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SEMINAL PLASMA GLUTATHIONE PEROXIDASE IN RELATION TO FUNCTIONAL COMPETENCE OF CRYOPRESERVED BUFFALO SPERMATOZOA

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SUMMARY

Glutathione peroxidase (GPx) has a central role in the defense against oxidative damage: however, data on GPx activity in the buffalo semen is limited. To expand this knowledge GPx activity in the seminal plasma of buffalo-bulls was studied. Fifty five semen samples were collected from 19 buffalo-bulls and evaluated before cryopreservation. Aspartate aminotransferase (AST) level was assayed in the seminal plasma before freezing and after freezing- thawing. The results revealed GPx activity (14.59 ± 0.50 U/ml) in seminal plasma that varied with age of the buffalo-bulls. Older age was associated with a decrease in GPx activity. Increasing GPx activity in the seminal plasma was accompanied by a significant decrease in sperm motility of freshly collected semen. The rate of increase in post-thaw sperm motility in response to 5 mM pentoxifylline was high in samples with high GPx activity before freezing. Increased GPx activity before freezing resulted in high percentages of abnormal midpiece and tails post-thawing. Positive correlations existed between AST levels in the seminal plasma (before freezing and after thawing) and frequency of post-thaw sperm cell abnormalities.

INTRODUCTION

The role of reactive oxygen species (ROS) in pathophysiologic functions of spermatozoa has been defined for many years (Bilodeau et al., 2000; Chatterjee and Gagnon, 2001). Mammalian semen is endowed with high levels of ROS scavengers including GPx (Li, 1975; Brown et al.,

1977). Previous investigations indicated that cryopreservation could reduce the activity of GPx in semen with a subsequent decrease in the longevity and fertilizing ability of spermatozoa (Hammerstedt, 1993; Bilodeau et al., 2000).

This investigation was designed to find out whether buffalo semen contains high levels of GPx. If so, what is the relationship between its specific activity in the seminal plasma and the quality of freshly ejaculated semen as well as the functional competence of cryopreserved spermatozoa?

MATERIALS AND METHODS

Animals:

Nineteen healthy buffalo-bulls (4 - 15 years old) from Abassia center for frozen buffalo semen, belonging to the General Organization for Veterinary Services, Ministry of Agriculture, Cairo, were used in this study. Semen collection was performed according to artificial insemination standard procedure. In most samples, the first and the second ejaculates from each animal were pooled. Otherwise, the first or the second ejaculate was used separately.

Semen evaluation and processing:

Fifty-five semen samples were used in this study. Immediately after collection, the semen samples were evaluated using conventional methods. An aliquot of one ml of each semen sample was centrifuged at 3000 rpm for 5 minutes and the supernatant fluid (seminal plasma) was collected and immediately frozen and kept at -20°C until analysis of GPx activity.

Following initial evaluation, semen samples were prepared for dilution in Laiciphos 477-egg yolk-glycerol diluent (Tayseer, 1993). They were diluted (one-step dilution) to a final concentration of 50 - 60 X 10^6 sperm / ml diluent and cooled down to 5°C during one hour (Talevi et al., 1994). After cooling , the diluted semen was packed in 0.5 ml straws during the equilibration period (4 hours). A cooled semen sample of four straws (2 ml) was centrifuged at 3000 rpm for 10 minutes and the supernatant fluid was frozen at -20°C pending analysis for AST before freezing. After equilibration, semen straws were frozen on liquid nitrogen vapor at about -80 to -120°C for 10 minutes, after which the straws were immersed into liquid nitrogen at - 196°C (Sansone et al., 2000).

Post-thaw sperm incubation:

Twenty four hours after freezing straws were thawed in a water bath at 40°C for 30 seconds and then transferred to a water bath (30°C) and incubated for 4 hours. Sperm motility % was evaluated every one hour and viability indices were calculated according to Milovanov et al. (1964). Immediately after thawing, smears stained with nigrosen - eosin stain (Dott and Foster, 1972) were examined for sperm abnormalities. The percentage of spermatozoa with abnormal acrosomes

was recorded in other smears of thawed semen stained by Fast green FCF according to the method of Wells and Awa (1970). In addition, an aliquot (2 ml) of thawed semen was removed just after thawing and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and stored at -20°C until analysis of AST. Following the incubation for four hours, a split sample of thawed semen was diluted (1:1) with tris-based buffer supplemented with 5 mM pentoxifylline (PTx) and sperm motility was determined after 15 minutes. Consequently, the rate of increase in sperm motility was calculated.

