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# Effect of Trehalose on Cryopreservation, Oxidative Stress and DNA Integrity of Buffalo Spermatozoa

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**Abstract:** Cryopreservation induces sublethal damage to the spermatozoa, thereby reduce their fertile life. In the present study, the cryoprotective effect of trehalose on buffalo semen freezability, DNA integrity, antioxidant activity and *in vitro* fertilizing potentials were evaluated. The semen was diluted with Tris based extender containing different trehalose concentrations (0, 25, 50, 100,150 and 200 mM), cooled to 5°C, loaded into 0.25-mL straws, frozen and stored in liquid nitrogen. The cryopreserved spermatozoa were assessed for post-thawing sperm motility, viability and acrosomal integrity. DNA damage was assessed by single cell gel electrophoresis (Comet assay) and the measurements performed were DNA damage, tail length and tail moment. Enzymes, antioxidant activity and lipid peroxidation were assessed. Results clearly indicated that addition of 100 mM trehalose to the semen extender significantly ($P<0.05$) improved the motility, viability and acrosomal integrity (61.25±1.25%, 172.25±5.58 and 10.25±2.39%, respectively) in comparison with the control (41.25±4.32%, 89.13±15.57 and 23.5±1.85%, respectively. Additionally, extender containing 100 mM trehalose significantly ($P<0.05$) reduced sperm DNA damage, tail length and tail moment of the frozen-thawed semen (1.83±0.71%, 2.09 ±0.48µm and 3.16±0.68, respectively) and diminished sperm cells apoptosis compared with the base extender (4.59±1.38%, 5.15±0.51 µm and 22.94±6.46, respectively). Moreover, extender containing 100 mM trehalose significantly ($P<0.05$) increased the level of glutathione reductase, speroxide dismutase and total antioxidant (110.64±5.39, 65.28±4.06 and 0.55±0.045 mµ/ml, respectively) and decreased the lipid peroxidation (62.01±5.74 nmol/ml) with respect to the base extender (62.01±5.74, 26.1±1.40,0.19±0.025 mµ/ml and 21.57±1.45 nmol/ml, respectively). Moreover, the addition of 100 mM trehalose significantly ($P<0.05$) improved the *in vitro* fertilization rate, morula and blastocyst development (57.25±2.63, 25.97±2.82 and 17.43±1.35%, respectively) compared with the control extender (38.41 ±3.48, 12.26±2.13 and 4.78±1.54, respectively). It was concluded that the addition of 100 mM trehalose to the freezing extender led to the reduction of cryodamage, enhanced viability of the buffalo spermatozoa, protected DNA of the sperm cells from deterioration, reduced the oxidative stress provoked by freezing and thawing and enhanced the *in vitro* fertilizing capacity of the cryopreserved buffalo spermatozoa.

**Key words:** Trehalose · Cryopreservation · Buffalo semen · DNA integrity · *In vitro* fertilization · Antioxidant activity

## INTRODUCTION

The enhancement of artificial insemination is a valuable tool in genetic improvement programs [1]. However, the biggest problem to exploiting cryopreserved buffalo semen is damage of sperm membrane structures during freezing and thawing, which leads to fewer viable and motile cells post-thawing [2]. An intact and functional plasma membrane is a key component of the cell and must be maintained during freezing conditions if the cell is to be kept alive [3]. Therefore, cryoprotectants are included in cryopreservation extender to reduce the damaging effects of the freezing process [4, 5]. An important factor in the efficacy of an extender is supplementation with

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disaccharides such as trehalose and sucrose, [6-8]. Trehalose, is a non-reducing disaccharide in which two glucose molecules are linked together in a 1, 1-glycosidic linkage (α-d-glucopyranosyl-1, 1-α-d-glucopyranoside), commonly found in high concentrations in many organisms such as yeast and fungal spores [9, 10], capable of surviving complete dehydration. Supplementation of semen extenders with trehalose is well known to improve the motility and viability of cryopreserved mammalian sperm cells [11, 12]. Trehalose probably plays a key role in preventing deleterious alteration to the membrane during reduced-water states and the action of trehalose appears to be connected with its ability to replace water at the membrane/solution interface [13]. In addition, trehalose have several functions in sperm extender, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluent, acting as a cryoprotectants and increase sperm membrane fluidity, rendering the spermatozoa capable of enduring freeze-thawing damage [14, 15]. Moreover, trehalose presumably involves a stabilization of certain cell proteins and/or lipid membranes during stresses such as cryopreservation, heat, desiccation or oxidative stress [16]. When trehalose was added in hypertonic conditions, it showed a synergic effect with glycerol used as a cryoprotectant in order to avoid intracellular ice crystal formation [17]. Therfore an important goal for the present study was to determine the cryoprotective mechanisms of trehalose on buffalo spermatozoa, through investigating the effects of trehalose on quality parameters of the cryopreserved spermatozoa, DNA integrity, apoptosis, antioxidant activities and *in vitro* fertilizing potentials of frozen-thawed buffalo semen.

