, as these radicals are generated during cell metabolism (Johnson and Nasr–Esfahani, 1994; Yu and Quinn, 1994). Moreover, studies on cattle bull (Snedeker and Gaunya, 1970) and rabbit buck (Bamba and Adams, 1990) spermatozoa have revealed that DMSO in combination with glycerol is superior in providing cryoprotection. Conversely, DMSO alone (1.5 or 3%) or in combination with glycerol (1.5 or 3% DMSO and 3 or 6% glycerol) added either at 4 or 37°C did

not provide cryoprotection to buffalo bull spermatozoa (Rasul et al., 2007). The harmful effect of DMSO during cryopreservation is linked with its toxicity rather than the osmotic shock (Rasul et al., 2007). A possibility to overcome these toxic and osmotic effects is to test new concentrations of DMSO and glycerol in the extender and the temperature of addition of these cryoprotectants for cryopreservation of buffalo spermatozoa. Moreover, it has been stated that buffalo spermatozoa are more prone to vulnerability during the freeze-thawing process than bovine spermatozoa, hence giving relatively lower *in vitro* quality and *in vivo* fertility (Andrabi, 2009). Therefore, the objective of present study was to work out a synergism between glycerol and DMSO and to reduce the level of glycerol and to overcome their toxic and osmotic effects in cryodiluent for buffalo spermatozoa. In addition, the effect of best evolved concentrations of glycerol and DMSO in extender on *in vivo* fertility of buffalo spermatozoa was assessed.

6.3. MATERIALS AND METHODS

Details of materials and methods have been described in Chapter 2 (Sections 2.1, 2.2.2-2.2.3.5, 2.4.7, 2.5, 2.5.1-2.5.5.1 and 2.6.4).

6.4. RESULTS

6.4.1. Effect of cryoprotectants (DMSO and/or glycerol) in extender on sperm motility, velocity distribution and kinematic parameters

Data on the effect of cryoprotectants (DMSO and/or glycerol) in extender on sperm motility, velocity distribution and kinematic parameters of buffalo bull spermatozoa during cryopreservation (post dilution, post-equilibration, and post–thawing) are shown in Table 6.1. At post dilution, mean value of progressive motility was greater ($P < 0.05$) in G2 than G1. At post-equilibration, mean value of progressive motility was greater ($P < 0.05$) in G4 as compared to G1 and control. At post–thawing, mean value of progressive motility was greater ($P < 0.05$) in G4 as compared to other treatment groups and control. At post dilution, rapid velocity was greater ($P < 0.05$) in G2 as compared to other treatment groups and control. At post-equilibration, rapid velocity was greater ($P < 0.05$) in G4 as compared to G1 and G3. At post–thawing,

rapid velocity was greater ($P < 0.05$) in G4 as compared to other treatment groups and control. At post-equilibration, mean value of medium velocity was greater ($P < 0.05$) in G1 as compared to control. At post–thawing, mean value of medium velocity was greater ($P < 0.05$) in the control as compared to G1, G3 and G4.

At post dilution, mean value of average path velocity was greater ($P < 0.05$) in G2 as compared to control and G4. At post-equilibration, mean values of average path velocity were greater ($P < 0.05$) in control as compared to G1 and G3. At post–thawing, mean value of average path velocity was greater ($P < 0.05$) in G4 as compared to all treatment groups and control. At post-equilibration, mean values of straight line velocity were greater ($P < 0.05$) in G4 and control as compared to G1 and G3. At post–thawing, mean value of straight line velocity was greater ($P < 0.05$) in G4 as compared to G1, G3 and control. At post dilution, mean value of curvilinear velocity was greater ($P < 0.05$) in G2 as compared to all treatment groups and control. At post-equilibration, mean value of curvilinear velocity was greater ($P < 0.05$) in the control when compared with G1, G3 and G4. At post–thawing, mean value of curvilinear velocity was greater ($P < 0.05$) in G4 compared to G1, G3 and control (Table 1).

6.4.2. Effect of cryoprotectants (DMSO and/or glycerol) on *in vitro* longevity

Data on the effect of cryoprotectants (DMSO and/or glycerol) on progressive motility and rapid velocity of buffalo bull spermatozoa from 0–2 h incubation (37°C) at post–thawing are presented in Table 6.2. The percentage recovery of progressive motility and rapid velocity were greater ($P < 0.05$) in G4 as compared to other treatment groups and control.

6.4.3. Effect of cryoprotectants (DMSO and/or glycerol) in extender on supra–vital plasma membrane integrity, mitochondrial trans-membrane potential, viable and intact acrosome, and DNA integrity

Data on the effect of cryoprotectants (DMSO and/or glycerol) in extender on supra–vital plasma membrane integrity, mitochondrial trans-membrane potential, viable and intact acrosome, and DNA integrity of buffalo bull spermatozoa during

cryopreservation (post dilution, post-equilibration, and post–thawing) are shown in Table 6.3. At post dilution, mean value of supra–vital plasma membrane integrity was greater ($P < 0.05$) in G4 as compared to other treatment groups and control except G2. At post-equilibration, mean value of supra–vital plasma membrane integrity was greater ($P < 0.05$) in G2 and G4 compared to G1 and G3. At post–thawing, mean value of supra–vital plasma membrane integrity was greater ($P < 0.05$) in G4 as compared to other treatment groups and control. Mean value of mitochondrial trans-membrane potential was greater ($P < 0.05$) in G4 as compared to other treatment groups and control at all stages of cryopreservation. Mean value of viable and intact acrosome was greater ($P < 0.05$) in G4 as compared to other treatment groups and control at all stages of except G2 at post dilution. At post–thawing, mean value of DNA integrity was greater ($P < 0.05$) in G4 as compared to other treatment groups and control.

6.4.4. Effect of cryoprotectants (DMSO and/or glycerol) in extender on *in vivo* fertility (%)

Data on the effect of cryoprotectants (DMSO and/or glycerol) in extender on *in vivo* fertility (%) of buffalo spermatozoa are given in Table 6.4. *In vivo* fertility (%) of buffalo spermatozoa was significantly greater ($P < 0.05$) in G4 as compared to control.

