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Impact of supplementation of semen extender with antioxidants on the quality of chilled or cryopreserved Arabian stallion spermatozoa

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ABSTRACT

The aim of the present study was to evaluate the effects of supplementation of semen extender with various non-enzymatic antioxidants on the quality of cooled or cryopreserved Arabian stallion spermatozoa. Semen collected from four pure Arabian stallions was centrifuged at 600*g* for 15 min. Spermatozoa were then diluted in INRA-82 extender supplemented with bovine serum albumin (BSA; 0, 10, 15 and 20 mg/mL) or trehalose (0, 75, 100 and 150 mM) or zinc sulphate (0, 100, 150 and 200 μM). The diluted semen was then either cooled at 5 °C or cryopreserved in 0.5−ml plastic straws. After cooling or thawing, sperm motility, viability, sperm abnormalities, viability index, and plasma membrane integrity were evaluated. The results showed that supplementation of semen extender with 150 mM trehalose or with 200 μM zinc sulphate significantly ($P < 0.05$) improved motility, viability, sperm membrane integrity and acrosome status in Arabian stallion spermatozoa after cooling or after freezing and thawing compared with controls (non-supplemented media) or with those supplemented with other concentrations of trehalose or zinc sulphate. Supplementation of semen extender with BSA did not improve sperm motility or cryosurvival of Arabian stallion spermatozoa after cooling or after freezing and thawing. In conclusion, supplementation of semen extender with non-enzymatic antioxidants (trehalose or zinc sulphate) improved the quality of chilled and frozen/thawed Arabian stallion spermatozoa. The most beneficial effects occur when semen diluent was supplemented with 150 mM trehalose or 200 μM zinc sulphate.

1. Introduction

Equine breeding has been improved over the past years largely because of advances in the cryopreservation of stallion semen [40,62,63,65]. Cryopreservation of stallion spermatozoa has many advantages; it allows long-term preservation and world-wide distribution of valuable genetic resources as well as it avoids the risk of disease transmission associated with transportation and natural mating [54]. However, compared with other domestic species; the fertility of frozen-thawed stallion sperm remains low [34,35]. Several factors have been reported to influence the cryosurvival of stallion spermatozoa include osmotic stress, ice crystal formation, toxicity of the cryoprotectants and the variability among stallions [54,59]. However, the physiological and biochemical causes related to this variability remain unclear [60].

It is well known that cooled or frozen-thawed spermatozoa experience a various degree of damage include reduction in sperm motility, viability and/or perturbations in membrane integrity and consequence loss of sperm fertilizing ability or even sperm death [71,73]. Among various causes, oxidative stress has been ascribed to affect the fertility and physiology of frozen/thawed spermatozoa [1,9,56,67,70]. Oxidative stress occurs as a consequence of imbalance between the levels of reactive oxygen species (ROS) production and the antioxidant capacity of the cell [37,38]. It has been demonstrated that equine spermatozoa have the ability to produce ROS [12] and this process is believed to involve an NADPH oxidase similar to that occurs in human sperm cells [2]. Although, excessive amounts of ROS are harmful to the sperm cells [39], low level of these molecules are required to induce sperm capacitation in

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human, a process that is required for the spermatozoa to acquire their fertilizing ability [27,57]. The same phenomenon has also been reported in equine spermatozoa [17]. Under oxidative stress, spermatozoa suffer extensive damage such as peroxidation of membrane lipids [69], oxidation of protein [52], DNA fragmentation, oxidation of bases [4,15], low mitochondrial membrane activity [32,44] and inactivation of enzymes associated with motility [25,26]. The negative effect of oxidative stress on equine sperm motility, viability and DNA integrity has been previously reported [16]. Therefore, the presence of antioxidant enzymes is important to circumvent oxidative damage in the spermatozoa [36,68,76].

Spermatozoa are susceptible to attack by oxidative stress due to deficiency in the levels of intracellular antioxidant enzymes as a result of their low amount of cytoplasm. Furthermore, unlike many other cell types, sperm membranes contain high levels of polyunsaturated fatty acids which give the membranes the fluidity enabling them to fuse with the oocytes and achieve fertilization [33,45]. The lipids in the spermatozoa are the main substrates for peroxidation and excess amounts of ROS and free radicals have negative influences on sperm motility and fertilizing ability [3]. Seminal plasma contains a variety of antioxidants that can fight against excess amount of ROS and subsequently protects spermatozoa from oxidative stress [16].

It has been reported that in stallions and in other mammalian species, preparation of the spermatozoa for cryopreservation involves removal of seminal plasma and subsequently removal of the main source of antioxidant protection. Previous studies reported that the antioxidant activity of bovine spermatozoa decreased after cryopreservation as compared with the fresh spermatozoa [18]. Moreover, freezing and thawing of equine spermatozoa has been reported to be associated with elevation in ROS levels [12]. The addition of either enzyme scavengers or non-enzymatic antioxidants during *in vitro* manipulation of spermatozoa has been reported as potent protectors against oxidative stress [16]. The aim of present study was to evaluate the effect of supplementation of semen extender with antioxidants; namely bovine serum albumin (BSA), trehalose or zinc sulphate on the quality of cooled or frozen-thawed spermatozoa of Arabian stallions.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated.