## MATRIAL AND METHODS

**Semen Collection and Processing:** Semen samples were collected from six buffalo bulls of proven fertility, kept at the Animal Reproduction Research Institute farm, Al-Harm, Egypt. Only semen samples of at least 70 % initial motility and 800.00X10$^6$sperm cells/ml were used. Immediately after collection, semen samples were pooled, split into 6 portions and diluted at a 1:8 ratio at 30°C with Tris-based extender supplemented with different trehalose concentrations (0, 25, 50, 100,150 and 200 mM). Immediately after dilution, the extended semen was cooled to 5°C throughout 60 minute in a cold cabinet. The cooled semen was loaded into 0.25 ml French straws (IMV, L'Aigle, France), then lowered into liquid nitrogen vapor inside foam box. The straws were then immersed into liquid nitrogen and stored until analysis. Frozen semen samples were thawed in a water bath at 37°C for 30 second. Classical semen quality tests (motility, viability and acrosomal integrity) were assessed according to Mohammed *et al.* [18].

**Assessment of Sperm DNA Integrity:** The DNA integrity and the incidence of DNA strand breaks or fragmentation was detected using alkaline comet assay according to Boe-Hansen [19].

**Detection of Sperm Cells Apoptosis:** Apoptosis, or physiological cell death, was evaluated by measurement of DNA fragmentation as described by Cohen and Duke [20]. Measurement of DNA fragmentation with the DPA colorimetric assay is very typical for the apoptotic process and preferentially used to evaluate apoptosis in the cell populations where DNA labeling is impossible or difficult.

**Biochemical Analysis:** Aspartate-aminotransferase (AST); alanine-aminotransferase (ALT) and alkaline phosphatase (AKP) enzymes leakage during cryopreservation was assessed spectrophotometrically according to Tietz [21] to evaluate the membrane stabilizing effect of trehalose. Additionally, Antioxidant activity, Glutathione peroxidase (GSH), superoxide dismutase (SOD) and the lipid peroxidation of the cryopreserved spermatozoa were measured as described by Cortassa *et al.* [22] to evaluate the antioxidant influence of trehalose.

**Evaluation of *in vitro* Fertilizing Potential of the Treated Semen:** The fertilizing potentials of the treated semen were assessed using *in vitro* fertilization technology as demonstrated by Totey *et al.* [23]. Three straws from each treatment were thawed in a water bath at 37°C for 30 sec. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's Albumin-Lactate-Pyruvate (TALP) containing 6 mg/ml bovine serum albumin (BSA), for 1 hour as recorded by Parrish *et al.* [24]. The uppermost layer of the medium containing the most spermatozoa was collected and washed twice by centrifugation at 2000 rpm for 10 minutes. The sperm pellet was resuspended in the fertilization TALP medium containing 10 μg/ml heparin. After appropriate dilution, 2 μl of sperm suspension was added to the fertilization drops, containing the *in vitro* matured buffalo oocytes, at a final concentration 2 X10$^6$

sperm cell/ml. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 hour at 39°C in an atmosphere of 5% $CO_2$ in air with maximum humidity. After that, the inseminated oocytes were freed from extra cumulus cells and attached spermatozoa by gentle pipetting and then cultured in TCM-199 medium with Hepes modification for seven days in the same previous conditions. The proportional of the cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded at 5-7 day post-insemination.

**Statistical Analysis:** All data were analyzed by using Costat Computer Program, Version 3.03 copyright (1986) Cottort Software and were compared by the least significant difference least (LSD) at 5 and 1% levels of probability. All data demonstrated as Means ± SE.

## RESULTS

Data presented in Table 1 reveal that, fortification of freezing extender with trehalose improved the freezability of buffalo semen as compared to the control semen in a dose-dependent trend. Addition of 100 mM trehalose to semen extender, appeared to be the best concentration that improved (P<0.05) the post-thawing sperm motility; viability indices and maintained the acrosomal integrity (61.25±1.25%, 172.25±5.58 and 10.25±2.39 %, respectively) as compared with the base extender (41.25±4.32%, 89.13±15.57 and 23.5±1.85%, respectively).