GPx assay:

GPx was assayed spectrophotometerically according to Paglia and Valentine (1967) modified by Hopkins and Tudhope (1973) using Ransel Reagents (Randox Laboratories Ltd.).

AST assay:

AST activity (IU / ml) in the seminal plasma before freezing and after thawing was measured spectrophotometerically according to the method of Tietz (1976). The rate of increase in post-thaw activity of extracellular AST (%) was calculated.

Statistical analysis:

The data was divided according to age of the buffalo-bulls into: young (4 - 6 years), middle (7 - 10 years) and old aged bulls (14 - 15 years). Correlation coefficients, t-test and analysis of variance (ANOVA) were calculated using a commercial software (Statistica for windows, 1993).

RESULTS

GPx activity in the seminal plasma of buffalo-bulls was 14.59 ± 0.50 U/ml (Table 1). Fresh semen parameters (motility % and % abnormal tails) differed significantly ($P<0.05$) between the young and old bulls (60.50 ± 0.34 Vs 63.33 ± 1.00 and 35.67 ± 5.19 Vs 14.90 ± 1.92, respectively). GPx activity was significantly ($P<0.05$) lower in old (13.07 ± 1.04 U/ml) than young (16.27 ± 0.21 U/ml) aged bulls (Table 1).

As noticed from Table 2, the rate of increase in post-thaw sperm motility after addition of 5 mM PTx was significantly ($P<0.05$) higher in both young (236.13 ± 59.27%) and middle aged (277.50 ± 76.04%) than old (112.11 ± 27.24%) buffalo-bulls. Concurrently, the activities of GPx in fresh semen samples were significantly ($P<0.05$) higher for young and middle aged than old aged buffalo-bulls (Table 2).

A positive linear correlation was found between frequency of tail abnormalities in fresh semen and GPx activity in seminal plasma (Table 3). The latter showed an inverse relation ($P<0.05$) with the post-thaw sperm motility and viability indices of buffalo spermatozoa (Table 3).

Analysis of variance (Table 4) revealed a significant ($P<0.05$) effect of GPx activity in seminal

plasma of fresh semen on post-thawing percentages of abnormal midpiece and proximal droplets of buffalo spermatozoa. Furthermore, a highly significant (P<0.01) effect of GPx on the rate of increase in AST activity post-thawing was noted (Table 4).

Table 1: Effect of age of buffalo-bulls on fresh semen attributes and GPx activity in seminal plasma (mean ± SEM).

Semen parameters	Buffalo-bulls ages						n	Overall mean
	n	Young (4-6 yr.)	n	Middle (7-10 yr.)	n	Old (14-15 yr.)		
Volume (ml)	20	2.65 ± 0.24	14	2.38 ± 0.20	21	2.71 ± 0.24	55	2.61 ± 0.13
PH	20	6.72 ± 0.02	14	6.74 ± 0.03	21	6.74 ± 0.02	55	6.73 ± 0.01
Conc. (x10^9/ml)	20	1.02 ± 0.10	14	1.22 ± 0.13	21	1.11 ± 0.09	55	1.10 ± 0.06
Mass motility	20	3.05 ± 0.05	14	3.14 ± 0.10	21	3.05 ± 0.0	55	53.07 ± 0.04
% Motility	20	60.50^a ±0.34	14	62.50^{ab} ±1.46	21	63.33^b ±1.00	55	62.09 ± 0.56
% Live sperm	9	86.78 ± 2.30	5	86.20 ± 2.11	10	85,10 ± 1.64	24	85.96 ± 1.14
% abn. acro.	9	0.33 ± 0.17	5	0.80 ± 0.58	10	1.00 ± 0.37	24	0.71 ± 0.20
% abn. head	9	3.67 ± 0.83	5	3.40 ± 1.17	10	2.50 ± 0.58	24	3.13 ± 0.46
% abn. midpiece	9	9.56 ± 1.19	5	13.20 ± 2.73	10	8.20 ± 0.88	24	9.75 ± 0.86
% abn. tail	9	35.67^a±5.19	5	24.60^{ab} ±4.27	10	14.90^b±1.92	24	24.71 ± 2.91
% proximal drop.	9	1.22 ± 0.43	5	1.40 ± 0.75	10	0.90 ± 0.28	24	1.13 ± 0.24
% distal drop.	9	1.33 ± 0.75	5	0.40 ± 0.40	10	0.10 ± 0.10	24	0.63 ± 0.31
GPx activity	17	16.27^a±0.21	14	14.63^a ± 0.89	19	13.07^b±1.04	50	14.59 ± 0.50

Means in the same row not sharing common superscript letters differ significantly P<0.05.

