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SEMINAL PLASMA SUPEROXIDE DISMUTASE IN RELATION TO FUNCTIONAL COMPETENCE OF CRYOPRESERVED BUFFALO SPERMATOZOA

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SUMMARY

Buffalo sperm fertilizing capacity principally depends on sperm motility and membrane integrity. Peroxidative damage induced by Reactive Oxygen Species (ROS) has been proposed as one of the major causes of defective sperm function. The objective of the present study was to investigate the presence of superoxide dismutase (SOD) in the buffalo semen and its role in the defense against oxidative damage. Fifty semen samples were collected from 16 buffalo-bulls (4 - 15 years old) and evaluated before cryopreservation. The activity of SOD and glutathione-S-transferase (GST) as well as the levels of glutathione (GSH) and malondialdehyde (MDA) were assayed in the seminal plasma before freezing. Aspartate aminotransferase (AST) and cholesterol contents were assayed in seminal plasma before freezing and after freezing- thawing. The results revealed the presence of SOD activity (0.07 ± 0.01 U/mg protein) in the seminal plasma of buffalo-bulls.The increasing SOD activity accompanied high sperm motility after dilution and before freezing. Semen evaluating parameters varied with the age of bulls and high values were found in young than old aged animals. Positive correlations existed between activities of SOD and GST in the seminal plasma and inverse relations were detected between SOD activity and both cholesterol efflux and frequency of post-thaw sperm cell abnormalities. The relations between post-thaw sperm cell abnormalities and each of MDA, AST and cholesterol were positive. The rate of increase in post-thaw sperm motility in response to 5 mM pentoxifylline was high in samples with low MDA level before freezing.

INTRODUCTION

There is growing evidence that oxidative stress significantly impairs sperm functions (Ochsendorf et al., 1998). Due to their high content of polyunsaturated fatty acids spermatozoa are susceptible to damage induced by reactive oxygen species (De Lamirande and Gagnon, 1995). The process of peroxidation is accompanied by extensive structural alterations, particularly in the acrosomal region of the sperm cell, a rapid and irreversible loss of motility, a profound change in metabolism, and a high rate of leakage of intracellular sperm constituents (Jones and Mann, 1977).

Bovine spermatozoa are poorly adapted to metabolize the toxic hydrogen peroxide (Bilodeau et al., 2000). Frozen-thawed bull spermatozoa are more easily peroxidized than fresh spermatozoa (Trinchero et al., 1990) and this is associated with production of oxygen free radicals that leads to reduction in sperm membrane fluidity and decrease in sperm function following cryopreservation (Chattergiee and Gagnon, 2001).

One of the main antioxidant enzymes involved in ROS detoxification in bovine semen is SOD (Bilodeau et al., 2000).

The present work was undertaken to investigate the presence of SOD in buffalo semen and its influence on the motility, viability, membrane integrity and morphologic abnormalities of spermatozoa after thawing.

MATERIALS AND METHODS

Animals:

Sixteen 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 procedures. In most experiments, the first and the second ejaculates from each animal were pooled. Otherwise, either the first or the second ejaculate was used separately.

Semen evaluation and processing:

Fifty semen samples were used in this study. Immediately after collection, 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, immediately frozen and kept at-20°C until analysis of SOD and GST activities and GSH and MDA levels.

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 within one hour (Talevi et al., 1994). After cooling , the diluted semen was packaged in 0.

ml straws during the equilibration period (4 hours). Semen from four straws (2 ml) was centrifuged at 3000 rpm for 10 minutes and the supernatant fluid was frozen at -20°C pending analysis for cholesterol and 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 cholesterol and AST. Following 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.

SOD and GST assays:

The activities of SOD (U/mg protein) and GST (U/mg protein/minute) were estimated as described by Habig et al. (1974) and Giannopolitis and Ries (1977) and the enzyme activities were expressed as unit / mg of protein. Protein was determined by the method of Bradford (1976).

GSH and MDA levels:

The level of GSH (µmol/ml) was measured according to the method of Srivastava and Beutler (1968). Seminal plasma MDA level (nmol/ml) was determined by the method of Yoshioka et al. (1979) and expressed as nmol MDA / ml tetramethoxy propane.

