QUALITY OF FROZEN BUFFALO-BULL SEMEN IN RELATION TO ADDITION OF ANTIOXIDANTS (GLUTATHIONE AND ASCORBIC ACID)

BY

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ABSTRACT

The study aimed to investigate the effect of antioxidants addition to buffalo-bull semen during deep freezing preservation on post thaw sperm motility, morphology, acrosomal membrane integrity, sperm penetration through cervical mucus, the release of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Lactate Dehydrogenase (LDH) enzymes from sperm cells to the medium. Semen samples were collected from three clinically normal buffalo-bulls using artificial vagina, twice weekly; two ejaculates per time, for two weeks. Ejaculates were assessed immediately after collection using standard routine evaluation criteria. Ejaculates had motility less than 60% were discarded. Other ejaculates were pooled and diluted to concentration of 100 million sperm/ml in egg yolk citrate glycerol diluent. Extended samples were divided into five equal fractions. 1st fraction served as control, 2nd and 3rd fractions, reduced glutathione (GSH) was added at concentrations of 2.5 and 5.0 mM respectively, 4th and 5th fractions, ascorbic acid was added at concentrations of 0.45 and 0.90 g/L respectively. All samples were packaged in minitubes (vol. 0.5 ml) containing 50 million sperm and frozen in liquid nitrogen vapour using instant freezing technique and preserved in liquid nitrogen for one month. The results indicated that, additions of GSH and ascorbic acid during deep freezing preservation of buffalo-bull semen increased significantly ($P < 0.05$) sperm motility percentages and penetration power of sperm through cervical mucus. The morphological abnormalities, acrosomal integrity damage, release of AST, ALT and LDH from sperm cells to medium were decreased in the thawed semen compared with control. Addition of GSH to deep freezing preserved buffalo-bull semen gave better results than ascorbic acid addition. Also, high concentrations of GSH (5mM) and ascorbic acid (0.90 g/L) improved the semen quality than the lower concentrations.

INTRODUCTION

It is well known that, during storage of mammalian spermatozoa, sperm phospholipids undergo peroxidation, which leads to formation of toxic fatty acid peroxides (Sinha et al., 1996). Bovine spermatozoa from frozen thawed semen are sensitive to lipid peroxidation (O'Flaherty et al., 1997) which leads to structural damage to the sperm cell accompanied by lowered motility and metabolism (Mann et al., 1980).

An important reason for the decrease in fertility during storage of semen is the formation of lipid peroxides in the presence of oxygen radicals (Aurich et al., 1997).

These lipid peroxides act as free radicals initiating an autocatalytic chain reaction, resulting in further damage to the cell membrane (Cotran et al., 1989). The sperm plasma membrane contains a high amount of unsaturated fatty acids and therefore particularly susceptible to peroxidative damage with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (Griveau et al., 1995).

Antioxidant in the semen, including ascorbic acid, tocopherol, β-carotene, catalase, glutathione peroxidase and superoxide dismutase, leading to balance the lipid peroxidation and prevent excessive peroxide formation (Alvarez et al., 1987; Kantola et al., 1988; Jeulin et al., 1989; Beconi et al., 1993 and Griveau et al., 1995). The endogenous antioxidative capacity of the semen may be insufficient during prolonged storage (Aurich et al., 1997).

The effects of antioxidant addition to diluted semen gave contradictory results, and positive as well as negative effects have been reported in sheep, human and cattle (Lindemann et al., 1988; Fraga et al., 1991 and Stojanov et al., 1994). So, this study aimed to investigate the effects of two antioxidants (GSH and ascorbic acid) when added to buffalo-bull semen during deep freezing preservation on post thaw sperm motility, morphology, acrosomal membrane integrity, sperm penetration into the cervical mucus, the release of AST, ALT and LDH enzymes from sperm cells to the medium.

MATERIALS AND METHODS

I. Semen samples collections:

Three clinically normal buffalo-bulls, presented in the faculty of veterinary medicine, Alexandria University, Egypt, were used in this study. Semen samples were collected, using artificial vagina and buffalo-cow as a teaser, twice weekly for two weeks, two ejaculates from each buffalo-bull per time. The ejaculates were rapidly transferred to the laboratory.

