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Effect of Various Antioxidants on the Quality of Frozen-Thawed Bull Semen

[1]O. Uysal, [2]M.N. Bucak, [1]İ. Yavaş and [1]Ö. Varışlı
[1]Department of Reproduction and Artificial Insemination,
Faculty of Veterinary Medicine, Ankara University, 06110, Diskapi, Ankara, Turkey
[2]Lalahan Livestock Research Institute, Ministry of Agriculture, Ankara, Turkey

Abstract: Free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages that may lead to decreased sperm motility or cell death. The aim of this study was to determine the effects of the addition of various of the supplements consist of glutathione (GSH), oxidized Glutathione (GSSG), cysteine, taurine, hypotaurine, Bovine Serum Albumin (BSA), trehalose and hyaluronan to freezing media on the post-thawing sperm characteristics, including motility, morphology, viability by propidium iodide (PI stain) with the aid of fluoresans microscope and membrane integrity by HOST. A total number of 20 ejaculates were collected using the artificial vagina from 2 bulls and the ejaculates were diluted with a Tris-based extender containing additives and no additives as control. GSH (5 mM), GSSG (5 mM), cysteine (5 mM), taurine (50 mM), hypotaurine (25 mM) and BSA (5 mg mL^{-1}), trehalose (50 mM) and hyaluronan (1000 μg mL^{-1}) showed more positive effect than other supplements and controls in protecting sperm characteristics after the freezing-thawing process ($p<0.001$). Many aspects of sperm protection e.g., sperm motility, viability and membrane stabilisation of the sperm cells during relative cryopreservation, are the key factors in determining the preservation of sperm function. The results of this study provide a new approach to the cryopreservation of sperm from bulls and related breeds. Further studies are necessary to obtain results to confirm present findings.

Key words: Antioxidants, bull semen, freezing, frozen-thawed, BSA

INTRODUCTION

Major steps of cryopreservation, such as cooling and freezing/thawing, exert physical as well as chemical stresses on the sperm membrane (Chatterjee *et al*., 2001). Cryopreservation of spermatozoa is associated with an oxidative stress induced by free radicals (Salvador *et al*., 2006). Sperm cells have a high content of unsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to Lipid Peroxidation (LPO) by O_2^- and H_2O_2 (Sinha *et al*., 1996). The freezing process produces physical and chemical stress on the sperm membrane which in turn that reduces sperm viability and fertilizing ability. In recent years, antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation and free radicals are eliminated by antioxidant systems (Baumber *et al*., 2000).

According to Bilodeu *et al.* (2001), thiols such as glutathione and cysteine prevent the loss of sperm motility in frozen-thawed bull semen. Szczesniak-Fabianczyk *et al.* (2006) reported that a semen extender with cysteine improved the viability, chromatin structure and membrane integrity of boar sperm cells during liquid preservation. GSSG, but not GSH, prevents increase in the mobility of sulfhydryl containing proteins due to the freezing/thawing of spermatozoa (Chatterjee *et al*., 2001). Taurine, hypotaurine among non-enzymatic antioxidants (Stradaioli *et al*., 2007) have been found to have beneficial effects by decreasing cellular damages (Foote *et al*., 1993). Matsuoka *et al.* (2006) reported that Bovine Serum Albumin (BSA) can be substituted for egg-yolk for in ram semen diluent and that it enhances the motility and viability of ram sperm cells following the freezing-thawing process. In addition, supplementation with trehalose of semen diluents is well known to improve the viability and motility of liquid or cryopreserved ram sperm cells (Bucak and Tekin, 2007). Hyaluronan, an essential component of the extracellular matrix and non-sulfated glycosaminoglycan, is involved in important physiological functions such as motility, capacitation of the spermatozoa (Ghosh and Datta, 2003) and preserves post-thaw spermatozoa viability and *in vitro* membrane stability (Pena *et al*., 2004).

A wide variety of antioxidants have been tested GSH, GSSG, cysteine, taurine, hypotaurine, bovine serum albumine, trehalose and hyaluronan to minimize the

Corresponding Author: Dr. Ongun Uysal, Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ankara University 06110, Diskapi, Ankara, Turkey

damage caused by freezing and thawing in this study. As the nature of the oxidative stress during the freezing/thawing cycle has not yet been ascertained, we investigated whether Reactive Oxygen Species (ROS) are generated during the various phases of the freeze-thaw cycle.