AST assay:

AST activity (U/L) 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.

Cholesterol level:

The cholesterol level (mg/dL) was measured according to the method described by Allain et al. (1974). The rate of increase in post-thaw level of cholesterol (%) 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

SOD activity in the seminal plasma of buffalo-bulls was 0.07± 0.01U/mg protein (Table 1). Fresh semen parameters (sperm cell concentration (X 10^9/ml), percent abnormal acrosomes and percent abnormal tails differed significantly (P<0.05) between young and old aged bulls (1.19 ± 0.09 Vs 0.86 ± 0.08, 0.25 ± 0.11 Vs 1.17± 0.27 and 29.56 ± 2.01 Vs 12.83 ±1.66, respectively). Concurrently, sperm motility % differed significantly (P<0.05) between middle and old aged buffalo-bulls. SOD activity was, however, non significantly, lower in old (0.05 ± 0.01 U/ mg protein) than in young (0.07 ± 0.02 U/ mg protein) and middle (0.08± 0.01 U/ mg protein) aged bulls (Table 1).

As presented in Table 2, sperm motility (%) after dilution as well as after cooling differed significantly (P<0.05) between the middle and old aged bulls (75.00 ±1.58 Vs 68.33 ± 1.53 and 75.00 ±1.58 Vs 68.33 ± 1.99, respectively). Simultaneously, the activities of SOD in fresh semen samples were higher in middle than in old aged buffalo-bulls (Table 2).

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

Semen parameters	Buffalo-bulls ages						Overall mean
	Young(4-6 years)		Middle(7-10 years)		Old (14-15 years)		
	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	
Volume (ml)	19	2.92 ± 0.21	16	2.45 ± 0.16	15	2.90 ± 0.20	2.77 ± 0.11
pH	19	6.69 ± 0.02	16	6.69 ± 0.02	15	6.71 ± 0.03	6.70 ± 0.01
Conc. (X10^9/ml)	19	1.19^a ± 0.09	16	1.12^a ± 0.12	15	0.86^b ± 0.08	1.07 ± 0.06
Mass motility	19	3.11 ± 0.11	16	3.25 ± 0.11	15	3.07 ± 0.07	3.14 ± 0.06
% Motility	19	64.47^a±1.32	16	68.44^b±1.35	15	63.33^a±1.26	65.40 ± 0.81
Live sperm	16	88.44±1.23	12	89.42 ±1.00	12	86.42 ±1.25	88.13 ±0.70
% abnormal acrosome	16	0.25^a± 0.11	12	0.75^b± 0.28	12	1.17^b± 0.27	0.68 ± 0.14
% abnormal head	16	2.81 ±0.53	12	3.50 ±0.72	12	3.08 ± 0.60	3.10 ± 0.34
% abnormal midpiece	16	9.56±0.89	12	11.25 ± 1.88	12	8.25 ± 0.96	9.68 ± 0.73
% abnormal tail1	16	29.56^a±2.01	12	20.67^b±1.63	12	12.83^b±1.66	21.88 ±1.52
% proximal droplets	16	1.38±0.26	12	1.58±047	12	1.08 ± 0.26	1.35 ± 019
% distal droplets	16	0.81±0.28	12	0.75 ± 0.22	12	0.42 ± 0.15	0.68 ± 0.14
SOD activity	19	0.70^a±0.02	16	0.08^b±0.01	15	0.05^{ab}±0.01	0.07 ± 0.01

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

Table (2): Effect of age of buffalo-bulls on post-thaw semen parameters and enzymes activity in seminal plasma (means ± SEM).