II. Semen evaluation:

The semen samples were directly evaluated for determining of:

1- Individual sperm motility (Salisbury et al., 1978).
2- Sperm cell concentration using Neubaur hemocytometer (Bearden and Fuquay, 1980).
3- Sperm abnormalities (secondary ones, free head and bent tail) using Alkaline Methyl Violet stain (Barth and Oko, 1989).
4- Acrosome integrity using Giemsa stain (Watson, 1975).
5- Sperm mucus penetration test (mm/30 minutes at 37° C) (Correa et al., 1997).

Ejaculates had motility less than 60% were discarded. Other ejaculates were pooled before dilution.

III. Semen dilution:

The pooled ejaculates were diluted to concentration of 100 million sperm/ml using egg yolk citrate glycerol diluent: Egg yolk: 20 ml, Sodium citrate 2.9%: 80 ml, glycerol: 20 ml, penicillin: 100,000 I.U. and streptomycin: 100 mg (Ahmad et al., 1996).

IV. Treatments:

Diluted semen was divided into 5 fractions:

1- 1st fraction used as a control.
2- 2nd and 3rd fractions, GSH (Sigma Chemical Co., St. Louis, MO, USA)was added in concentrations of 2.5 and 5 mM respectively.
3- 4th and 5th fractions, Ascorbic acid (Pharco Pharmaceutical Co., Egypt) was added in concentrations of 0.45 and 0.90 g/L respectively.

All fractions were packaged in minitubes (vol. 0.5 ml) containing 50 million sperm then frozen in liquid nitrogen vapour using instant freezing technique (Gravance et al., 1997) and preserved in liquid nitrogen for one month.

V. **Evaluation of thawed semen:**

Semen was thawed in water bath at 37° C for 60 minutes (Gravance et al., 1997). Thawed semen was evaluated for sperm motility percentages, sperm abnormalities, acrosomal integrity and sperm mucus penetration test as described before.

Content of every 10 minitubes was pooled and centrifuged. The supernatant was used for determination of the extracellular activity of AST, ALT (Reitman and Frankel, 1957) using reagents received from "Biochemical Trade Inc., Miami, Florida, USA" and LDH (Cabaud et al., 1958) using reagents received from "ELITECH diagnostics, Egypt".

IV. **Statistical analysis:**

Analysis Of Variance (ANOVA) and Duncan's Multiple Range Test were carried out according to SAS (1988).

RESULTS

The results are presented in table 1 and Figures 1-7.

The results of this study revealed that, addition of antioxidants (GSH or ascorbic acid) to the semen of the buffalo-bulls during deep freezing improved quality of thawed semen (increased significantly "$P < 0.01$" sperm motility percentages and sperm penetration into estrus mucus and decreased significantly "$P < 0.01$" sperm abnormalities percentages, acrosomal damage and the content of the medium from AST, ALT and LDH. Addition of GSH gave better results than ascorbic acid. Also, high GSH concentration (5mM) gave better semen quality than the lower one (2.5mM). Besides, high Ascorbic acid concentration (0.90 g/L) was better than the lower (0.45g/L).

DISCUSSION

Dead and abnormal spermatozoa have adverse effects on companion cells (Shannon and Curson, 1972 and Lindemann et al., 1982) and consequently on fertilization potential of the semen (Saacke and White, 1972). Bovine semen should not possess more than 15 to 18 % abnormal spermatozoa to achieve optimum fertility (Linford et al., 1976).

This study revealed that, addition of antioxidants (GSH or ascorbic acid) to frozen buffalo-bull semen increased significantly ($P < 0.01$) post thaw sperm motility and penetration through cervical mucus while, sperm abnormalities, acrosomal damage and the content of the medium from AST, ALT and LDH enzymes were decreased significantly ($P < 0.01$). Addition of antioxidants (GSH and Ascorbic acid) to frozen

thawed buffalo-bull semen, in this study, lead to stabilization of the spermatozoa membrane as indicated by the decrease in permeability to AST, ALT and LDH enzymes and increased their motility. These results were more obvious with GSH 5mM concentration.