A negative linear correlation (r = - 0.61; P<0.05) was detected between AST level before freezing and rate of increase in AST activity post-thawing (Table 5). However, the relation between AST level before freezing and AST level post-thawing in total and middle aged buffalo-bulls was positive (r = 0.93; P<0.05). A positive relation (r = 0.85; P<0.05) was found between AST level post-thawing and post-thaw abnormal tail percent in young buffalo-bulls. Furthermore,

a positive linear correlation (r = 0.68; P<0.05) was calculated between rate of increase in AST activity post-thawing and post-thaw abnormal head percent in old buffalo-bulls (Table 5).

Table 2: Effect of age of buffalo-bulls on post-thaw semen parameters and increase in the AST level in seminal plasma (mean ± SEM).

Post-thaw Semen parameters	Buffalo-bulls ages						n	Total
	n	Young (4-6 yr.)	n	Middle (7-10 yr.)	n	Old (14-15 yr.)		
Motility after dil.	20	70.25 ± 1.68	14	69.64±1.77	21	71.67±0.80	55	70.64±0.81
Motility after cool.	20	70.00 ± 1.74	14	69.64±1.77	21	71.43±0.86	55	70.46±0.83
Post-thaw motility	20	41.00 ± 2.01	14	45.00±2.10	21	42.86±1.94	55	42.73±1.17
Motility after 4 h	20	18.50 ± 2.74	14	17.14±3.09	21	19.76±2.22	55	18.64±1.50
Viability indices	20	125.25±10.73	14	118.21 ± 12.42	21	136.91±10.19	55	127.91 ± 6.32
Motility after PTx	20	40.50 ± 2.83	14	44.64 ± 4.37	21	36.19 ± 3.22	55	39.91 ± 1.97
% increase in motility	20	236.13^{a}± 59.27	14	277.50^{a}± 76.04	21	112.11b± 27.24	55	199.31± 31.63
% abn. acro.	9	40.67 ± 4.13	5	34.80 ± 2.56	10	38.30 ± 3.66	24	38.46 ± 2.25
% abn. head	9	6.44 ± 1.02	5	6.60 ± 2.01	10	8.10 ± 1.32	24	7.17 ± 0.77
% abn. midpiece	9	13.56 ± 1.42	5	13.20 ± 3.51	10	10.80 ± 0.89	24	12.33 ± 0.95
% abn. tail9	9	37.22 ± 5.13	5	27.60 ± 7.22	10	23.70 ± 3.66	24	29.58 ± 3.02
% proximal drop.	9	00.22 ± 0.15	5	0.00 ± 0.00	10	0.50 ± 0.27	24	0.29 ± 0.13
% distal drop.	9	00.33 ± 0.24	5	0.20 ± 0.20	10	0.00 ± 0.00	24	0.17 ± 0.10
GPx activity	17	16.27^{a} ±0.21	14	14.63^{a} ± 0.89	19	13.07^{b} ±1.04	50	14.59 ± 0.50
AST before freez.	17	00.14 ± 0.01	14	0.12 ± 0.01	19	0.13 ± 0.01	50	0.13 ± 0.01
AST after freez.	17	00.16 ± 0.01	14	0.14 ± 0.01	19	0.14 ± 0.01	50	0.15 ± 0.01
% AST increase	17	22.76 ± 9.89	14	27.19 ± 10.97	19	21.00 ± 8.66	50	23.33 ± 5.51

Means in the same row not sharing a common superscript letter differed significantly P<0.05

Table 3: Correlation Coefficients between GPx activity in seminal plasma and semen parameters of buffalo-bulls.