Post-thaw Semen parameters	Buffalo-bulls ages						Total
	Young(4-6 years)		Middle(7-10 years)		Old (14-15 years)		
	n	Mean ± SEM	n	Mean ± SEM	n	Mean ± SEM	
Motility after dilution	19	70.79^{ab} ± 0.21	16	75.00^{a} ± 1.58	15	69.33^{b} ± 1.53	71.70 ± 1.03
Motility after cooling	19	70.79^{ab} ± 1.93	16	75.00^{a} ± 1.58	15	68.33^{b} ± 1.99	71.20 ± 1.12
Post-thaw motility	16	36.88 ± 2.62	12	37.92 ± 3.17	12	37.50 ± 2.42	37.38 ± 1.55
Motility after	16	14.06 ± 2.71	12	15.83 ± 4.39	12	14.17± 3.36	14.63 ± 1.93
Viability indices	16	102.19±11.60	12	114.79±19.12	12	99.58 ±14.77	105.19±8.44
Motility after PTx	16	42.50 ± 3.85	12	41.25± 5.33	12	34.58± 5.49	39.75 ± 2.74
% increase in motility	16	343.33± 77.13	12	305.05± 74.09	12	289.58±113.22	315.72± 49.78
% abnormal acrosome	16	37.81 ± 1.88	12	35.75 ± 2.25	12	34.42±1.96	36.18± 1.16
% abnormal head	16	07.31 ± 0.66	12	07.25 ± 1.30	12	09.58 ± 139	07.98 ± 0.63
% abnormal midpiece	16	13.25 ± 0.89	12	14.83 ± 1.67	12	11.50 ± 0.72	13.20 ± 0.67
% abnormal tail1	16	35.98^{a} ± 1.87	12	29.42^{ab} ± 3.24	12	24.08^{b} ± 3.32	30.43 ± 1.37
SOD activity	19	00.07^{a}± 0.02	16	00.08^{b}± 0.01	15	0.05^{ab}± 0.01	00.07 ± 0.01
GSH level	19	08.41 ± 0.93	15	08.22 ± 0.96	15	08.30 ± 0.87	08.32 ± 0.53
GST activity	19	00.23 ± 0.05	14	00.19 ± 0.02	15	00.15 ± 0.02	00.19 ± 0.02
MDA level	19	02.41 ± 0.23	16	02.05 ± 0.16	15	02.75 ± 0.28	02.40 ± 0.14
Cholesterol before freezing	16	79.64 ± 5.55	12	84.41 ± 4.94	12	86.31 ± 3.96	83.07 ± 2.89
Cholesterol After thawing	19	101.22 ± 4.61	16	101.72 ± 3.37	15	102.03 ± 3.43	101.62± 2.26
Cholesterol increase	16	29.59 5.27	12	28.02 ± 6.41	12	21.48 ± 5.26	26.68 ± 3.22
AST before freezing	16	223.20±21.25	12	264.91±25.64	12	218.70±24.07	234.36± 13.58
AST after thawing	19	320.61±24.08	16	332.61±32.81	15	327.94 ± 27.90	326.65±15.91
AST increase	16	56.39±14.32	12	36.26 ± 11.43	12	73.02 ± 22.12	55.34 ± 9.45

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

A positive linear correlation (P<0.05) was found between SOD activity and both of GSH level and GST activity in seminal plasma (Table 3). Conversely, SOD activity showed an inverse relations (P<0.05) with cholesterol level before freezing, after thawing, and post-thaw abnormal tails (%) of buffalo spermatozoa (Table 3).

Table 4 shows a negative linear correlation (P<0.05) between GSH level in seminal plasma and percent abnormal tails in fresh and post-thawed ejaculates. Moreover, negative correlations (P<0.05) were found between GST activity and both of cholesterol level and AST activity in fresh and post-thawed ejaculates (Table 4). GST activity showed a negative correlation (P<0.05) with percent post-thaw abnormal tails and a positive correlation (P<0.05) with percent increase in motility after addition of PTx to the thawed samples (Table 4).