2.1. Semen collection and processing

Semen was collected from four Pure Arabian Stallions individually housed at Al-Zahraa horse stud, Cairo, Egypt. All stallions were of proven paternity and their age ranged from 10 to 12 years. The stallions were maintained according to the institutional and Ministry of Agriculture, Egyptian Government regulations with semen being collected on a regular basis (one collection per week) during the period between August 2015 to October 2015. Semen was collected using a Missouri model artificial vagina (All Vet. Supply Inc., USA) lubricated and pre-warmed to 45–50 °C and fitted with an inline filter to separate the gel fraction. In order to reduce the extra-gonadal sperm reserves; semen was collected over a period of five consecutive days before the start of the experiments. After that, ten ejaculates were collected from each stallion over a period of ten consecutive weeks. Semen collection was performed early in the morning and a mare in estrus was used as a mount animal. Immediately after collection, ejaculates were transported to the laboratory and kept at 37 °C for 20 min until processing. Sperm motility, concentration, viability and sperm morphology were evaluated. Only ejaculates with motility higher than 60% and more than 250×10^6 sperm cells/mL were used in the experiments. The filtered ejaculate was diluted 1:1 (v/v) with INRA-82 extender and then centrifuged at 600*g* for 15 min. After centrifugation, the supernatant was discarded and the pellet was re-suspended in the freezing medium (INRA-82 contained 15% egg yolk plus 5% glycerol) to a final concentration of 100×10^6 sperm cells/mL. The freezing extender was supplemented with- or without-various antioxidants at different concentrations according to the experimental design. For sperm cooling, the cryotubes filled with diluted samples were kept in refrigerator at 5 °C for 90 min. For cryopreservation, diluted spermatozoa were equilibrated at 5 °C for 15 min and then loaded in 0.5–ml plastic straws. After sealing the straws with polyvinyl powder, they were placed 4 cm over liquid nitrogen (LN_2) vapor for 10 min and then directly plunged into LN_2 for storage [23]. After at least one week storage, thawing was done by transferring the frozen straws into water bath at 37 °C for 30 s for analysis.

2.2. Experimental design

To evaluate the effects of supplementation of semen extender with different antioxidants on the quality of chilled or cryopreserved spermatozoa, each ejaculate was divided into 10 aliquots and each aliquot was diluted with cryopreservation medium as described above. The semen extender was supplemented with either BSA (0, 10, 15 and 20 mg/ml) or trehalose (0, 75, 100 and 150 mM) or zinc (Zn; 0, 100, 150 and 200 μM). Sperm motility, viability, morphology, sperm membrane integrity and acrosome status were evaluated at 1, 2 and 3 h post cooling and post thawing for estimation of viability index.

2.3. Evaluation of sperm motility

A small aliquot of diluted cooled or cryopreserved spermatozoa treated with different antioxidants was applied onto a pre-warmed glass slide for motility evaluation under a microscope. Motile and immotile sperm (200 in total) were counted from the recorded results and the percentage of motile population was calculated.

2.4. Determination of sperm viability and sperm abnormalities

Sperm viability and sperm morphological evaluation were conducted after eosin–nigrosin staining according to the method described previously [29]. Slides were examined under a microscope and live spermatozoa remained unstained indicating intact plasma membrane, however, dead sperm cells absorbed the stain indicating disrupted sperm plasma membranes. Two-hundred spermatozoa were counted under a microscope and the percentage of live spermatozoa was calculated as well as viability index for each sample was estimated as described before [51].

2.5. Determination of acrosome status

The integrity of the acrosome was detected in spermatozoa after cooling and thawing by using Spermac (FertiPro N.V., Beernem, Belgium) stain according to the method described previously [22]. Briefly, an air-dried sperm smear prepared from each sample after cooling and after freezing and thawing was fixed in a solution composed of 10% formalin for 10 min. Each slide was then processed through stain solutions A, B and C for 1 min at room temperature. After air drying the slides were analyzed under oil immersion (x 1000). Two-hundred sperm cells were counted and the percentage of sperm cells with intact acrosome characterized by normal oval-shaped head with dark green anterior acrosome regions and red-pink postacrosomal areas. Sperm heads with shed membranes or

with partially green were considered abnormal acrosome.

2.6. Hypo-osmotic swelling (HOS) assay

The HOS assay in equine sperm is based on the observation that intact sperm membranes swell upon incubation of the gametes in a hypo-osmotic buffer and, as the membranes do not return to their original state, this results in the curling of the tail towards the head [55]. Following sperm cooling or thawing, 100 μl of sperm aliquot were re-suspended in 1 mL of pre-warmed 100 mOsm (1.712 gm sucrose dissolved in 50 mL of sterile deionized water). The mixture was incubated at 37 °C for 60 min. A small drop was then placed on a microscope slide and covered with a cover slip. The slides were examined under phase contrast microscope at ×400. One hundred cells were observed for evidence of plasma membrane swelling (tail coiling). The percentage of spermatozoa with tail coiling (HOS+) was recorded for each sample.