Data presented in Table 2 indicate that, semen cryopreservation increased the DNA damage, tail length and the tail moment. However, *in vitro* provision of semen extender with 100 mM trehalose significantly (P<0.01) reduced the DNA fragmentation, tail length and tail moment of the frozen-thawed semen (1.83±0.71%, 2.09±0.48µm and 3.16±0.68, respectively) as compared with the control frozen semen (4.59±1.38%, 5.15±0.51 µm and 22.94±6.46, respectively) as illustrated in Fig (1, A).

The current results showed a significant negative correlation between DNA damage and post-thawing motility (R=- 0.48, P<0.05) and viability index (R=- 0.54, P<0.01) and a significant positive correlation between DNA damage and acrosomal integrity (R= 0.45, P<0.05). Furthermore, the present results revealed that, cryopreservation induced a harmful apoptotic effect on the buffalo semen as shown Fig (1, B); meanwhile, trehalose treatment protected the sperm cells from apoptosis as shown in Fig (1, C).

Data regarding the antioxidant activity and the membrane stabilizing effect of trehalose during cryopreservation are verified in Table 3. Semen cryopreservation increased (P<0.05) the leakage of AST, ALT and AKP enzymes of the frozen-thawed spermatozoa (106±5.95, 23.0±2.49 and 23.28±1.75 U/L, respectively). However, *in vitro* provision of semen extender with 100 mM trehalose significantly (P<0.05) reduced the enzyme leakage (61.25±5.55, 11.5±1.04 and 14.65±2.79 U/L, respectively). Moreover, the results presented in Table 3 demonstrated that, trehalose had a persuasive antioxidant

Table 1: Effect of freezing extender fortification with trehalose on the buffalo semen freezability

| Treatments | Dilution motility (%) | Post-thawing motility (%) | Reduction motility (%) | Viability index | Acrosomal integrity (%) |
|---|---|---|---|---|---|
| Control | 76.25±2.40$^{a}$ | 41.25±4.32$^{d}$ | 53.84±4.55$^{a}$ | 89.13±15.57$^{d}$ | 23.5±1.85$^{a}$ |
| Trehalose 25mM | 77.5±3.23$^{a}$ | 43.75±2.39$^{cd}$ | 42.88±5.51$^{ab}$ | 109.5±7.03$^{cd}$ | 17.75±1.89$^{b}$ |
| Trehalose 50mM | 78.75±1.25$^{a}$ | 51.25±1.25 $^{bc}$ | 34.89±1.57$^{bc}$ | 145.5±3.33$^{ab}$ | 15.5±1.32$^{bc}$ |
| Trehalose 100mM | 82.50±1.45$^{a}$ | 61.25±1.25$^{a}$ | 25.64±2.52$^{c}$ | 172.25±5.58$^{a}$ | 10.25±2.39$^{d}$ |
| Trehalose 150mM | 80.00±2.04$^{a}$ | 56.25±2.39$^{ab}$ | 30.56±3.66$^{bc}$ | 146.5±12.43$^{ab}$ | 12.00±1.29$^{cd}$ |
| Trehalose 200mM | 81.25±2.39$^{a}$ | 52.5±4.79$^{abc}$ | 35.25±5.94$^{bc}$ | 127.25±1.03$^{bc}$ | 12.75±1.49$^{cd}$ |
| Over all mean | 79.38±0. 95 | 51.04±1.81 | 37.18±2.44 | 131.69±6.52 | 15.29±1.11 |

Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Table 2: Effect of freezing extender fortification with trehalose on the buffalo DNA integrity

| Treatments | DNA damage (%) | Tail length (µm) | Tail moment |
|---|---|---|---|
| Control | 4.59±1.38 $^{a}$ | 5.15±0.51 $^{a}$ | 22.94±6.46 $^{a}$ |
| Trehalose 25mM | 2.64±0.69 $^{ab}$ | 3.06±0.92 $^{b}$ | 6.88±1.85 $^{b}$ |
| Trehalose 50mM | 2.56±0.56 $^{ab}$ | 2.42±0.58 $^{b}$ | 6.94±3.03 $^{b}$ |
| Trehalose 100mM | 1.83±0.71 $^{b}$ | 2.09±0.48 $^{b}$ | 3.16±0.68 $^{b}$ |
| Trehalose 150mM | 2.70±0.68 $^{ab}$ | 2.89±0.58 $^{b}$ | 7.49±2.37 $^{b}$ |
| Trehalose 200mM | 2.33±0.57 $^{ab}$ | 2.71±0.72 $^{b}$ | 5.52±1.05 $^{b}$ |
| Over all mean | 2.77±0.33 | 3.03±0.31 | 8.82±1.79 |

Values with different superscript letters in the same columns are significantly different at least (P<0.05).