MATERIALS AND METHODS

Animals and semen collection: Semen samples from 2 mature Holstein bulls (4 years of age) were used in this study. A total number of 20 ejaculates were collected from the bulls using an artificial vagina twice a week. The ejaculates were evaluated and accepted for evaluation if the following criteria were met: volume varying between 5.0-6.5 mL, sperm concentration more than 1×10^9 sperm mL^{-1}, the motile sperms percentage higher than 70% and less than 10% abnormal sperm in total. Semen samples were pooled to eliminate individual differences. Ten pooled ejaculates were included in the study (Paulenz *et al*., 2002).

Semen processing and evaluation: All the reagents used were analytical grade (Sigma, St. Louis). Since the additives (Sigma Chemical Co.), GSH, GSSG, cysteine, taurine, hypotaurine, BSA, trehalose and hyaluronan were available in different purities, the following samples of stated catalogue designations were used throughout the study: GSH (G-6013), GSSG (G-2140), taurine (T-4571), hypotaurine (H-1384), BSA (Sigma Fraction V, A-9647), cysteine (C-7352), trehalose (T 0167), hyaluronan (Fluka 53747). In this trial, a Tris-based extender (CTR) was used (Tris 36.3 g L^{-1}, fructose 10 g L^{-1}, citric acid 5 g L^{-1}, egg yolk 10 mL /100 mL, glycerol 7%, penicillin 100.000 iu/100 mL, streptomycin 100 mg 100 mL Penovil/VİLSAN)-pH 6.8, 300 mOsm] for all the ejaculates. Each ejaculate was split into 9 equal aliquots and diluted with the CTR extender with GSH (5 mM) GSSG (5 mM), cysteine (5 mM), taurine (50 Mm), hypotaurine (25 mM) and BSA (5 mg mL^{-1}), trehalose (50 mM), hyaluronan (1000 μg mL^{-1}) or no additives (controls) for a total of 9 experimental semen groups (37°C) at a final concentration of 50×10^6 spermatozoa per mL. Diluted semen samples were drawn into 0.25 mL French straws and frozen in liquid nitrogen vapour (-100 to -120°C) and then stored in liquid nitrogen (-196°C). Post-thawing sperm motility, morphologic sperm abnormalities, acrosome damages, the functional membrane integrity of sperm cells by Hypoosmotic-Swelling Test (HOST) and viability by PI stain with the aid of fluoresans microscope and were determined in samples.

Statistical analyses: The sperm evaluations were repeated 15 times and the results were expressed as the mean±SEM. Means were analyzed by Analysis of Variance (ANOVA), followed by the Duncan test to determine significant differences between the 8 experimental groups and control group- with additives or no additive after the freezing-thawing process for sperm characteristics using the SPSS/PC version 12.0 software (SPSS, Chicago). Differences with values of $p < 0.05$ were considered to be statistically significant (Daniel, 1991).

RESULTS AND DISCUSSION

Ejaculat volume (mL), sperm motility (%), sperm concentration ($X10^9$ mL^{-1}), total abnormality (%), viability (%), membrane integrity by HOST (%) and pH in fresh bull semen are set out in Table 1. Differences between bulls are not significant ($p>0.05$) for sperm characteristics. The effect of various antioxidants on post-thawing sperm motility, total abnormality, acrosome damage, membrane integrity by HOST and viability by fluorescent staining in frozen-thawed bull spermatozoa is set out in Table 2. The antioxidant GSH at 5 mM had a significant effect in maintaining sperm motility and membrane integrity, when compared to the control and other groups, respectively. Taurine at 50 mM concentrations showed a most positive effect when compared to the other treatments and control group in protecting sperm morphology during the freezing-thawing process of bull semen significantly. Cysteine at 5 mM had a significant effect in maintaining sperm viability and hyaluronan at 1000 μg mL^{-1} concentration exhibited the lowest post-thawing acrosomal damage, when compared to the control and other groups significantly ($p<0.001$).

The sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility and eventually loss in fertility, resulting from reactive oxygen species during aerobic incubation (Alvarez *et al*., 1987).

