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EFFECT OF SOME ANTIOXIDANTS ON VIABILITY OF FROZEN BUFFALO SEMEN

(With 2 Tables)

By

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تأثير بعض مضادات الأكسدة على حيوية السائل المنوي المجمد للجاموس

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إن إضافة البنتوكسفلين ، إنزيم Superoxide dismutase، EDTA، ثيوسلفات الصوديوم، حامض الأسكوربيك، السستين ، المثيونين أو كلوريد المنجنيز إلى مخففي السترات والترس لطلائق الجاموس أدى إلى تحسن معنوي في خواص الحيوانات المنوية المجمدة والمسالة كالنسبة المئوية للحركة الأمامية بعد الإسالة وكذلك معدل الحيوية. وقد أسفرت النتائج على أن افضل مضاد للتأكسد في مخفف السترات هو إنزيم Superoxide dismutase عند تركيز ١٠٠ وحدة/مللي وفي مخفف الترس كان البنتوكسفلين عند تركيز ١,٤ مجم/مللي.

SUMMARY

Supplementation of Tris and citrate buffalo semen extenders with pentoxifylline, superoxide dismutase, EDTA, sodium thiosulfate, ascorbic acid, cystine, methionine, or manganous chloride resulted in a significant ($P<0.01$) improvement in the motility and viability of frozen-thawed buffalo spermatozoa. Superoxide dismutase (100 Units/ml) in citrate-based diluents and pentoxifylline (1.40 mg/ml) in Tris-based diluents were the most effective semen treatments.

Key words: *Buffalo, Semen, Sperm, Antioxidants, Cryopreservation.*

INTRODUCTION

The widespread application of artificial insemination in buffalo and realization of its full potential depends largely on the use of frozen semen (Crudeli *et al.*, 1999). Nevertheless, the reduction and increase in temperature during freezing and thawing of buffalo semen inevitably procures structural and biochemical damage to a significant proportion of spermatozoa (Sansone *et al.*, 2000). Lipid peroxidation is a major biochemical insult that plays a key role in eliciting of defective sperm function (Mammoto *et al.*, 1996) and limiting the viability of frozen-thawed buffalo (Goyal *et al.*, 1998) and bull (Bilodeau *et al.*, 1999, 2000) spermatozoa. Like wise, it has been suggested that buffalo spermatozoa contain comparatively more unsaturated fatty acids than in other species which may render them more vulnerable to the oxidative stress and lipid peroxidation (Singh *et al.*, 1992).

Since inhibition of sperm lipid peroxidation by providing anaerobic conditions during processing of mammalian semen for hypothermic storage was proved to be technically difficult (Salamon and Maxwell, 1995), therefore, inclusion of reactive oxygen species scavengers in buffalo semen extenders has been proposed as an alternative strategy to improve the viability of cryopreserved spermatozoa (El-Sheltawi *et al.*, 1999 and Sarlos *et al.*, 2002).

In this connection, the current study was undertaken to investigate the effect of prestorage fortification of semen extenders with some putative antioxidants on the motility and viability of frozen-thawed buffalo spermatozoa.

MATERIALS and METHODS

Chemical reagents:

Antioxidants and all other chemical reagents used for preparation of buffers and diluents were of the highest commercially available purity and were purchased from Sigma-Aldrich Co,. Deisenhofen, Germany.

Animals and semen collection:

Semen samples were collected by means of an artificial vagina from five buffalo bulls aged 5 to 6 years. These animals were kept at Animal Reproduction Research Institute (ARRI), Al-Haram, Giza Province.

Semen extenders:

Two types of diluents were used for cryopreservation of buffalo semen; i) Egg yolk-citrate diluent (Dhami *et al.*,1995) ; and ii) Egg yolk-Tris buffered diluent (Abdel-Malak *et al.*,1994).

Semen processing and experimental procedures:

Immediately after collection, the ejaculates were evaluated for volume, mass activity, individual motility and sperm concentration according to the standard methods reported by El-Menoufy (1974).

In addition, sperm morphological abnormalities were examined in smears stained with Spermac™ stain (Fertipro N.V. Sint-Martens-Latem, Belgium) according to Oettle (1986). Only ejaculates of at least 70% initial motility and $600x10^6$ sperm cells/ml were used in two in vitro experiments. In each trial, semen ejaculates obtained from all bulls were pooled to yield one semen sample with a total volume of 17 to 20 ml.

