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INFLUENCE OF COMBINATIONS OF SOME PERMEABLE CRYOPROTECTANTS WITH CHICKEN AND DUCK EGG YOLKS ON FREEZABILITY AND DNA INTEGRITY OF BUFFALO SPERMATOZOA

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ABSTRACT

The present study aimed to investigate the cryoprotective effect of different combinations of glycerol (G), dimethyl sulphoxide (DMSO) and dimethylformamide (DMF) with chicken egg yolk (CEY) and duck egg yolk (DEY) on freezability of buffalo spermatozoa. Semen samples were collected from 4 buffalo bulls and diluted with Tris-based extender supplemented with 7% (V/V) cryoprotectant including G, DMSO, DMF or a mixture of 3.5% G + 3.5% DMSO, 3.5% G + 3.5% DMF and 3.5% DMSO + 3.5% DMF. All the fore-mentioned aliquots were divided into two equal portions; one is supplemented with chicken's egg yolk (CEY) and the other with duck's egg yolk (DEY). Post-thawing sperm motility, viability and acrosomal, plasmatic membrane and DNA integrities were assessed. The results clearly indicated that adding a mixture of 3.5% G + 3.5% DMSO with DEY to Tris extender significantly improved *($p<0.01$)* post-thawing sperm motility, viability index and percentage of spermatozoa with intact acrosomes, plasma membrane and DNA (58.33%±1.66%, 173.33±5.46, 69.00±1.58%, 65.00±1.15% and 96.91±0.13%, respectively) compared with the use of 7% G and CEY (51.50±1.44%, 146.25±6.49, 61.00±2.30, 58.00±1.15% and 91.40±0.17, respectively). In conclusion, motility and functional integrity of cryopreserved buffalo spermatozoa could be enhanced by using Tris extender containing a mixture of 3.5% glycerol + 3.5% DMSO with duck's egg yolk.

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INTRODUCTION

Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material using artificial insemination **(Bucak and Uysal, 2008).** The success of an AI program depends on the proper management of semen collection, storage and use **(Leboeuf *et al*., 2000).** There are many factors, which play a role in the successful cryopreservation of semen such as type of extender, the concentration of cryoprotectant, freezing methods, equilibration times and the temperatures used **(Purdy, 2006).** The cryopreservation process is known to cause damage to sperm cell morphology, resulting in a loss of approximately 40-50% of viable sperm and leading to a lower fertility rate **(Lei *et al*., 2007).** Consequently,

cryoprotectants are added to sperm cryodiluents to protect spermatozoa against this damage **(Purdy, 2006)**. The cryoprotective effect of cryoprotectants against freezing damage acts through several mechanisms, such as lowering the freezing point of intracellular and extracellular water **(Royere *et al*., 1996),** penetrating and interacting with cytoplasmic components, as well as forming a protective layer around sperm membranes (**García *et al*., 2012**). Generally, cryoprotectants are classified into two groups, permeable and non-permeable. Permeable cryoprotectants including glycerol, DMSO, acetaldehyde, propylene glycol and ethylene glycol [EG] **(Di Santo *et al*., 2012).**

Glycerol is the permeating cryoprotectant most frequently used to freeze semen from different species **(Baren *et al*., 2004; García *et al*., 2012).** However, it has been well documented that glycerol, depending on the concentration used, has chemical and osmotic toxicities to the plasma membrane and metabolism of cryopreserved sperm of mammals, which contributes to the disorganization of the sperm plasma membrane, as well as the reduction in motility and fertilizing ability of sperm **(Hammerstedt *et al*., 1990).** Even when using adequate glycerol concentrations, detrimental effects on sperm are not avoided **(Moffet *et al*., 2003; Vidament, 2005).** In addition, glycerol at high concentrations can lead to programmed cell death **(Wundrich *et al*., 2006),** resulted in a significant increase in the percentage of necrotic spermatozoa **(García *et al*., 2012)** and has a deleterious effect on sperm DNA **(El-Badry *et al*., 2014)**. In buffalo bulls, many authors demonstrated that spermatozoa were better preserved in cryodiluents containing glycerol and DMSO but without EG **(Rohilla *et al*., 2005; El-Harairy *et al*., 2011; Almadaly *et al*., 2019).**

Egg yolk (depends on containing cholesterol, phospholipids, and low-density lipoprotein) prevents the formation of ice crystals, thus protecting the integrity of sperm plasma membranes against cold shock during the freeze-thaw process **(Hu *et al*., 2010).** Chicken EY is the most commonly used EY because of its easy availability **(Bathgate *et al*., 2006).** However, the extenders containing EYs from other avian species yielded high post-thawing results in buffalo **(Akhter *et al*., 2017)**, equine **(Webb *et al*., 2011)** bull **(Su *et al*., 2008),** and ram sperm **(Ali *et al*., 2013)**. It is suggested that these improvements are attributed to the differences in the composition of the yolks.