2.7. Statistical analysis

Forty ejaculates were used in each experimental group and each experiment was repeated four times. Data were normalized using arcsine transformation and were presented as mean ± SEM. Data were analyzed by using one-way ANOVA followed by Tukey's multiple comparisons test using the SPSS package version 22 software (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of supplementation of semen extender with BSA on the quality of chilled Arabian stallion spermatozoa

The effect of supplementation of semen extender with different concentrations of BSA (10, 15 and 20 mg/mL) on sperm motility, viability, sperm abnormalities and viability index of cooled Arabian stallion spermatozoa was presented in Table 1. No significant differences were observed in sperm motility, percentages of abnormal spermatozoa and viability indices in the spermatozoa cooled in a medium supplemented with 10 mg/mL BSA and the control group (spermatozoa cooled in a medium without BSA supplement). The percentage of live spermatozoa was significantly higher ($P < 0.05$) in 10 mg/mL BSA supplemented group compared with the control and with 15 and 20 mg/mL supplemented groups. Supplementation of semen extender with 15 or 20 mg/mL BSA significantly reduced ($P < 0.05$) sperm motility as compared with the controls. These results indicate that supplementation of semen extender with BSA has no beneficial effects on motility of cooled stallion spermatozoa.

3.2. Effect of supplementation of semen extender with BSA on the quality of frozen/thawed Arabian stallion spermatozoa

Supplementation of semen extender with 10 mg/mL BSA significantly improved ($P < 0.05$) sperm motility, membrane integrity (HOS+) and viability indices in frozen/thawed spermatozoa compared with the controls (without BSA supplement) and with 15 and 20 mg/mL BSA supplemented medium (Table 2). No significant differences were observed in sperm viability between 10 mg/mL BSA supplemented group and control ones. Supplementation of semen extender with 15 or 20 mg/mL BSA significantly reduced ($P < 0.05$) the proportions of live spermatozoa as compared to those supplemented with 10 mg/mL and with the control group. These results indicate that supplementation of semen extender with higher concentrations of BSA (15 or 20 mg/mL) were detrimental to the sperm viability after freezing and thawing. No significant effect of BSA supplementation on sperm abnormalities and acrosome integrity as compared with the controls.

3.3. Effects of supplementation of semen extender with trehalose on the quality of cooled Arabian stallion spermatozoa

The quality of Arabian stallion spermatozoa after cooling in media supplemented with different concentrations of trehalose (75, 100 or 150 mM) was evaluated and summarized in Table 3. Supplementation of semen extender with 150 mM trehalsoe significantly ($P < 0.05$) increased motility, percentage of live spermatozoa and viability index of cooled spermatozoa as compared with the controls or with 75 or 150 mM trehalose supplementation. The percentage of abnormal spermatozoa was significantly lower ($P < 0.05$) in 150 mM trehalose supplemented group than in other groups.

3.4. Effects of supplementation of semen extender with trehalose on the quality of Arabian stallion spermatozoa after freezing and thawing

The effects of supplementation of semen diluent with trehalose (75, 100 or 150 mM) on different functions of frozen/thawed Arabian stallion spermatozoa were evaluated and presented in Table 4. The results showed that sperm motility, percentage of live spermatozoa, sperm membrane integrity (percentage of HOS + spermatozoa) and viability indices were significantly higher ($P < 0.05$) in 150 mM trehalose supplemented group as compared with the controls and with the other trehalose supplemented groups (75 and 100 mM). The percentages of sperm with reacted acrosome did not significantly differ among the four groups and the values ranged from 76.7% to 79.9%.

3.5. Effects of supplementation of semen diluent with zinc (Zn) on the quality of cooled Arabian stallion spermatozoa

Supplementation of semen extender with 200 μM zinc significantly ($P < 0.05$) increased sperm motility, percentage of live spermatozoa and viability index in chilled semen as compared with the controls (cooled in zinc free medium) and with 100 and 150 μM zinc supplemented groups (Table 5). Sperm abnormalities were significantly higher in the control and in 100 μM Zn supplemented groups than that in 150 and 200 μM Zn supplemented groups.

Table 1
Effects of supplementation of semen extender with bovine serum albumin (BSA) on quality of cooled Arabian stallion spermatozoa.

BSA (mg/ml)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	Viability index (mean ± SEM)
0	62.6 ± 0.7[a]	78.9 ± 0.3[b]	21.5 ± 0.5[ab]	177.1 ± 3.1[ab]
10	63.6 ± 0.7[a]	81.8 ± 0.5[a]	20.0 ± 0.3[b]	184.3 ± 3.3[a]
15	59.0 ± 0.9 [b]	77.0 ± 0.4[b]	22.4 ± 0.5[a]	172.1 ± 4.0[b]
20	57.3 ± 0.8[b]	79.3 ± 0.5[b]	22.8 ± 0.5[a]	169.6 ± 3.9[b]

Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 2
Effect of supplementation of semen extender with bovine serum albumin (BSA) on the quality of frozen/thawed Arabian stallion spermatozoa.

BSA (mg/ml)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	HOS+ (mean % ± SEM)	Intact acrosome (mean % ± SEM)	Viability index (mean ± SEM)
0	48.6 ± 0.9[b]	73.5 ± 0.6[a]	26.4 ± 0.5[a]	74.7 ± 0.5[b]	77.7 ± 0.5[a]	136.2 ± 2.9[b]
10	52.4 ± 1.2[a]	75.1 ± 0.7[a]	22.9 ± 0.5[a]	78.8 ± 0.6[a]	78.9 ± 0.5[a]	149.5 ± 3.4[a]
15	46.5 ± 1.1[b]	71.6 ± 0.7[b]	26.6 ± 0.7[a]	73.2 ± 0.5[b]	79.2 ± 0.5[a]	128.8 ± 3.7[b]
20	47.0 ± 1.2[b]	73.2 ± 0.6[b]	25.5 ± 0.6[a]	72.9 ± 0.6[b]	79.5 ± 0.6[a]	128.7 ± 3.7[b]

HOS + means the percentages of spermatozoa with tail coiling in hypo-osmotic swelling (HOS) assay.
Values with dissimilar superscripts in the same column are significantly different at $P < 0.05$.

