

EFFECTS OF HYPOTAURINE AND CYSTEINE IN WASHING SOLUTIONS ON COOLED AND FROZEN THAW GOAT SPERMATOZOA

A. A. Memon¹, H. Wahid², Y. Rosnina², Y. M. Goh² and A. N. Tunio¹

¹Faculty of Animal Husbandry and Veterinary Sciences,
Sindh Agriculture University Tandojam, Pakistan

²Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM,
Serdang, Selangor Darul Ehsan, Malaysia

ABSTRACT

This study was conducted to determine the effect of hypotaurine and cysteine (antioxidant) in washing solution (Tris citric acid fructose extender without egg yolk) on motility, membrane integrity, morphology, acrosome integrity and viability of cooled and frozen thaw boer goat spermatozoa. Significantly better results in terms of motility and morphology of cooled spermatozoa have been observed when antioxidants were added in Tris citric acid fructose extender washing solution. Furthermore, significant improvement was observed by supplementation of tris citric acid fructose extender in terms of motility and acrosome integrity compared to non-supplemented groups after freezing. In conclusion, antioxidants can be added in washing solution in order to reduce the oxidative stress in cooled and frozen boer goat spermatozoa.

Keywords: Antioxidants, cryopreservation, cysteine, hypotaurine, solutions, washing.

INTRODUCTION

Use of artificial insemination (AI) technology in the goat has been limited. There are several reasons for this, including the relative difficulty in freezing goat buck sperm. Goat buck spermatozoa are poorly resistant to cryopreservation stress compared to spermatozoa from other domesticated mammals, notably bulls (White, 1993). In goat bucks, the presence of bulbourethral gland secretions in seminal plasma has negative interactions between the phospholipids of the egg yolk or the milk based extenders for liquid or frozen storages. Therefore in goat bucks there are some differences in the cryopreservation method from other domestic species such as bull, boar and ram (Leboeuf *et al.*, 2000; Purdy, 2006). Seminal plasma removal is an additional step for goat spermatozoa cryopreservation. However, results of seminal plasma removal are quite variable

(Leboeuf *et al.*, 2000; Azeredo *et al.*, 2001; Purdy, 2006; Peterson *et al.*, 2007; Kozdrowski *et al.*, 2007; Ustuner *et al.*, 2009; Naing *et al.*, 2010; Sariozkan *et al.*, 2010).

During washing process, not only seminal plasma separated but also other beneficial component and compounds such as inorganic compounds, organic components and antioxidants are also removed. These substances are essential for functionality and survival as well as preventing lipid peroxidation of spermatozoa after ejaculation. The dilution of semen considerably caused in decrease of the concentration of antioxidants in spermatozoa because of limited capacity of antioxidants to scavenger the oxidants (Bucak *et al.*, 2008). The total antioxidants concentration in the extender and cells could be reduced when semen was diluted many folds in extenders (Kumar and Das, 2005). It is apparent that dilution of semen for washing reduces the amount of antioxidants which is already insufficient for prevention of oxidative stress.

Therefore our hypothesis was that early addition of antioxidants in washing could be advantages in reducing the lipid peroxidation and improving the quality of chilled and frozen boer goat semen and preventing the lipid peroxidation at earlier stage. Thus, this study was planned to investigate the effects of adding antioxidants in washing solution on motility, membrane integrity, morphology, acrosome integrity and viability of chilled frozen thaw boer goat spermatozoa.

MATERIALS AND METHODS

Animals

Four Boer goat bucks (approximately 3-4 years old) were used in the study. These animals were raised on the farm as semen donor for AI purpose. They were maintained under uniform feeding, housing and lighting conditions. Experimental animals were fed twice daily at 8 am and 4 pm in order to achieve a predetermined feed intake of 2.5 % body weight (on dry matter basis) per goat per day. Water was available at libitum.

Immediately after collection each ejaculate was immersed into a water bath maintained at 37°C prior to evaluation. The semen samples were evaluated for volume, color, consistency, mass activity, sperm motility, sperm concentration and sperm morphology. The volume of ejaculate was determined by collecting semen into a graduated tube. Color was evaluated by visual observations. The consistency was scored 1 = watery-cloudy, 2 = milky, 3 = thin creamy, 4 = creamy and 5 = creamy-grainy (Shamsuddin *et al.*, 2000). To evaluate the mass activity (wave motion) a drop (20 µl) of undiluted semen was placed on a pre-warmed slide 37°C without a coverslip and examined under phase contrast microscope (100 x) (Nikon, Eclipse, E200, Japan). The mass activity was scored 0 = no motility, 1 = few sperm with weak movement (< 20%), 2 = some motile spermatozoa (20-40%) without wave movement, 3 = slow wave movement (40-60%) with motile spermatozoa, 4 = rapid wave movement without whirlpool (60-

80%) with motile spermatozoa and 5 = very rapid wave movement with clear whirlpools (>80%) motile spermatozoa (Avdi *et al.*, 2004).