Table 1: Principle spermatological characteristics in fresh bull semen (means±SEM)

Bulls	Ejaculate volume	Sperm motility	Sperm concentration	Total abnormality	Viability	HOST	pH
1	5.03±0.2	83.50±2.4	1.48±0.1	5.40±0.6	82.30±2.8	83.80±3.3	6.59±0.0
2	4.80±0.2	81.00±1.9	1.83±0.1	5.20±0.9	85.50±1.6	81.90±2.5	6.51±0.0n: 10

$p>0.05$: Differences between the groups are not significant

Table 2: Spermatological characteristics in frozen-thawed bull spermatozoa (means %±SEM)

Groups	Motility	Total abnormality	Acrosome damage	HOST	Fluorescent staining
Hyaluronan(1000 μg mL^{-1})	55.5±2.5b	8.1±0.7ab	3.8±1.1a	60.0±2.5b	49.2±3.7ab
GSH (5 mM)	71.0±3.1a	10.8±1.0bcd	8.9±0.8b	79.3±3.7c	42.3±2.5a
GSSG (5 mM)	51.0±3.9bc	15.1±1.4de	10.3±0.5b	58.3±3.7ab	58.4±2.64cd
Cysteine(5 mM)	44.0±3.1bc	11.0±1.5bcde	9.1±1.1b	51.3±3.3ab	66.5±2.5d
Taurine(50 mM)	49.0±4.2bc	6.0±0.6a	4.4±0.5a	54.5±4.62ab	56.6±3.3bc
Trehalose(50 mM)	45.0±2.0c	10.5±2.1bc	9.7±2.0b	50.1±2.2a	59.6±2.6cd
Hypotaurine(25mM)	54.0±1.4b	14.5±2.2cde	11.6±1.9b	54.7±1.7ab	61.2±2.2cd
BSA (5 mg mL^{-1})	58.0±1.3b	17.8±1.7e	9.6±1.0b	57.3±1.6ab	59.8±2.3cd
Kontrol	53.0±3.2bc	17.8±0.9de	10.0±1.3b	59.2±3.4ab	56.0±3.0bc
P	***	***	***	***	***

n: 10; (a, b, c, d, e, ab, bc, cd, de, bcd, cde, bcde): Different letters within the same column showed significant differences among the groups (** * p<0.001)

Therefore, free radicals must be eliminated by supplementation with antioxidants such as GSH, cysteamine and taurine during the freezing-thawing or liquid storage of semen (Bucak *et al*., 2007).

Cryopreservation alters the membrane sulfhydryl status of spermatozoa. GSSG reduces the mobility of sulfhydryl-containing proteins in the sperm membrane. Sulfhydryl groups are under redox control and a change in the redox status of the membrane can be linked to the ROS production that occurs during cooling and freezing-thawing of spermatozoa (Mazur *et al*., 2000). The GSH/GSSG pair plays important roles both as a redox sensor and protector against ROS induced damages in many cell types (Halliwell and Gutteridge, 1998).

Based on our results, we can hypothesize that additives displayed cryoprotective influence on improving post-thawed sperm motility, sperm morphology, acrosome and membrane integrity and sperm viability. But, all of the additives did not give significant positive effect on sperm characteristics after thawing in the same way. In the present study, it was observed that the highest post-thawing sperm motility and membrane integrity were obtained from 5 mM concentration of GSH. But, GSH was determined to exert the poorest protective effect on post-thawing sperm viability throughout the study. In our previous study, GSSG (5 mM), BSA (20 mg mL^{-1}), cysteine (10 mM) and lycopene (800 μg) showed more positive effect than other concentrations of the supplements and controls in protecting all sperm characteristics after the freezing-thawing process in rams (Uysal and Bucak, 2007).

Taurine as an antioxidant have been found to protect ram (Uysal *et al*., 2000) sperm membrane against lipid peroxidation and loss of sperm motility. Epididymal compound, sulfonic amino acid, taurine is present in the epididymis at high concentrations, has been reported to improve post-thawing sperm motility of frozen ram semen (Sanchez-Partida *et al*., 1997). However, addition of HPT and taurine failed to improve post-thaw motility of bull sperm frozen in whole milk (Chen *et al*., 1993).

In this study, taurine at 50 mM concentration showed a most positive effect in protecting sperm morphology during the freezing-thawing process of bull semen. Conversely, supplementation of extender with taurine did not improve post-thawing sperm motility in the same way.

Thiol compounds, such as cysteine, are precursors of intracellular glutathione biosynthesis and cysteine protects sperm cells from toxic oxygen metabolites causing lipid peroxidation of sperm plasma membranes under *in vitro* conditions (Meister and Tate, 1976). Funahashi and Sano (2005) reported that a semen extender with 5 mM cysteine improved the viability and membrane integrity of boar sperm cells during liquid storage.