Table 3
Effects of supplementation of semen extender with trehalose on quality of cooled Arabian stallion spermatozoa.

Trehalose (mM)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	Viability index (mean ± SEM)
0	61.6 ± 0.7[c]	77.9 ± 0.3[a]	20.6 ± 0.5[a]	176.1 ± 3.1[a]
75	58.4 ± 0.7[a]	78.8 ± 0.5[a]	20.9 ± 0.4[a]	169.4 ± 2.9[a]
100	59.4 ± 0.7[a]	79.2 ± 0.4[a]	20.3 ± 0.5[a]	173.5 ± 2.9[a]
150	65.8 ± 0.8[b]	82.0 ± 0.4[b]	17.4 ± 0.3[b]	200.4 ± 3.1[b]

Values with dissimilar superscripts in the same column are significantly different at $P < 0.05$.

Table 4
Effect of supplementation of semen extender with trehalose on the quality of frozen/thawed Arabian stallion spermatozoa.

Trehalose (mM)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	HOS+ (mean % ± SEM)	Intact acrosome (mean % ± SEM)	Viability index (mean ± SEM)
0	47.6 ± 0.9[b]	72.5 ± 0.6[a]	25.4 ± 0.5[a]	73.7 ± 0.5[a]	76.7 ± 0.5[a]	137.2 ± 2.9[b]
75	44.8 ± 0.9[a]	73.3 ± 0.6[a]	25.5 ± 0.6[a]	73.5 ± 0.5[a]	77.1 ± 0.5[a]	130.5 ± 2.8[a]
100	48.8 ± 0.8[b]	75.2 ± 0.5[b]	23.9 ± 0.5[b]	75.2 ± 0.5[b]	77.9 ± 0.5[a]	140.4 ± 2.7[b]
150	56.5 ± 0.9[c]	77.8 ± 0.4[c]	21.5 ± 0.5[c]	79.0 ± 0.5[c]	79.9 ± 0.4[a]	165.0 ± 3.4[c]

HOS + means the percentages of spermatozoa with tail coiling in hypo-osmotic swelling (HOS) assay.
Values with dissimilar superscripts in the same column are significantly different at $P < 0.05$.

Table 5
Effects of supplementation of semen extender with zinc on the quality of cooled Arabian stallion spermatozoa.

Zinc (μM)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	Viability index (mean ± SEM)
0	61.6 ± 0.7[b]	77.9 ± 0.3[a]	20.6 ± 0.5[a]	176.1 ± 3.1[a]
100	58.4 ± 0.6[a]	78.6 ± 0.4[a]	20.6 ± 0.5[a]	166.0 ± 2.7[a]
150	60.9 ± 0.6[b]	80.0 ± 0.4[b]	18.9 ± 0.4[b]	177.6 ± 2.9[a]
200	67.9 ± 0.8[c]	82.7 ± 0.4[c]	18.0 ± 0.3[b]	208.3 ± 2.8[b]

Values with dissimilar superscripts in the same column are significantly different at $P < 0.05$.

3.6. Effects of zinc (Zn) supplementation to semen extender on the quality of frozen/thawed Arabian stallion spermatozoa

Sperm motility, percentage of live spermatozoa, proportions of HOS + spermatozoa, acrosome reaction and viability index were significantly ($P < 0.05$) higher in frozen/thawed spermatozoa that was diluted in a medium supplemented with Zn at 200 μM concentration than those in controls (medium without any Zn supplementation) or 100 and 150 μM Zn supplemented groups. The percentage of abnormal spermatozoa was significantly ($P < 0.05$) lower in 150 μM Zn supplemented group compared with those in the controls and in 100 μM supplemented ones. These values were further decreased by supplementation of semen diluent with 200 μM Zn (Table 6).

4. Discussion

Cryopreservation of spermatozoa has potential in preserving genetic materials in both humans and domestic animals [11]. It is well documented that sperm experience various degrees of damage during freezing and thawing include perturbations to the sperm organelles and changes in membrane fluidity and enzymatic

Table 6
Effects of supplementation of semen extender with zinc on different functions of Arabian stallion spermatozoa after freezing and thawing.

Zinc (μM)	Motility (mean % ± SEM)	Live sperm (mean % ± SEM)	Sperm abnormalities (mean % ± SEM)	HOS+ (mean % ± SEM)	Intact acrosome (mean % ± SEM)	Viability index (mean ± SEM)
0	47.6 ± 0.9[a]	72.5 ± 0.6[a]	25.4 ± 0.5[a]	73.7 ± 0.5[a]	76.7 ± 0.5[a]	137.2 ± 2.9[a]
100	46.1 ± 1.0[a]	73.9 ± 0.7[a]	25.2 ± 0.6[a]	75.8 ± 0.4[ab]	78.2 ± 0.5[a]	132.0 ± 2.4[a]
150	51.9 ± 0.8[b]	76.8 ± 0.4[b]	23.4 ± 0.5[b]	77.5 ± 0.4[b]	78.1 ± 0.5[a]	148.2 ± 3.1[b]
200	58.3 ± 0.9[c]	79.1 ± 0.4[c]	21.0 ± 0.5[c]	81.0 ± 0.5[c]	79.7 ± 0.4[b]	173.0 ± 3.2[c]

