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An Endeavor to Improve Longevity of Cryopreserved Equine Sperm

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Abstract: Exposure of sperm cells to the oxidative stress pending hypothermic storage of semen has been suggested to be responsible, in part, for the decline of their motility and fertility. This study was conducted to evaluate the *in-vitro* effects of antioxidants (AOs) and/or caffeine on longevity of cryopreserved stallion spermatozoa. Aliquots from the gel-free fraction of semen ejaculates (n=12) collected from 5 Arabian stallions (9-18 years old) of unknown sperm freezability, were mixed 1:1 with a Tris-egg yolk extender (TEYE), centrifuged at 500 x g for 5 min and sperm cells were frozen in the form of 0.25 ml concentrated pellets after 2-step addition of TEYE supplemented with or without AOs (0.50 mg ml^{-1} Na pyruvate, 1 mg ml^{-1} Na thiosulfate, 5 mg ml^{-1} bovine serum albumin, 0.15 mg ml^{-1} zinc chloride and 0.50 mg ml^{-1} ferulic acid). The final pre-freeze concentrations of glycerol and sperm cells were 5% and 562-924 x 10^6 ml^{-1}, respectively. Frozen pellets from non-AOs and AOs-treated sperm were thawed in a Tris-citric acid-glucose solution (40°C) containing 0, 0.49, 0.97 or 1.94 mg ml^{-1} caffeine and incubated (140-230 x 10^6 sperm ml^{-1}) at 30°C for 3 h. Sperm progressive motility (%) was assessed after centrifugation, before freezing and after 0, 1, 2 and 3 h of thawing. The results revealed significant ($p<0.05$) effects of sperm treatments only on post-thaw motility. Neither AOs alone nor caffeine alone could significantly ameliorate the maintenance of sperm motility. AOs plus 0.97 or 1.94 mg ml^{-1} caffeine were the superior supplements in improving the longevity of stallion spermatozoa.

Key words: Stallion · semen · antioxidants · caffeine

INTRODUCTION

Stallion spermatozoa are endowed with an extreme liability to the oxidative stress in view of their absolute reliance on the aerobic metabolism to meet their ATP requirements [1], their inability to repair membranes or to synthesize AOs [2], their exuberant content of polyunsaturated fatty acids (PUFA) [3], their unique capacity to generate O_2^- and H_2O_2 through mitochondrial respiration or fueling the activity of NADPH oxidase system [4, 5] and their restricted endogenous antioxidant defense mechanism, viz., as spermatozoa discard the majority of their cytoplasm during final stages of spermatogenesis, they lose most of the cytoplasmic defense enzymes which safeguard somatic cells from the peroxidative damage [6, 7].

Cryoconserved stallion sperm experience a shorter lifetime and a lower fertility than their fresh coordinates [8]. This was partially accredited to the outstanding imparities between fresh and frozen sperm in the generation rate of superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) [4] or in the intracellular concentration of free calcium ions (Ca^{2+}) [8, 9].

The objective of the present study was, therefore, to investigate if pre-freeze inclusion of antioxidants (AOs) in Tris-egg yolk extenders and/or post-thaw supplementation of sperm cells with caffeine could ameliorate freezability of stallion semen.

MATERIALS AND METHODS

All chemicals utilized in preparation of media presented in table 1 were purchased from Sigma-Aldrich Co., Deisenhofen, Germany. A mixture of sodium pyruvate (0.50 mg ml^{-1}), sodium thiosulfate (1 mg ml^{-1}), BSA (5 mg ml^{-1}), zinc chloride (0.15 mg ml^{-1}) and ferulic acid (0.50 mg ml^{-1}), were used in conformity with a preliminary dose-response study that was conducted on 28 water- and lipid-soluble AOs for selection of the optimal combination of them in Tris-egg yolk extenders based on motility, viability, plasma membrane integrity and morphological normalcy of stored (5°C for 96 h) stallion spermatozoa.