Type of correlation	n	Correlation Coefficients
GPx activity X % abnormal tail in fresh semen	24	r = 0.46*
GPx activity X post-thaw sperm motility % at 0 h	24	r = - 0.61*
GPx activity X post-thaw sperm motility % at 1 h	24	r = - 0.53*
GPx activity X post-thaw sperm motility % at 2 h	24	r = - 0.59*
GPx activity X post-thaw sperm motility % at 3 h	24	r = - 0.46*
GPx activity X post-thaw sperm motility % at 4 h	24	r = - 0.51*
GPx activity X post-thaw viability indices	24	r = - 0.58*
GPx activity X post-thaw motility % at 0 h in old aged bulls	19	r = - 0.70*
GPx activity X post-thaw motility % at 2 h in old aged bulls	9	r = - 0.66*
GPx activity X post-thaw motility % at 4 h in old aged bulls	19	r = - 0.72*
GPx activity X post-thaw viability indices in old aged bulls	19	r = - 0.65*
GPx activity X motility response to PTx in old aged bulls	19	r = - 0.82*

* P<0.05

Table 4: Analysis of variance for the effect of GPx activity on post-thaw semen parameters of buffalo-bulls.

Variables	DF	MS	F-value
Post-thaw abnormal midpiece %	4	59.11	4.10*
Error	13	14.41	
Post-thaw proximal droplets %	4	1.11	4.06*
Error	13	0.27	
Rate of AST increase	6	3747.09	4.46**
Error	33	840.10	
Post-thaw abnormal midpiece % in old aged buffalo-bulls	3	8.79	10.54*
Error	3	0.83	

* P<0.05

** P<0.01

Table 5: Correlation Coefficients between seminal plasma AST activities and semen parameters in the buffalo-bulls.

Type of correlation	n	Correlation Coefficients
AST activity before freezing X rate of AST increase post-thawing	50	r = - 0.61*
AST activity before freezing X AST activity post-thawing	50	r = 0.93*
AST activity before freezing X post-thaw AST activity in middle aged bulls	14	r = 0.93*
AST activity post-thawing X post-thaw abnormal tail% in young aged bulls	8	r = 0.85*
Rate of AST increase post-thawing X post-thaw abnormal head % in old aged bulls	10	r = 0.68*

* P<0.05

DISCUSSION

The current study, for the first time, provides evidence for the presence of GPx activity in seminal plasma of buffalo-bulls. Several authors (Brown et al., 1977; Smith et al., 1979; Bilodeau et al., 2000) reported similar findings in seminal plasma of bulls. In fact, bovine seminal plasma possesses more than tenfold the GPx activity present in seminal plasma of human, ram and stallion (Kantola et al., 1988; Saaranen et al., 1989).

In the present work, there was a marked reduction in GPx activity in seminal plasma with increasing age of buffalo-bulls. Similar finding was reported in bulls (Kelso et al., 1997).

The current study, indeed, revealed higher motility of spermatozoa and lower percentage of abnormal tails in old than young buffalo-bulls. On the contrary, Kelso et al. (1997) found a lower (P<0.05) sperm motility in old than young aged bulls.

In this study, addition of 5 mM PTx exerted a marked increase in post-thaw sperm motility. PTx appeared to be effective for preserving sperm motility in vitro (Okada et al., 1997; Gradil and Ball, 2000). The discrepancy between the rates of increase in sperm motility on using PTx in the different age groups of buffalo-bulls might be attributed to the GPx activity in seminal plasma before freezing. GPx may provide a mechanism that protects the sperm membranes from oxidative damage and subsequent cell death (Senger, 1980). The enzyme may also contribute to good viability of bovine spermatozoa in various steps of cryopreservation (Kantola et al., 1988).

Data presented in this study suggest that increase of GPx activity in seminal plasma before freezing

accompanied with a decrease of sperm motility after freezing - thawing and incubation at 30°C. Similar results were reported in bulls (Slaweta et al., 1988). Also from the present results, GPx activity exerted a significant ($P<0.05$) effect on the percent of abnormal midpiece post-thawing. This is indicated by the tendency (however non significant) of increase in post-thaw abnormal midpiece percent with increasing activity of GPx. Nevertheless, Kendall et al. (2000) found a significant effect of GPx activity on increasing proportion of live sperm and proportion of intact sperm membranes in rams.

In the current work, there was a highly significant ($P<0.01$) effect of GPx activity on the rate of AST increase post-thawing. This may be explained by the reduction in post-thaw sperm motility and viability indices with increasing GPx activity before cryopreservation.

In view of our findings, GPx is not potent enough to prevent sperm damage during cryopreservation. Freezing and thawing significantly reduce GPx activity and this is detrimental to sperm function (Bilodeau et al., 2000). This is indicated, in the present study, by the positive correlations between AST activity in seminal plasma before freezing and after thawing and sperm cell abnormalities.

It is now generally recognized that buffalo seminal plasma contains high activity of GPx enzyme that varied with the age of the buffalo-bulls.

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