HOS + means the percentages of spermatozoa with tail coiling in hypo-osmotic swelling (HOS) assay.
Values with dissimilar superscripts in the same column are significantly different at $P < 0.05$.

activity that subsequently resulted in a reduction in sperm motility, viability and fertilizing ability [7]. Two main factors namely; formation of intracellular ice crystals and osmotic stress have been described to be responsible for the cell death and subcellular damage that occur during freezing and thawing [18,59,73]. However, it has been indicated that the intracellular ice crystals is unlikely to occur at the freezing rates currently applied in sperm cryopreservation, with most damage being mainly due to osmotic stress [53]. Sperm cryopreservation is also associated with an increase in ROS production and a decrease in the antioxidant levels, which in turn increases the susceptibility of the frozen/thawed spermatozoa to the lipid peroxidation (LPO; [5]). Furthermore, LPO and apoptosis-like mechanisms have been reported to be linked with premature aging and DNA fragmentation in cryopreserved equine spermatozoa [54,58]. Oxidative damage is then well recognized as a major factor in sperm cryodamage and therefore, justifies the use of antioxidants to improve the outcomes of sperm cryopreservation is an important procedure. In the present study we evaluated the impact of supplementation of semen extender with different concentrations of BSA, trehalose or zinc on motility, viability, morphology, membrane integrity, and acrosome status of cooled or frozen-thawed spermatozoa of Arabian stallions.

Our results show that supplementation of semen extender with BSA has no beneficial effects on sperm motility of Arabian stallion spermatozoa especially after cooling. However, it does improve (at 10 mg/mL) motility and plasma membrane integrity in frozen/thawed spermatozoa (Table 2). Beneficial effects of supplementation of semen extender with BSA on the quality of frozen/thawed spermatozoa have been previously reported in different species include equine [28,43,47], bovine [72,74], ovine [31,50,74] and caprine [8,75]. The axosome and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. It is well established that ROS can induce axonemal and mitochondrial damage, resulting in the immobilization of sperm [2,61]. Disruption of membrane integrity causes a raise in membrane permeability and decreases the ability of the spermatozoon to control the intracellular concentrations of ions involved in the sperm motion [16]. The present results suggest that supplementation of semen extender with BSA at a concentration of 10 mg/mL enhances some of the functions such as motility and membrane integrity of frozen/thawed Arabian stallion spermatozoa; although, it did not improve sperm viability. The results also show that supplementation of semen extender with BSA at concentrations higher than 10 mg/mL were detrimental to sperm viability. Extreme doses of antioxidants in the freezing medium can counteract the oxidative stress and it can also affect the normal sperm functions connected with the ROS. Therefore, the effective concentration of BSA must be used in the freezing extender.

Various mechanisms have been speculated to explain the positive impact of BSA on the quality of frozen/thawed spermatozoa. First, BSA has the ability to adhere to the sperm plasma membranes leads to modifications in the lipid composition of the membranes and subsequently changes in the membrane fluidity through an increase in the cholesterol efflux [19,24]. BSA was also reported to have an antioxidant effect through its ability to scavenge the free radicals [13].

Trehalose, a non-permeating disaccharide, acts as non-enzymatic scavenger and plays an important role in the protection of the spermatozoa against ROS [66]. Trehalose induces its protective effects against oxidative stress through osmotic effect and specific interactions with phospholipids in the sperm plasma membranes, rendering hypertonic media, causing cellular osmotic dehydration before freezing and then decreasing the amount of cell injury by its crystallization [20]. In the present study we demonstrated dose dependent effects of trehalose supplementation to semen extender on the quality of chilled and frozen/thawed Arabian stallion spermatozoa with the highest percentages of sperm motility and viability occurred at 150 mM trehalose supplementation (Tables 3 and 4). Similar beneficial effects of trehalose on the quality of cryopreserved spermatozoa have been reported in different species include buffalo bulls [66], rams [7,50], boars [41] and rabbits [20]. Contrary to our results, previous studies showed no beneficial effects of trehalose supplementation to semen extender on the quality of chilled stallion spermatozoa [10]. The differences between the results may be ascribed to the individual variations in the stallion spermatozoa, the breeds, and the type of the diluent used in each study.

It is well known that zinc plays an imperative role in the process of sperm production and differentiation [64]. Furthermore, previous studies reported the role of zinc in protecting spermatozoa against oxidative stress and subsequent improving male fertility [14,30]. In the present study we demonstrated that supplementation of semen diluent (INRA-82) with 200 μM zinc sulphate significantly improved motility, viability, plasma membrane integrity and acrosome status of Arabian stallion spermatozoa after cooling at 5 °C or after freezing and thawing as compared with the controls and with those supplemented with 100 and 150 μM zinc sulphate (Tables 5 and 6). Consistent with our results, the positive impact of zinc sulphate on the quality of the spermatozoa have been previously reported in stallions [42], buffalo bulls [6], goats [48] and humans [49]. Various mechanisms have explored the positive role of zinc on the quality of the cryopreserved spermatozoa for example, zinc as an antioxidant and ROS scavenger can protect the spermatozoa against the delirious effects of LPO maintaining the integrity of the plasma membrane [21]. Moreover, it has been reported that zinc could protect the membrane potential of mitochondria in cryopreserved spermatozoa which is important to maintain the energy that requires for sperm motility [46].