These results are in agreement with Gupta and Tripathi (1984) and Slaweta and Laskowska (1987) who observed improvement in progressive motility of diluted bovine semen in presence of glutathione. Also, Kim and parthasarathy (1998) recorded that, addition of antioxidants in the media of human semen brought beneficial effects in preventing loss of motility and inhibiting lipid peroxidation besides, treating patients with antioxidants has shown to have a positive effect on improving fertilization. Parinaud et al. (1997) showed that, incubation of human semen with antioxidants during liquefaction and centrifugation increases recovery of motile spermatozoa. Donoghue and Donoghue (1997) found that, addition of antioxidants to extended turkey semen improves sperm survival, membrane integrity and reduces the loss of motility after 48-hrs storage. Also, Maxwell and Stojanov (1996) recorded that, the antioxidants improved the motility and acrosome integrity of spermatozoa of liquid storage ram semen. Sinha et al. (1996) found that, addition of GSH (5mM) during deep freezing preservation of goat semen, increased the motility of thawed spermatozoa and decreased the percentage of acrosomal abnormalities also, decreased the release of AST, ALT and LDH from sperm cell into the medium.

Aurich et al. (1997) found that, ascorbic acid increased the percentage of membrane-intact spermatozoa in diluted semen of stallion. They reported that, ascorbic acid inhibits peroxidation of membrane lipids during storage and thus has protective effects on sperm membrane while, the progressive motile spermatozoa during storage did not influenced. Also, Askari et al. (1994) found that, ascorbic acid did not result in any improvement in sperm motility of freez-thaw human semen. While, Dawson et al. (1992) recorded that, men receiving ascorbic acid showed improvement in sperm quality.

The sudden increase in oxygen utilization by spermatozoa during thawing, following the dormant metabolic stage, might be responsible for increased production of free radicals, leading to increased lipid peroxidation and thus spermatozoal membrane damage (Sinha et al., 1996) so, addition of antioxidants to frozen semen should prevent this phenomenon.

Glutathione as a substrate of glutathione peroxidase present in the sperm head, slows down the peroxidation process and hence maintains sperm motility (Christopherson, 1968). Glutathione plays an active role in sperm fructolysis. It is a coenzyme of 1,3-diphosphoglyceric aldhyde dehydrogenase that leads to oxidation of triose phosphate to phosphoglyceric acid, which is later reduced to pyruvic acid and then lactic acid (Slaweta and Laskowska, 1987). This may be the reason of improving sperm motility during this study with GSH addition.

Ascorbic acid reduces oxygen radicals (Luck et al., 1995) and neutralizes reactive oxygen species (Anderson and Luckey, 1987). Besides, it protects the activity of superoxide dismutase (Beconi et al., 1993) and regenerates other antioxidative systems (Buettner , 1993).

Sperm functional parameters such as ATP content in sperm, cervical mucus penetration, sperm penetration assay into oocytes and acrosomal membrane integrity along with acrosine activity were used as reliable predictors of sperm fertilizing ability

(World Health Organization, 1992). So, increased cervical mucus penetration ability by spermatozoa and decreased acrosomal integrity damage, in this study as a result of GSH and ascorbic acid addition, are indicative for good fertilizing capacity of the spermatozoa. This also is in agreement with Harrison and Vickers (1990) and Harkema and Boyle (1992) who recorded that, assessment of sperm motility and membrane integrity allows for good estimates of fertilizing capacity.

Conclusion: *Antioxidants addition during deep freezing preservation of buffalo-bull semen improved the quality of thawed semen and the best results were obtained with GSH (5mM) concentration.*

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Table 1. Effect of GSH (2.5 and 5mM) and Ascorbic acid (0.45 and 0.90 g/L) on quality of frozen thawed buffalo-bull semen (mean ± SE)