The assessment of motility alone is inadequate for the evaluation of sperm survival after thawing (Uysal *et al*., 2006). The integrity and functional activity of the sperm membrane is the major importance in the fertilization process and assessment of membrane function may be a useful indicator of the fertilizing ability of spermatozoa (Uysal and Korkmaz, 2004). Because highly motile cells can have damage in structures or functions which can be performed by combined (hypoosmotic-supravital staining) test (HE-test), evaluating head and tail membrane behaviour. Conversely, highly nonmotil sperm cells can have intact plasmelemma and so viability. It is possible to evaluate using Eosine Exclusion Test (EET), HOS, Water Test (WT) and fluorescent staining with PI the structural and functional membrane integrity and viability correlated with the *in vitro* fertilizing ability of sperms in frozen ram, boar and bull semen (Pintado *et al*., 2000; Uysal *et al*., 2005b). Just so, although post-thawing sperm motility obtained from GSSG, cysteine, taurine and trehalose was found significantly lower than other treatments, HOST (membrane integrity) and fluorescent staining (viability) results from GSSG, cysteine, taurine and trehalose were determined higher than post-thawing sperm motility values respectively in this study.

BSA is known to eliminate free radicals generated by oxidative reactions and therefore to protect the membrane integrity of sperm cells from lipid peroxidation during the semen freezing process (Lewis *et al*., 1997). In our study,

there was improvement in sperm motility, membrane integrity and viability of bull spermatozoa with BSA (5 mg mL^{-1}) in the cryopreservation medium after thawing. But, the highest morphologic abnormality was determined by extender containing BSA in this study. Moreover, it was observed that total sperm abnormality value with BSA was very close to that of control group.

Hyaluronan improves sperm motility, viability and membrane integrity after freezing and thawing procedures (Pena *et al*., 2004). According to our findings, although supplementation of cryopreservation medium with hyaluronan at 1000 μg mL^{-1} concentration caused to decrease post-thawing sperm motility in bull semen, it protected acrosome integrity and improved membrane integrity of bull spermatozoa.

CONCLUSION

In conclusion, this study demonstrated that supplementation with antioxidants of semen diluents, depending on various concentrations, during semen cryopreservation attempts, may exert beneficial effects on the quality of the freezing-thawing of ram semen. This study has shown that many aspects of sperm protection e.g. sperm motility, viability and membrane stabilisation of sperm cells during relative cryopreservation, are of prime importance, the antioxidants GSH (5 mM), taurine (50 mM), cysteine (5 mM) and hyaluronan (1000 μg mL^{-1}) provided a near-optimal concentration for improved sperm survival during the freezing-thawing process. The results of this study therefore, provide a new approach to the cryopreservation of sperm from bulls of different breeds. Further studies are necessary to obtain results to confirm present findings.

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REFERENCES

Alvarez, J.G., J.C. Touchstone, L. Blasco and B.T. Storey, 1987. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa: Superoxide dismutase as a major enzyme protectant against oxygen toxicity. J. Androl., 23: 338-348.

Baumber, J., B.A. Ball, C.G. Gravance, V. Medina and M.C.G. Davies-Morel, 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. J. Androl., 21: 895-902.

Bilodeau, J.F., S. Blanchette, C. Gagnon and M.A. Sirard, 2001. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology, 56: 275-286.

Bucak, M.N. and N. Tekin, 2007. Protective effect of Taurine, Glutathione and Trehalose on the liquid storage of ram semen. Small Rum. Res. (In Press).

Bucak, M.N., A. Ateşşahin, Ö. Varı şlı, A. Yüce, N. Tekin and A. Akçay, 2007. The influence of trehalose, taurine, cysteamine and hyaluronanon ram semen: Microscobic and oxidative parameters after freze-thawing process. Theriogenology, 67: 1060-1067.

Chatterjee, S., E. De Lamirande and C. Gagnon, 2001. Cryopreservation alters membrane sulphydryl status of bull spermatozoa: Protection by oxidized glutathione. Mol. Reprod. Dev., 60: 498-506.

Chen, Y., R.H. Foote and C.C. Brockett, 1993. Effect of sucrose, trehalose, hypotaurine, taurine and blood serum on survival of frozen bull sperm. Cryobiology, 30: 423-431.