In conclusion, supplementation of semen extender with non-enzymatic antioxidants (Trehalose or Zinc) improved the quality of chilled and frozen/thawed Arabian stallion spermatozoa. The results demonstrated that both type and concentration of the antioxidant influence the quality of the spermatozoa after cooling and after cryopreservation and the most beneficial effects were observed when semen extender; INRA-82, was supplemented with 150 mM trehalose or 200 μM zinc sulphate. BSA supplementation during freezing and thawing seems to be less beneficial on the cryosurvival of Arabian stallion spermatozoa. This study highlights the positive role of antioxidants in improving different functions of Arabian stallion spermatozoa after cooling or after cryopreservation that could correlate with sperm fertilizing ability.

Declaration of interest

Authors of this study have no conflict of interest.

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References

[1] A. Agarwal, K. Makker, R. Sharma, Clinical relevance of oxidative stress in male factor infertility: an update, Am. J. Reproductive Immunol. 59 (2008) 2–11.
[2] R.J. Aitken, J.S. Clarkson, Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa, J. Reprod. Fertil. 81 (1987) 459–469.

[3] R.J. Aitken, J.S. Clarkson, S. Fishel, Generation of reactive oxygen species, lipid peroxidation, and human sperm function, Biol. Reprod. 41 (1989) 183–197.
[4] R.J. Aitken, E. Gordon, D. Harkiss, J.P. Twigg, P. Milne, Z. Jennings, D.S. rvine, Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa, BioloReprod 59 (1998) 1037–1046.
[5] R.J. Aitken, C. Krausz, Oxidative stress, DNA damage and the Y chromosome, Reproduction 122 (2001) 497–506.
[6] S.M. Alavi-Shoushtari, S.A. Rezai, M.H. Ansari, A. Khaki, Effects of the seminal plasma zinc content and catalase activity on the semen quality of water buffalo (Bubalus bubalis) bulls, Pak J. Biol. Sci. 12 (2009) 134–139.
[7] J.G. Alvarez, B.T. Storey, Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility, Biol. Reprod. 29 (1983) 548–555.
[8] F. Amidi, A. Farshad, A.K. Khor, Effects of cholesterol-loaded cyclodextrin during freezing step of cryopreservation with TCGY extender containing bovine serum albumin on quality of goat spermatozoa, Cryobiology 61 (2010) 94–99.
[9] J.B. Anderson, R.C. Williamson, Testicular torsion in Bristol: a 25-year review, Br.J Surg. 75 (1988) 988–992.
[10] P.B. Arifiantini R.I., T.L. Yusufa, D. Sajuthib, The quality of stallion semen in skim milk and dimitropoulos extenders preserved at 5°C and ambient temperature supplemented with different sugar, Media Peternakan 36 (2013) 45–51.
[11] E. Axner, U. Hermansson, C. Linde-Forsberg, The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa, Anim. Reprod. Sci. 84 (2004) 179–191.
[12] B.A. Ball, A.T. Vo, J. Baumber, Generation of reactive oxygen species by equine spermatozoa, Am. J. Vet. Res. 62 (2001) 508–515.
[13] A.K. Bansal, G.S. Bilaspuri, Impacts of oxidative stress and antioxidants on semen functions, Vet. Med. Int. 2010 (2010) pii: 686137.
[14] V. Barbato, R. Talevi, S. Braun, A. Merolla, S. Sudhakaran, S. Longobardi, R. Gualtieri, Supplementation of sperm media with zinc, D-aspartate and coenzyme Q10 protects bull sperm against exogenous oxidative stress and improves their ability to support embryo development, Zygote 25 (2017) 168–175.
[15] G. Barroso, M. Morshedi, S. Oehninger, Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa, HumReprod 15 (2000) 1338–1344.
[16] J. Baumber, B.A. Ball, J.J. Linfor, Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants, Am. J. Vet. Res. 66 (2005) 772–779.
[17] J. Baumber, B.A. Ball, J.J. Linfor, S.A. Meyers, Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa, J. Androl. 24 (2003) 621–628.
[18] J.F. Bilodeau, S. Chatterjee, M.A. Sirard, C. Gagnon, Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing, Mol. Reprod. Dev. 55 (2000) 282–288.
[19] M. Blank, L. Soo, J.S. Britten, Adsorption of albumin on rabbit sperm membranes, J. Membr. Biol. 29 (1976) 401–409.
[20] M.N. Bucak, A. Atessahin, O. Varisli, A. Yuce, N. Tekin, A. Akcay, The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process, Theriogenology 67 (2007) 1060–1067.
[21] H.I. Calvin, Electrophoretic evidence for the identity of the major zinc-binding polypeptides in the rat sperm tail, Biol. Reprod. 21 (1979) 873–882.
[22] P.J. Chan, J.U. Corselli, J.D. Jacobson, W.C. Patton, A. King, Spermac stain analysis of human sperm acrosomes, Fertil. Steril. 72 (1999) 124–128.
[23] M.J.A. Cristanelli, R.P. Squires, E.L., B.W. Pickett, Effects of egg yolk and glycerol level in lactose–EDTA–egg yolk extender and of freezing rate on the motility of frozen–thawed stallion spermatozoa, Theriogenology 23 (1985) 14.
[24] B.K. Davis, R. Byrne, K. Bedigian, Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during in vitro incubation of rat sperm cells, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 1546–1550.
[25] E. de Lamirande, C. Gagnon, Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes, J. Androl. 13 (1992) 368–378.
[26] E. de Lamirande, C. Gagnon, Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility, J. Androl. 13 (1992) 379–386.
[27] E. de Lamirande, C. Gagnon, Capacitation-associated production of superoxide anion by human spermatozoa, Free Radic. Biol. Med. 18 (1995) 487–495.
[28] K.E. Dixon, J.L. Kreider, Effect of calcium and fatty acids on the isolation of stallion spermatozoa in BSA, Theriogenology 15 (1981) 335–344.