Evaluation items	Control	GSH		Ascorbic acid	
		2.5mM	5mM	0.45 g/L	0.90 g/L
Sperm Motility %	41.00± 0.98^{D}	49.40± 1.52^{B}	55.10± 1.34^{A}	44.10± 1.16^{CD}	46.20± 0.98^{BC}
2ndary Sperm abnormalities %	15.90± 0.82^{A}	12.60± 0.71^{BC}	10.90± 0.64^{C}	14.40± 0.80^{AB}	12.70± 0.74^{BC}
Damaged acrosome %	11.30± 0.71^{A}	7.10± 0.64^{CD}	5.60± 0.58^{C}	9.30± 0.53^{B}	8.60± 0.52^{BC}
Cervical Mucus Penetration (mm)	17.90± 1.12^{C}	24.90± 1.15^{B}	31.10± 1.21^{A}	21.90± 0.79^{B}	24.20± 0.89^{B}
AST (U/L)	70.20± 1.35^{A}	44.20± 1.08^{C}	39.10± 0.70^{D}	54.70± 0.85^{B}	45.10± 0.69^{C}
ALT (U/L)	304.40± 3.39^{A}	255.10± 3.15^{D}	219.60 ±2.34^{E}	290.70± 2.22^{B}	279.20± 2.08^{C}
LDH (U/L)	1111.26 ±21.83^{A}	846.87± 15.93^{C}	630.0± 16.07^{D}	962.02± 8.67^{B}	871.16± 10.62^{C}

Means in the same row having different letters are significantly different (P <0.0 1).

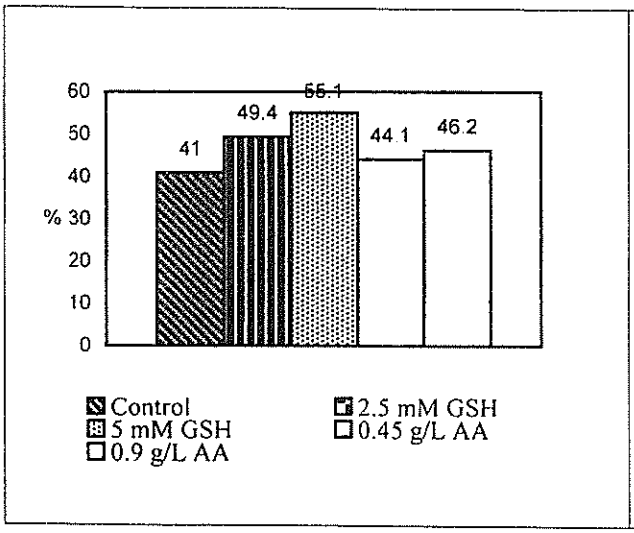

Fig.1 : Effect of GSH and Ascorbic acid (AA) on post thaw sperm motility of frozen buffalo bull semen

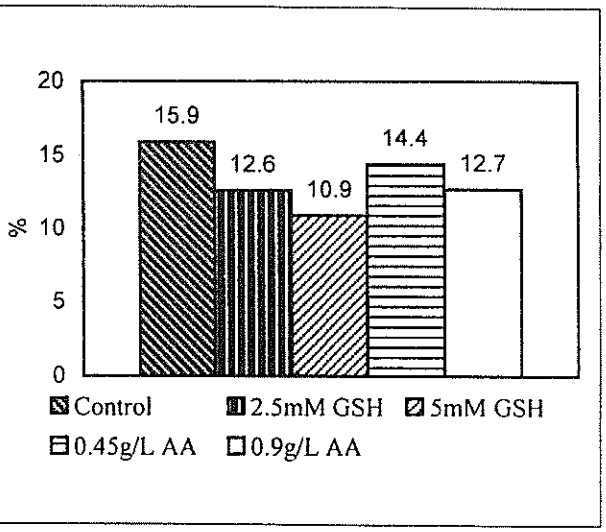

Fig. 2: Effect of GSH and Ascorbic acid (AA) on post thaw secondary sperm abnormalities percentages of frozen buffalo bull semen

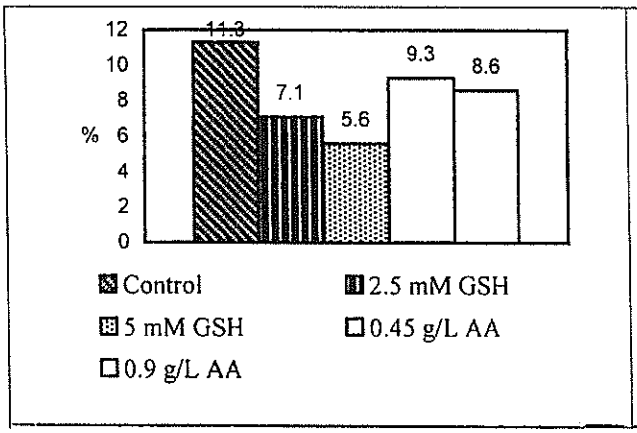

Fig.3 : Effect of GSH and Ascorbic acid (AA) on post thaw sperm acrosomal damage of frozen buffalo- bull semen