On a once biweekly collection schedule (May to August 2003), twelve ejaculates were obtained via a CSU-style artificial vagina from 5 Arabian stallions

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Table 1: Composition and temperature of media used in centrifugation of semen, two-step addition of extenders and freeze/thaw processing of stallion sperm in the form of 0.25 ml pellets

	Extenders				
Components	A Control 30°C	B Antioxidants 30°C	C Control 5°C	D Antioxidants 5°C	Thawing solution 40°C
Tris (hydroxymethyl) amino methane* (g)	2.40	2.40	2.40	2.40	2.40
Citric acid anhydrous* (g)	1.25	1.25	1.25	1.25	1.25
D-Glucose monohydrate* (g)	0.45	0.45	0.45	0.45	0.45
Penicillin G sodium* (IU)	50000	50000	50000	50000	50000
Streptomycin sulfate* (mg)	50	50	50	50	50
Fresh chicken egg yolk* (ml)	22	22	22	22	-
Sodium pyruvate (mg)	-	50	-	50	-
Sodium thiosulfate, $5H_2O$ (mg)	-	100	-	100	-
Bovine serum albumin (BSA) Fraction V, Fatty acid-free (mg)	-	500	-	500	-
Zinc chloride (mg)	-	15	-	15	-
Ferulic acid (3-Hydroxy-3-methoxycinnamic acid) (mg)	-	50	-	50	-
Glycerol (ml)	-	-	10	10	-
Glass-distilled water* (ml)	100	100	100	100	100

* The basic ingredients of Tris extender [12]

Single batches of these media were formulated and reposited in 50 ml vials at -20°C until used

(9-18 years old) of unknown sperm freezability and belonging to El-Zahra Arab Horse Stud, Cairo, Egypt. At the time of collection (early in the morning before offering the daily ration), an estrous mare was used as a mount animal. Pre-warmed collection bottles fitted with nylon filters were used for seclusion of gel from semen. Each animal was housed in a hygienic separate stall and fed 2 to 4 kg of a balanced grain ration plus 5 to 7 kg of barseem hay daily. All stallions had sired foals and their overall per-cycle pregnancy rate pending the preceding natural service program was 61.19%.

After collection, the gel-free fraction of the ejaculate was evaluated and only samples with more than 50% initial motility and 250×10^6 sperm cells ml^{-1} were processed. Two aliquots of gel-free semen from each ejaculate were transferred to 14 ml conical centrifuge tubes. The first aliquot (6 ml, control) was diluted with 6 ml of extender A, centrifuged (500 x g for 5 min) at room temperature, the supernatant was aspirated and sperm pellet was reconstituted in 1.50 ml of extender A. The second aliquot (6 ml, AOs) was treated as described above but sperm pellet was resuspended in 1.50 ml of extender B. Both aliquots were then cooled to 5°C over a 4 h period. The cooling rates were rapid from 30 to 19°C (-0.70°C /min), slow from 19 to 8°C (-0.05°C/min) and again rapid from 8 to 5°C (-0.70°C/min). Cooled spermatozoa were glycerinated by adding 1.50 ml of extender C to the first aliquot and 1.50 ml of extender D to the second aliquot and then equilibrated at 5°C for 3-4 h. The final concentrations of glycerol and spermatozoa in equilibrated aliquots were 5% and 562-924 x 10^6 ml^{-1}, respectively. Next, spermatozoa were frozen in the form of 0.25 ml pellets onto a plate made of polytetrafluoroethylene that was cooled beforehand by immersing it in liquid nitrogen for 10 min and raising it up to be exposed to the vapor of liquid nitrogen. After 3 min, frozen pellets were plunged in liquid nitrogen for 10 min and promptly thawed by dropping one pellet into a test tube containing 0.75 ml of a pre-warmed (40°C) thawing solution (Table 1) which was supplemented with 0, 0.49, 0.97 or 1.94 mg ml^{-1} caffeine. Afterwards, frozen-thawed spermatozoa (140-230 x 10^6 ml^{-1}) were incubated at 30°C for 3 h. Two pellets were used for each post-thaw sperm treatment.

Sperm progressive motility was examined in gel-free semen, after centrifugation and resuspension of sperm in glycerol-free extenders, just before freezing and after thawing as well as at hourly intervals of post-thaw incubation period. All estimations of motility percentages were carried out by a single observer using a phase-contrast microscope (400 x) equipped with a thermal stage maintained at 37°C. The viability index of frozen-thawed spermatozoa was computed from the following equation [10].

$$VI = \sum \left[M \times \frac{T - R}{2} \right]$$

where; VI is the viability index, Σ is a sign for the sum total, M is the percentage of sperm motility, T is the time of next determination of motility and R is the time of previous determination of motility. The concentration of sperm cells in gel-free semen, equilibrated aliquots and in thawed pellets was measured by a Neubauer haemocytometer.