The sperm motility was estimated subjectively by preparing a wet mount of diluted semen by placing a 5 µl drop of fresh semen under coverslip at magnification of 200X under phase contrast microscope. At least 200 spermatozoa, selected randomly from a minimum of four microscopic fields, were examined. The mean of four successive estimations were recorded as the final motility. The sperm concentration was determined by means of a haemocytometer. Morphologically normal spermatozoa were assessed using nigrosin-eosin stain (Evans and Maxwell, 1987). Sperm viability of the samples was assessed by means of the eosin-nigrosin staining (Evans and Maxwell, 1987). The stain was prepared as: Eosin-Y 1.67 g, Nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 ml distilled water. The sperm suspension smears were prepared by mixing a drop of the semen sample with 2 drops of the stain on a warm slide and spreading the stain with a second slide immediately. The viability was assessed by counting 200 cells under the phase-contrast microscope at magnification 1000X. Sperm showing partial or complete purple coloring was considered non-viable and only sperm showing strict exclusion of the stain were considered to be alive. The percentage of acrosome integrity (normal apical ridges) was determined from sperm smears stained with nigrosin-eosin examined under phase contrast microscope at 1000X magnification under oil immersion objective and bright field (Yildiz *et al.*, 2000). A total of 200 spermatozoa were counted in at least four microscopic fields. The sperm membrane integrity was assessed by hypo-osmotic swelling test. It was performed by incubating 20 µl of semen in 200 µl of a 100mOsm hypo-osmotic solution containing 9.0 g fructose, 4.9 g sodium citrate at 37°C for 60 minutes. After incubation 10 µl of the mixture was spread on a warm microscopic slide. A total of 200 spermatozoa were counted in at least four different microscopic fields. The percentage of sperm with swollen and curled tails were then recorded (Revell and Mrode, 1994; Buckett *et al.*, 1997).

Semen dilution and freezing

All chemicals were reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO). The Tris egg yolk (TEY) based extender consisted of 250 mM Tris, 88.5 mM citric acid, 69.38mM fructose, 18% (v/v) egg yolk and antibiotic (80000 IU penicillin and 100000 µg streptomycin per ml) was used as cooling extender. The freezing extender comprised of 250 mM Tris, 88.5 mM citric acid, 69.38 mM fructose, 18% (v/v) egg yolk and glycerol 8 % (v/v) (Liu *et al.*, 1998).

In present experiment, washing solution supplemented with antioxidants was tested for cryopreservation of boer goat semen. Two antioxidants (hypotaurine and cysteine) were included in washing solution Tris citric acid fructose extender without egg yolk. After initial evaluation, ejaculates qualifying the standard criteria as volume 1 and 2 ml, concentration of $> 2.5 \times 10^9$ sperm/ml, having >75% progressive motility and >85% of sperms with normal morphology were pooled and selected for extension and freezing. Pooled ejaculates were equally divided

into 3 aliquots and then each aliquot were transferred into centrifuge tube containing Tris with or without antioxidants. Briefly aliquots A diluted with Tris buffers without egg yolk contains hypotaurine 10 mM antioxidants, aliquots B diluted with Tris buffers without egg yolk contains cysteine 5mM, aliquots C were served as control mixed with Tris citric acid fructose (TCF) without egg yolk and antioxidants at the ratio of (v/v1:1). Diluted spermatozoa were centrifuged at 1500 x g for 5 min. The supernatants were discarded then semen was processed with the Tris citric acid fructose egg yolk extender supplemented with their respective antioxidants in two steps and cooled into cooling chamber at 4°C for 2.5hrs. Cooled semen were diluted in tris citric acid egg yolk glycerol extender and maintained for 30 minutes. Final concentration was adjusted at 120×10^6 sperm /straw. Straws were filled and sealed by automatic filling and sealing machine (MRSI-CE, IM, France). The straws were equilibrated in a horizontal position in cold cabinet for 30 minutes. After equilibration, straws were placed in contact with liquid nitrogen (LN₂) vapors 3 cm above the surface of LN₂ (-120°C) for 10 minutes in an expandable polystyrene box. Then straws were immersed in liquid nitrogen (-196°C) for storage (Fig. 1).

