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ORIGINAL ARTICLE

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

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Introduction

Cryopreservation is a non-physiological process, involving the adaptability of spermatozoa to the osmotic and temperature shocks (Holt 2000a, b). 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 during cryopreservation result in 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 retrieval of cryopreserved buffalo spermatozoa (Andrabi 2014).

Suitable membrane-permeable 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 based extender at 37 °C for buffalo spermatozoa (Andrabi 2009). The physiological role of glycerol as cryoprotectant takes place by lowering the freezing point of water, binding with metallic ions, replacing intracellular water, and by reducing the electrolyte profile in the unfrozen portion (Holt 2000b; Medeiros et al. 2002). Beside this, glycerol has also demonstrated 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 having lower molecular weight than glycerol (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). Therefore, there is a possibility to overcome these effects by testing new concentrations of DMSO and glycerol in extender for cryopreservation of buffalo spermatozoa. Moreover, it is reported that buffalo spermatozoa are more prone to hazards during the freeze-thawing process than bovine spermatozoa, thus resulting in relatively lower in vitro quality and in vivo fertility (Andrabi 2009). Therefore, the objective of this study was to devise a synergism between glycerol and DMSO and to reduce the level of glycerol in cryodiluent for buffalo spermatozoa. Additionally, the effect of best evolved concentrations of glycerol and DMSO in extender was assessed on in vivo fertility of buffalo spermatozoa.

Materials and methods

Chemicals

All the chemicals were procured from Merck (Darmstadt, Germany).

Experiment I

Animals

Five healthy Nili-Ravi buffalo (*Bubalus bubalis*) bulls were used in this study. The bulls were kept at National Agricultural Research Centre, Islamabad, Pakistan under suitable nutritional and housing conditions. Moreover, the prevalent ethical rules and guidelines of animal welfare were followed during the study.

Preparation of extender

A base extender comprising of *tris*-(hydroxymethyl)aminomethane (15.0 g), citric acid (7.8 g), fructose (1.0 g), streptomycin sulphate (0.5 g), fresh hen egg yolk (100 ml) and 400 ml of distilled water (Andrabi et al. 2008a) was prepared. The osmolarity of the base extender without egg yolk was 320 mOsmol/kg. 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 7.00 and they were stored at −20 °C until use.

Semen collection and processing

Semen collection was carried out for a period of 6 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 × 10^6/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 having 7 % glycerol (control, C). The second aliquot was diluted at 37 as well as at 4 °C in extender having 3.5 % DMSO (Group 1, G1). The 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, G2). The 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, G3). The fifth aliquot was diluted in extenders having 1.75 % glycerol and 1.75 % DMSO at 37 as well as at 4 °C (Group 4, G4). The final concentration of viable spermatozoa in all experimental groups was 50 × 10^6/ml.

Sperm suspension was cooled from 37 to 4 °C in 2 h and equilibrated at 4 °C for 4 h. Cooled semen samples were then loaded into 0.54 ml French straws (IMV, L'Aigle, France) and frozen in programmable freezer at ultra-fast freezing rate (Planer Kryo 550-16, 110 Windmill, Sunbury, Middlesex, UK) with initial holding of straws at +4 °C for 2 min, from 4 to −10 °C at the rate of −10 °C/min, from −10 to −20 °C at the rate of −15 °C/min, from −20 to −120 °C at the rate of −60 °C/min and final holding for 30 s at −120 °C (Shah et al. 2016). The straws were then plunged into liquid nitrogen (LN_2). After 24 h of storage in LN_2 tank, semen straws (n = 3 per group) were thawed in a water bath at 37 °C for 30 s.

Evaluation of sperm quality

Sperm quality techniques were applied at post-dilution (37 °C), post-equilibration (4 °C) and post-thawing (37 °C) stages of cryopreservation.

Computer-assisted sperm analysis (CASA)

Sperm motility parameters, velocity distribution and kinematics were evaluated by using CASA (CEROS, version 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA) as described by Shah et al. (2016). A drop (5 µl) of each type of semen sample (post dilution, post-equilibration, and post-thawing) was loaded on a pre-warmed (37 °C) glass slide then cover-slipped and positioned in a portable MiniTherm stage (37 °C) of microscope. About two hundred sperm per evaluation period (post dilution, post-equilibration, and post-thawing) were analyzed (magnification, ×10) in two to five fields for progressive motility (%), rapid velocity (%), medium velocity (%), average path velocity (µm/s), straight line velocity (µm/s), and curved line velocity (µm/s).