Table 6.1 Effect of cryoprotectants (glycerol and/or DMSO in extender [Control (C; (7% glycerol at 37°C), Group 1 (G1; 3.5% DMSO at 37°C as well as at 4°C), Group 2 (G2; 3.5% glycerol at 37°C and 3.5% DMSO at 4°C), Group 3 (G3; 3.5% DMSO at 37°C and 3.5% glycerol at 4°C), Group 4 (G4; 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C)] on sperm motility, velocity distribution and kinematic parameters in buffalo bull (five bulls, six replicates, 30 ejaculates) during cryopreservation

Variable	**Stage**	**Experimental groups**				
		C	G1	G 2	G 3	G 4
PM	PD	29.17±2.75[ab]	26.33±3.07[b]	41.60±5.50[a]	29.50±2.53[ab]	33.83±2.18[ab]
	PE	18.42±0.89[b]	17.33±1.50[b]	20.17±1.96[ab]	17.00±0.58[ab]	25.53±1.60[a]
	PT	14.33±0.84[b]	06.22±0.53[c]	16.33±1.68[b]	06.96±0.18[c]	21.16±1.07[a]
RV	PD	48.50±4.09[b]	42.50±4.59[b]	75.33±0.85[b]	50.67±2.34[a]	51.20±1.49[b]
	PE	31.67±1.32[ab]	31.40±1.07[b]	33.00±1.16[ab]	31.25±1.22[b]	39.11±1.40[a]
	PT	21.83±1.07[b]	06.83±0.60[c]	18.50±2.11[b]	8.53±0.42[c]	28.72±1.96[a]
MV	PD	47.33±3.43[a]	47.33±6.40[a]	29.40±6.68[a]	45.17±2.50[a]	47.17±2.47[a]
	PE	46.00±3.20[b]	60.50±2.12[a]	51.17±2.24[ab]	57.17±2.52[ab]	54.36±3.24[ab]
	PT	71.67±1.47[a]	18.50±3.43[c]	65.00±2.30[ab]	22.96±2.29[c]	58.42±3.86[b]
VAP	PD	85.65 ±3.03[b]	90.02±4.86[b]	100.7 ±5.38[a]	87.13 ±0.68[ab]	86.90±1.64[b]
	PE	80.93 ±2.69[a]	70.52 ±0.80[b]	76.02 ±1.41[ab]	68.78 ±2.23[b]	76.31±1.83[ab]
	PT	63.80±0.57[b]	62.39±1.83[b]	61.78±1.37[b]	60.30±0.44[b]	69.33±2.34[a]
VSL	PD	67.13 ±2.69[a]	67.62 ±3.66[a]	79.30 ±5.67[a]	68.33 ±1.23[a]	70.72±1.31[a]
	PE	61.43 ±1.68[a]	53.83 ±0.88[b]	58.42 ±2.05[ab]	53.13 ±1.45[b]	63.55±1.39[a]
	PT	51.10±0.85[b]	51.78±1.42[b]	53.83±1.77[ab]	51.64±0.57[b]	58.83±1.00[a]
VCL	PD	144.00 ±3.29[b]	131.60 ±5.38[b]	166.10 ±2.28[a]	141.22 ±2.44[b]	133.06±4.98[b]
	PE	142.17 ±4.77[a]	125.78 ±3.46[b]	131.58 ±0.84[ab]	119.98±4.84[b]	118.53±3.26[b]
	PT	101.54±1.96[b]	103.97±2.64[b]	92.45±1.67[c]	97.30±0.51[b]	115.05±2.70[a]

Values are shown as mean ± SEM. Values without a common superscript in a row indicate significant ($P < 0.05$) difference among the treatments at a given stage. Post dilution (PD, 37°C); post-equilibration (PE, 4°C), and post–thawing (PT, 37°C); Progressive motility (PM, %), rapid velocity (RV, %), medium velocity (MV, %), average path velocity (VAP, µm sec^{-1}), straight line velocity (VSL, µm sec^{-1}) and curvilinear velocity (VCL, µm sec^{-1}).

Table 6.2 Effect of cryoprotectants glycerol and/or DMSO in extender on sperm longevity (% recovery) based on CASA progressive motility (%) and rapid velocity (%)] of buffalo bull (five bulls; six replicates) during 2 h of incubation period (37°C) at post–thawing

Variable	Experimental groups	% recovery
Progressive motility	C	14.18 ± 0.77^{b}
	G1	16.23 ± 2.06^{b}
	G2	09.81 ± 1.93^{c}
	G3	14.43 ± 0.39^{bc}
	G4	33.99 ± 1.00^{a}
Rapid velocity	C	12.34 ± 3.84^{b}
	G1	14.90 ± 1.52^{b}
	G2	14.63 ± 2.85^{b}
	G3	13.62 ± 2.26^{b}
	G4	33.26 ± 2.42^{a}

Values are shown as Mean±S.E.M. Values without a common superscript in % recovery column indicate significant ($P < 0.05$) difference among the experimental groups at a particular incubation time.

Control (C; (7% glycerol at 37°C), Group 1 (G1; 3.5% DMSO at 37°C as well as at 4°C), Group 2 (G2; 3.5% glycerol at 37°C and 3.5% DMSO at 4°C), Group 3 (G3; 3.5% DMSO at 37°C and 3.5% glycerol at 4°C), Group 4 (G4; 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C.

Table 6.3 Effect of cryoprotectants (DMSO and/or glycerol) in extender [Control (C; 7% glycerol at 37°C), Group 1 (G1; 3.5% DMSO at 37°C as well as at 4°C), Group 2 (G2; 3.5% glycerol at 37°C and 3.5% DMSO at 4°C), Group 3 (G3; 3.5% DMSO at 37°C and 3.5% glycerol at 4°C), Group 4 (G4; 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C)] on supra–vital plasma membrane integrity (%), mitochondrial trans-membrane potential (%), viable and intact acrosome (%) and DNA integrity (%) of buffalo bull spermatozoa (five bulls, six replicates, 30 ejaculates) during cryopreservation

Variable	**Stage**	**Experimental groups**				
		C	**G1**	**G 2**	**G 3**	**G 4**
Supra–vital plasma membrane integrity	PD	63.83 ± 0.65^{c}	63.17 ± 0.60^{c}	68.33 ± 0.71^{ab}	65.67 ± 0.88^{bc}	71.00 ± 0.73^{a}
	PE	62.50 ± 0.76^{ab}	60.33 ± 0.56^{bc}	65.00 ± 0.68^{a}	57.83 ± 0.70^{c}	64.17 ± 0.95^{a}
	PT	18.17 ± 0.48^{c}	07.67 ± 0.49^{d}	21.50 ± 0.76^{b}	10.00 ± 0.26^{d}	26.83 ± 0.75^{a}
Mitochondrial trans-membrane potential	PD	67.17 ± 0.70^{b}	62.33 ± 0.42^{c}	68.17 ± 0.75^{b}	62.33 ± 1.12^{c}	72.17 ± 0.60^{a}
	PE	60.33 ± 0.67^{c}	58.33 ± 0.84^{c}	64.83 ± 0.48^{b}	59.83 ± 0.60^{c}	68.00 ± 0.97^{a}
	PT	28.17 ± 0.70^{b}	10.83 ± 0.48^{c}	31.17 ± 0.75^{b}	11.67 ± 0.42^{c}	36.00 ± 1.13^{a}
Viable and intact acrosome	PD	62.17 ± 0.60^{b}	57.50 ± 0.43^{c}	63.17 ± 0.60^{b}	63.17 ± 0.60^{b}	67.17 ± 0.48^{a}
	PE	59.00 ± 0.58^{b}	59.83 ± 0.60^{b}	68.00 ± 0.58^{a}	61.33 ± 0.76^{b}	69.17 ± 0.48^{a}
	PT	44.17 ± 0.83^{b}	26.83 ± 1.08^{c}	46.67 ± 1.20^{b}	24.50 ± 1.20^{c}	60.83 ± 0.75^{a}
DNA integrity	PD	93.00 ± 0.36^{a}	93.50 ± 0.34^{a}	93.50 ± 0.22^{a}	93.50 ± 0.56^{a}	93.50 ± 1.37^{a}
	PE	93.00 ± 0.25^{a}	93.17 ± 0.47^{a}	93.33 ± 0.21^{a}	93.33 ± 0.21^{a}	93.50 ± 0.42^{a}
	PT	89.50 ± 0.43^{b}	89.50 ± 0.56^{b}	90.67 ± 0.21^{ab}	89.50 ± 0.22^{b}	92.00 ± 0.26^{a}