[29] M.W. Evans G, Salamon 's Artificial Insemination of Sheep and Goats, Butterworths, Sydney. Australia, 1987.
[30] C. Foresta, A. Garolla, I. Cosci, M. Menegazzo, M. Ferigo, V. Gandin, L. De Toni, Role of zinc trafficking in male fertility: from germ to sperm, Hum. Reprod. 29 (2014) 1134–1145.
[31] Y. Fukui, H. Kohno, T. Togari, M. Hiwasa, Fertility of ewes inseminated intrauterinally with frozen semen using extender containing bovine serum albumin, J. Reprod. Dev. 53 (2007) 959–962.
[32] F. Gallon, C. Marchetti, N. Jouy, P. Marchetti, The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability, Fertil. Steril. 86 (2006) 1526–1530.
[33] P. Gharagozloo, A. Gutierrez-Adan, A. Champroux, A. Noblanc, A. Kocer, A. Calle, S. Perez-Cerezales, E. Pericuesta, A. Polhemus, A. Moazamian, J.R. Drevet, R.J. Aitken, A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models, Hum. Reprod. 31 (2016) 252–262.
[34] Z. Gibb, T.J. Butler, L.H. Morris, W.M. Maxwell, C.G. Grupen, Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and non-sorted stallion sperm, Theriogenology 79 (2013) 1001–1009.
[35] Z. Gibb, C.G. Grupen, W.M. Maxwell, L.H. Morris, Field fertility of liquid stored and cryopreserved flow cytometrically sex-sorted stallion sperm, Equine Vet. J. 49 (2017) 160–166.
[36] S. Gong, M. San Gabriel, A. Zini, P. Chan, C. O'Flaherty, Low Amounts and High Thiol Oxidation of peroxiredoxins in spermatozoa from infertile men, J. Androl. 33 (2012) 1342–1351.
[37] B. Halliwell, Oxidative stress and neurodegeneration: where are we now? J. Neurochem. 97 (2006) 1634–1658.
[38] B. Halliwell, J. Gutteridge, The chemistry of free radicals and related reactive species, in: B. Halliwell, J. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press, New York, 2007, pp. 30–74.
[39] B. Halliwell, J. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, New York, 2007.
[40] N. Hoffmann, H. Oldenhof, C. Morandini, K. Rohn, H. Sieme, Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing, Anim. Reprod. Sci. 125 (2011) 112–118.
[41] J.H. Hu, Q.W. Li, Z.L. Jiang, H. Yang, S.S. Zhang, H.W. Zhao, The cryoprotective effect of trehalose supplementation on boar spermatozoa quality, Reprod. Domest. Anim. 44 (2009) 571–575.
[42] T.A.A. Khalifa, M.M. Waheed, A.G. Lymberopoulos, An endeavor to improve longevity of cryopreserved equine sperm, J. Hell. Vet. Med. Soc. 57 (2006) 195–204.
[43] M.E. Klem Jr., J.L. Kreider, J.B. Pruitt, G.D. Potter, Motility and fertility of equine spermatozoa extended in bovine serum albumin and sucrose, Theriogenology 26 (1986) 569–576.
[44] A.J. Koppers, G.N. De Iuliis, J.M. Finnie, E.A. McLaughlin, R.J. Aitken, Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa, J. Clin. Endocrinol. Metabolism 93 (2008) 3199–3207.
[45] A.J. Koppers, M.L. Garg, R.J. Aitken, Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa, Free Radic. Biol. Med. 48 (2010) 112–119.
[46] A.P. Kotdawala, S. Kumar, S.R. Salian, P. Thankachan, K. Govindraj, P. Kumar, G. Kalthur, S.K. Adiga, Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function, J. Assist. Reprod. Genet. 29 (2012) 1447–1453.
[47] J.L. Kreider, W.C. Tindall, G.D. Potter, Inclusion of bovine serum albumin in semen extenders to enhance maintenance of stallion sperm viability, Theriogenology 23 (1985) 399–408.
[48] P.Y. Kumar, B. Yadav, S, Effect of zinc and selenium supplementation on semen quality of barbari bucks, Indian J. Anim. Res. 48 (2014) 366–369.
[49] U. Kvist, L. Bjorndahl, Zinc preserves an inherent capacity for human sperm chromatin decondensation, Acta Physiol. Scand. 124 (1985) 195–200.
[50] T. Matsuoka, H. Imai, H. Kohno, Y. Fukui, Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa, J. Reprod. Dev. 52 (2006) 675–683.
[51] V.K. Milovanov, Biology of Reproduction and Artificial Insemination of Farm Animals, Seljhozizdat, Moscow, 1962.
[52] T. Morielli, C. O'Flaherty, Oxidative stress impairs function and increases redox protein modifications in human spermatozoa, Reproduction 149 (2015) 113–123.
[53] G.J. Morris, K. Faszer, J.E. Green, D. Draper, B.W. Grout, F. Fonseca, Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation, Theriogenology 68 (2007) 804–812.
[54] D.M. Neild, B.M. Gadella, M.G. Chaves, M.H. Miragaya, B. Colenbrander, A. Aguero, Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation, Theriogenology 59 (2003) 1693–1705.
[55] G.J. Nie, J.G. Wenzel, Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes, Theriogenology 55 (2001) 1005–1018.
[56] C. O'Flaherty, Iatrogenic genetic damage of spermatozoa, in: E. Baldi, M. Muratori (Eds.), Genetic Damage in Human Spermatozoa, Springer, New York, 2014, pp. 117–135.
[57] C. O'Flaherty, N. Beorlegui, M.T. Beconi, Participation of superoxide anion in the capacitation of cryopreserved bovine sperm, Int. J. Androl. 26 (2003) 109–114.
[58] C. Ortega-Ferrusola, Y. Sotillo-Galan, E. Varela-Fernandez, J.M. Gallardo-Bolanos, A. Muriel, L. Gonzalez-Fernandez, J.A. Tapia, F.J. Pena, Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm, J. Androl. 29 (2008) 213–221.
[59] C. Ortega Ferrusola, L. Gonzalez Fernandez, B. Macias Garcia, C. Salazar-Sandoval, A. Morillo Rodriguez, H. Rodriguez Martinez, J.A. Tapia, F.J. Pena, Effect of cryopreservation on nitric oxide production by stallion spermatozoa, Biol. Reprod. 81 (2009) 1106–1111.
[60] C. Ortega Ferrusola, L. Gonzalez Fernandez, J.M. Morrell, C. Salazar Sandoval, B. Macias Garcia, H. Rodriguez-Martinez, J.A. Tapia, F.J. Pena, Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes,