Sperm in vitro longevity based on CASA (% recovery)

The sperm longevity in terms of % recovery in post-thaw sperm characteristics i.e., progressive motility and rapid velocity was evaluated during 2 h of incubation at 37 °C by using the following formula (Anzar et al. 2011):

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

Sperm supra-vital plasma membrane integrity (%)

Structural and functional integrity of spermatozoa was evaluated according to Chan et al. (1991). An aliquot of sperm suspension (50 µl) was mixed with hypo-osmotic solution (500 µl; 0.735 g sodium citrate and 1.351 g fructose; osmolarity 190, mOsmol/kg) in falcon tubes for each experimental group and control, and was incubated at 37 °C for 30–40 min. Later on, an equal drop (5 µl) of eosin solution (0.5 % tri-sodium citrate dihydrate [wt:vol] 2.92 %) and incubated semen was mixed on slide for 10 s, and finally cover-slipped. Two hundred spermatozoa per evaluation period (post dilution, post-equilibration, and post-thawing) were evaluated under phase contrast microscope (magnification, ×400; Leica, Leitz Wetzlar, Germany). Sperm with unstained heads and swollen tails were considered as structurally and functionally intact, whereas sperm with pink heads and un-swollen tails were classified as non-viable.

Sperm mitochondrial transmembrane potential (%)

Mitochondrial trans-membrane potential was evaluated with JC-1 (the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide) as described by Ahmed et al. (2016) in buffalo. Twenty µl of JC-1 solution (0.15 mM in DMSO) was added in 100 µl of each

sperm suspension (5×10^6 total cells) and was incubated at 37 °C for 10 min. Two hundred sperm per evaluation period (post dilution, post-equilibration, and post-thawing) with orange mid-piece (high mitochondrial transmembrane potential) and with green mid-piece (low mitochondrial transmembrane potential) were evaluated under epifluorescence microscope (magnification, ×400; Nikon (Tokyo, Japan) Optiphot; 490/550 nm excitation/barrier filter).

Viable sperm with intact acrosome (%)

Protocol of Kovács and Foote (1992) was followed for dual staining of sperm with Trypan blue-Giemsa stain. An equal volume (5 µl) of Trypan-blue solution (0.2 %) and semen was placed on a slide at room temperature, and was mixed well using edge of the cover slip. The prepared smears were then air-dried vertically, and fixed in formaldehyde-neutral red solution (86 ml 1 N HCl + 14 ml 37 % formaldehyde 0.2 g Neutral red) for 2–3 min. After rinsing with distilled water and air-drying, the slides were placed in Giemsa stain (7.5 %) for 2.5 h at 37 °C. Later on, slides were washed with distilled water and air-dried, then Balsam of Canada was applied for mounting and the fields were cover slipped. Two hundred sperm per evaluation period (post dilution, post-equilibration, and post-thawing) were evaluated under phase contrast microscope (magnification, ×400; Leica, Leitz Wetzlar, Germany). Sperm having white head region and purple acrosomal region were classified as viable with intact acrosome, whereas sperm having blue head region and pale lavender acrosomal region were considered as nonviable with damaged acrosome.

Sperm DNA integrity (%)

Sperm DNA integrity was evaluated by acridine orange test according to Martins et al. (2007). The smears from each experimental group were prepared on glass slides and air-dried. The fixing of smears in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion) was made for 2 h at room temperature instead of overnight as modification. The slides were air-dried again, and incubated in buffer solution (80 mmol/l citric acid and 15 mmol/l $Na_2HPO_4 \cdot 7H_2O$, pH 2.5) at 75 °C for 5 min to evaluate DNA intactness. Accordingly, slides were stained with acridine orange stain (0.2 mg/ml distilled water) and washed with water for removing background staining. Slides were cover slipped and evaluated in wet condition. Two hundred spermatozoa per stage (post dilution, post-equilibration, and post-thawing) were evaluated under epifluorescence microscope (Nikon Optiphot; 490/550 nm excitation/barrier filter). Sperm with intact DNA emitted green fluorescence while sperm with damaged DNA emitted yellow to red fluorescence.

Experiment II

In vivo fertility (%)

As per the statistical analysis of data, more promising results were found with G4 treatment on post-thawed in vitro sperm quality variables. Additionally, no interaction was found between bull ($n = 5$) and treatment (glycerol and/or DMSO) 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 an extender containing 7 % glycerol (C) or the extenders containing 1.75 % glycerol and 1.75 % DMSO at 37 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.

Post-thawed sperm quality was evaluated after 24 h of storage in LN_2 prior to use for in vivo fertility trial (Ahmed et al. 2016). A total of four hundred and seventeen buffaloes in their 2nd to 3rd lactation (C, $n = 214$; G4, $n = 203$) were inseminated with frozen-thawed semen. The buffaloes had clinically normal reproductive tract with true signs of oestrus. The oestrus detection was based on the mucus discharge and decrease in milk. All experimental inseminations were carried out approximately 24 h after the onset of oestrus. The rectal palpation at day 60 post-AI was undertaken to assess the pregnancy.