Daniel, W.W., 1991. Analysis of Variance. In: Biostatistic: Daniel, W.W. (Ed.). A Foundation for Analysis in the Health Sciences. John Wiley and Sons, Hoboken, pp: 274-320.

Foote, R.H., Y. Chen, C.C. Brockett and M.T. Kaproth, 1993. Fertility of Bull Spermatozoa Frozen in Whole Milk Extender with Trehalose, Taurine, or Blood Serum. J. Dairy Sci., 76: 1908-1913.

Funahashi, H. and T. Sano, 2005. Select antioxidants improve the function of extended boar semen stored at 10°C. Theriogenology, 6: 1605-1616.

Ghosh, I. and K. Datta, 2003. Sperm surface hyaluronan binding protein (HABP1) interacts with zona pellucida of water buffalo (*Bubalus bubalis*) through its clustered mannose residues. Mol. Reprod Dev., 64: 235-244.

Halliwell, B. And J.M.C. Gutteridge, 1998. Antioxidant Defences. In: Free radicals in Biology and Medicine. Oxford: Halliwell, B. and J.M.C. Gutteridge (Eds.). University Press, (London), pp: 155-158.

Lewis, S.E., E.S. Sterling, I.S. Young and W. Thompson, 1997. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril, 67: 142-147.

Matsuoka, T., H. Imai, H. Kohno and Y. Fukui, 2006. Effects of bovine serum albumine and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. J. Reprod. Dev., 52: 675-683.

Mazur, P., I.I. Katkov, N. Katkova and J.K. Critser, 2000. The enhancement of the ability of mouse sperm to survive freezing and thawing by the use of high concentrations of glycerol and the presence of an Escherichia coli membrane preparation (Oxyrase) to lower the oxygen concentration. Cryobiology, 40: 187-209.

Meister, A. and S.S. Tate, 1976. Glutathione and related gamma-glutamyl compounds: Biosynthesis and utilization. Annu. Rev. Biochem., 45: 559-604.

Paulenz, H., L. Söderquist, R. Pérez-Pé and K.A. Berg, 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology, 57: 823-836.

Pena, F.J., A. Johannisson, M. Wallgren and H. Martinez, 2004. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. Theriogenology, 61: 63-70.

Pintado, B., J. de la Fuente and E.R.S. Roldan, 2000. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin: Accuracy in the assessment of cell viability J. Reprod. Fertil., 118: 145-152.

Salvador, I., J. Yanız, M.P. Viudes-De-Castro, E.A. Gomez and M.A. Silvestre, 2006. Effect of solid storage on caprine semen conservation at 5°C. Theriogenology, 64: 252-260.

Sanchez-Partida, L.G., B.P. Setchell and W.M.C. Maxwell, 1997. Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. Reprod. Fertil. Dev., 9: 689-696.

Sinha, M.P., A.K. Sinha, B.K. Singh and P.I. Prasad, 1996. The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. Theriogenology, 41: 237-243.

Stradaioli, G., T. Noro, L. Sylla and M. Monaci, 2007. İ Decrease in Glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. Theriogenology, 15: 1249-1255.

Szczesniak-Fabianczyk, B., M. Bochenek, Z. Smorag and M.A. Silvestre, 2006. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. Reprod. Biol., 3: 81-87.

Uysal, O., H. Kinet, M. Çevik and S. Çetinkaya, 2000. Fertility obtained from frozen ram semen with different extenders containing varied antioxidants. Vet. J. Ankara Üniv., 47: 177-189.

Uysal, O. and T. Korkmaz, 2004. Evaluation of Membrane Integrity by hypo-osmotic swelling eosine test in canine spermatozoa. Indian Vet. J., 81: 1229-1231.

Uysal, O., M.N. Bucak, İ. Yavaş, Ö. Varışlı and İ.S. Gürcan, 2005b. Evaluation of ram sperm frozen with various taurine concentrations. Indian Vet. J., 82: 1059-1061.

Uysal, O., T. Korkmaz, İ. Yavaş and N.M. Bucak, 2006. Evaluation by hypoosmotic swelling-eosine test of cryopreserved bovine spermatoza. Indian Vet. J., 83: 557-559.

Uysal, O. and M.N. Bucak, 2007. Effect of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta. Vet. Brno., No: 3 (In Press).