Reproduction 138 (2009) 55–63.
[61] S.I. Peris, J.F. Bilodeau, M. Dufour, J.L. Bailey, Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm, Mol. Reprod. Dev. 74 (2007) 878–892.
[62] E. Pillet, G. Duchamp, F. Batellier, V. Beaumal, M. Anton, S. Desherces, E. Schmitt, M. Magistrini, Egg yolk plasma can replace egg yolk in stallion freezing extenders, Theriogenology 75 (2011) 105–114.
[63] T. Pojprasath, C. Lohachit, M. Techakumphu, T. Stout, T. Tharasanit, Improved cryopreservability of stallion sperm using a sorbitol-based freezing extender, Theriogenology 75 (2011) 1742–1749.
[64] M. Saaranen, U. Suistomaa, M. Kantola, S. Saarikoski, T. Vanha-Perttula, Lead, magnesium, selenium and zinc in human seminal fluid: comparison with semen parameters and fertility, Hum. Reprod. 2 (1987) 475–479.
[65] J.C. Samper, T. Plough, Techniques for the insemination of low doses of stallion sperm, Reprod. Domest. Anim. 45 (Suppl 2) (2010) 35–39.
[66] N. Shiva Shankar Reddy, G. Jagan Mohanarao, S.K. Atreja, Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (Bubalus bubalis) sperm quality following cryopreservation, Anim. Reprod. Sci. 119 (2010) 183–190.
[67] R. Smith, H. Kaune, D. Parodi, M. Madariaga, R. Rios, I. Morales, A. Castro, Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress, Hum. Reprod. 21 (2006) 986–993.
[68] R. Smith, D. Vantman, J. Ponce, J. Escobar, E. Lissi, Andrology: total antioxidant capacity of human seminal plasma, Hum. Reprod. 11 (1996) 1655–1660.
[69] B.T. Storey, Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa, Mol. Hum. Reprod. 3 (1997) 203–213.
[70] T.T. Turner, The study of varicocele through the use of animal models, Hum. Reprod. Update 7 (2001) 78–84.
[71] O. Uysal, M.N. Bucak, Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen, ACTA Vet. BRNO 76 (2007) 383–390.
[72] O. Uysal, M.N. Bucak, I. Yavas, O. Varisli, Effect of various antioxidants on the quality of frozen-thawed bull semen, J. Animal Veterinary Adv. 6 (2007) 5.
[73] P.F. Watson, The causes of reduced fertility with cryopreserved semen, Anim. Reprod. Sci. 60–61 (2000) 481–492.
[74] C.A. Wolfe, P.S. James, A.P. Gunning, S. Ladha, Y. Christova, R. Jones, Lipid dynamics in the plasma membrane of ram and bull spermatozoa after washing and exposure to macromolecules BSA and PVP, Mol. Reprod. Dev. 59 (2001) 306–313.
[75] H. Yamashiro, K. Kumamoto, H. Wang, Y. Yamashita, T. Terada, Effect of semen collection in extender solution on the characteristics of goat spermatozoa, J. Reprod. Dev. 52 (2006) 397–406.
[76] A. Zini, M.A. Fischer, V. Mak, D. Phang, K. Jarvi, Catalase-like and superoxide dismutase-like activities in human seminal plasma, Urol. Res. 30 (2002) 321–323.