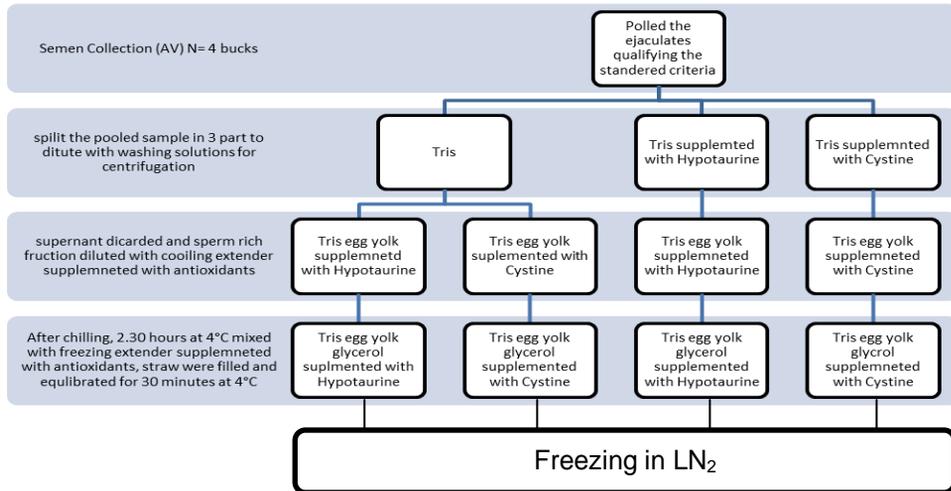


Figure 1. Flow chart for processing boer goat spermatozoa.

Immediately after cooling, semen samples were evaluated for motility, membrane integrity, acrosome integrity, morphology and viability. For post thaw examination, after 24 hrs of freezing, thawing of the frozen straws were carried out, 4 straws were thawed at 37°C for 30 sec and pooled to perform evaluation.

Statistical analysis

The effect of Tris citric acid fructose washing solution supplemented with antioxidants on sperm quality parameters was analyzed by one-way analysis of variance (ANOVA) using procedure of general linear model (PROC GLM) of SAS version 9.1. All data were compared across treatment groups. Significantly different means were then further differentiated using the least significant

difference (LSD) comparison procedures. All statistical tests were conducted at 95% confidence level. Results were expressed as mean \pm S.E.M.

RESULTS AND DISCUSSION

Data for the effect of washing solutions supplemented with antioxidants before freezing are shown in Table 1. Significant differences were observed in motility and morphology of cooled boer goat semen, when semen washed with tris citric acid fructose extender supplemented with either hypotaurine or cysteine than non-supplemented group ($P < 0.05$) while there was no significant difference ($P > 0.05$) in other traits.

Table 1. Effects of washing solution (Tris citric acid fructose extender supplemented antioxidants) on motility, membrane integrity, acrosome integrity and viability in pre freezing boer goat spermatozoa.

Washing solution with and without antioxidants	Motility (%)	Membrane Integrity ^{ns} (%)	Morphology (%)	Acrosome Integrity (%) ^{ns}	Viability ^{ns} (%)
TCF with Hypotaurine	75.37 \pm 0.60 _a	61.12 \pm 1.4 ₉	85.87 \pm 0.79 ^a	66.00 \pm 1.0 ₂	88.12 \pm 0.6 ₉
TCF without Hypotaurine	71.50 \pm 0.76 _b	60.62 \pm 1.6 ₁	82.75 \pm 0.65 ^b	64.62 \pm 0.8 ₂	87.37 \pm 0.5 ₃
TCF with Cysteine	76.00 \pm 0.63 _a	63.12 \pm 2.4 ₅	84.00 \pm 1.02 ^a _b	66.12 \pm 0.9 ₀	88.87 \pm 0.5 ₂
TCF without Cysteine	72.00 \pm 0.96 _b	59.25 \pm 1.4 ₅	83.25 \pm 0.59 ^b	64.87 \pm 0.7 ₄	87.25 \pm 0.4 ₉

Values with different superscripts within column differ significantly at $P < 0.05$; ns No significant difference.