Values are shown as Mean±SEM. Values without a common superscript in a row indicate significant ($P < 0.05$) difference among the treatments at a given stage. Post dilution (PD, 37°C); post-equilibration (PE, 4°C), and post–thawing (PT, 37°C)

Table 6.4 Effect of cryoprotectants (DMSO and/or glycerol) in extender [Control (C; 7% glycerol at 37°C) and Group 4 (G4; 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C)] on *in vivo* fertility of buffalo bull spermatozoa

Experimental groups	**Buffaloes inseminated (n)**	**Buffaloes pregnant (n)**	**Buffaloes non pregnant (n)**	**Pregnancy rate (%)**	**Chi-square value**	**P value**
Control	214	128	86	59.81^{b}	4.102	0.040
G4	203	141	62	69.45^{a}		

Values without a common superscript in a column differ significantly ($P < 0.05$).

6.5. DISCUSSION

Determination of an optimal membrane–permeable cryoprotectant in the addition and dilution protocol is an important aspect of developing an efficient sperm cryopreservation procedure in water buffalo. To the best of author's knowledge, this is the first study that has found cryoprotection synergism between glycerol and DMSO during the cryopreservation of water buffalo spermatozoa.

Motility is generally considered to be one of the most vital variables for assessing the quality of spermatozoa during cryopreservation (Shah et al., 2016). In the present study, a higher progressive motility in G4 compared with other treatment groups and control at post-thawing shows the cryoprotection synergism between glycerol and DMSO, which facilitated the sperm to adapt and tolerate the freeze-thawing cycle. Similarly, Snedeker and Gaunya (1970) found valuable effect of 6% glycerol with 1% DMSO in tris–citric acid extender on post-thaw subjective motility of cattle bull spermatozoa. A cryoprotection synergism between 3% glycerol and 12% DMSO was also reported in terms of improved sperm motility in rabbit buck (Bamba and Adams, 1990). Similarly, the use of DMSO (6%) added in one-step, lowered the PM of Angora buck spermatozoa at post-thawing (Büyükleblebici et al., 2014). In contrast, Rasul et al., (2007) could not find any synergism between glycerol and DMSO in improving the progressive motility of buffalo spermatozoa during cryopreservation. The addition of DMSO alone or in combination with glycerol proved to be toxic for buffalo spermatozoa rather than creating the osmotic shock. Moreover, the lack of glycerol in the extender rendered the movement of spermatozoa, despite the insignificant protection to plasma membrane and acrosome (Rasul et al., 2007). The toxicity of DMSO at certain concentration and temperature could be due to its ability to induce non-lamellar structures in phospholipids and to augment membrane permeability that might compromise the sperm structures and functions (Sum and de Pablo, 2003). Moreover, glycerol at greater concentrations can cause osmotic damage to spermatozoa because it passes through the sperm membrane at slower rate (Guthrie et al., 2002). Whereas, DMSO has lower molecular weight and can rapidly cross the plasma membrane, thus protects spermatozoa from the osmotic stress during cryopreservation (Yu and Quinn, 1994). It is, therefore, suggested that the contrasting results of effectiveness of glycerol and DMSO either alone or in combined form are

mainly due to the different concentrations/combinations of glycerol and DMSO and their temperatures of addition during cryopreservation of buffalo bull spermatozoa.

Besides being predictors of fertility, the percentages of motile spermatozoa with rapid velocity and medium velocity are also considered to be of significance in routine evaluation, but the primary importance is always of the rapid velocity (Barratt et al., 2011). In this study, higher rapid velocity in G4 at post-thawing compared with other treatment groups and control at post-thawing confirms the cryoprotection synergism between glycerol and DMSO.

Regarding the distribution of motile spermatozoa in medium velocity, its mean values were increased or decreased with the corresponding values of rapid velocity or slow velocity. Interestingly, there is no study in buffalo that has evaluated the effect of glycerol in combination with DMSO on velocity distribution of motile spermatozoa. Moreover, rapid velocity at post-thawing is supported by progressive motility i.e., a similar pattern of effect of cryoprotectants is observed on progressive motility of buffalo spermatozoa at post-thawing.

The CASA systems also provide information about the velocities (average path velocity, straight line velocity and curvilinear velocity), as they move along their trajectories (Holt et al., 2007). In this study, at post-thawing, it can be stated that the higher average path velocity, straight line velocity and curvilinear velocity in G4 compared to other treatment groups (except in G2 in case of straight line velocity) and control demonstrates the cryoprotection synergism between glycerol and DMSO. Likewise, the use of DMSO (6%) in one-step during cryopreservation resulted in lower kinematics of Angora buck spermatozoa (Büyükleblebici et al., 2014). Similarly, Rasul et al. (2007) reported that higher concentration of glycerol (6%) lowered the linear motility and accordingly enhanced the circular motility, depicting that after thawing, glycerol influenced negatively on the motion characteristics of buffalo sperm. These changes due to glycerol may be due to glycerol-related osmotic and/or toxic shocks to buffalo sperm, as suggested in ram (Fiser and Fairfull, 1989). However, glycerol and DMSO at certain concentrations and temperatures demonstrate strong affinity with the cells' phospholipids head groups during freezing thus resulting in improved sperm kinematics.

The *in vitro* longevity of spermatozoa is assessed to envisage the viability of spermatozoa in the female reproductive tract (Akhter et al., 2008). The current study found significantly higher percentage recovery of progressive motility and rapid velocity in G4 compared to other treatment groups and control. From the cryobiological perspective, DMSO can more efficiently improve the super cooling of the intracellular media compared glycerol. As a result, the ice crystallization may be delayed effectively when DMSO exists intracellularly. Therefore, this suggests that combination of glycerol and DMSO at certain concentrations can improve the longevity and survivability of buffalo spermatozoa.

The sperm plasma membrane is the primary site being affected during the process of freezing-thawing (Hammerstedt et al., 1990). Thus, the evaluation of structural and functional integrity of plasma membrane is pivotal for predicting the fertility potential of spermatozoa (Brito et al., 2003). In the current study, greater supra–vital plasma membrane integrity in G4 as compared to other treatment groups and control at post–thawing appears to be the result of cryoprotection synergism between glycerol and DMSO. Likewise, the use of DMSO (6%) in one-step resulted in higher damage to plasma membrane of Angora buck spermatozoa (Büyükleblebici et al., 2014). Conversely, Rasul et al. (2007) reported that even lower concentration of DMSO (1.5%) antagonized the cryoprotective effect of 3 or 6% glycerol, thus resulting in compromised post–thawing integrity of buffalo sperm plasmalemma. The effect of DMSO on protein organization and strength is both concentration as well as temperature dependant. Reversible changes in the protein organization are the main effect of revelation of subunit proteins to lower concentrations of DMSO at low temperatures, while unalterable denaturation of subunit proteins may be a dominant result at higher temperatures and higher concentrations of DMSO (Yu and Quinn, 1994). The DMSO has temperature dependent toxicity that is believed to be due to its hydrophobic/hydrophilic balance (Arakawa et al., 1990). In addition, DMSO was found to be noticeably less efficient when liposomes contained lipids carried a net negative charge. This implied that electrostatic interactions between the polar sulphoxide moiety of DMSO and phospholipid membranes may be of paramount importance with respect to cryoprotective effect of DMSO. Again, it is affirmed that these differences are mainly due to the optimum combinations of glycerol and DMSO

and their temperatures of addition during semen cryopreservation (Yu and Quinn, 1994).