In the first experiment, semen samples were split and diluted (1:4) at 30°C with egg yolk-citrate extenders supplemented with or without 1.40 mg/ml pentoxifylline, 100.00 Units/ml superoxide dismutase 1.00 mg/ml EDTA, 1.00 mg/ml sodium thioulfate, 0.10 mg/ml ascorbic acid, 1.00 mg/ml cystine, 2.00 mg/ml methionine, and 0.80 mg/ml manganous chloride. In the second experiment, semen samples were split and diluted (1:4) at 30°C with egg yolk-Tris extenders supplemented with or without the same concentrations of the above mentioned antioxidants except methionine was added to the diluents at a concentration of 1.00 mg/ml. The concentrations of antioxidants in citrate and Tris-based extenders were used according to the best results obtained by (Khalifa, 2001). Within 5 minutes after dilution, the extended semen in the 1^{st} and 2^{nd} experiment was cooled to 5°C over a period of 60 and loaded into 0.25 ml French straws at 5°C. The number of progressively motile spermatozoa per straw was 30 to $40x10^6$. The straws were then arranged horizontally on cold (5°C) freezing racks and lowered into liquid nitrogen vapour inside a foam box according to Mohammed *et al.*, (1998). The straws were then immersed and stored in liquid nitrogen. Frozen semen was thawed in a water bath at 40°C for 30 seconds. Sperm motility was subjectively assessed immediately after dilution, before freezing and after thawing as well as after 1,2 and 3 hours of incubation in water bath at 37°C. The postthaw viability index was calculated according to Milovanov (1962).

Statistical anaysis:

All data were subjected to analysis of variance (ANOVA) by using the general linear models procedures of the Statistical Analysis Systems (SAS, 1990).

RESULTS

Examination of freshly collected semen samples from each buffalo bull revealed that the overall mean values of progressive motile sperm, sperm concentration and total abnormalities were 71.00%, 665.68 x 10^6/ml and 15.25% respectively.

Table 1&2 demonstrate the influence of elected doses of antioxidants on the motility and viability of buffalo spermatozoa during the different stages of freeze thaw processing of semen in citrate and Tris-based extenders. Fortification of citrate- and Tris-based extenders with pentoxifylline, superoxide dismutase, EDTA, sodium thiosulfate, ascorbic acid, cystine, methionine or even with monganous chloride significantly ($p<0.01$) improved sperm motility percentages and augmented the viability indices of frozen-thawed spermatozoa.

Analysis of variance clarified a highly significant effect ($p<0.01$) for both semen treatments and stages of semen processing on sperm motility percentages as well as viability indices of frozen thawed buffalo semen. While the maximum values of postthaw sperm motility (57.00±3.39%) and viability index (150.50±11.76) in citrate-based diluents were recorded for superoxide dismutase-treated semen (Table 1) On the other hand, it was clear that the highest percentages of sperm motility after thawing (54.00±1.87%) as well as the superior value of postthaw viability index (158.00±6.44) were achieved by inclusion of pentxifylline in Tris-based diluents (Table 2).

DISCUSSION

It is well known that eutherian spermatozoa are particularly susceptible to the peroxidative damage because they contain an extremely high concentration of polyunsaturated fatty acids (predominantly docosahexaenoic acid), exhibit no capacity for membrane repair, and possess a significant ability to generate superoxide anion radicals from at least two sources (O'Flaherty *et al.*,1997, 1999). The first source is sperm mitochondrial respiration via oxidation of NADH by NADH-dependent oxidoreductase system (Vernet *et al.*, 1999) . The second source is the cytoplasm of sperm midpiece through

oxidation of NADPH by a membrane-bound NADPH-oxidase system (Ball and Vo, 1999).

Despite normally there is a balance between reactive oxygen species produced and destroyed in spermatozoa, under specific conditions such as hypothermic storage of semen (Cerolini *et al.*, 2000; Roca *et al.*, 2000), this balance can be upset resulting in a dramatic attenuation of the antioxidant defense system (chiefly superoxide dismutase and glutathione) in spermatozoa as well as a profuse increase in the generation of superoxide anions and hydrogen peroxide in the preserved semen (Toniolli *et al.*, 1998; Bilodeau *et al.*, 2000). The major sources of reactive oxygen species in the frozen-thawed semen are the oxidative deamination of aromatic amino acids by aromatic L- amino acid oxidase released from dead and damaged sperm (Shannon and Curson, 1982; Upreti *et al.*, 1998), and the presence of catalytically active iron in seminal plasma and egg yolk which mediates free radicals production (Vishwanath and Shannon, 1997). Consequently, it seems that cryopreserved spermatozoa suffer oxidative stress due to their inability to scavenge superoxide anions and hydrogen peroxide (Bilodeau and Bras, 1999; Roca *et al.*, 2000). In turn, hydrogen peroxide interacts with superoxide anions to give rise to the formation of hydroxyl radicals which are powerful initiators of lipid peroxidation cascade in spermatozoa (Calamera and Quiros, 1996). The accumulation of lipid peroxidation products (malonaldehyde) by spermatozoa has been correlated with inactivation of sperm metabolic enzymes as well as loss of sperm membrane integrity, motility and genomic integrity (Vishwanath and Shannon , 1997 ; Krzyzosiak *et al.*, 2000).

