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IMPACT OF ANTIOXIDANTS ON VIABILITY OF CHILLED AND CRYOPRESERVED STALLION SPERMATOZOA

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SUMMARY

A series of factorially arranged six experiments were conducted to study the effects of twenty-eight antioxidants on maintenance of viability of stallion spermatozoa in a chemically - defined stallion semen extender (Tris - egg yolk) at 5 °C and - 196°C. Sixty-nine ejaculates were collected from nineteen Arabian horses (5 - 21 years old). In four preliminary experiments (n = 40), the appropriate concentrations of the best four antioxidants in terms of sperm viability at 5°C were selected. In the fifth experiment (n = 19), a comparison between the selected antioxidants and a mixture (cocktail) of them was studied on bases of sperm viability, sperm morphology and changes in levels of extracellular aspartate and alanine aminotransferase. The sixth experiment (n = 10) was carried out for using the best antioxidant that could improve the viability of frozen - thawed stallion spermatozoa. Results of the preliminary experiments revealed that four antioxidants viz., sodium pyruvate, sodium thiosulfate, bovine serum albumin and zinc chloride could induce beneficial effect on viability of stallion spermatozoa. A mixture of the previous four antioxidants was most effective for improving spermatozoal morphology and viability at 5°C. Extracellular level of alanine aminotransferase was minimal on using the mixture of antioxidants after cold - storage of spermatozoa for 72 hours at 5 °C. The results of frozen - thawed stallion semen revealed that viability indices were superior when using the mixture of antioxidants specially when caffeine was added to the thawing solution.

INTRODUCTION

Preservation of liquid semen at 5°C is an important technique in the breeding management of horses (Ball et al., 2001). Stallion sperm have little endogenous antioxidants (by removing most seminal plasma) to protect them against reactive oxygen species (ROS) that may be present during hypothermic storage of semen (Ball et al., 2000). There is growing evidence that oxidative damage of spermatozoa by ROS impairs stallion sperm function in terms of declined motility and fertility (Ball et al., 2001).

Accordingly, the objective of the current study was to evaluate the effect of using antioxidants on motility and viability of stallion spermatozoa during storage at 5°C and - 196 °C.

MATERIALS AND METHODS

All kits and chemicals used in this study were purchased from Sigma-Aldrich Co. Germany and Stanbio laboratory Co.

Animals and ejaculates:

In this study, nineteen healthy Arabian horses (5- 21 years old) belonging to El-Zahraa Arab Horse Stud, Cairo, were used. These stallions have been used as sires in the regular breeding program of the stud. The study lasted for two years from September 2001 to August 2003.

Sixty - nine ejaculates were collected from the stallions using the CSU model artificial vagina. Immediately after collection, semen samples were transferred to the laboratory and evaluated by the conventional methods.

Experimental design:

In this investigation, twenty - eight antioxidants (in two concentrations) were tested in six laboratory experiments. A series of four preliminary experiments was conducted for selecting of the appropriate concentration for each antioxidant in terms of sperm motility and viability. In each experiment (10 ejaculates); seven antioxidants were used.

In experiment five (19 ejaculates), we compared between the selected four antioxidants resulted from the previous experiments and a mixture (cocktail) of them. The comparison was based on evaluation of sperm viability, sperm morphology and changes in the extracellular levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

The sixth experiment (10 ejaculates) was carried out for using the most effective antioxidant elected from experiment five that could induce a beneficial influence on functional competence of frozen - thawed spermatozoa in terms of sperm motility and viability.

Experimental procedure:

Following initial evaluation, each semen sample was prepared for preservation in Tris - egg yolk extender (Samper et al., 1988) at 5°C for 72 hours after being supplemented with antioxidants. In experiment 1, the used antioxidants were catalase, glutathione peroxidase, sodium pyruvate, pyruvic acid, ascorbic acid, ferulic acid and 4-aminosalicylic acid. In experiment 2, we tested hypotaurine, taurine, cysteine hydrochloride, sodium thiosulfate, thiourea, ethylenediaminetetraacetic acid (EDTA) free acid and sodium salt of EDTA. In the third experiment, we used dimethyl sulfoxide (DMSO), mannitol, sodium benzoate, bovine serum albumin (BSA), pentoxifylline, reduced nicotinamide adenine dinucleotide (β-NADH) and glutathione reductase . In experiment 4, the used antixidants were DL-α-tocopherol acetate, butylated hydroxytoluene in 0.50% DMSO, cytochrome c, superoxidae dismulatase, β-carotene in 0.50% DMSO,L-penicillamine and zinc chloride. The viability indices were calculated for each experiment according to Milovanov et al. (1964).