The objective of this study was to assess the effects of permeable cryoprotectants (G, DMSO & DMF) and egg yolk source (CEY & DEY) and their combinations on post-thaw parameters of buffalo bull spermatozoa.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Cairo University, Faculty of Science.

Preparation of extenders

Tris-based buffer was prepared according to **Reddy *et al*. (2010),** which comprised Tris 33.2 g/l, citric acid 18.3 g/l, dextrose 7.8 g/l and supplemented with 100 mMol of trehalose **(Badr *et al*., 2010).** Antibiotics (gentamycin sulphate 500µg/mL, tylosin tartrate 100µg/mL, lincomycin HCl 300µg/mL, and spectinomycin HCl 600µg/mL) were added to the diluent according to **Akhter *et al*. (2011).** Aliquots of Tris extender were supplemented with 7% (vol/vol) cryoprotectant including glycerol (G), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) or a mixture of 3.5% G + 3.5% DMSO, 3.5% G + 3.5% DMF and 3.5% DMSO + 3.5% DMF. All the fore-mentioned aliquots were divided into two equal portions; one is supplemented with 20% chicken's egg yolk (CEY) and the other with 20% duck's egg yolk (DEY).

Animals and semen collection

Four healthy buffalo bulls of known fertility, maintained on the experimental farm of Animal Reproduction Research Institute (ARRI), Agriculture Research Center (ARC), were used in the present study. Their age and body weight ranged between ٣-٢ years and 400-550 Kg, respectively. Early in the morning, using a pre-warmed artificial vagina (40-42 ^{o}C), twice a day ejaculates were collected from each bull once per week for five consecutive weeks with a total of 40 ejaculates.

Semen Processing

Pooled semen samples were diluted with tris-based extender (containing different cryoprotectants and different egg yolks) at 37^{o}C in the incubator in an appropriate dilution rate to obtain a final concentration of 50 x 10^{6} sperm cell/ml. Diluted semen was then cooled slowly to 5^{o}C in a cold cabinet for a period of 1.5 h. Semen was loaded in 0.25 ml straws (IMV, France) and placed 4 cm above liquid nitrogen in the vapour phase in a foam box for 15 minutes before being plunged into the liquid phase **(Khalifa, 2001).** Straws were stored in liquid nitrogen until thawing (one week after freezing) at 37^{o}C in a water bath for 30 sec. Two straws of each treatment (N =12) and replicate (N=5) were thawed at 37^{o}C in a water bath for 30 sec and pooled for evaluation.

Evaluation of frozen-thawed semen

Motility estimations were done at hourly intervals for 3 hours. The viability index was calculated according to **Milovanov (1962)** to be equal to half of

the post-thaw sperm cells motility in addition to the summation of recorded motility at 1st, 2nd and 3rd hours post-thawing. The procedure described by **Jeyendran *et al*. (1984)** was used to determine the percentage of HOST-positive sperm cells in each semen sample. A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed hypo-osmotic solution (0.735 g of sodium citrate dihydrate and 1.351 g of fructose in 100 ml of sterile, de-ionized water). The mixture was incubated at 37°C for 30 minutes in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of the sample was placed on a clean microscope slide and cover-slipped for examination using phase-contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes of the sperm tail. Acrosome integrity was evaluated by a dual staining procedure **(Didion *et al*., 1989).** Briefly, spermatozoa were incubated with an equal volume of 0.2% trypan blue for 10 min and washed twice (centrifugation at 700g for 6 min) with saline. Smears were made on glass slides and dried quickly on a warm stage. Slides were stained with 10% Giemsa stain for 40 min. They were rinsed by distilled water, air-dried, and covered with coverslips. Spermatozoa were classified as acrosome intact [light purple - dark pink acrosome] and damaged/lost acrosome [unstained or blue acrosome].