In accord with the forementioned disputations, the current study recorded a pronounced improvement in the motility and viability of frozen-thawed buffalo spermatozoa after inclusion of antioxidants in semen extenders. These results are agree with Beconi, *et al.*, 1993; Sarlos *et al.*, 2002 and Badr, *et al.*, 2003.

Pentoxifylline as an inhibitor of superoxide anions generation (Gavella *et al.*, 1991) , was detected to have the ability to prolong the viability of cryopreserved buffalo (Ramesha *et al.*, 2000) and human (Kolon *et al.*, 1995) spermatozoa. As superoxide dismutase was considered a potent scavenger of superoxide anion radicals (Mennella and Jones, 1980), it was found that this enzyme could improve the viability of frozen-thawed bull (O'Flaherty *et al.*, 1997, 1999) and ram (Maxwell and Watson, 1996) semen.

So, it is concluded that Addition of antioxidants to the freezing extenders of buffalo semen could improve postthaw sperm motility and viability. Superoxide dismutase in citrate-based diluents and pentoxifylline in Tris-based diluents were the most effective semen treatments.

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Table 1 : Effect of antioxidants on freezability of buffalo semen in citrate-based extenders (Means ±SE).

Semen treatments	Sperm motility(%)			Overall means	Postthaw viability index
	After dilution	Before freezing	After thawing		
Control	74.00±1.87	70.00±0.00	34.00±1.87	59.33±4.88 [a]	80.00±8.91 [a]
Pentoxifylline	78.00±2.00	75.00±1.58	44.00±1.87	65.67±4.22 [b]	118.00±5.49 [b]
Superoxide dismutase	80.00±1.58	76.00±1.87	57.00±3.39	71.00±2.98 [c]	150.50±11.76 [c]
EDTA	79.00±1.87	77.00±2.00	51.00±3.32	69.00±3.66 [bc]	137.50±3.35 [bc]
Sodium thiosulfate	81.00±1.00	76.00±1.00	48.00±4.06	68.33±4.10 [bc]	122.00±9.33 [b]
Ascorbic acid	80.00±1.58	75.00±1.58	52.00±3.39	69.00±3.49 [bc]	134.00±10.02 [bc]
Cystine	80.00±3.16	76.00±1.87	53.00±4.06	69.67±3.60 [c]	131.50±13.38 [bc]
Methionine	81.00±1.87	79.00±1.87	47.00±3.00	69.00±4.34 [bc]	116.50±5.68 [b]
Manganous chloride	80.00±1.58	74.00±1.87	52.00±3.74	68.67±3.50 [bc]	136.00±11.93 [bc]

Means with different superscripts in the same column are significantly different ($p<0.01$).

Table 2 : Effect of antioxidants on freezability of buffalo semen in Tris-based extenders (Means ±SE).

Semen treatments	Sperm motility(%)			Overall means	Postthaw viability index
	After dilution	Before freezing	After thawing		
Control	73.00±1.22	71.00±1.00	41.00±1.00	61.67±3.95 [a]	108.60±4.86 [a]
Pentoxifylline	81.00±2.45	81.00±1.87	54.00±1.87	72.00±3.58 [b]	158.00±6.44 [b]
Superoxide dismutase	76.00±1.00	75.00±0.00	47.00±2.00	66.00±3.66 [c]	145.00±6.89 [bcd]
EDTA	75.00±1.58	74.00±1.87	48.00±3.39	65.67±3.58 [c]	130.00±8.66 [cd]
Sodium thiosulfate	77.00±2.00	72.00±1.22	47.00±2.00	65.33±3.63 [c]	140.00±6.89 [bcd]
Ascorbic acid	78.00±2.00	77.00±2.00	49.00±3.32	68.00±3.84 [c]	147.00±6.25 [bc]
Cystine	77.00±1.22	76.00±1.87	47.00±3.00	66.67±3.89 [c]	126.00±9.41 [ad]
Methionine	77.00±2.00	74.00±1.87	44.00±2.45	65.00±4.14 [c]	131.00±6.40 [cd]
Manganous chloride	78.00±1.22	76.00±2.45	51.00±1.00	68.33±3.40 [c]	128.00±5.15 [cd]

Means with different superscripts in the same column are significantly different ($p<0.01$).