Reduction in sperm mitochondrial trans-membrane potentialhas been mostly linked with lowered fertility (Gallon et al., 2006). It appears that the high mitochondrial trans-membrane potential in G4 at post dilution, post-equilibration and post–thawing was due to cryoprotection between glycerol and DMSO. This rapid loss in sperm mitochondrial trans-membrane potentialat different stages of cryopreservation favors earlier studies that have proposed that glycerol can disrupt ATP generation at higher concentrations (Hammerstedt and Graham, 1992), thus resulting in lowered motility and velocities. Again, the divergent effects of cryoprotectants on mitochondrial trans-membrane potential of buffalo spermatozoa could be attributed to the dose dependent cytotoxicity at the particular temperature of addition.

The acrosomal intactness of sperm is crucial for fertilization because it carries hydrolytic enzymes required for oocyte penetration (McLeskey et al., 1998). It appears that higher viable and intact acrosome in G4 compared with other treatment groups and control at post–thawing was due to the cryoprotection synergism between glycerol and DMSO. Similarly, the use of DMSO (3 and 6%) in one-step resulted in higher acrosomal damage of Angora buck spermatozoa (Büyükleblebici et al., 2014). However, Rasul et al. (2007) and Bamba and Adams (1990) found nontoxic effect of DMSO in combination with glycerol on acrosome integrity of buffalo (3% DMSO) and rabbit (3–15% DMSO) spermatozoa, respectively. These studies evaluated only the acrosomal integrity without the viability of spermatozoa whereas both aspects of acrosomes were studied in the current study.

Normal genetic material of spermatozoa is essential for successful fertilization, and as well as for the embryo and fetal development if a healthy offspring is to be born (Andrabi, 2007). This study found significantly higher DNA integrity in G4 as compared to other treatment groups and control at post–thawing. It appears that higher sperm DNA integrity in G4 may have been sustained due to lesser membrane damage and lipid peroxidation and resultantly sperm viability was enhanced. Moreover, spermatozoa suddenly generate an array of reactive oxygen species (ROS), comprising the superoxide anion, hydrogen peroxide and nitric oxide (Aitken et al.,

2010) and extra ROS are generated during freeze-thawing (Guthrie and Welch, 2006) thus these may have compromised the DNA integrity in other experimental groups and control.

Outcome of the fertilization process depends on the functional competence of spermatozoa (Dayem et al., 2009). The present study found significantly greater *in vivo* fertility (%) of buffalo spermatozoa in G4 (69.45) as compared to control (59.81). It is noteworthy that a fertility rate higher than 50% with frozen–thawed spermatozoa is considered as an acceptable result of AI in water buffalo (Andrabi, 2014). Thus, the current findings of *in vivo* fertility are rational and do suggest that G4 can be used in the freezing protocol for cryopreservation of water buffalo spermatozoa and fertility trials.

6.5.1. CONCLUSION

This part of the study concludes that cryoprotection synergism exists between glycerol and DMSO in Group 4 (1.75% glycerol and 1.75% DMSO at 37 as well as at 4°C) in improving the frozen thawed quality of buffalo spermatozoa and *in vivo* fertility. Moreover, it is asserted that least toxic combination of cryoprotectants has been identified by reducing the concentration of glycerol for buffalo spermatozoa. It is further suggested that least toxic combinations of glycerol and DMSO can be tested in freezing protocols of other livestock species to improve the outcome of cryopreservation.

GENERAL DISCUSSION

Pakistan is an agriculture based country and livestock plays a significant role in its economy by providing necessary items of human diet particularly in the form of milk, and meat. Agriculture sector in Pakistan is confronting definite challenges which require instantaneous and focused consideration both at research and policy level for improving the livestock in order to cater the growing needs of human. Accordingly the livestock contributed about 55.9 percent of the agricultural value, which is equivalent to 11.8 percent of the national GDP during 2013-14 compared to 55.5 percent and 11.9 percent during the 2012-13. The buffalo population in Pakistan was 32.7, 33.7, 34.6 and 35.6 million during the years 2011-12, 2012-13, 2013-14, 2014-15, respectively (Economic Survey of Pakistan, 2014-15). The milk gross production of buffalo population was 29,473, 30,350, 31,252 and 32,180 tons during the years 2011-12, 2012-13, 2013-14, and 2014-15, respectively (Economic Survey of Pakistan, 2014-15).

Generally, buffalos are reproducing well, but some reproductive disorders that have been indicated in buffaloes, hinder their reproductiveness. These mainly include late onset of puberty, poor estrus expression, longer postpartum ovarian quietness, and most prominently reduced conception rates due to poor nutrition (Perera, 2011; Andrabi, 2014; Lyashenko, 2015). Although, buffaloes can breed round the year, seasonal patterns (tropical and temperate) such as rainfall and thermal stress may lead to elevation of prolactin and melatonin levels and which influence the reproductive efficiency of the buffalo (Perera, 2011; Ahmed et al., 2016a). In order to overcome these issues, artificial insemination (AI), a salient reproductive biotechnology, is now playing a pivotal role in the field of animal breeding and genetics (Adams et al., 2015; Singh and Balhara, 2016). The AI practices that are undertaken currently in Pakistan need to be strengthened effectively. This would help in eradicating the low fertility bulls and thus accelerate the pace of the genetic improvement of buffaloes. There is a need to improve the prevailing semen cryopreservation practices by focusing on the semen storage and transportation facilities at all AI centers in Pakistan. To make AI more successful and vibrant, testing of different cryodiluents, in the freezing extenders, is therefore indispensable so that a freezing protocol could be evolved for

better *in vitro* quality of spermatozoa and ultimately considerable *in vivo* fertilization potential of water buffalo.

The current study was conducted to test and evaluate a series of cryodiluents with most appropriate cooling, equilibration, freezing and thawing rates for improving both the *in vitro* quality and as well as the *in vivo* fertility of the water buffalo spermatozoa. Curcumin was tested for its efficacy as an antioxidant in the freezing extender. UV-C irradiated egg yolk plasma was used as a replacement of the conventional whole egg yolk in the tris-citric acid based freezing extender. Glycerol and DMSO were tested to ascertain the synergism between these cryoprotectants in the tris-citric acid based freezing extender. The *in vitro* quality of buffalo spermatozoa was ascertained by employing different techniques and assays comprising computer assisted sperm analyzer (CASA), supra-vital plasma membrane integrity (SV-PMI, %), viable and intact acrosome (V-IACR, %), mitochondrial trans-membrane potential (MMP, %) and DNA integrity (DNA-I, %). The experimental extenders that turned out significantly better post-thaw *in vitro* quality of buffalo spermatozoa in this study were used in field fertility trials.