Sperm cell DNA integrity by Comet assay (Single cell gel electrophoresis)

The alkaline comet assay for spermatozoa was carried out according to **Hughes *et al*. (1996)**. Fully frosted glass slides were covered with 100 µl of 0.5% normal melting point agarose (Sigma), a coverslip was added and the agarose was allowed to solidify. The coverslips were removed and $1x10^5$sperm cells in 50 µl PBS (7.2 pH) were mixed with 50 µl of 1.2% low melting point agarose and used to form the second layer. The slides with coverslips removed were then placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton X at a pH of 10). The slides were then incubated at 37°C in 100 µl/ml of proteinase K in lysis buffer overnight. After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA for 20 min to allow the DNA to denature.

Electrophoresis was performed at room temperature, at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. The slides were then washed with a neutralizing solution of 0.4 M Tris at pH 7 to remove alkali and detergents. After neutralization, the slides were each stained with 50 µl of 20 µg/ml ethidium bromide and mounted with a coverslip. A total of 200 sperm cells were examined under a fluorescent microscope (400X). The intensity of the stain in the comet tail region is presumed to be related to the DNA content, and DNA damage is estimated from measurements of the per cent DNA in tail, tail length and tail moment, using an image analysis system (Comet-Score program). Spermatozoa with fragmented DNA (damaged) display increased migration of the DNA from the nucleus towards the anode, while spermatozoa with non-fragmented DNA (undamaged) do not form a "comet" **(Fraser, 2004)**.

Statistical analysis

One-way analysis of variance and Duncan's multiple range tests were done for the obtained data after the angular transformation of percentages to their corresponding arcsin values **(Snedecor and Cochran, 1989).** Data were analyzed using SPSS program (version 22.0), and the level of statistical significance was set at $P \leq 0.05$.

RESULTS

As presented in table 1, semen samples extended in the extender containing a combination of G and DMSO with Duck egg yolk has a significantly highest *(p<0.01)* post-thaw motility and viability index (58.33 ± 1.66% & 173.33 ± 5.46, respectively). Intermediate significant *(p<0.01)* values of post-thaw motility and viability indices were shown with diluents containing either G or DMSO in addition to chicken egg yolk (52.50 ± 1.44 & 146.25 ± 6.49 and 47.50 ± 4.33% & 124.58 ± 10.58, respectively) or duck egg yolk (57.50 ± 4.33% & 196.58 ± 5.12 and 50.00 ± 5. 77% & 135.00 ± 8.66, respectively). The significantly lowest *(p<0.01)* values of post-thaw motility and viability indices were recorded for diluents supplemented with DMF either containing chicken egg yolk (30.00 ± 2.88% & 68.33 ± 5.83, respectively) or duck egg yolk (30.00 ± 5.77% & 73.33 ± 4.40, respectively).

Data presented in table 2 showed that the inclusion of a mixture of G + DMSO + duck egg yolk significantly improved the freezability of buffalo spermatozoa in terms of the highest membrane, acrosomal and DNA integrities (65.00 ± 1.11%, 69.16 ± 1.58 and 94.28 ± 0.15%, respectively) and the lowest DNA damage (3.08 ± 0.13%, 5.01 ± 0.10 pixels and 0.46 ± 0.00 for the percentage of DNA in the tail of Comet, tail length and Olive tail moment, respectively, Fig. 1). The significantly lowest *(p <0.01)* values of spermatozoa with the membrane, acrosomal and DNA integrities (Fig. 2) were recorded for diluents supplemented with DMF either containing chicken or duck egg yolk.

Table 1: Effect of different cryoprotectants and egg yolk source on the post-thaw total motility and viability of frozen-thawed buffalo spermatozoa (Mean ±SEM).