Table (3): Correlation Coefficients between SOD activity in seminal plasma and semen parameters of buffalo-bulls

Type of correlation	n	Correlation Coefficients
SOD activity X GSH level in all ejaculates	49	r = 0.36*
SOD activity X GST activity in all ejaculates	50	r = 0.66*
SOD activity X cholesterol level before freezing in all ejaculates	40	r = - 0.40*
SOD activity X GST activity in young aged bulls	19	r = 0.65*
SOD activity X GST activity in middle aged bulls	16	r = 0.68*
SOD activity X post-thaw cholesterol level in middle aged bulls	16	r = - 0.72*
SOD activity X post-thaw abnormal tails (%) in middle aged bulls	12	r = - 0.62*
SOD activity X GST activity in old aged bulls	15	r = 0.77*

* P<0.05

Table (4): Correlation Coefficients between GSH level, GST activity in seminal plasma and semen parameters of buffalo-bulls.

Type of correlation	n	Correlation Coefficients
GSH level X abnormal tails % in all ejaculates	39	r = - 0.35*
GSH level X post-thaw abnormal tails % in all ejaculates	39	r = - 0.54*
GSH level X post-thaw abnormal tails % in young aged bulls	16	r = - 0.62*
GSH level X post-thaw abnormal tails % in middle aged bulls	11	r = - 0.66*
GST activity X cholesterol level before freezing in all ejaculates	40	r = - 0.43*
GST activity X % motility increase due to PTx in young aged bulls	16	r = 0.52*
GST activity X post-thaw abnormal tails % in middle aged bulls	12	r = - 0.61*
GST activity X cholesterol level before freezing in old aged bulls	12	r = - 0.59*
GST activity X post-thaw cholesterol level in old aged bulls	15	r = - 0.59*
GST activity X AST activity before freezing in old aged bulls	12	r = - 0.69*
GST activity X post-thaw AST activity in old aged bulls	15	r = - 0.64*

* P<0.05

Table (5): Correlation Coefficients between MDA, AST and cholesterol levels in seminal plasma and semen parameters of buffalo-bulls.

Type of correlation	n	Correlation Coefficients
MDA level X percent motility increase due to PTx in young aged bulls	16	r = - 0.65*
MDA level X post-thaw abnormal acrosomes (%) in young aged bulls	16	r = 0.55*
MDA level X post-thaw abnormal tails (%) in old aged bulls	12	r = 0.61*
AST activity before freezing X post-thaw abnormal tails	12	r = 0.76*
Cholesterol level before freezing X post-thaw abnormal tails (%) in old aged bulls	12	r = 0.60*

* P<0.05

A positive linear correlation (P<0.05) was found between post-thaw sperm cell abnormalities and each of MDA level, AST activity and cholesterol level (Table 5). However, the relation between MDA level and the % increase in sperm motility after addition of PTx to the thawed samples was negative (r = - 0.65; P<0.05).

Analysis of variance (Table 6) revealed a significant (P<0.05) effect of MDA level in seminal plasma of fresh semen on post-thaw sperm motility % at 0 hour in all ejaculates, viability indices and % increase in sperm motility after addition of PTx to thawed samples in young bulls. Furthermore, this effect was highly significant (P<0.01) on post-thaw sperm motility % at 2 hours in young bulls (Table 6).

As shows in Table 7, analysis of variance revealed a highly significant (P<0.01) effect of cholesterol level before freezing on sperm motility %. This effect was significant (P<0.05) on post-thaw abnormal midpiece % in all ejaculates (Table 7).

Table(6): Analysis of variance for the effect of MDA level on post-thaw semen parameters

Variables	DF	MS	F-value
Post-thaw motility % at 0 h in all ejaculates	4	236.18	2.87*
Error	34	82.30	
Post-thaw motility % at 2 h in young aged bulls	2	625.00	10.71**
Error	12	58.30	
Viability indices in young aged bulls	2	5498.60	4.19*
Error	12	1311.90	
% motility increase due to PTx in young aged bulls	2	281193.10	4.21*
Error	12	66850.50	

* P<0.05

Table (7): Analysis of variance for the effect of cholesterol level before freezing on semen parameters of buffalo-bulls.

Variables	DF	MS	F-value
% sperm motility in all ejaculates	9	58.90	13.20**
Error	7	4.50	
Post-thaw abnormal miidpiece % in all ejaculates	9	34.90	4.97*
Error	7	7.00	

* P<0.05

** P<0.01

Table (8): Analysis of variance for the effect of post-thaw cholesterol level on semen parameters of buffalo-bulls.