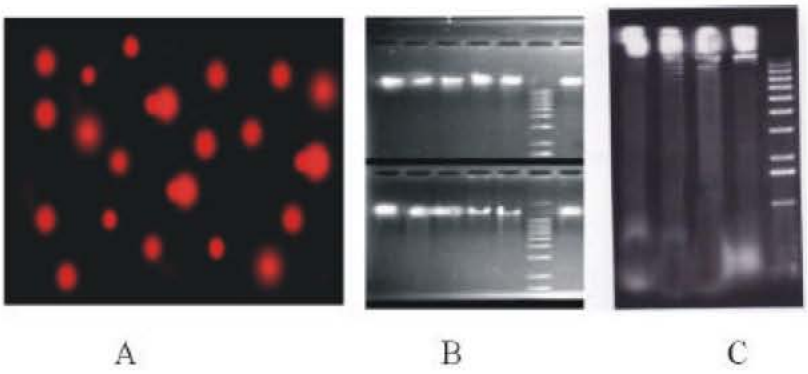


**Fig. 1: A. Buffalo spermatozoa after the single cell gel electrophoresis (comet) assay. Increased DNA fragmentation is represented by an increasing amount of DNA present in the comet tail. B. Buffalo spermatozoa showed normal apoptotic cells after cryopreservation with 100 mM trehalose enriched extender, C. Buffalo spermatozoa showed drastic apoptotic effect on the buffalo semen**

Table 3: Effect of freezing extender fortification with trehalose on the antioxidant activity, lipid peroxidation and enzyme leakage (Means ± SE)

| Treatments | TO | SOD | GSH | Malandiahyde | AST | ALT | AKP |
|---|---|---|---|---|---|---|---|
| Control | 0.19±0.025$^{d}$ | 26.1±1.40$^{e}$ | 62.01±5.74$^{c}$ | 21.57±1.45$^{a}$ | 106±5.95$^{a}$ | 23.0±2.49$^{a}$ | 23.28±1.75$^{a}$ |
| Trehalose 5mM | 0.27±0.025$^{cd}$ | 30.63±1.47$^{de}$ | 70.51±3.14$^{c}$ | 18.6±1.12$^{a}$ | 100.25±5.54$^{a}$ | 22.25±1.49$^{ab}$ | 19.9±1.38$^{ab}$ |
| Trehalose 50mM | 0.29±0.015$^{c}$ | 37.15±1.79$^{d}$ | 64.55±4.93$^{c}$ | 14.39±2.58$^{b}$ | 97.5±4.21$^{a}$ | 17.75±1.25$^{c}$ | 18.55±1.34$^{ab}$ |
| Trehalose 100mM | 0.55±0.045$^{a}$ | 65.28±4.06$^{a}$ | 110.64±5.39$^{a}$ | 9.18±1.46$^{c}$ | 61.25±5.55$^{b}$ | 11.5±1.04$^{d}$ | 14.65±2.79$^{b}$ |
| Trehalose 150mM | 0.42±0.035$^{b}$ | 53.75±1.88$^{b}$ | 88.78±4.57$^{b}$ | 12.79±1.44$^{bc}$ | 78.25±6.24$^{b}$ | 15.25±1.80$^{cd}$ | 16.43±1.00$^{b}$ |
| Trehalose 200mM | 0.31±0.010$^{c}$ | 44.4±1.53$^{c}$ | 86.42±4.33$^{b}$ | 12.69±2.15$^{bc}$ | 74.75±8.83$^{b}$ | 21.0±1.50$^{ab}$ | 15.48±2.35$^{b}$ |
| Over all mean | 0.34±0.0245 | 42.88±2.92 | 80.48±3.92 | 14.87±1.06 | 86.33±4.02 | 18.46±1.04 | 18.04±1.00 |

Values with different superscript letters in the same columns are significantly different at least (P<0.05).