CPA	Post-thaw motility (%)				Viability index
	Zero h.	One h.	Two h.	Three h.	
G+CEY	52.50±1.44^{ab}	47.50±1.44^{abc}	40.00±2.88^{abc}	32.50±1.44^{bc}	146.25±6.49^{bc}
DMSO+CEY	47.50±4.33^{abc}	40.00±5.77^{cd}	33.3±1.66^{c}	27.50±1.44^{c}	124.58±10.85^{d}
DMF+CEY	30.00±2.88^{e}	22.50±4.33^{f}	18.33±1.66^{d}	12.50±1.44^{d}	68.33±5.83^{g}
G+DMSO+CEY	55.00±2.88^{ab}	50.00±2.88^{ab}	42.50±1.44^{ab}	35.00±2.88^{b}	155.00±8.66^{ab}
G+DMF+CEY	45.00±2.88^{bcd}	40.83±2.20^{cd}	33.33±1.66^{c}	27.50±1.44^{c}	124.17±3.63^{d}
DMSO+DMF+CEY	35.00±4.33^{de}	30.00±2.88^{ef}	25.00 ±2.88^{d}	15.00±2.88^{d}	87.50±4.38^{ef}
G+DEY	57.50±4.33^{ab}	52.50±1.44^{a}	46.66±1.66^{a}	41.66±1.66^{a}	169.58±5.12^{a}
DMSO+ DEY	50.00±5.77^{ab}	42.50±1.44^{bc}	37.50±1.44^{bc}	30.00±2.88^{bc}	135.00±8.66^{cd}
DMF + DEY	30.00±5.77^{e}	25.00±2.88^{ef}	20.00±2.88^{d}	13.33 ±1.66^{d}	73.33 ±4.40^{fg}
G+DMSO+DEY	58.33±1.66^{a}	52.50 ±1.44^{a}	46.66±1.66^{a}	45.00 ±2.88^{a}	173.33 ±5.46^{a}
G+DMF+DEY	45.00±0.00^{bcd}	42.50 ±1.44^{bc}	35.00±2.88^{c}	30.00±2.88^{bc}	130.00±1.44^{cd}
DMSO+DMF+DEY	37.50±4.33^{cde}	32.50±1.44^{de}	25.00±2.88^{d}	18.33±1.66^{d}	94.58±3.70^{e}

Within columns, means with different alphabetical superscripts (a, b, c) are significantly different at least at $p < .05$.

Table 2: Effect of different cryoprotectants and egg yolk source on DNA, membrane and acrosomal integrities of buffalo spermatozoa (Mean ±SEM).

CPA	HOST	Acrosomal integrity	DNA Integrity				
			Sperm with non-fragmented DNA	DNA in the head of comet %	DNA in the tail of comet%	Tail length (pixel)	Olive tail moment
G+CEY	58.00± 1.15^{bc}	61.00± 2.30^{bc}	91.40± 0.17^{de}	95.83± 0.14^{bc}	4.16± 0.14^{de}	8.66± 0.11^{c}	0.72± 0.02^{d}
DMSO+CEY	52.00± 1.73^{e}	54.33± 0.88^{d}	90.80± 0.57^{ef}	94.88± 0.36^{d}	5.11± 0.36^{c}	9.53± 0.08^{b}	1.15± 0.09^{b}
DMF+CEY	43.33± 1.45^{fg}	40.66± 2.02^{f}	91.88± 0.21^{cd}	96.00± 0.28^{b}	4.00± 0.28^{e}	7.35± 0.57^{d}	0.67± 0.00^{de}
G+DMSO+CEY	60.33± 1.45^{b}	60.90± 1.24^{bc}	93.06± 0.31^{b}	96.33± 0.20^{ab}	3.66± 0.20^{ef}	5.91± 0.15^{d}	0.56± 0.01^{ef}
G+DMF+CEY	53.33± 0.88^{de}	57.33± 1.45^{cd}	92.81± 0.18^{bc}	96.15± 0.08^{b}	3.85± 0.08^{e}	6.40± 0.11^{d}	0.73± 0.01^{d}
DMSO+DMF+CEY	41.00± 1.73^{g}	43.33± 1.45^{ef}	90.30± 0.40^{e}	92.66± 0.08^{d}	7.33± 0.08^{a}	10.44± 0.57^{a}	1.41± 0.07^{a}
G + DEY	60.66± 2.02^{c}	64.00± 1.15^{c}	94.05± 0.16^{a}	95.86± 0.08^{bc}	4.13± 0.08^{de}	7.52± 0.11^{d}	0.66± 0.02^{de}
DMSO+ DEY	53.66± 0.88^{cde}	52.66± 1.45^{d}	91.40± 0.23^{de}	95.33± 0.20^{cd}	4.66± 0.20^{cd}	8.40± 0.23^{c}	0.97± 0.01^{c}
DMF + DEY	46.33± 1.45^{f}	46.66± 2.02^{e}	92.10± 0.51^{bcd}	95.76± 0.14^{bc}	4.23± 0.14^{de}	7.33± 0.20^{d}	0.58± 0.00^{ef}
G+DMSO+DEY	65.00± 1.15^{a}	69.16± 1.58^{a}	94.28± 0.15^{a}	96.91± 0.13^{a}	3.08± 0.13^{f}	5.01± 0.10^{f}	0.46± 0.00^{f}
G+DMF+DEY	56.66± 0.88^{bcd}	59.33± 1.45^{bc}	93.00± 0.28^{b}	96.33± 0.22^{ab}	3.66± 0.22^{ef}	6.08±0. 13^{d}	0.79± 0.05^{d}
DMSO+DMF+DEY	43.00± 1.73^{fg}	46.00± 1.73^{e}	91.33± 0.20^{de}	93.30± 0.11^{e}	6.70± 0.11^{b}	9.66± 0.20^{ab}	1.22± 0.05^{b}

Within columns, means with different alphabetical superscripts (a, b, c) are significantly different at least at $p < .05$.