Statistical analysis of the results was performed according to Snedecor and Cochran [11]. Results are

Table 2: Effect of antioxidants and/or caffeine on motility (%) of frozen-thawed stallion sperm

Semen treatments	Caffeine (mg ml^{-1})	Post-thaw incubation time (h) 0	1	2	3
Control	0.00	41.25±2.27^{a}	31.88±4.53^{a}	16.25±3.50^{a}	10.63±3.20^{a}
	0.49	46.88±3.53^{ab}	34.38±4.95^{ab}	17.50±3.54^{ab}	12.50±3.78^{a}
	0.97	47.50±4.63^{ab}	36.88±5.08^{ab}	18.75±4.30^{ab}	12.50±3.27^{a}
	1.94	53.13±2.82^{b}	40.00±3.66^{ab}	20.00±6.05^{abc}	11.88±3.89^{a}
Antioxidants	0.00	49.38±3.20^{ab}	36.88±3.53^{ab}	22.50±3.78^{ab}	15.63±3.33^{a}
	0.49	51.25±3.50^{b}	41.25±3.24^{ab}	24.38±4.06^{abc}	16.88±4.32^{a}
	0.97	55.63±2.20^{b}	43.75±2.95^{b}	32.50±2.67^{c}	15.00±3.41^{a}
	1.94	55.00±2.31^{b}	45.00±2.83^{b}	30.00±4.91^{bc}	18.13±2.66^{a}

Means ± SEM within columns having dissimilar superscripts are significantly different at $p<0.05$ (n=12)

presented as means ± SEM. Differences between motility percentages of untreated and AOs-treated sperm (post-centrifugation and pre-freeze) were determined by an unpaired two-tailed *t*-test. Analysis of variance (two-way ANOVA) and the least significant difference (LSD) test were used to examine the effects of semen treatments and incubation time on post-thaw motility percentages. One-way ANOVA and LSD test were applied in order to analyze the influence of sperm treatments on post-thaw viability indices. All P-values less than 0.05 were considered statistically significant.

RESULTS

The overall means of gel-free fraction volume, sperm motility percentage and sperm concentration of semen ejaculates were 28.35±3.25 ml, 77.50±3.27% and 358.08±32.91 x 10^{6} ml^{-1}, respectively.

Apart from sperm treatments, the motility percentages after centrifugation, before freezing, after 0, 1, 2 and 3 h of thawing averaged 84.38±2.28, 81.88±2.49, 50.00±1.18, 38.75±1.41, 22.73±1.55 and 14.14±1.20%, respectively. Incubation time had a significant ($p<0.0005$) effect on post-thaw sperm motility percentages.

Post-centrifugation (84.38±3.20 and 85.38±3.46 %) and pre-freeze (80.00±3.41 and 83.75±3.75%) motility did not differ ($p>0.05$) among untreated and AOs-treated sperm, respectively. Nonetheless, immediately after thawing (Table 2), the motility percentages of sperm treated with 1.97 mg ml^{-1} caffeine alone or with AOs plus increasing concentrations of caffeine were significantly ($p<0.05$) higher than those of untreated sperm. Meanwhile, upon incubation of semen for 1 or 2 h, the amelioration in motility percentages remained ($p<0.05$) solely for sperm supplemented with AOs plus 0.97 or 1.94 mg ml^{-1} caffeine. At 3 h of incubation, motility percentages of sperm treated with AOs plus 1.94 mg ml^{-1} caffeine tended ($p>0.05$) to be higher than that of other sperm treatments. The interaction between incubation time and semen treatments was significant ($p<0.05$).

Values of post-thaw viability indices were not significantly improved by addition of AOs alone or caffeine alone at any concentration. However, the viability indices of sperm exposed to AOs plus caffeine at 0.97 mg ml^{-1} (119.06±7.68) or 1.94 mg ml^{-1} (120.63±8.73) were superior ($p<0.05$) to those of untreated sperm (79.38±7.82) and sperm treated with 0.49 mg ml^{-1} caffeine alone (87.81±10.92), but were not significantly different from those of sperm supplemented with AOs alone (99.69±8.86) or with caffeine alone at 0.97 mg ml^{-1} (91.88±11.79) and 1.94 mg ml^{-1} (98.44±12.35).