In the fifth experiment, semen samples were preserved in Tris - egg yolk extender (Samper et al., 1988) at 5 °C for 144 hours. The used antioxidants were sodium pyruvate (0.5 mg/ml), sodium thiosulfate (1.0 mg/ml), BSA (5.0 mg/ml), zinc chloride (0.15 mg/ml) and a mixture (cocktail) of them. Viability indices of preserved spermatozoa were calculated. Aliquot of 0.5 ml semen was removed after dilution and after 72 hours of incubation at 5°C. These aliquots were centrifuged (3000 rpm) and the supernatants were stored at -20°C pending analysis. AST (IU/L) and ALT (IU/L) were assayed spectrophotometrically under the conditions specified by the commercial kit systems. Smears of stored semen (at 5°C) were stained with nigrosin - eosin solution (Dott and Foster, 1972) and examined under oil-immersion lens (1000x) for abnormal sperm percent and ratio of live spermatozoa at 0 and 72 hours of incubation.

In experiment six, only semen samples with at least 70% initial motility and 250 x 10^6 sperm cell / ml were used in two parallel tests, control (A) and treated (B) samples. In clean screw caped test tubes, semen samples were diluted (6 ml semen + 6 ml Tris - buffer) at 30 °C with a single stepwise addition of Tris - buffer solution. The diluted semen samples were then centrifuged at 3000 rpm for 3 minutes. Most of the supernatants were decanted and the formed sperm pellets were resuspended in 1.5 ml of Tris - egg yolk extender (A) and 1.5 ml of Tris - egg yolk cocktail extender (B) and were reconstituted gently. Both diluted samples were cooled slowly to 5 °C (0.05 - 0.1°C / minute) in the refrigerator along with another two tubes, one of which (A*) contained 1.5 ml Tris - egg yolk glycerol (5%) extender and the other (B*) contained 1.5 ml Tris - egg yolk cocktail glycerol (5%) extender. After cooling to 5 °C, we mixed tube (A*) with tube (A) and tube

(B*) with tube (B) to form 3 ml of control (A) sample and 3 ml of treated (B) sample, respectively. The cooled samples were stored at 5°C for 2 - 3 hours (equilibration period). The cooled semen (A and B) was frozen in the form of pellets (0.25 ml / pellet) on a Fluorethene plate cooled by immersion in liquid nitrogen inside a foam box (34x 22x 17 cm, containing 5 liters of liquid nitrogen) for 15 minutes and then the plate was lifted to the surface of liquid nitrogen. After 2 - 3 minutes, the frozen pellets were immersed in liquid nitrogen, transferred into the liquid nitrogen container and stored as control (A) and treated (B) pellets for 3 hours before thawing.

Thawing of frozen stallion semen pellets was carried out using a Tris - buffer solution and different concentrations of caffeine. Eight frozen pellets [4 (A) and 4 (B)] were plunged into eight prewarmed (40°C for 15 minutes) 7 ml glass tubes containing 0.75 ml / tube of Tris - buffer solution and caffeine in the following manner: tubes A1 and B1 contained Tris - buffer solution; tubes A2 and B2 contained Tris - buffer solution + 2.5 mmol % caffeine; tubes A3 and B3 had Tris - buffer solution + 5 mmol % caffeine; tubes A4 and B4 contained Tris - buffer solution + 10 mmol % caffeine. All tubes were held in a water bath at 40°C and were shaken gently until melting of semen pellets. The thawed semen in control and treated samples was then transferred into prewarmed, clean tubes (2 ml) and incubated in a water bath at 30°C for 3 hours. Viability indices of thawed semen were calculated.

Statistical analysis:

Analysis of data was performed using a commercial software Statistica for windows, 1993.