7.1. EQUILIBRATION, FREEZING AND THAWING OF BUFFALO SPERMATOZOA

The equilibration time (Dhami et al., 1996;Foote et al., 2002; Andrabi, 2014), freezing rates (Woelders and Chaveiro, 2004; Andrabi, 2007; Day et al., 2008; Anzar et al., 2010) and thawing rates (Mazur, 2010) are the key to successful freezing of spermatozoa. In the Experiment 1, three equilibration times (E1, 2 h; E2, 4 h; E3, 6 h), two freezing rates (FR1, manual, 5 cm over liquid nitrogen (LN_2) for 10 min, plunging in LN_2; FR2, programmable ultra-fast, holding at 4°C for 2 min, from 4 to −10°C at −10°C/min, from −10 to −20°C at −15°C/min, from −20 to −120°C at −60°C/min, holding at −120°C for 30 sec, plunging in LN_2), and three thawing rates (T1, 37°C for 30 sec; T2, 50°C for 15 sec; T3, 70°C for 7 sec) were evaluated.

This study provided significantly higher post-thaw sperm progressive velocity, rapid velocity, average path velocity, straight line velocity, curvilinear velocity, straightness, linearity, supra-vital plasma membrane integrity, mitochondrial trans-

membrane potential, and viable with intact acrosome with 4 h , FR2 and T3 compared to other equilibration times, freezing rate (FR1) and thawing rates (2 and 6 h), respectively. There was no significant effect of equilibration times on sperm DNA integrity similar to that has also been reported by Shahverdi et al. (2014) in buffalo. DNA integrity was significantly better in programmable ultra-fast freezing rate (FR2) as compared to the conventional freezing rate (FR1). The current study observed higher DNA integrity after thawing with T1 (37°C for 30 sec, 95.56%) as compared to T2 (50°C for 15 sec, 93.83%) and T3 (70°C for 7 sec, 93.93%); this statistical difference is unlikely to be of any biological relevance as Kumar et al. (2012) have already reported that buffalo bulls classified with high-fertility had about 90% cryopreserved spermatozoa with intact DNA.

This is worthy to mention that the present study, being pioneering study, was successful in freezing the buffalo bull spermatozoa using −60°C/min freezing rate during the critical temperature which determines the equilibrium status of spermatozoa between intracellular and extracellular surroundings (Holt, 2000; Kumar, 2003). Presently, it appears that thawing at faster rate cryopreserved the spermatozoa well in parallel with the fast freezing rate in this study. This observation complies with the bifactorial theory of cryoinjury suggested by Mazur (1965) which states that "optimum cell survival needs that the freezing rate be coupled with the thawing rate". This part of the study concluded that inclusion of 4 h equilibration time, programmable ultra-fast freezing rate, and rapid thawing at 70°C for 7 sec in the cryopreservation protocol improves the post thaw quality of buffalo bull spermatozoa.

7.2. CURCUMIN AS AN ANTIOXIDANT IN FREEZING EXTENDER

In order to minimize the effects of oxidative stress on spermatozoa during the process of cryopreservation, addition of antioxidants in semen extender is required (Andrabi, 2007; Andrabi et al., 2008a; Griesser et al., 2011). In Experiment 2, the effects of curcumin (diferuoyl methane) as antioxidant by diluting it in tris-citric acid extender containing supplementations of 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM and control (without curcumin) were evaluated on freezability of water buffalo bull spermatozoa. The equilibration time (4 h), freezing rate (FR2 as used in Experiment 1), and thawing rate were 37°C for 30 sec. Outcomes of spermatozoa cryopreserved with curcumin in

extender have been reported in Angora buck (Bucak et al., 2010), Holstein bull (Bucak et al., 2012), miniature boar (Jeon and Kim, 2013) and albino Wistar rats (Soleimanzadeh and Saberivand, 2013). Moreover, oral administration of curcumin have been reported to improve the morphologic features of spermatozoa in New Zealand White rabbit buck (Seadawy et al., 2014) and mice (Mathuria and Verma, 2008; Głombik et al., 2014; Lin et al., 2015). To the best of author's knowledge, this is the first report that used curcumin as antioxidant in semen extender for cryopreservation of water buffalo spermatozoa.

The lipid peroxidation (LPO) cascade is initiated with the attack of ROS on polyunsaturated fatty acids of the sperm plasmalemma (Van Loon et al., 1991; Baumber et al., 2003; Andrabi, 2009). The integrities of plasma membrane and acrosome of spermatozoa are essential criteria to sustain spermatozoal functions in the female's reproductive tract and oocyte penetration (Holt, 2000; Esteves et al., 2007).

Addition of curcumin at the concentrations of 1.5 and 2.0 mM in cryodiluents alleviated the seminal TAC during the cooling and freezing processes. Curcumin doses of 1.5 and 2.0 mM not only sustained the seminal TAC capacity but also restricted the generation of LPO during cryopreservation. The higher post-thaw turnout of progressive motility, rapid velocity, average path velocity, straight-line velocity, curvilinear velocity, straightness, linearity, supravital plasma membrane integrity, and percentage of viable with intact acrosome of spermatozoa were found with 1.5 mM curcumin as compared to other concentrations and control. These variables also showed a positive correlation with TAC and negative association with LPO at post-thaw.

Most of the CASA variables were significantly correlated with supravital plasma membrane integrity, percentage of viable sperm with intact acrosome, DNA integrity and biochemical assays particularly at the post-thawing stage of cryopreservation. This signifies the efficacy of CASA in evaluating the buffalo bull spermatozoa in this study. *In vitro* longevity of buffalo spermatozoa was also assessed at post-thawing of treatments during 2 h of incubation period at 37°C in a water bath. Progressive motility and rapid velocity were greater with 1.5 mM curcumin at 1 h of incubation compared to 0.5 and 2.0 mM curcumin and control. After 2 h incubation period, both

the progressive motility and rapid velocity were greater ($P < 0.05$) with 1.5 mM curcumin than the control. The curcumin with concentration of 1.5 mM gave significant output as compared to other concentrations and control due the maintenance of seminal total antioxidant capacity and prevention of lipid peroxidation. The present study concluded that freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of 1.5 mM curcumin (diferuoyl methane) as antioxidant in semen extender.

7.3. CHICKEN EGG YOLK PLASMA (EYP) AS AN ALTERNATE TO WHOLE CHICKEN EGG YOLK (WCEY) IN FREEZING EXTENDER

Earlier studies have demonstrated that WCEY has inconsistent composition, HDLs and egg yolk granules that interfere with sperm motility (Akhter et al., 2011). It was suggested that these disadvantages can be minimized with the use of EYP (Pillet et al., 2011). This part of study was primarily designed to assess the effect of different concentrations of UV-C irradiated chicken EYP (v/v; 10% or 15% or 20%) in tris−citric acid based extender on water buffalo sperm quality during cryopreservation (post–dilution, PD; post–equilibration, PE; post–thawing, PT). The effect of best evolved concentration of UV-C irradiated EYP in extender on *in vivo* fertility of buffalo spermatozoa was also evaluated.