Statistical analysis

Experiment I was repeated six times for all groups (five bulls, six replicates, and thirty ejaculates). No interaction was observed between bull ($n = 5$) and treatment (glycerol and/or DMSO) with the GLM procedure, so bull data were pooled and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's method 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. All statistical analyses were conducted using Minitab (Release 17.3.1®, Minitab, Inc., Pine Hall Road, State College, PA, United States).

Results

The data on effect of cryoprotectants (Glycerol and/or DMSO) 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 1. At post dilution, mean value of progressive motility was higher ($P < 0.05$) in G2 than G1. At post-equilibration, mean value of progressive motility was higher ($P < 0.05$) in G4 compared to G1 and control. At post-thawing, mean value of progressive motility was higher ($P < 0.05$) in G4 compared to other treatment groups and control. At post dilution, rapid velocity was higher ($P < 0.05$) in G2 compared to other treatment groups and control. At post-equilibration, rapid velocity was higher ($P < 0.05$) in G4 compared to G1 and G3. At post-thawing, rapid velocity was higher ($P < 0.05$) in G4 compared to other treatment groups and control. At post-equilibration, mean value of medium velocity was higher ($P < 0.05$) in G1 compared to control. At post-thawing, mean value of medium velocity was higher ($P < 0.05$) in control compared to G1, G3 and G4.

At post dilution, mean value of average path velocity was higher ($P < 0.05$) in G2 compared to control and G4. At post-equilibration, mean values of

Table 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 of buffalo bull (five bulls; six replicates) during cryopreservation [post-dilution (PD, 37 °C), post-equilibration (PE, 4 °C), and post-thawing (PT, 37 °C)]

Variable	Stage	Experimental group				
		C	G1	G2	G3	G4
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}	6.22 ± 0.53^{c}	16.33 ± 1.68^{b}	6.96 ± 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}	6.83 ± 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 expressed as Mean ± SEM. Values lacking common superscript in a row indicate significant ($P < 0.05$) difference among the treatments at a given stage. Progressive motility (PM, %), rapid velocity (RV, %), medium velocity (MV, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s) and curved line velocity (VCL, μm/s)

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

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

Table 2 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 in vitro longevity [% recovery based on CASA progressive motility (%) and rapid velocity (%)] of buffalo bull (five bulls; six replicates) during 2 h incubation (37 °C) at post-thawing

Variable (%)	Experimental group	% recovery
Progressive motility	C	14.18 ± 0.77^{b}
	G1	16.23 ± 2.06^{b}
	G2	9.81 ± 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 expressed as Mean ± SEM. Values lacking common superscript in % recovery column indicate significant ($P < 0.05$) difference among the experimental groups in a particular variable

The data on effect of cryoprotectants (Glycerol and/or DMSO) 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 3. At post dilution, mean value of supra-vital plasma membrane integrity was higher ($P < 0.05$) in G4 compared to other treatment groups and control except G2. At post-equilibration, mean value of supra-vital plasma membrane integrity was higher ($P < 0.05$) in G2 and G4 compared to G1 and G3. At post-thawing, mean value of supra-vital plasma membrane integrity was higher ($P < 0.05$) in G4 compared to other treatment groups and control. Mean value of mitochondrial trans-membrane potential was higher ($P < 0.05$) in G4 compared to other treatment groups and control at all stages of cryopreservation. Mean value of viable and intact acrosome was higher ($P < 0.05$) in G4 compared to other treatment groups and control at all stages of cryopreservation except G2 at post-equilibration. At post-thawing, mean value of DNA integrity was higher ($P < 0.05$) in G4 compared to other treatment groups (except G2) and control.

In vivo fertility (%) of buffalo spermatozoa was significantly higher ($P < 0.05$) in G4 compared to control (Table 4).

Discussion

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. To the best of our knowledge, this is the first study that has found 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 (Shah et al. 2016). In the present study, higher sperm 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-extender on post-thaw subjective motility of cattle bull

Table 3 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 supra-vital plasma membrane integrity (%), mitochondrial trans-membrane potential (%), viable and intact acrosome (%) and DNA integrity (%) of buffalo bull spermatozoa (five bulls; six replicates) during cryopreservation [post-dilution (PD, 37 °C), post-equilibration (PE, 4 °C), and post-thawing (PT, 37 °C)]

Variable	Stage	Experimental group				
		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}	7.67 ± 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 expressed as Mean ± SEM. Values lacking a common superscript in a row indicate significant ($P < 0.05$) difference among the treatments at a given stage