SOD: sueroxide dismutase, GSH: Glutathione reductase, AST: aspartate-aminotransferase, ALT:alanine-aminotransferase, AKP: alkaline phosphotase TO: Total antioxidant

Table 4: Effect of fortification of freezing extender with trehalose on the *in vitro* fertilization rate

| Treatments | NO. of oocytes | Penetration rate (%) | Fertilization rate (%) |
|---|---|---|---|
| Control | 91 | 61.19± 3.7 1$^{a}$ | 38.41 ±3.48$^{d}$ |
| Trehalose 25mM | 89 | 66.31± 1.66$^{a}$ | 40.14±2.61$^{cd}$ |
| Trehalose 50mM | 90 | 63.70± 1.07$^{a}$ | 45.42±2.85$^{bcd}$ |
| Trehalose 100mM | 89 | 68.03± 1.88$^{a}$ | 57.25±2.63$^{a}$ |
| Trehalose 150mM | 84 | 65.44± 1.31$^{a}$ | 51.66±2.77$^{ab}$ |
| Trehalose 200mM | 84 | 68.55± 3.57$^{a}$ | 47.83±2.04$^{bc}$ |
| Over all mean | | 65.53±1.11 | 46.78±1.68 |

Values with different superscript letters in the same columns are significantly different at least (P<0.05)

Table 5: Effect of trehalose addition to the freezing extender on the *in vitro* embryo development

| Treatments | No. of oocytes | Cleavage rate (%) | Morula stage (%) | Blastocyst stage (%) |
|---|---|---|---|---|
| Control | 103 | 32.18±2.99$^{b}$ | 12.26±2.13$^{c}$ | 4.78±1.54$^{d}$ |
| Trehalose 25mM | 114 | 35.66±3.29$^{b}$ | 13.13±1.83$^{c}$ | 7.93±1.62$^{cd}$ |
| Trehalose 50mM | 90 | 46.26±4.49$^{a}$ | 18.76±1.26$^{b}$ | 12.11±1.67$^{bc}$ |
| Trehalose 100mM | 109 | 52.37±2.25$^{a}$ | 25.97±2.82$^{a}$ | 17.43±1.35$^{a}$ |
| Trehalose 150mM | 108 | 46.2±2.64$^{a}$ | 20.23±1.08$^{b}$ | 16.53±1.69$^{ab}$ |
| Trehalose 200mM | 113 | 45.00±2.06$^{a}$ | 19.62±1.41$^{b}$ | 13.86±1.84$^{ab}$ |
| Over all mean | | 42.94±1.81 | 18.33±2.87 | 12.11±1.11 |

Values with different superscript letters in the same columns are significantly different at least (P<0.05)

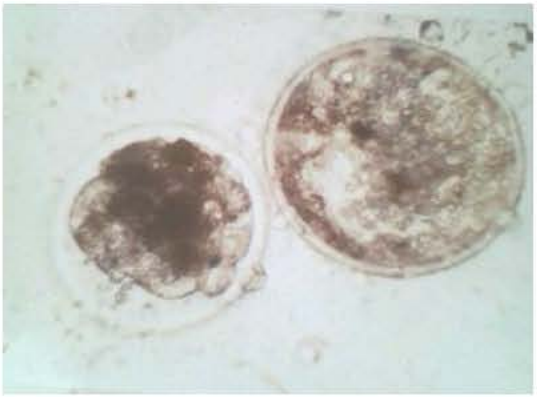

Fig. 2: *In vitro* fertilized buffalo oocytes developed to the morula and the blastocyst stages after insemination with 100mM trehalose treated semen

activity in a dose dependent trend. Addition of 100 mM trehalose to the freezing extender; significantly ($P< 0.05$) increased the total antioxidant, SOD and GSH activity (0.55±0.045, 65.28±4.06 and 110.64±5.39 mμ/ml, respectively) and decreased the Malandiahyde (lipid peroxidation) of the frozen-thawed spermatozoa (9.18±1.46 nmol/ml) as compared with the base extender (0.19±0.02, 26.1±1.40, 62.01±5.74 mμ/ml and 21.57±1.45 nmol/ml, respectively).

Data regarding the effect of replenishing of semen extender with trehalose on the *in vitro* fertilizing potentials and embryo development are presented in Tables 4 and 5. The current results revealed that, addition of 100 mM trehalose to the freezing extender resulted in higher *in vitro* fertilization rate, cleavage rate, morula and blastocyst development (57.25±2.63, 52.37±2.25, 25.97±2.82 and 17.43±1.35, respectively) compared with the control semen (38.41 ±3.48, 32.18±2.99, 12.26±2.13 and 4.78±1.54, respectively) as shown in Fig.(2).