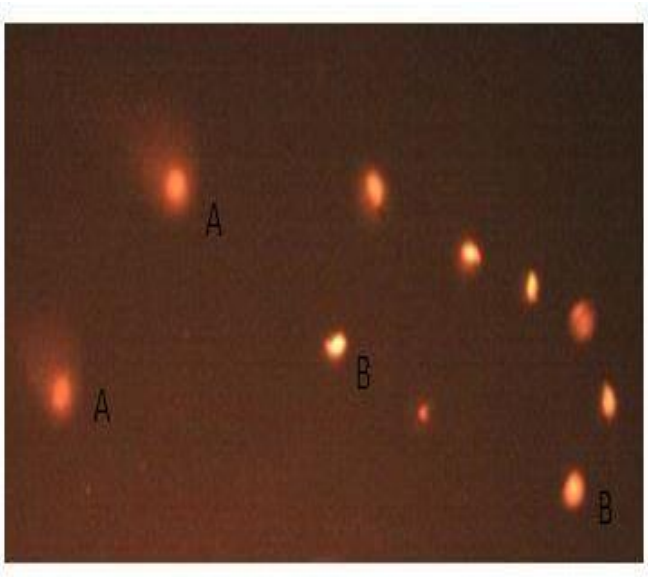

Fig.1: Comet picture of buffalo spermatozoa cryopreserved in Tris diluent containing a mixture of G + DMSO + duck egg yolk with (A) or without (B) no DNA fragmentation.

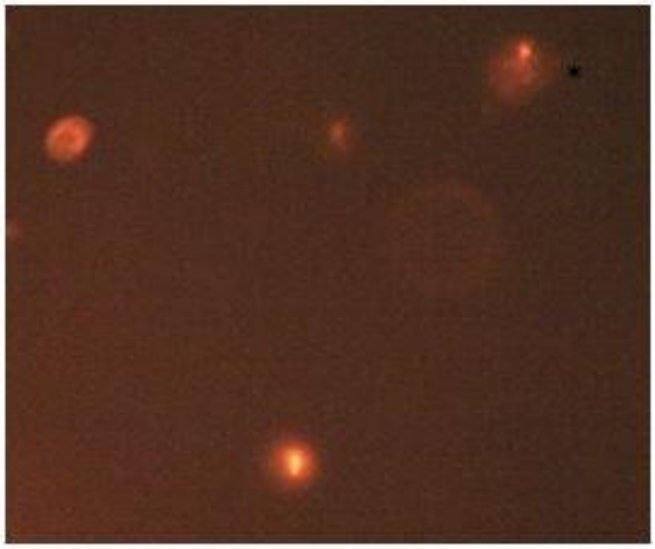

Fig.2: Comet picture of buffalo spermatozoa cryopreserved in Tris diluent supplemented with DMF and chicken egg yolk showing DNA fragmentation (Asterix).

DISCISSION

In the current study combinations of penetrative cryoprotectants including glycerol, DMSO and DMF with two egg yolk sources (CEY & DEY) were evaluated aiming to improve the quality of frozen-thawed buffalo-bull spermatozoa. Our results revealed that buffalo spermatozoa were better cryopreserved using 7% glycerol as CPA rather than using the same concentration of DMF and DMSO in terms of post-thaw motility and DNA, membrane and acrosomal integrities. Similarly, many researchers considered glycerol (at 6 to 7% concentration) to be the most suitable CPA for buffalo semen cryopreservation **(Rasul *et al*., 2007; Sianturi *et al*., 2012; Shahverdi *et al*., 2014; Kumar *et al*., 2016; Herbowo *et al*., 2019).** Generally, the glycerol is being routinely used as a major cryoprotectant for mammalian sperm freezing (**García *et al*., 2012**) due to its slow permeability across the plasma membrane **(Gao and Critser, 2000; García *et al*., 2012; Vafaei *et al*., 2019)**.