Table 4 Effect of cryoprotectants (Glycerol and/or DMSO) 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 group	Pregnancy rate (%)	Chi square value	*P* value
C	59.81^{b}	4.102	0.040
G4	69.45^{a}		

Values lacking common superscript in pregnancy rate column differ significantly ($P < 0.05$)

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 progressive motility of Angora buck spermatozoa at post-thawing (Büyükleblebici et al. 2014). In contrast, Rasul et al. (2007) could not find the 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 absence of glycerol in the extender led to a complete cessation of sperm movement, despite negligible 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 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 protect spermatozoa from the osmotic stress during cryopreservation (Yu and Quinn 1994). It is, therefore, put forward that the above stated contrast

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

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 of the rapid velocity (Barratt et al. 2011). In this study, higher rapid velocity in G4 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, the mean values were increased or decreased with the corresponding values of rapid velocity or slow velocity (data not shown) or static (data not shown). 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, the present data of rapid velocity at post-thawing are supported with that of 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 curved line velocity), as they move along their trajectories (Holt et al. 2007). In our study, at post-thawing, it can be stated that the higher average path velocity, straight line velocity and curved line 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 %) reduced linear motility and consequently increased circular motility, indicating that glycerol had its negative effect on the fine motion characteristics of frozen-thawed buffalo sperm. These changes 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 predict the viability of spermatozoa in female reproductive tract (Akhter et al. 2008). We found significantly higher percentage recovery of sperm progressive motility and rapid velocity in G4 compared to other treatment groups and control. From the cryobiological perspective, DMSO at certain concentration can efficiently improve the super cooling of the intracellular media compared to 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 freezing-thawing process (Hammerstedt et al. 1990). Thus, the assessment of structural and functional integrity of plasma membrane is pivotal for predicting the fertility potential of spermatozoa (Brito et al. 2003). In the present study, higher supra-vital plasma membrane integrity in G4 compared with other treatment groups and control at post-thawing appears to be as a 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. Regarding DMSO, its influence on protein structure and stability is concentration and temperature dependant. Reversible alterations in protein structure are the major effect of exposure of subunit proteins to low DMSO levels at low temperatures, while irreversible denaturation of subunit proteins may be a dominant effect at higher temperatures and higher DMSO concentrations (Yu and Quinn 1994). The temperature dependence of toxicity of DMSO was believed to be due to its hydrophobic/hydrophilic balance in regard to temperature (Arakawa et al. 1990). Furthermore, DMSO was found to be markedly less effective when liposomes contained lipids carrying a net negative charge. This suggested that electrostatic interactions between the polar sulphoxide moiety of DMSO and phospholipid membranes may be an important factor in the cryoprotective effect of DMSO (Yu and Quinn 1994). 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.

Reduction in sperm mitochondrial trans-membrane potential has been mostly associated with lowered fertility (Gallon et al. 2006). It appears that higher mitochondrial trans-membrane potential in G4 compared with other treatment groups and control at post-thawing was due to cryoprotection synergy between glycerol and DMSO. This rapid loss in sperm mitochondrial trans-membrane potential at post-thawing supports previous studies that have suggested that glycerol at higher concentrations can disrupt ATP production (Hammerstedt and Graham 1992), thus resulting in lowered motility and velocities. Again, the divergent affects 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 in carrying hydrolytic enzymes required for oocyte penetration and thus aids in fertilization (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. It is worth to mention that both of these studies had limitations by evaluating only the acrosomal integrity minus the viability of spermatozoa.

Normal sperm genetic material is required for successful fertilization, as well as for embryo and fetal development that will ultimately result in a healthy offspring (Andrabi 2007). We found significantly higher sperm DNA integrity in G4 compared to other treatment groups (except G2) 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, sperm spontaneously produce a variety of reactive oxygen species (ROS), including 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). This study found significantly higher in vivo fertility (%) of buffalo spermatozoa in G4 (69.45) compared to control (59.81). It is worth to mention that a fertility rate >50 % with frozen-thawed spermatozoa is considered as an acceptable outcome of AI in water buffalo (Andrabi 2009). Thus, our findings of in vivo fertility are reasonable and do suggest that G4 can be used in freezing protocol for cryopreservation of water buffalo spermatozoa.

This study concludes that cryoprotection synergism exists between glycerol and DMSO (G4; 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, we can assert that the least toxic combination of cryoprotectants has been identified by reducing the concentration of glycerol for buffalo spermatozoa. It is further suggested that the least toxic combinations of glycerol and DMSO may also be tested in freezing protocols of other livestock species to improve the outcome of cropreservation.

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Compliance with ethical standards

Conflict of interest Authors have no conflict of interest to declare.

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