RESULTS

The results of the preliminary experiments are shown in Tables 1 - 4. The best four antioxidants with significantly ($P<0.01$) higher means of viability indices for cold - stored stallion semen were sodium pyruvate (56.42 ± 7.04), sodium thiosulfate (61.02 ± 4.22), BSA (49.82 ± 5.66) and zinc chloride (53.75 ± 4.47). The appropriate concentrations of these antioxidants were 0.5, 1.0, 5.0 and 0.15 mg / ml extender, on seriatim.

Table 1: Viability indices of cold – stored stallion semen using antioxidants of experiment 1 (mean ± SEM)

serial	Treatments	Concentration	Viability indices
1	Control	0	42.03 ± 7.44
2	Catalase	450 U / ml	48.69 ± 6.69**
3	Catalase	900 U / ml	51.75 ± 6.37**
4	Glutathione peroxidase	0.5 mg / ml	51.92 ± 6.47**
5	Glutathione peroxidase	1 mg / ml	51.83 ± 6.96**
6	sodium pyruvate	0.5 mg / ml	56.42 ± 7.04**
7	sodium pyruvate	1 mg / ml	52.65 ± 7.18**
8	Pyruvic acid	0.5 mg / ml	53.13 ± 7.02**
9	Pyruvic acid	1 mg / ml	4.62 ± 1.13**
10	Ascorbic acid	0.5 mg / ml	45.95 ± 6.20
11	Ascorbic acid	1 mg / ml	32.22 ± 5.98**
12	Ferulic acid	0.5 mg / ml	53.18 ± 6.82**
13	Ferulic acid	1 mg / ml	53.03 ± 6.81**
14	4-aminosalicylic acid	0.5 mg / ml	50.45 ± 7.23**
15	4-aminosalicylic acid	1 mg / ml	50.19 ± 7.46**

** Significantly different from control (P < 0.01)

Table 2: Viability indices of cold – stored stallion semen using antioxidants of experiment 2 (mean ± SEM)

Serial	Treatments	Concentration	Viability indices
1	Control	0	49.37 ± 4.63
2	Hypotaurine	0.1 mg / ml	57.03 ± 4.65
3	Hypotaurine	0.2 mg / ml	56.27 ± 4.72
4	Taurine	5 mg / ml	56.79 ± 4.45**
5	Taurine	10 mg / ml	57.32 ± 4.28**
6	Cysteine hydrochloride	0.5 mg / ml	52.85 ± 4.76
7	Cysteine hydrochloride	1 mg / ml	45.12 ± 5.48
8	Sodium Thiosulfate	0.5 mg / ml	60.93 ± 4.26**
9	Sodium Thiosulfate	1 mg / ml	61.02 ± 4.22**
10	Thiourea	0.5 mg / ml	56.57 ± 3.68**
11	Thiourea	1 mg / ml	49.88 ± 5.20
12	EDTA (free acid)	0.5 mg / ml	54.99 ± 4.95
13	EDTA (free acid)	1 mg / ml	47.09 ± 5.07
14	Sodium salt EDTA	0.5 mg / ml	55.41 ± 4.80
15	Sodium salt EDTA	1 mg / ml	55.08 ± 4.34

** Significantly different from control (P < 0.01)

Table 3: Viability indices of cold – stored stallion semen using antioxidants of experiment 3 (mean ± SEM)

serial	Treatments	Concentration	Viability indices
1	Control	0	39.89 ± 5.55
2	DMSO	2.00 %	45.26 ± 5.53
3	DMSO	4.00 %	41.22 ± 5.33
4	Mannitol	1.00 mg / ml	41.76 ± 5.39
5	Mannitol	2.00 mg / ml	42.80 ± 5.42
6	Sodium benzoate	0.20 mg /ml	44.49 ± 5.64
7	Sodium benzoate	0.40 mg /ml	47.13 ± 5.71**
8	Bovine serum albumin	5.00 mg / ml	49.82 ± 5.66**
9	Bovine serum albumin	10.00 mg / ml	45.41 ± 6.25
10	Pentoxifylline	0.20 mg /ml	41.34 ± 5.51
11	Pentoxifylline	0.40 mg /ml	10.29 ± 1.87**
12	B- NADH	0.25mg / ml	7.88 ± 1.36**
13	B- NADH	0.50mg / ml	6.58 ± 1.52**
14	Glutathione reductase	0.007U / ml	12.74 ± 3.02**
15	Glutathione reductase	0.014U / ml	8.04 ± 2.09**