In a recent study, glycerol was found to improve the post-thaw buffalo sperm characteristics including motility, viability, plasma membrane integrity, acrosomal membrane integrity as well as yielded higher pregnancy rate in comparison with EG and DMSO **(Almadaly *et al*., 2019).** Furthermore, **Tasdemir *et al*. (2013)** reported that there was no advantage in using EG or DMSO for freezing of buffalo spermatozoa as G yielded better post-thaw sperm characteristics as compared to EG and DMSO. Also, **Guerrero (2006)** found that the post-thaw motility and membrane intactness of bull spermatozoa frozen in 7% G-Tris-based extender were significantly higher than those of sperm frozen in 7% EG-Tris-based extender. In bovines, **El-Harairy *et al*. (2011)** found that post-thaw motility and the conception rate of bull spermatozoa frozen in a lactose-yolk-citrate extender containing 7% G or 7% DMSO was similar. On the contrary to our results, **Swelum *et al*. (2011)** reported that 5% EG resulted in greater post-thaw intact-acrosome, intact-plasma membrane and conception rate of buffalo-bull spermatozoa than 7% G. This discrepancy might be due to different animal breed and semen extender as well as diverse cooling and freezing protocols.

There were marked species differences in the effect of DMF on sperm freezability. The use of DMF improved cryo survival of frozen-thawed semen in equine **(Ghallab *et al*., 2019)**, caprine **(Ariantie *et al*. 2013)**, and swine **(Bianchi *et al*. 2008)**; however, DMF is not an effective cryoprotectant for ram **(Moustacas *et al*., 2011)** canine **(Mota-Filho *et al*. 2011)** sperm. The differences in the composition of sperm membrane among species are more likely to be the reason for the different results. **Mota-Filho *et al*. (2011)** attributed the sperm intolerance to DMF to either biochemical effects or due to osmotic imbalance.

Permeable cryoprotectants including glycerol, DMSO and DMF pass through the plasma membrane and replace the water in the sperm cell **(Di Santo *et al*., 2012)**. Such cryoprotectants are toxic at higher concentrations and numerous reports have shown that sperm fertility potential is dramatically decreased in freezing medium supplemented with high concentrations of these permeable agents **(Gilmore *et al*., 1997)**. So, the use of two permeable cryoprotectants rather than a single one is recommended to enables the use of lower concentrations of each cryoprotectant , thereby reducing potential toxicity **(Pugh *et al*., 2000).** In the present study DNA integrity was better maintained by the use of a combination of G + DMSO. DNA integrity is a concern during cell freezing because cryopreservation easily changes mitochondrial membrane properties and increases the production of ROS (Reactive Oxygen Species), which may subsequently result in the oxidation of DNA, producing high frequencies of single and double-strand DNA breaks **(Said *et al*., 2010).**

Currently, egg yolk is a common component of most semen extenders for domestic animals as it protects the plasma membrane and acrosome against temperature-related injury **(Amirat *et al*., 2004).** Chicken EY is the most commonly used EY because of its easy availability **(Bathgate *et al*., 2006).** However, the extenders containing EYs from the avian species other than chickens resulted in significantly high post-thawing parameters in buffalo sperm **(Andrabi *et al*., 2008; Waheed *et al*., 2012; Akhter *et al*., 2017),** bulls **(Su *et al*., 2008)**, equine **(Webb *et al*., 2011)**, and rams **(Ali *et al*., 2013).** It is suggested that this improvement in post-thaw sperm quality is attributed to the differences in the biochemical composition of the yolks. Our finding that the cryo survival of buffalo spermatozoa is more improved by adding DEY rather than CEY came in accordance with those recorded in buffalo by **Waheed *et al*. (2012)** and **El-Sharawy *et al*. (2015)** in buffalo bulls. According to **Bathgate *et al*. (2006)**, the basic components of CEY and DEY did not differ, but the ratios of fatty acids and phospholipid were different. The yolk from duck eggs had more monounsaturated fatty acids than yolk from chicken eggs. Moreover, yolk from duck eggs contained more phosphatidylinositol than CEY.

CONCLUSIONS

It is suggested that the interaction between permeating and nonpermeating CPAs had improved most of the post-thaw sperm parameters studied herein and it may be related to the possible synergism among these compounds. So, it could be concluded that Tris extender containing a mixture of 3.5% glycerol+3.5% DMSO with duck's egg yolk is recommended as it enhances post-thaw motility and functional integrity of cryopreserved buffalo spermatozoa

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