Computer-assisted sperm analysis acquires detailed digital data of sperm movement in many microscopic fields and presents an objective assessment of velocity distribution and kinematics, which are not possible to measure and/or observe manually (Verstegen et al., 2002; Partyka et al., 2012; Amann and Waberski, 2014; Lu et al., 2014). Progressive motility and rapid velocity were significantly higher ($P < 0.05$) in the extenders containing 20% EYP compared to 10% EYP and 20% WCEY at post-equilibration and post-thawing. Medium velocity was significantly greater ($P < 0.05$) in control compared to other experimental groups at all the stages of cryopreservation, which indicates that EYP treatments had greater rapid velocities as compared to control. Sperm kinematics (average path velocity, VAP; and straight line velocity, VSL) were significantly greater ($P < 0.05$) in the extenders containing 15% EYP and 20% EYP at post-equilibration and post-thawing.as compared to 20% WCEY. The spermatozoa longevity was also assessed in this study after thawing the treatments

and incubating them for a period of 2 h at 37°C in a water bath. It was seen that 20% EYP had significantly lower ($P < 0.05$) loss in prospect of the progressive motility and rapid velocity as compared to 20% WCEY during 2 h of incubation period.

Supra–vital HOST gives an assessment of both functional and structural PMI of spermatozoa simultaneously (Chan et al., 1991). This study found significantly higher SV-PMI with P2 and P3 compared to P1 and WCEY at PD and with P2 and P3 compared to WCEY at PE and PT. Mitochondria are the energy centers of spermatozoa (Cossarizza and Salvioli, 2001), and low MMP is considered as a sign of early sperm apoptosis (Barroso et al., 2006). Significantly higher MMP was found with P2 and P3 as compared to P1 and WCEY during PD and PT, and with P3 as compared to P1 and WCEY at PE. The assessment of acrosomal integrity provides an estimate of sperm fertility (Graham and Moce, 2005). A higher V–IACR protection to buffalo spermatozoa in P3 as compared to control was observed during PD and PE and in P2 and P3 as compared to WCEY at PT. Sperm DNA integrity is a vital evaluation parameter and is associated with fertilization potential in buffalo (Ahmed et al., 2016a). This study found significantly higher DNA integrity in P2 and P3 as compared to control at PT. A significantly higher *in vivo* fertility was found with P3 as compared to control (76.61% vs. 64.49%, $P < 0.05$).

The dynamic UV–C irradiation is a remarkable technology to lower the microbial loads without harming the pertinent quality attributes of chicken egg products (de Souza and Fernandez, 2011). The UV–C irradiation dose ranging from 2,000 to 8,000 $\mu W \cdot s/cm^2$ is reported to be lethal for 90% of bacteria and viruses (Kowalski et al., 2000). In the present study, the dynamic UV–C irradiation dose given to chicken EYP was calculated to be 19920000 $\mu W\ sec/cm^2$. It is therefore, put forward that the dose of dynamic UV–C irradiation was high enough to culminate the microorganisms from chicken EYP. Thus, the EYP being enriched with LDLs (Anton, 2013; Strixner and Kulozik, 2013), may also have met the sanitary requirements (Andrabi et al., 2016) for addition in extender as a non-membrane permeable cryoprotectant. This study concluded that WCEY (20%, v/v) can be replaced with UV-C irradiated chicken EYP (20%, v/v) in tris citric acid extender to improve the post-thaw *in vitro* quality, and *in vivo* fertility of water buffalo spermatozoa. The method of EYP extraction is simple and can be used in routine buffalo semen cryopreservation process. Moreover, the

protocol of dynamic UV-C irradiation adopted for sterilization of EYP may be used to meet the sanitary requirements.

7.4. ALONE AND COMBINED USE OF GLYCEROL AND DMSO IN A FREEZING EXTENDER

Determination of an optimal membrane–permeable cryoprotectant addition and dilution protocol is an important aspect of developing an efficient sperm cryopreservation procedure in water buffalo. The objective of this part of study was to find out a cryoprotection synergism between glycerol and DMSO (dilution with 7% glycerol, control; dilution with 3.5% DMSO at 37°C as well as at 4°C, Group 1; dilution with 3.5% glycerol at 37°C and 3.5% DMSO at 4°C, Group 2); dilution with 3.5% DMSO at 37°C and 3.5% glycerol at 4°C, Group 3; dilution with 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C, Group 4) for water buffalo spermatozoa. To the best of author’s knowledge, this is the first study that has found that there exists cryoprotection synergism between glycerol and DMSO for water buffalo spermatozoa.

Motility is generally believed to be one of the most vital parameter for evaluating the quality of spermatozoa during cryopreservation (Kumar et al., 2012; Shah et al., 2016). Besides being the predictors of fertility, the percentages of motile spermatozoa with rapid velocity and medium velocity are also considered to be of significance in routine evaluation, but the primary importance is always given to rapid velocity (Tomlinson et al., 2010; Barratt et al., 2011). The CASA systems also provide information about the velocities (average path velocity, straight line velocity and curvilinear velocity), as they move along their trajectories (Holt et al., 2007; Amann and Waberski, 2014). The evaluation of structural and functional integrity of plasma membrane is pivotal for predicting the fertility potential of spermatozoa (Brito et al., 2003; Crespilho et al., 2014). Reduction in sperm mitochondrial trans-membrane potential has been mostly related with lower fertility (Gallon et al., 2006).

This study observed higher progressive motility, rapid velocity, average path velocity, straight line velocity, curvilinear velocity, supra–vital plasma membrane integrity , mitochondrial trans-membrane potential, viable and intact acrosome and DNA

integrity in extender with Group 4 as compared to the other treatment groups (except in Group 2 in case of straight line velocity) and control, respectively. Normal genetic material of sperms is a pre-requisite for successful fertilization, and development of embryo and fetus that will ultimately grow in a healthy offspring (Andrabi, 2007; Kumar et al., 2012). Regarding the distribution of motile spermatozoa in medium velocity, its mean values were increased or decreased with the corresponding values of rapid velocity or slow velocity. Interestingly, no study is available in buffalo that has evaluated the effect of glycerol in combination with DMSO on velocity distribution of motile spermatozoa. Moreover, the present data of rapid velocity at post-thawing are supported with that of progressive motility i.e., a parallel pattern of effect of cryoprotectants is observed on progressive motility of buffalo spermatozoa at post-thawing. Snedeker and Gaunya (1970) found valuable effect of 6% glycerol with 1% DMSO in tris–extender on post-thaw subjective motility of cattle bull spermatozoa. Rasul et al. (2007) could not find synergism between glycerol and DMSO in improving the progressive motility of buffalo spermatozoa during cryopreservation. The toxicity of DMSO at certain concentration and temperature could be due to its ability to induce non-lamellar structures in phospholipids and to enhance membrane permeability that might compromise the sperm structures and functions (Sum and de Pablo, 2003). Moreover, glycerol at higher concentrations can cause osmotic damage to spermatozoa because it passes through the sperm membrane at slower rate (Guthrie et al., 2002). Whereas, DMSO has lower molecular weight and can rapidly cross the plasma membrane, thus protects spermatozoa from the osmotic stress during cryopreservation (Yu and Quinn, 1994). It is, therefore, suggested that the contrasting results of effectiveness of glycerol and DMSO either alone or in combined form in these studies are mainly due to the different concentrations/combinations of glycerol and DMSO, their temperatures of addition in the semen extenders and freezing rates during cryopreservation of buffalo bull spermatozoa.