The variables	DF	MS	F-value
Post-thaw abnormal tails % in all ejaculates	14	173.20	5.57*
Error	6	31.11	

* P<0.05

Analysis of variance (Table 8) revealed a significant (P<0.05) effect of post-thaw cholesterol level on post-thaw abnormal tails % in all semen samples. Moreover, as shown in data presented in Table 9 there is a significant (P<0.05) effect of post-thaw increase in cholesterol rate on post-thaw abnormal acrosomes % in all ejaculates. Analysis of variance (Table 10) revealed a significant (P<0.05) effect of post-thaw % increase in AST rate on both post-thaw sperm motility % at 4 hours in all ejaculates and post-thaw motility response to PTx in all semen samples.

Table (9): Analysis of variance for the effect of post-thaw % increase in cholesterol rate on semen parameters of buffalo-bulls.

The variables	DF	MS	F-value
Post-thaw abnormal acrosomes % in all ejaculates	12	45.40	36.30*
Error	2	1.30	

* P<0.05

Table (10): Analysis of variance for the effect of post-thaw % increase in AST rate on semen parameters of buffalo-bulls.

Variables	DF	MS	F-value
Post-thaw motility % at 4 h in all ejaculates	3	622.20	74.67*
Error	2	8.33	
Post-thaw motility response to PTx in all ejaculates	3	594.40	71.33*"
Error	2	8.33	

* P<0.05

DISCUSSION

Literature survey did not clarify any information regarding the distribution and properties of SOD in buffalo semen. However, the present study identified a high activity of this enzyme in the seminal plasma of buffalo-bulls.

In this study, we found a clear reduction in SOD activity in seminal plasma with increasing age of buffalo-bulls. Kelso et al. (1997) reported similar finding in bulls.

The present results clearly showed a lower sperm cell concentrations, motility and abnormal tails percent in old than young and middle aged buffalo-bulls. These findings accompanied a reduction in SOD activity in seminal plasma with increasing animals age. Similar findings were published in bulls (Kelso et al., 1997).

The marked increase in sperm motility after dilution (and before freezing) in middle than old aged buffalo-bulls could be attributed to the high activity of SOD in seminal plasma of middle aged bulls. SOD was found to increase the duration of motility in sperm preparations (Lindemann et al., 1988) as it is on the front line of defense mechanism against effects of reactive oxygen species (Bilodeau et al., 2000).

Results of our study clarified that the increase in SOD activity in seminal plasma was accompanied with concomitant increase in GSH level and GST activity. Also, there was a significant inverse relation between both SOD activity and GSH level in seminal plasma and both of cholesterol levels and post-thaw sperm tails abnormalities. GSH and SOD could have therapeutic potential for better functional competence of spermatozoa (Griveau and Le-Lannou, 1994). Concerning the influence of SOD on functional competence of buffalo spermatozoa, It exerted a significant improvement in post-thaw sperm motility and plasma membrane integrity as well as a significant decrease in post-thaw sperm abnormalities (Lindemann et al., 1988; O' Flaherty et al, 1997, 1999). The presence of GSH in seminal plasma resulted in protection of sperm motility (UcKun et al., 2002) and restoring the functional constitution of polyunsaturated fatty acids in the cell membrane of spermatozoa (Lenzi et al., 1996).

In the present study, there was a synergistic effect between GST and the added PTx for increasing post-thaw sperm motility. GST is a member of the glutathione cycle that is responsible for detoxification of hydrogen peroxide (Li, 1975). The added PTx, on the other hand, keeps sperm motility, prevents membrane lipid peroxidation (Bell et al., 1993; Yovich, 1993; McKinney et al., 1996; Okada et al., 1997) and significantly increases sperm intrinsic relative force (Patrizio et al., 2000).