Table 2. Effects of washing solution (Tris citric acid fructose extender supplemented with antioxidants) on motility, membrane integrity, acrosome integrity and viability in post thaw boer goat spermatozoa.

Washing solution with and without antioxidants	Motility (%)	Membrane Integrity ^{ns} (%)	Morphology ^{ns} (%)	Acrosome Integrity (%)	Viability ^{ns} (%)
TCF with Hypotaurine	67.75 \pm 0.70 ^a	59.37 \pm 1.59	79.87 \pm 1.04	63.25 \pm 0.96 ^a	86.75 \pm 0.98
TCF without Hypotaurine	65.25 \pm 0.94 ^b	57.25 \pm 1.25	76.62 \pm 1.08	60.00 \pm 0.71 ^b	85.87 \pm 0.67
TCF with Cysteine	67.25 \pm 0.82 ^a	61.37 \pm 2.20	78.00 \pm 1.46	62.75 \pm 0.59 ^a	86.50 \pm 0.78
TCF without Cysteine	65.00 \pm 0.57 ^b	56.87 \pm 1.16	77.87 \pm 0.93	60.12 \pm 0.40 ^b	85.62 \pm 0.80

Values with different superscripts within column differ significantly at $P < 0.05$; ns No significant difference.

Data for the influence of washing solution supplemented with antioxidants after freezing are summarized in Table 2. Significant ($P < 0.05$) improvement was observed by supplementation of tris citric acid fructose extender in term of motility and acrosome integrity compared to non-supplemented groups.

Semen cryopreservation allows the widespread dissemination of valuable genetic material, even to small flocks by means of AI, leading to an increased rate of genetic gain (Atessahin *et al.*, 2008). However, mammalian sperm membranes incorporate many unsaturated fatty acids therefore are highly sensitive to lipid peroxidation, which occurs as a result of the oxidation of the membrane lipids by the partially reduced oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals, leading to decreased sperm quality (Bucak *et al.*, 2007; Lenzi *et al.*, 2002). This antioxidant capacity in sperm cells may however, be insufficient in preventing lipid peroxidation during the freeze thawing process. Thus mammalian spermatozoa lack a significant cytoplasmic component, which contains antioxidants that counteract the damaging effects of reactive oxygen species and lipid peroxidation (Aurich *et al.*, 1997; Storey, 1997). There are number of strategies to overcome this among them inclusion of antioxidants in the cryopreservation media is one of them. It has been reported by many studies (Sariozkan, 2000; Memon *et al.*, 2011) that addition of antioxidants improved the quality of semen against Reactive Oxygen Species - induced damage. Various antioxidants have been used for this purpose. Hypotaurine and cysteine are also categorized as antioxidants and used in the cooling and cryopreservation media to protect spermatozoa against Lipid per Oxidation (LPO). Hypotaurine is a precursor of taurine which exists in mammalian sperm including men (Van *et al.*, 1966), hamster (Meizel *et al.*, 1980), rat (Fraster, 1986) bull (Guerin *et al.*, 1995) and boar (Johnson *et al.*, 1972). It is essential for sperm functions such as capacitation, motility, fertilizing ability and early embryonic development. It is known to neutralize hydroxyl radicals produced during LPO and thus, to protect the thiol groups in the sperm plasma membrane, preventing sperm from damage due to oxidation (Feilman *et al.*, 1987; Barnett and Bavister, 1992).

Hypotaurine binds avidly with the hydroxyl ion (Pasantes-Morales and Fellman, 1989) which could play an important role in the protection of the sperm membrane lipid as its high unsaturated fatty acid content is susceptible to lipid peroxidation injuries (Huxtable, 1992). Cysteine is a precursor of intracellular glutathione (Uysal and Bucak, 2007). It has been shown to penetrate the cell membrane easily, enhancing the intracellular reduced glutathione (GSH) biosynthesis both *in vivo* and *in vitro* and protecting the membrane lipids and proteins due to indirect radical scavenging properties. It is also thought that GSH synthesis under *in vitro* conditions may be impaired because of deficiency of cysteine in the media, due to its high instability and auto oxidation to cysteine (Bucak *et al.*, 2008). Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post thawed sperm motility in many species i.e., ram (Uysal and Bucak, 2007) goat (Bucak and Uysal, 2008), bull (Bilodeau *et al.*, 2001) and boar semen (Szczesniak-Fabianczyk, 2006). Addition of antioxidants in extenders aimed to reduce the oxidative stress and prevent the cold shock and cryo injury to spermatozoa.