## DISCUSSION

Cryopreservation adversely affects the cryosurvival of spermatozoa, which leads to reduce their fertile life [25, 26]. Irreversible damage during this process reduces motility and fertility of spermatozoa and has been attributed to different reasons [27]. One of the main reasons for reduced quality of spermatozoa is intracellular ice crystallization during cryopreservation [28], which has been minimized when isotonic extenders are supplemented with non-penetrated sugars.

The present study revealed that addition of trehalose to semen extender improve buffalo semen freezability, in a dose dependant manner. These results were in consistent with the findings of other investigators [14, 26, 29, 30], who found that the incorporation of trehalose in semen diluents protects the spermatozoa of many species against cryodamage. The precise mechanism by which trehalose preserve the sperm membrane during cryopreservation remains a subject of debate, nevertheless, for reproductive purposes, it is important that the sperm cell be able to perform, as well as withstand a rapid loss of a large fraction of its intracellular water during freezing. Water plays an important role in the maintenance of the structural and functional integrity of biological membranes. The removal of this water by dehydration or freezing often results in vast structural and functional alteration in the biological membranes. The presence of trehalose may render the membrane less vulnerable to the rapid physical and morphological changes that occur during the rapid efflux of water. Moreover, the cryoprotective effect of trehalose results primarily from the formation of a hydrogen bond between the sugar hydroxyl group and the phospholipid polar head group, thus substituting for the water molecules under cryopreservation or dehydration [31-33]. Therfore, the observed protection of the sperm by trehalose indicates that trehalose probably plays a key role in preventing deleterious alteration to the membrane during reduced water states [7]. It is conceivable that the ability of trehalose itself into bilayer phospholipids could increase membrane fluidity, induce depression in the membrane phase transition temperature of dry lipids and form a glass drying, leading to greater endurance of the spermatozoa against freeze-thawing damage [34-36]. Taken together with this information, trehalose creates an osmotic pressure, inducing cell dehydration and lowers the incidence of intracellular ice formation [4, 6]. Additionally, trehalose may act as a stabilizer and protectant of proteins and cell membranes, whose fluidity decreases during temperature downshift. This may emphasize the current results that indicated that trehalose provision to the freezing extender diminished the enzymes leakage. Cells with membrane damage lose essential metabolites and enzymes. Numerous enzymes such as aspartate-aminotransferase and alkaline phosphatase have been determined in semen of several species [37]. Aspartate-aminotransferase is present in the acrosome of the intact sperm cell and in seminal plasma. Membrane damage to the mid-piece results in release of AST to the seminal plasma. As a result, ATP production is blocked, immobilizing the sperm cell [38]. Moreover, it was speculated that, trehalose prevents the denaturation and aggregation of specific proteins at cold temperatures [39].

Likewise, the current study evidently revealed that trehalose acts as a free radical scavenger and the accumulation of trehalose reduces lipid peroxidation and enhances cellular resistance to the oxygen radicals and to the oxidative damage. These results were in harmony with Hu *et al.* [30] who found that, extender supplemented with trehalose reduced the oxidative stress induced by cryopreservation, protected cells against oxygen radicals and improved measures of bovine semen quality.

Previous studies speculated that, abnormal chromatin structure might impede the survival of bull spermatozoa during freezing and thawing [40] and fertilize a very low percentage of ova *in vitro* or fail to fertilize, even after direct injection of spermatozoa into the ovum [41]. Little is known about the effect of trehalose on the DNA deterioration and sperm cells apoptosis. The present data strongly indicated that trehalose protect sperm DNA from damage and extensively diminish spermatozoal cells apoptosis during cryopreservation. This may clarify a recent mechanism by which trehalose could improve the freezability of buffalo spermatozoa. In conclusion, the results of the present study indicated that, the addition of trehalose to the freezing extender led to reduction of cryodamage to the buffalo spermatozoa in a dose dependent trend. Addition of 100 mM trehalose to the freezing extender led to the reduction of cryodamage, enhanced viability of the buffalo spermatozoa, protected DNA of the sperm cells from deterioration, reduced the oxidative stress provoked by freezing and thawing and enhanced the *in vitro* fertilizing capacity of the cryopreserved buffalo spermatozoa.

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