** Significantly different from control (P < 0.01)

Table 4: Viability indices of cold – stored stallion semen using antioxidants of experiment 4 (mean ± SEM)

Serial	Treatments	Concentration	Viability indices
1	Control	0	38.34 ± 4.54
2	DL-α-tocopherol acetate	0.75 mg / ml	20.57 ± 4.41**
3	DL-α-tocopherol acetate	1.50 mg / ml	13.66 ± 3.09**
4	BHT in 0.50% DMSO	0.55 mg / ml	42.83 ± 4.35
5	BHT in 0.50% DMSO	1.10 mg / ml	50.10 ± 5.13**
6	Cytochrome c	0.05 mg /ml	41.72 ± 4.72
7	Cytochrome c	0.10 mg /ml	36.15 ± 4.61
8	Superoxide dismutase	25.00 U / ml	47.30 ± 5.01**
9	Superoxide dismutase	50.00 U / ml	49.55 ± 5.20**
10	β-carotene in 0.50% DMSO	0.05 mg /ml	46.10 ± 4.56
11	β-carotene in 0.50% DMSO	0.10 mg /ml	51.14 ± 4.44**
12	L-penicillamine	0.25mg / ml	47.49 ± 3.80
13	L-penicillamine	0.50mg / ml	41.81 ± 4.00
14	Zinc chloride	0.15 mg / ml	53.75 ± 4.47**
15	Zinc chloride	0.30 mg / ml	48.86 ± 5.16**

** Significantly different from control (P < 0.01)

Table 5 shows the effect of the elected antioxidants from the previous experiments on motility and viability of cold - stored stallion semen during the incubation periods. Bovine serum albumin, zinc chloride and a mixture of them (cocktail) exerted a beneficial effect ($P<0.05$) on motility and viability of stallion semen stored at 5 °C. Furthermore, the best result was obtained when a cocktail of the antioxidants was used (Table 5).

As shown in Table 6, the rate of increase of AST in the extracellular medium was significantly higher for controls (123.91%) in comparison to the use of antioxidants during storage of stallion semen at 5°C. Also, the rate of increase of ALT in the extracellular medium was more pronounced for controls (82.12 %) without antioxidants during cold - storage of stallion semen (Table 7).

Table 5: Effect of elected antioxidants on motility and viability of the cold – stored stallion semen (mean ± SEM)

Treatments	Sperm motility during incubation at 5 °C				Viability indices
	0 hour	6 hours	72 hours	144 hours	
Control	61.32^{a} ± 4.12	57.63^{a} ± 4.55	30.00^{a} ± 4.44	5.79^{a} ± 2.21	47.46^{a} ± 5.41
Sodium pyruvate	67.11^{bc} ± 4.31	63.16^{bc} ± 4.56	35.00^{bd} ± 4.62	10.00^{bc} ± 2.81	56.10^{b} ± 5.91
Sodium thiosulfate	63.42^{ac} ± 4.12	60.53^{ac} ± 4.36	31.58^{ab} ± 4.65	7.89^{ab} ± 1.77	51.17^{c} ± 5.27
BSA	68.95^{b} ± 4.00	65.79^{b} ± 4.36	37.63^{def} ± 4.95	12.89^{c} ± 2.89	61.00^{d} ± 6.19
Zinc chloride	67.89^{bc} ± 4.77	65.53^{bc} ± 4.87	41.32^{ef} ± 4.71	11.32^{bc} ± 2.86	62.28^{d} ± 6.23
Cocktail	72.89^{d} ± 4.26	71.58^{d} ± 4.31	42.37^{f} ± 4.85	12.37^{bc} ± 3.32	66.09^{d} ± 5.85

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

Table 6: The rate of increase of AST (IU/L) in the extracellular medium during storage of stallion semen at 5 °C