The present results showed that lipid peroxidation potential (MDA formation) correlated positively with sperm cell abnormalities and negatively with post-thaw sperm motility after the addition of PTx. Also, MDA level exerted a significant ($P<0.05$) effect on post-thaw sperm motility. Simlar findings were reported in human (Rao et al., 1989; Gomez et al., 1998). Poor sperm motility was linked with membrane fragility and spermatozoa with abnormalities probably had membrane and/or cytoplasmic antioxidant system defects (Slaweta et al., 1988; Rao et al., 1989; Aitken et al., 1993).

In the current work, cholesterol levels and AST activities showed a significant ($P<0.05$) effect on sperm motility and sperm cell abnormalities. Both cholesterol efflux and AST activities are indicators of sperm membrane damage by oxidative stress of superoxide anions (Gagnon and Lamirande, 1995).

It is tempting to speculate that high SOD activities and low MDA levels in buffalo semen before freezing affect sperm motility and sperm cell abnormalities after freezing-thawing and incubation at 30°C. SOD activity of a fresh semen sample appears to be a good predictor of the life time of that particular sample and so may prove useful in semen analysis.

REFERENCES

Aitken, R.J., Buckingham, D. and Harkiss, D. (1993): Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J. Reprod. Fert., 97: 441 - 450.

Allain, C.C., Poom, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974): Enzymatic determination of total serum cholesterol. Clin. Chem., 20 (4): 470 -475.

Bell, M., Wang, R., Hellstrom, W.J. and Sikka, S.C. (1993): Effect of cryoprotective additives and cryopreservstion protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. J. Androl., 14 (6): 472 - 478.

Bilodeau, J., Chatterjee, S., Sirard, M. and Gagnon, C. (2000): Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev., 55: 282 - 288.

Bradford, M.M. (1976): A rapid and sensitive method of the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.,72:248 - 254.

Chatterjee, S. and Gagnon, C. (2001): Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev., 59: 451 - 458.

De-Lamirande, E. and Gagnon, C. (1995): Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum. Reprod. 10 Suppl 1: 15 - 21.

Dott, H.M. and Foster, G.C. (1972): A technique for studying the morphology of mammalian spermatozoa which

are cosinophilic in a differential "live / dead" stain. J. Reprod. Fert., 29: 4443 - 4445.

Gagnon, C. and Lamirande, E. (1995): The effect of reactive oxygen species on sperm motility. Comptes Rendus de l' Academie d' Agriculture de France, 81: 159 - 165.

Giannopolitis, C.N. and Ries, S.K. (1977): Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol., 59: 309 - 314.

Gomez, E., Irvine, D.S. and Aitken, R.J. (1998): Evaluation of spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. Int. J. Androl., 21 (2): 81 - 94.

Griveau, J.F. and Le-Lannou, D. (1994): Effects of antioxidants on human sperm preparation techniqqes. Int. J. Androl., 17 (5): 225 - 231.

Habig, H.W., Pabest, J.M. and Jakoby, B.W. (1974): Glutathione-S-transferase. J. Biol. Chem., 249 (22): 7130 - 7139.

Jones, R. and Mann, T. (1977): Damage to ram spermatozoa by peroxidation of endogenous phospholipids. J. Reprod. Fert., 50: 261- 268.

Kelso, K.A., Redpath, A., Noble, R.C. and Speake, B.K. (1997): Lipid and antioxidant changes in spermatozoa and seminal plasma throughout the reproductive period of bulls. J. Reprod. Fert., 109 (1): 1 - 6.

Lenzi, A., Picardo,M., Gandini, L. and Dondero, F. (1996): Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum. Reprod. Update. 2 (3): 246- 256.

Li, T.K. (1975): The glutathione and thiol content of mammalian spermatozoa and seminal plasma. Biol. Reprod. 12: 641-646.

Lindemann, C.B., O'Brien, J.A. and Giblin, F.J. (1988): An investigation of the effectiveness of certain antioxidants in preserving the motility of reactivated bull sperm models. Biol. Reprod. 38 (1): 114 - 120.

McKinney, K.A., Lewis, S.E. and Thomposon, W. (1996): The effects of pentoxifylline on the generation of reactive oxygen species and lipid peroxidation in human spermatozoa. Andrologia, 28 (1): 15 - 20.