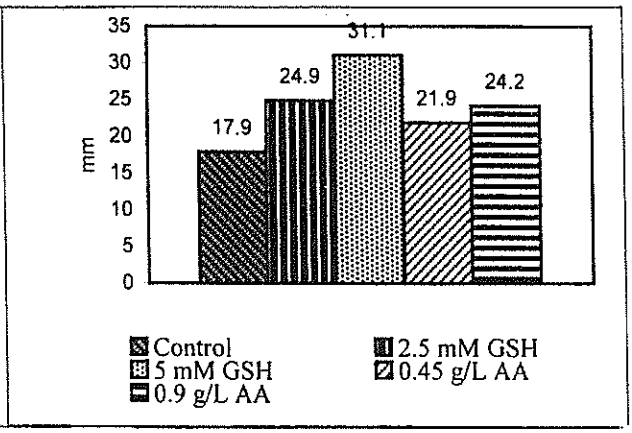

Fig. 4: Effect of GSH and Ascorbic acid (AA) on post thaw sperm cervical mucus penetration (mm) of frozen buffalo-bull semen

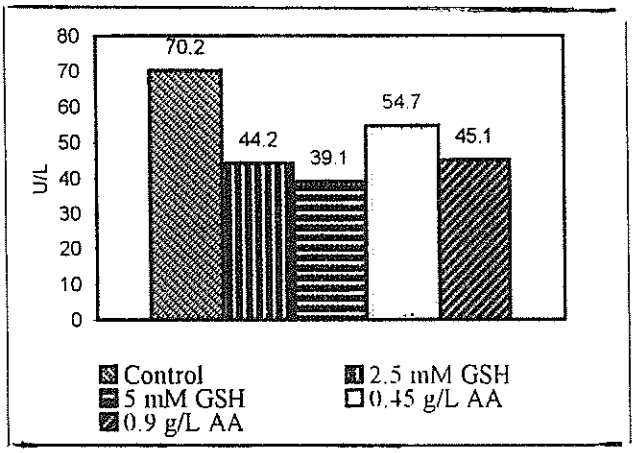

Fig. 5: Effect of GSH and Ascorbic acid (AA) on post thaw extracellular activity of AST (U/L) of frozen buffalo-bull semen

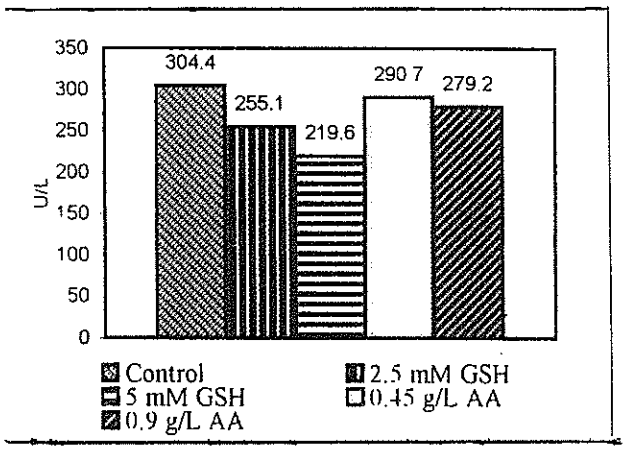

Fig. 6: Effect of GSH and Ascorbic acid (AA) on post thaw extracellular activity of ALT (U/L) of frozen buffalo-bull semen