This study also carried out the longevity of the spermatozoa after thawing the treatments at 37°C in a water bath during 2 h of incubation period. The present study found significantly higher percentage recovery of progressive motility and rapid velocity in Group 4 as compared to other treatment groups and control. From the cryobiological perspective, DMSO can more efficiently improve the super cooling of

the intracellular media as compared to glycerol. As a result, the ice crystallization may be delayed effectively when DMSO exists intracellularly. This suggests that combination of glycerol and DMSO at certain concentrations can improve the longevity and survivability of buffalo spermatozoa by counteracting their toxic as well as osmotic effects on the spermatozoa during the cryopreservation.

Outcome of the fertilization process depends on the functional competence of spermatozoa (Dayem et al., 2009; DeJarnette and Amann, 2010). This study found significantly higher *in vivo* fertility (%) of buffalo spermatozoa in Group 4 (69.45) as compared to the control (59.81). Importantly, fertility rate higher than 50% with frozen–thawed spermatozoa is considered as an acceptable result of AI in water buffalo (Vale, 1997). This study concluded that cryoprotection synergism exists between glycerol and DMSO in Group 4 (1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C) in improving the frozen–thawed quality of buffalo spermatozoa and *in vivo* fertility. It can be asserted that least toxic combination of cryoprotectants has been identified by reducing the concentration of glycerol for buffalo spermatozoa.

Overall, this study concluded that 4 h-equilibration time, programmable ultra-fast freezing rate (+4°C for 2 min, from 4 to −10°C at −10°C/min, from −10 to −20°C at −15°C/min, from −20 to −120°C at −60°C/min, holding at −120°C for 30 sec), and rapid thawing at 70°C for 7 sec in the cryopreservation protocol improves the post-thaw quality of buffalo bull spermatozoa. Furthermore, this freezing protocol was successfully used in the other experiments in the current study and significantly better results were obtained in prospects of *in vitro* quality and longevity of spermatozoa during cryopreservation and satisfactory field fertility rates were also achieved. The supplementation of 1.5 mM curcumin as antioxidant in the extender provided better cryoprotection for freezability of water buffalo spermatozoa and this finding may be used for achieving good fertility rates in the buffalo. The UV-C irradiated chicken EYP (20%) can be effectively used as alternate to WCEY (20%) in tris–citric acid extender for cryopreservation and *in vivo* fertility of water buffalo spermatozoa. A synergism between glycerol and DMSO (addition of 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C, Group 4) potentially improves the quality and *in vivo* fertility of cryopreserved water buffalo spermatozoa. The significant *in vitro* and in vivo outcome from the current study would be helpful in overcoming the

cryopreservation related issues of buffalo spermatozoa and therefore these would further support in improving the AI protocols currently practiced for increasing buffalo population in Pakistan.

7.5. RECOMMENDATIONS

Cryopreservation of spermatozoa is performed at very low temperatures in the freezing protocols. The key challenge during cryopreservation, depending on the type of cells, is the use of appropriate cryoprotectant, cooling, equilibration time, freezing rate and thawing rate. The cryoinjury can be minimized by controlling the development of intracellular ice crystals and also the changes that happen in the composition of liquid phase during cryopreservation. Keeping in view these fundamentals of cryopreservation, this study was designed in four different settings and beneficial results were obtained for improving *in vitro* quality and in vivo fertility of buffalo spermatozoa. Based on the findings of the current study, following recommendations are proposed to apply for the cryopreservation of buffalo spermatozoa:

1. There is need to adopt 4 h equilibration time with programmable ultra-fast freezing rate (FR2), and rapid thawing at 70°C for 7 sec in cryopreservation protocol for improving quality of buffalo bull spermatozoa. The freezing rate (FR2) could also be used for maintaining the sperm quality for longer storage period.
2. There is need to validate the efficacy of 1.5 mM curcumin (diferuoyl methane) as antioxidant in semen extender of buffalo bull. It is proposed that future studies may test this cryodiluent for field fertility trials in buffalo.
3. It is suggested that use of UV-C irradiated chicken egg yolk plasma (EYP, 20%, v/v) in tris citric acid extender is greatly beneficial for improving both *in vitro* quality at post-thaw, and *in vivo* fertility of water buffalo spermatozoa compared to conventional whole chicken egg yolk (WCEY, 20%, v/v). Using chicken EYP is appropriate due to its simple extraction/centrifugation technique and UV-C treatment fulfills the sanitation requirements of chicken EYP. This procedure is cost-effective for using in freezing extender as compared to the commercially available semen extenders.
4. In order to minimize osmotic damage of glycerol and toxicity of DMSO to spermatozoa, these were used either alone &/or in combined form during

cryopreservation. This study was successful in finding out a cryoprotection synergism level of both cryoprotectants in terms of sperm *in vitro* quality and *in vivo* fertility. It is, therefore, recommended that adding 1.75% glycerol and 1.75% DMSO at 37°C as well as at 4°C is helpful in improving the frozen thawed quality of buffalo spermatozoa and *in vivo* fertility. It is further suggested that least toxic combinations of glycerol and DMSO can be tested in freezing protocols of other livestock species to improve the outcome of cryopreservation.

PUBLICATIONS OUT OF PhD THESIS

Full length Papers

1. **Syed Aftab Hussain Shah**, Syed Murtaza Hassan Andrabi and Irfan Zia Qureshi. (2016). Effect of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. ***Andrology***, 4: 972–976. (Impact Factor **2.515**)
2. **Syed Aftab Hussain Shah**, Syed Murtaza Hassan Andrabi and Irfan Zia Qureshi. Freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of curcumin (diferuoyl methane) in semen extender. Accepted on August 1, 2016. ***Andrologia***, Early View. DOI: 10.1111/and.12713. (Impact Factor **1.441**)
3. **Syed Aftab Hussain Shah**, Syed Murtaza Hassan Andrabi, Hussain Ahmed and Irfan Zia Qureshi. (2017). Chicken egg yolk plasma in tris-citric acid extender improves the cryopreserved quality and *in vivo* fertility of water buffalo bull (*Bubalus bubalis*) spermatozoa. ***Theriogenology***, 89: 32–40. (Impact Factor **1.838**)
4. **Syed Aftab Hussain Shah**, Syed Murtaza Hassan Andrabi, Hussain Ahmed and Irfan Zia Qureshi. (2016). Cryoprotection synergism between glycerol and dimethyl sulfoxide improves mitochondrial transmembrane potential, plasmalemma, acrosomal, DNA integrities and *in vivo* fertility of water buffalo (*Bubalus bubalis*) spermatozoa. ***Cytotechnology***, 68: 2335–2344. (Impact Factor**1.864)**

Note: For face page of published papers, please see Appendix.