In routine extension and freezing procedures for semen cryopreservation of buck, antioxidants added into either cooling, freezing or in both media after washing. During washing process, not only seminal plasma separated but also other beneficial components are also removed. These substances are essential for functionality and survival and preventing lipid peroxidation of spermatozoa after ejaculation. The dilution of semen critically affects the sperm survival during cryopreservation. The total antioxidants concentration in the extender and cells could be reduced when semen was diluted many folds in extenders (Kumar and Das, 2005). The dilution of semen considerably caused in decrease of the concentration of antioxidants in spermatozoa because of limited capacity of antioxidants to scavenger the oxidants (Bucak *et al.*, 2008). Hence, effectiveness of supplementation of cryopreservation media with antioxidants depends on dilution and time of antioxidant addition. It is apparent that dilution reduces the amount of antioxidants which is already insufficient for prevention of oxidative stress. Furthermore, washing with a media containing no antioxidants may further deteriorate the quality after freezing. In the present study, significantly ($P < 0.05$) better results in terms of motility and morphology of cooled spermatozoa have been observed when spermatozoa washed with a media (tris citric acid fructose) supplemented with either hypotaurine or cysteine. Significant ($P < 0.05$) improvement was also observed in term of motility and acrosome integrity compared to non-supplemented groups after freezing. To our knowledge, this is first report of improving frozen spermatozoa quality by means of semen washing with media supplemented with antioxidants.

CONCLUSION

In conclusion, from present study it is observed that early addition of antioxidants in washing solution could be advantages in improving the quality of chilled and frozen boer goat semen.

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REFERENCES

- Atessahin, A., M. N. Bucak, P. B. Tuncer and M. Kizil. 2008. Effects of antioxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process. *Small Rum. Res.*, 77: 38-44.
- Aurich, J. E., U. Schonherr, H. Hoppe and C. Aurich. 1997. Effect of antioxidants on motility and membrane integrity of chilled-stored stallion semen. *Theriogenology*, 48: 185-192.

Avdi, M., B. Leboeuf and M. Terqui. 2004. Advanced breeding and "buck effect" in indigenous Greek goats. *Livestock Prod. Sci.*, 87: 251-257.

Azeredo, G. A., C. R. Esper and K. T. Resende. 2001. Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. *Small Rum. Res.*, 41: 257-263.

Barnett, D. K. and B. D. Bavister. 1992. Hypotaurine requirement for in vitro development of golden hamster one-cell embryos into morulae and blastocysts, and production of term offspring from in vitro-fertilized ova. *Biol. Reprod.*, 47: 297-304.

Bilodeau, J. F., S. Blanchette., I. C. Gagnon, and M. A. Sirard. 2001. Thiols prevent H₂O₂-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*, 56: 275-286.

Bucak, M. N., A. Atessahin and A. Yuce. 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rum. Res.*, 75: 128-134.

Bucak, M. N. and O. Uysal. 2008. The role of antioxidants in freezing of Saanen goat semen. *Indian Vet. J.*, 85: 148-150.

Bucak, M. N. and N. Tekin. 2007. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Rum. Res.*, 73: 103-108.

Buckett, W. M., M. J. Luckas, I. A. Aird, R. G. Farquharson, C. R. Kingsland and D. I. Lewis-Jones. 1997. The hypo-osmotic swelling test in recurrent miscarriage. *Fertil. Steril.*, 68: 506-509.

Evans, G. and W. M. C. Maxwell. 1987. Handling and examination semen. In: Maxwell, W.M.C. (Ed.), *Salamon's Artificial Insemination of Sheep and Goat*. Butterworths, Sydney, pp. 93-106.

Feilman, J. H., T. R. Green and A. U. Eicher. 1987. The oxidation of hypotaurine to taurine: bis-aminoethyl-alpha-disulfone, a metabolic intermediate in mammalian tissue spermatozoa. *Adv. Exp. Med. Biol.*, 217: 39-48.

Fraster, L. R. 1986. Both taurine and albumin support mouse sperm motility and fertilizing ability invitro but there is no obligatory requirement for taurine. *J. Reprod. Fertil.*, 77: 271-280.