Treatments	After dilution	After 72 hours at 5°C	Rate of increase (%)	Statistical significance
Control	$35.67^{a} \pm 7.16$	$79.87^{b} \pm 17.81$	123.91	P<0.05
Sodium pyruvate	$40.61^{a} \pm 7.20$	$54.69^{b} \pm 6.79$	34.67	P<0.01
Sodium thiosulfate	$38.50^{a} \pm 6.56$	$55.49^{b} \pm 6.71$	44.13	P<0.001
BSA	$36.36^{a} \pm 5.95$	$60.70^{b} \pm 8.29$	66.94	P<0.01
Zinc chloride	$41.32^{a} \pm 5.25$	$65.35^{b} \pm 11.86$	58.16	P<0.05
Cocktail	$40.63^{a} \pm 6.72$	$55.60^{b} \pm 10.47$	36.84	P<0.05

Means with dissimilar superscripts in the same row are significantly different

Table 7: The rate of increase of ALT (IU/L) in the extracellular medium during storage of stallion semen at 5 °C

Treatments	After dilution	After 72 hours at 5°C	Rate of increase (%)	Significant values
Control	$13.20^{a} \pm 1.94$	$24.04^{b} \pm 1.37$	82.12	P<0.01
Sodium pyruvate	$21.22^{a} \pm 2.62$	$30.48^{b} \pm 2.10$	43.64	P<0.01
Sodium thiosulfate	$19.05^{a} \pm 3.05$	$25.84^{b} \pm 3.49$	35.64	P<0.001
BSA	$18.15^{a} \pm 1.35$	$26.47^{b} \pm 1.90$	45.84	P<0.001
Zinc chloride	$15.28^{a} \pm 1.73$	$23.64^{b} \pm 1.71$	54.71	P<0.001
Cocktail	$19.73^{a} \pm 2.46$	$29.24^{b} \pm 2.95$	48.20	P<0.01

Means with dissimilar superscripts in the same row are significantly different

As shown in Table 8, the cocktail of antioxidants exerted an explicit effect ($p<0.05$) on sperm morphology of cold - stored stallion semen in terms of percentages of unstained sperm and abnormal heads.

In experiment six, the viability indices (mean ± SEM) of diluted semen were similar (87.00 ± 1.53 and 88.00 ± 1.86) for the control and treated samples, respectively. Nevertheless, the viability indices differed significantly ($p<0.05$) after cooling to 5°C (76.50 ± 3.88 and 82.00 ± 3.96) for the control and treated trials, respectively.

The results of frozen - thawed stallion semen are given in Table 9. Superior viability indices were recorded using the cocktail of antioxidants specially when caffeine was supplemented in the thawing solution. The effective concentration of caffeine in the thawing solution was 10 mmol % (Table 9).

Table 8: Effect of antioxidants on sperm morphology of cold – stored stallion semen (mean ± SEM)

Incubation periods	Treatments	Sperm morphology (%)				
		unstained sperm	abnormal acrosome	abnormal heads	abnormal midpiece	abnormal tails
0 hour at 5°C	Control	$85^{a} \pm 3$	1 ± 0	$2^{ab} \pm 1$	$1^{ab} \pm 0$	$7^{a} \pm 2$
72 hours at 5 °C	Control	$74^{b} \pm 2$	1 ± 1	$3^{a} \pm 1$	$0^{a} \pm 0$	$14^{b} \pm 1$
	Sodium pyruvate	$75^{b} \pm 2$	0 ± 0	$2^{ab} \pm 1$	$1^{ab} \pm 0$	$13^{b} \pm 2$
	Sodium thiosulfate	$72^{b} \pm 4$	0 ± 0	$1^{b} \pm 1$	$1^{ab} \pm 1$	$17^{b} \pm 3$
	BSA	$74^{b} \pm 3$	1 ± 0	$2^{ab} \pm 1$	$2^{b} \pm 0$	$18^{b} \pm 3$
	Zinc chloride	$75^{b} \pm 3$	0 ± 0	$1^{b} \pm 1$	$1^{ab} \pm 0$	$15^{b} \pm 2$
	Cocktail	$78^{ab} \pm 3$	0 ± 0	$1^{b} \pm 0$	$1^{ab} \pm 1$	$13^{b} \pm 2$