Milovanov, V.K., Trubkin, G.D., Chubenko, N.S., Tsvetkov, I.V., Erzin, Z.K. and Meschankin, A.B. (1964): Artificial insemination of livestock in the U.S.S.R. Israel Program For Scientific Translations, Jerusalem, pp. 102-104.

Ochsendorf, F.R., Buhl, R., Bastlein, A. and Beschmann, H. (1998): Glutathione in spermatozoa and seminal plasma of infertile men. Hum. Reprod., 13 (2): 353 - 359.

O'Flaherty, C.M., Beorlegui, N.B. and Beconi, M.T. (1999): Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology, 52 (2): 289 - 301.

O'Flaherty, C.M., Beconi, M.T. and Beorlegui, N.B. (1997): Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia, 29: 269-275.

Okada, H., Tatsumi, N., Kanzaki, M., Fujisawa, M., Arakawa, S. and Kamidono, S. (1997): Formation of reactive oxygen species by spermatozoa from asthenospermic patients: response to treatment with pentoxifylline. J. Urol., 157 (6): 2140- 2146.

Patrizio, P., Liu, Y., Sonek, G.J., Berns, M.W. and Tadir, Y.(2000): Effect of pentoxifylline on the intrinsic swimming forces of human sperm assessed by optical tweezers. J. Androl. 21 (5): 753 - 756.

Rao, B., Soufir, J.C., Martin, M. and David, G. (1989): Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. Gamete Res. 24 (2): 127 - 134.

Sansone, G., Nastri, M.J.F. and Fabbrocini, A. (2000): Storage of buffalo (Bubalus bubalis) semen. In Animal reproduction science, Special issue, K.L. Macmillan (ed.). Elsevier co.

Slaweta, R., Wasowicz, W. and Laskowska, T. (1988): Selenium content, glutathione peroxidase activity, and lipid peroxide level in fresh bull semen and its relationship to motility of spermatozoa after freezing - thawing. J. Vet. Med. A, 35: 455 - 460.

Srivastava, S.K. and Beutler, E. (1968): Accurate measurement of oxidized glutathione content of human, rabbit and rat red blood cells and tissues. Anal. Biochem., 25 (1): 70 - 76.

Statistica for Windows (1993): Release 4.5 A, statsoft, Inc.

Talevi, R., Pelosi, S., Sansone, G., Grasso, F. and Matassino, D. (1994): Effects of different pre-freezing rates on buffalo sperm. Motility and ultrastructure preservation. Proc. 4th Int. Buffalo Congr., Sao Paulo, Brazil, Vol. 3, pp. 537 - 539.

Tayseer, I.M. Ismail (1993): Studies on thawing of frozen buffalo semen. Ph. D. Thesis, Faculty of Veterinary Medicine, Cairo University.

Tietz, N.W. (1976): Fundamentals of clinical chemistry. W.B. Saunders Company, Philadelphia.

Trinchero, G.D., Affranchino, M.A., Schang, L.M. and Beconi, M.T. (1990): Antioxidant effect of bovine spermatozoa on lipid peroxidation. Com. Biol., 8: 339 - 350.

Uckun, F.M., Liu, X.P. and D'Crus, O.J. (2002): Human sperm immobilizing activity of aminophenyl arsenic acid and its- -N-substituted quinazoline, pyrimidine, and purine derivatives: protective effect of glutathione. Reprod. Toxicol., 16 (1): 57- 64.

Wells, M. E. and Awa, O.A. (1970): New technique for assessing acrosomal characteristics of spermatozoa. J. Dairy Sci., 53: 227 - 232.

Yoshioka, T., Kawada, K., Shimada, T. and Mori, M (1979): Lipid peroxidation in maternal and cord blood and protective mechanism against activated oxygen toxicity in the blood. Am. J. Obstet. Gynacol., 135 (3): 372 - 376.

Yovich, J.L. (1993): Pentoxifylline actions and applications in assisted reproduction. Hum. Reprod., 8 (11): 1786 -1791