DISCUSSION

Many factors may impose an extra oxidative load on cryopreserved sperm cells, such as 1) the dilution rates and availability of oxygen during semen processing [13]; 2) the presence of significant populations of dead and damaged spermatozoa [4, 14] as well as immature germ cells and leukocytes [15, 16] in semen; 3) centrifugation and removal most of seminal plasma [7, 17]; 4) excessive influx of Ca^{2+} into spermatozoa, particularly in milk-based extenders (ME), which is accompanied by intracellular production of H_2O_2 and premature capacitation [5, 9] and 5) the reduction of antioxidant defenses observed in sperm cells after cryopreservation of semen in egg yolk-based extenders (EYE) which not only have a very limited capacity to eliminate H_2O_2 [18] but also contain L-aromatic amino acids and free ions of iron (Fe^{2+}) and copper (Cu^{1+}) that key up the extracellular formation of H_2O_2 and hydroxyl radicals ($^{\bullet}OH$) in semen [14].

Considering the above-mentioned vulnerability of preserved semen to an antioxidant/pro-oxidant disequilibrium, it seems that exposure of frozen-thawed stallion spermatozoa to superphysiological levels of O_2^{-}, H_2O_2 and $^{\bullet}OH$ is inevitable and may lead to a peroxidative damage to plasma and inner mitochondrial membranes, a decline in ATP, cAMP and cGMP content and a subsequent decrease in sperm longevity [14, 18, 19].

Consequently, the outcomes of our study revealed that concentration and pellet-freezing of sperm cells in EYE containing AOs, followed by thawing, dilution and motility stimulation of sperm cells with caffeine (0.97 or 1.94 mg ml^{-1}) as a phosphodiesterase inhibitor mediating intracellular increases of cAMP levels [2], had a beneficial impact on their motility and longevity.

Pyruvate is a potent scavenger of H_2O_2 [20] and its supplementation at a concentration of 5 mM (~0.55 mg ml^{-1}) to chilled-stored stallion semen in ME [21] or to frozen-thawed bull semen in EYE [18] resulted in a significant augmentation of sperm motility and ATP levels. Ferulic acid (1.6 mM ~ 0.31 mg ml^{-1}), an effective constituent in various medicinal herbs, has been shown to scavenge oxygen free radicals, reduce membrane lipid peroxidation, increase intracellular cGMP level and to improve the motility and viability of human spermatozoa incubated in Ham's F-10 medium for 6 h at 37°C [19].

Oxidation of thiols (SH) in sperm proteins by O_2^- and H_2O_2 was found to be associated with inhibition of sperm motility and fertilizing ability [22]. Cryopreservation of bull sperm in EYE significantly reduced the intracellular level of SH [23] and post-thaw treatment of frozen semen with SH containing AOs prevented H_2O_2-mediated loss of sperm motility [24]. Supporting the results of our study, in a previous study on frozen buffalo semen, supplementation of EYE with sodium thiosulfate (1 mg ml^{-1}), as a source of SH to sperm cells, led to a remarkable improvement of their motility and plasma membrane stability [25].

Luxuriant entry of Ca^{2+} into sperm cells was found to coincide with activation of membrane-bound phospholipases [26] and peroxidative damage of their membranes [27]. The stabilizing influence of zinc on sperm membranes was attributed to its ability to suppress lipid peroxidation via inhibition of phospholipases and to protect SH and PUFA in biomembranes from Fe^{2+}-mediated oxidation [28]. In accordance with our results, addition of zinc chloride (1 mM ~ 0.14 mg ml^{-1}) to EYE brought about a significant enhancement in the motility of frozen buffalo sperm [29].

Albumin is a crucial extracellular antioxidant [30] through its propensity to bind transition metal ions (Fe^{2+} and Cu^{1+}) in EYE and, thereby, to minimize formation of $^{\bullet}OH$, the powerful initiator of lipid peroxidation cascade in sperm [14, 31]. Despite incubation of stallion [9] and bull [18] spermatozoa in media containing BSA and sodium pyruvate prolonged significantly their functional lifespan compared with ME and EYE, some researchers did not observe any improvement in the motility of chilled-stored stallion sperm after inclusion of BSA (3% w/v) in ME [32].

CONCLUSIONS

In view of the current study, we conclude that freeze/thaw processing of stallion semen in the presence of both AOs and 0.97 or 1.94 mg ml^{-1} caffeine achieved a significant amelioration in the maintenance of sperm motility.

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