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

Table 9: Effect of the antioxidants mixture (cocktail) on viability of post-thawed stallion semen in presence of caffeine (mean ± SEM)

Control (Tris – yolk glycerol) extender				Cocktail (Tris – yolk cocktail glycerol) extender			
A1 Tris – buffer (T)	A2 T + 2.5 mmol caffeine %	A3 T + 5 mmol caffeine %	A4 T + 10 mmol caffeine %	B1 T	B2 T + 2.5 mmol caffeine %	B3 T + 5 mmol caffeine %	B4 T + 10 mmol caffeine %
62.75^{c} ± 12.19	73.75^{bc} ± 13.84	75.50^{bc} ± 15.17	80.75^{bc} ± 15.95	$75.00^{a\,c}$ ± 16.37	83.75^{ab} ± 16.53	90.50^{ab} ± 18.19	93.25^{b} ± 18.07

Means with dissimilar superscripts in the same row are significantly different at $P<0.05$

DISCUSSION

Lipid peroxidation is recognized as a damaging process to spermatozoa of many species including stallion (Lenzi et al., 1996; Upreti et al., 1998; Ball et al., 2001; Chatterjee and Gagnon, 2001). The mechanism by which ROS disrupt the sperm function is believed to involve the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane (Aitken, 1994) and this process plays an important role in the pathophysiology of male infertility (Aitken et al., 1993). ROS increase DNA fragmentation (Lopes et al., 1998) and affect the sperm axoneme development (De Lamirande and Gagnon, 1992).

A complex antioxidant system is present in equine seminal plasma in form of a high activity of ergothioneine (Mann, 1964) and catalase (Ball et al., 2000). Nevertheless, processing of equine semen for in vitro storage typically requires removal of most, if not, all seminal plasma. Such processing may reduce the ability to scavenge hydrogen peroxide (H_2O_2) and thereby increase the susceptibility of spermatozoa to oxidative stress (Ball et al., 2000).

The present results showed that liquid storage of stallion semen has been associated with a significant decrease in sperm viability that occurred in the absence of any antioxidant. At the same time, it was clear that sodium pyruvate in its active an-

tioxidant concentration could improve sperm motility and viability during incubation at 5°C. A similar finding has been previously reported in rams (Upreti et al., 1998), bulls (Bilodeau et al., 2002) and stallions (Bruemmert et al., 2002).

Also from the present results, it was evident that inclusion of sodium thiosulfate in Tris - based extenders prevented the dramatic decrease in stallion sperm motility and viability during liquid storage. Similar results were obtained during freezing and thawing of bull, ram and boar semen (Nauck, 1988).

The current findings indicated also that bovine serum albumin maintained stallion sperm viability during cold storage in comparison to the other six antioxidants used. Bovine serum albumin has been recognized as a very potent inhibitor of lipid peroxidation when used in human (Alvarez and Storey, 1995; Kouoh et al., 1999) and ram (Ollero et al., 1998) semen.

In the current study, a highly significant viability index was found when zinc chloride was included in the extender used for liquid storage of stallion semen. The inhibitory effect of zinc on superoxide anion was hypothesized (Gavella and Lipovac, 1998). Similarly, pre-freeze supplementation of ram (Erokhin and Epishina, 1996) and buffalo (El - Sheltawi et al., 1999) semen extenders with zinc chloride led to a significant enhancement of post-thaw sperm motility and viability.

As expected, a mixture (cocktail) of the above mentioned antioxidants viz., sodium pyruvate, sodium thiosulfate, BSA and zinc chloride offered a better protection for stallion sperm motility and viability during storage at 5°C than the individual antioxidants. This protection has been reflected in the increased rate of extracellular AST and ALT in extenders used without antioxidants and better findings of sperm morphology on using the antioxidants mixture.

It is of interest to note that stallion sperm viability has been improved by adding a powerful antioxidants mixture to the semen extender during the freezing - thawing process. In addition, post - thaw sperm motility was augmented by using a motility stimulant - caffeine at 10 mmol %. In this respect, Andersson and Katila (1992) and Gradil and Ball (2000) recorded similar findings in post - thaw stallion semen by using caffeine and pentoxifylline, respectively.

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