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Effect of equilibration times, freezing, and thawing rates on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa

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SUMMARY

The effects of equilibration times (E1, 2 h; E2, 4 h; E3, 6 h), freezing rates (FR1, manual, 5 cm above liquid nitrogen (LN_2) for 10 min, plunging in LN_2; FR2, programmable ultra-fast, holding at +4 °C for 2 min, from 4 to −10 °C at −10 °C/min, from −10 to −20 °C at −15 °C/min, from −20 to −120 °C at −60 °C/min, holding at −120 °C for 30 sec, plunging in LN_2), and thawing rates (T1, 37 °C for 30 sec; T2, 50 °C for 15 sec; T3, 70 °C for 7 sec) were evaluated on quality of buffalo bull spermatozoa. Progressive motility (%), rapid velocity (%), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), and mitochondrial transmembrane potential (%) were higher ($p < 0.05$) with E2, FR2, and T3 compared to other groups. Sperm curved line velocity (VCL, μm/s) was higher ($p < 0.05$) with E2 and FR2 compared to other groups. Sperm straightness (%) and linearity (LIN, %) were higher ($p < 0.05$) with E2 compared to other groups. Sperm LIN was affected ($p < 0.05$) with T3 compared to other groups. Supravital-plasma membrane integrity (%), viability and acrosome integrity (%) of spermatozoa were higher ($p < 0.05$) with E2 and FR2 compared to other groups. Sperm DNA integrity (%) was higher ($p < 0.05$) with FR2 and T1 compared to other groups. We concluded that inclusion of 4 h-equilibration time, programmable ultra-fast freezing rate, and rapid thawing at 70 °C for 7 sec in cryopreservation protocol improves the post-thaw quality of buffalo bull spermatozoa.

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Freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of curcumin (diferuoyl methane) in semen extender

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Summary

Effects of curcumin as antioxidant in extender were evaluated on freezability of buffalo spermatozoa. Semen from each of the five bulls (*n* = 3 replicates, six ejaculates/bull, a total of 30 ejaculates) was diluted in *Tris*-citric acid extender containing curcumin (0.5, 1.0, 1.5 or 2.0 mM) or control. At pre-freezing and post-thawing, total antioxidant contents (µM/L) and lipid peroxidation levels (µM/ml) were higher ($p < .05$) and lower ($p < .05$) respectively, with 1.5 and 2.0 mM compared to 0.5 and 1.0 mM curcumin and control. At post-thawing, progressive motility (PM, %) and rapid velocity (RV, %) were higher ($p < .05$) with 1.5 mM compared to other doses of curcumin and control (except in case of RV, 1.5 was similar with 1.0 mM). Kinematics (average path velocity, µm/s; straight-line velocity, µm/s; curved-line velocity, µm/s; straightness, %; linearity, %), *in vitro* longevity (%, PM and RV) and DNA integrity (%) at post-thawing were higher ($p < .05$) with 1.5 mM compared to control. At post-thawing, supravital plasma membrane integrity (%) and viable spermatozoa with intact acrosome (%) were higher with 1.5 compared to 2.0 mM curcumin and control. We concluded that freezability of water buffalo spermatozoa is improved with the addition of 1.5 mM curcumin in extender.

KEYWORDS

antioxidant, buffalo, CASA, curcumin, spermatozoa

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Chicken egg yolk plasma in tris-citric acid extender improves the quality and fertility of cryopreserved water buffalo (*Bubalus bubalis*) spermatozoa

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A B S T R A C T

This study was primarily designed to evaluate the effect of different concentrations of ultraviolet (UV)-C–irradiated chicken egg yolk plasma (EYP; v:v; 10%, P1; 15%, P2; 20%, P3) or 20% (v:v) of whole chicken egg yolk (WCEY) in tris-citric acid (TCA) extender on water buffalo sperm quality during cryopreservation (postdilution, PD; postequilibration, PE; post-thawing, PT). Also the effect of best evolved concentration of UV-C–irradiated EYP in extender on *in vivo* fertility of buffalo spermatozoa was evaluated. At PE and PT, computer-assisted sperm analysis progressive motility (PM, %) was significantly higher in P3 compared with P1 and WCEY. Rapid velocity (RV, %) was higher ($P < 0.05$) in P3 compared with P1 and WCEY during cryopreservation (PD, PE, and PT). Average path velocity (μm/s) and straight line velocity (μm/s) were higher ($P < 0.05$) in P2 and P3 than WCEY at PE and PT. The decline percentage (%, longevity) in PM and RV was lower ($P < 0.05$) in P3 compared with WCEY during 2 hours incubation under *in vitro* condition at PT. Supravital plasma membrane integrity (%) was higher ($P < 0.05$) in P2 and P3 compared with control at different stages (PE and PT). Mitochondrial transmembrane potential (%) was higher ($P < 0.05$) in P2 and P3 compared with P1 and WCEY at different stages (PD and PT). Percentage of viable sperm with intact acrosome, and sperm DNA integrity (%) were higher ($P < 0.05$) in P2 and P3 compared with WCEY at PT. The *in vivo* fertility rate (%) was significantly higher with P3 compared with WCEY (76.61 vs. 64.49). In conclusion, WCEY (20%) can be replaced with UV-C–irradiated chicken EYP (20%) in TCA extender for cryopreservation of water buffalo spermatozoa.

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Cryoprotection synergism between glycerol and dimethyl sulfoxide improves the mitochondrial transmembrane potential, plasmalemma, acrosomal and DNA integrities, and in vivo fertility of water buffalo (*Bubalus bubalis*) spermatozoa

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Abstract The objective of the study was to devise a cryoprotection synergism between glycerol and dimethyl sulfoxide (DMSO) for water buffalo spermatozoa. Additionally, the effect of best evolved concentrations of glycerol and DMSO in extender was assessed on in vivo fertility of buffalo spermatozoa. Ejaculates (n = 30) were equally distributed into five aliquots; first aliquot was diluted at 37 °C in extender having 7 % glycerol (control); second aliquot was diluted at 37 °C as well as at 4 °C in extender having 3.5 % DMSO (Group 1); third aliquot was diluted at 37 °C in extender having 3.5 % glycerol and then at 4 °C in extender having 3.5 % DMSO (Group 2); fourth aliquot was diluted at 37 °C in extender having 3.5 % DMSO and then at 4 °C in extender having 3.5 % glycerol (Group 3); fifth aliquot was diluted in extenders having 1.75 % glycerol and 1.75 % DMSO at 37 as well as at 4 °C (Group 4). At post thawing, sperm progressive motility (%), rapid velocity (%), average path velocity (µm/s), curved line velocity (µm/s), in vitro longevity (%), structural and functional integrity of plasmalemma (%), mitochondrial transmembrane potential (%) and viable sperm with intact acrosome (%) were higher ($P < 0.05$) in Group 4 compared to other treatment groups and control. Regarding sperm DNA integrity (%); it was higher ($P < 0.05$) in Group 4 compared to Group 1, 3 and control. The in vivo fertility (%) of buffalo spermatozoa was significantly higher with Group 4 compared to control (69.45 vs. 59.81). In conclusion, synergism exists between glycerol and DMSO (Group 4) in improving the quality and in vivo fertility of cryopreserved water buffalo spermatozoa.

Keywords Glycerol · DMSO · Synergism · Cryopreservation · Buffalo spermatozoa

Introduction