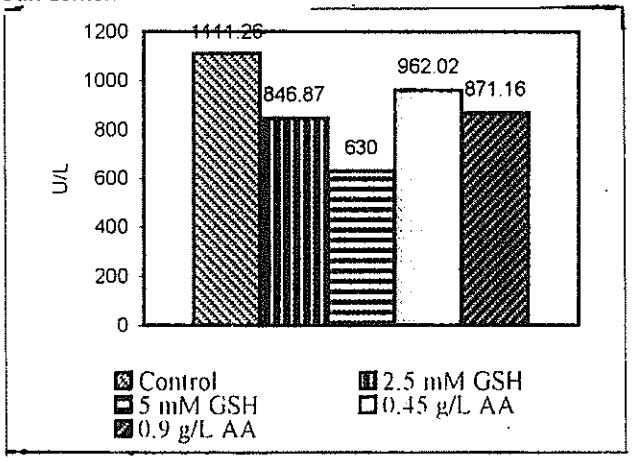

Fig. 7: Effect of GSH and Ascorbic acid (AA) on post thaw extracellular activity of LDH (U/L) of frozen buffalo-bull semen

الملخص العربى

علاقة جودة السائل المنوي المجمد لطلائق الجاموس بإضافة مضادات الأكسدة (الجلوتاثيون وحمض الاسكوربيك)

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استهدفت هذه الدراسة تقييم تأثير إضافة مضادات الأكسدة للسائل المنوي لطلائق الجاموس عندما يتم تجميده على حركة الحيوانات المنوية ومظهرها وسلامة القلنصوة واختبار اختراق الحيوانات المنوية لمخاط عنق الرحم ومحتوى الوسط من الاسبرتات امينوترانسفيراز (A ST) والالانين امينوترانسفيراز (A L T) واللاكتات ديهيدروجيناز (LD H) وذلك بعد ذوبان السائل المنوي المجمد. تم تجميع عينات السائل المنوي باستخدام المهبل الصناعي من ثلاثة طلائق جاموسي سليمة إكلينيكيا مرتين أسبوعيا قذفتين في كل مرة لمدة أسبوعين .

تم تقييم السائل المنوي مباشرة بعد التجميع باستخدام الطرق المعتادة وتم استبعاد القذفات التي بها اقل من ٦٠ % حركة فردية للحيوانات المنوية .أما باقي العينات فقد تم خلطها وتخفيفها باستخدام مخفف صفار البيض وسترات الصوديوم والجليسرول إلى ١٠٠ مليون حيوان منوي /مل . تم تقسيم السائل المنوي المخفف إلى خمسة أجزاء . الأول تم استخدامه كضابط أما الثاني والثالث فتم إضافة الجلوتاثيون إليهما بتركيز ٢،٥ و ٠, ٥ مللي مكافئ جرامى على الترتيب أما الرابع والخامس فتم إضافة حمض الاسكوربيك إليهما بتركيز ٠,٤٥ و ٠,٩٠ جم /لتر على الترتيب . تم تعبئة كل الأجزاء في قشات سعة كل واحدة ٠،٥ مل وتحتوى على ٥٠ مليون حيوان منوي وتم التجميد في بخار النيتروجين السائل وتم الحفظ في النيتروجين السائل .

أظهرت النتائج أن إضافة الجلوتاثيون أو حمض الاسكوربيك للسائل المنوي لطلائق الجاموس قبل تجميده أدت إلى زيادة معنوية في الحركة الفردية للحيوانات المنوية وكذلك قدرتها على اختراق مخاط عنق الرحم أما نسبة تشوهات الحيوانات المنوية ونسبة عدم سلامة القلنصوة ومحتوى الوسط من A ST , A L T , L D H فقد نقصت معنويا وذلك بعد ذوبان السائل المنوي المجمد. كما أظهرت النتائج أن إضافة الجلوتاثيون للسائل المنوي المجمد لطلائق الجاموس أدى إلى تحسن نوعيته اكثر من إضافة حمض الاسكوربيك له . كما أن التركيزات الأعلى للجلوتاثيون (٠, ٥ مللي مكافئ جرامى) وحمض الاسكوربيك (٠,٩٠ جم / لتر) كانت افضل من التركيزات الأقل .

الخلاصة : إضافة مضادات الأكسدة (الجلوتاثيون أو حمض الاسكوربيك) للسائل المنوي لطلائق الجاموس الذي يتم حفظه بالتجميد أدت إلى تحسن نوعيته وتم الحصول على افضل النتائج بإضافة الجلوتاثيون بتركيز ٠, ٥ مللي مكافئ جرامى .