Guerin, P., J. Guillaud and Y. Menezo. 1995. Hypotaurine in spermatozoa and genital secretions and its production by oviduct epithelial cells in vitro. *Hum Reprod.*, 10: 866-872.

Huxtable, R. J. 1992. Physiological action of taurine. *Physiol Rev.*, 72: 101-163.

Johnson, L. A., V. G. Pursel., R. J. Gerrits, and C. H. Thomas. 1972. Free amino acids composition of porcine seminal, epididymal and seminal vesicals fluids. *J Anim Sci.*, 34: 430-434.

Kumar, S., and G. K. Das. 2005. Frozen sperm quality with reference to reactive oxygen species: A review. *Indian. J. Anim. Sci.*, 75: 874-884.

Lenzi, A., L. Gandini., F. Lombardo., M. Picardo., V. Maresca, and E. Panfili. 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione and glutathione-dependent enzyme-PHGPx: from basic to clinic. *Contraception*, 65: 301-304.

Liu, Z., R. H. Foote and C. C. Brackett. 1998. Survival of bull sperm frozen at different rates in media varying in osmolarity. *Cryobiology*, 37: 219-230.

Meizel, S., C. W. Liu., P. K. Working and R. J. Murny. 1980. Taurine and Hypotaurine: their effects on motility, capacitation, and acrosome reaction of hamster sperm in vitro and their presence in sperm and reproductive tract fluid of several mammals. *Dev. Growth Differ.* 22: 483-494.

Memon, A. A., H. Wahid, Y. Rosnina, Y. M. Goh, M. Ebrahimi, F. M. Nadia and G. Audrey. 2011. Effect of hypotaurine and cysteine on sperm cytological parameters of cooled and post thaw boer goat semen. *Elixir Bio Tech.*, 38: 4100-4104.

Naing, S. W., H. Wahid, K. M. Azam, Y. Rosnina, A. B. Zuki, S. Kazhal, M. M. Bukar, M. Thein, T. Kyaw and M. M. San. 2010. Effect of sugars on characteristics of Boer goat semen after cryopreservation. *Anim. Reprod. Sci.*, 122: 23-28.

Pasantes-Morales, H., and J. H. Fellman. 1989. Taurine and Hypotaurine and membrane lipid peroxidation. In: Miquel J, Quintanilha, A.T., Weber, H., editors. *CRC handbook of freeredicals and antioxidants in biomedicine*, Vol. II Boca Raton, FLL CRC press, pp. 105-117.

Peterson, K., M. A. P. M. Kappen, P. J. F. Ursem, J. O. Nothling, B. Colenbrander and B. M. Gadella. 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: Effect of semen processing procedures and their correlation to fertility. *Theriogenology*, 67: 863-871.

Purdy, P. H. 2006. A review on goat sperm cryopreservation. *Small Rum. Res.*, 63: 215-225.

Revell, S. G. and R. A. Mrode. 1994. An osmotic resistance test for bovine semen. *Anim. Reprod. Sci.*, 36: 77-86.

Sariozkan, S., M. N. Bucak, P. B. Tuncer, P. A. Ulutas and A. Bilgen. 2000. The influence of cysteine and taurine on microscopic-oxidative stress parameters and

fertilizing ability of bull semen following cryopreservation. *Cryobiology*, 2: 134-138.

Shamsuddin, M., Y. Amiri and M. M. U. Bhuiyan. 2000. Characteristics of Buck Semen with Regard to Ejaculate Numbers, Collection Intervals, Diluents and Preservation periods. *Reprod. Dom. Anim.*, 35: 53-57.

Storey, B. T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol. Hum. Reprod.*, 3: 203-213.

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 chromatine structure. *Reprod. Biol.*, 3: 81-87.

Ustuner, B., U. Gunay and Z. Nur. 2009. Effect of seminal plasma, egg yolk, and season on the freezability of saanen buck semen. *Bull Vet. Inst. Pulawy*, 53: 369-374.

Uysal, O. and M. N. Bucak. 2007. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Veterinaria Brno*, 76: 383-390.

Van der Horst, C. J. G. and H. J. G. Grooten. 1966. The occurrence of hypotaurine and other sulphur containing amino acids in seminal plasma and spermatozoa of boar, bull and dog. *Biochim Biophys Acta*, 117: 495-497.

White, I. G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod. Fertil. Dev.*, 5: 639-658.

Yildiz, C., A. Kaya, M. Aksoy and T. Tekeli. 2000. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology*, 54: 579-585.

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