Indian Journal of Animal Sciences **91** (8): 623–627, August 2021/Article
https://doi.org/10.56093/ijans.v91i8.115918

Antioxidants improve the semen quality following cryopreservation in Indian yak bulls

B K D BORAH[1], B C DEKA[2], R K BISWAS[2], P CHAKRAVARTY[2], S SINHA[3], K AHMED[3] and S DEORI[3]✉

Assam Agricultural University, Khanapara, Guwahati, Assam 781 022 India

Received: 23 July 2020 ; Accepted: 2 August 2021

ABSTRACT

The present study was conducted to elucidate the effect of different antioxidants on the semen quality of cryopreserved yak semen. The ejaculates found suitable were extended (1:10) with 4 different Tris extenders by split sample technique containing different antioxidants in each of 3 extenders, viz. Taurine @ 50 mM, Trehalose @ 100 mM or vitamin E @ 2 mM and one control (without additive). The sperm motility, live sperm, HOST-reacted sperm, total incidence of acrosomal changes and extracellular release of ALT and AST differed significantly between antioxidants. Total sperm motility, live sperm and HOST-reacted sperm were significantly higher and total incidence of acrosomal changes and extracellular release of AST were significantly lower in extender containing antioxidants than that in control. The difference between taurine, trehalose and vitamin E was not significant for parameters like sperm motility, HOST-reacted sperm and extracellular release of ALT. The per cent live sperm was significantly higher and total incidence of acrosomal changes was significantly lower for taurine than for trehalose and vitamin E. The extracellular release of ALT was significantly lower in extender containing taurine or trehalose than in control while the extracellular release of AST was significantly lower in the extender containing taurine than that in containing trehalose, vitamin E and control. The findings of the present study revealed that addition of taurine, trehalose and vitamin E in Tris extender significantly improved the post thaw quality of yak semen. Among the antioxidants evaluated, 50 mM of taurine in Tris extender could be effectively used to obtain better quality of frozen thawed yak semen.

Keywords: Antioxidants, Cryopreservation, Extender, Semen, Yak

The gradual decline of yak population in the country has become a cause of concern among the development authorities as these animals largely cater to almost all the needs of the highlanders. Geographical isolation of yak herds in hilly inaccessible terrains increased the risk of inbreeding which adversely affected the genetic potential of these animals, resulting in poor economic return. Cryopreservation of semen and use of artificial insemination (AI) can be one of the effective tools for overcoming the inbreeding problem in yaks (Deori *et al*. 2016). Sperm cryopreservation not essentially only preserves the genetic resources, but also supports transportation of species between remote locations. Semen from Indian yak was successfully cryopreserved in Tris extender with 6.4% glycerol and 20% egg yolk following an equilibration period of 4 hr (Deori 2017). However, during the process of cryopreservation, spermatozoa are exposed to cold shock which render them susceptible to lipid peroxidation. Bansal and Bilaspuri (2011) reviewed the impact of oxidative stress and antioxidants on sperm function and explained effects of various types of antioxidants. Earlier research has shown that supplementation of antioxidants like alpha-tocopherol, butylated hydroxytoulene, superoxide dismutase and catalase, cysteine or glutathione to the semen extenders reported to improve the semen quality in both chilled and frozen-thawed semen in boar (Pena *et al.* 2003, Roca *et al*. 2005, Satorre *et al*. 2007), bull (Bilodeau *et al*. 2001), turkey (Donoghue and Donoghue 1997), stallion (Ball *et al*. 2001) and ram (Uysal and Bucak 2007). However, there is no such study with yak semen. Taurine is a non-enzymatic scavenger that protects the spermatozoa against reactive oxygen species (ROS) and thus exerted beneficial effects by decreasing cellular damage. Trehalose, interacts with phospholipids in the plasma membrane and increases membrane fluidity leading to greater resistance of spermatozoa against freeze-thawing damage (Reddy *et al.* 2010). Vitamin E protects the spermatozoa by preventing endogenous oxidative DNA and membrane damage, thereby helping them to overcome the oxidative attack. In the present study, antioxidants namely taurine, trehalose and vitamine E were incorporated in Tris extender and their

Present address: [1]Krishi Vigyan Kendra, Namsai, Arunachal Pradesh. [2]ICAR-NRC on Yak, Dirang, Arunachal Pradesh. [3]ICAR Research Complex for NEH region, Umiam, Meghalaya. ✉Corresponding author e-mail: drsdeori@gmail.com

relative effects in improving the quality of frozen yak semen was studied.

MATERIALS AND METHODS

Four healthy yak bulls aged 3 to 4.5 years were used to conduct the present study at institutional farm of ICAR-National Research Centre on Yak, Dirang, West Kameng district of Arunachal Pradesh located at an altitude of above 2,730 msl. Artificial vagina (AV) method was applied to collect 20 ejaculates from each of 4 bulls at weekly intervals. Immediately after collection, the collection tubes containing semen was placed in a water bath maintained at 35°C and initial evaluations were done as per standard methods (Borah 2013).

The ejaculates having minimum volume of 1.00 ml, mass activity 3^+ and initial sperm motility 70% were selected for further processing and freezing. The ejaculates found suitable were extended (1:10) with 4 different Tris extenders (Foote 1970) by split sample technique containing different additives in each of 3 extenders, viz. Taurine @ 50 mM, Trehalose @ 100 mM or vitamin E @ 2 mM and 1 control (no additive) based on available literature. The extended semen was filled into 0.5 medium straws @ 30 million spermatozoa per straw and then cooled @ 1°C/3 min and equilibrated for 4 h at 5°C before vapour freezing and finally stored in liquid nitrogen.

Based on the evaluation of total sperm motility, live sperm per cent, HOST-reacted spermatozoa, total acrosomal changes, and extracellular release of alanine transferase (ALT) and aspartate transferase (AST) both after equilibration and freezing, the influence of the different antioxidants on the post thaw quality of the semen was found out. Total sperm motility was examined under a phase contrast microscope at a magnification of 400× and recorded from 0 to 100 based on the percentage of motile sperm. Percentage of live spermatozoa was determined using Eosin-Nigrosin staining technique. A thin smear was prepared on a clean grease-free glass slide and 200 spermatozoa were examined in different areas of the smear at a magnification of 1000× of microscope for determining the percentage of live spermatozoa. Stained or partially stained spermatozoa were considered as dead. The HOST-reacted spermatozoa were determined as per Jeyendran *et al*. (1984) by using hypo-osmotic solution. Total acrosomal changes were studied in stained semen smear using Giemsa staining technique (Watson 1975). The ALT and AST activity was estimated by enzymatic colorimetric method using commercial ENZOKIT for ALT or AST (RFCL Limited, Dehradun and was expressed in IU/L.

Analyses were performed using the Statistical System software package (SAS, Cary, NC, USA, 2010). Data on different parameters were analyzed using two-factorial analysis of variance (ANOVA) (GLM procedure).

RESULTS AND DISCUSSION

The result of mean sperm motility, percentage of live sperm and HOST-reacted spermatozoa at different stages of processing of yak semen are presented in Table 1 and mean total incidence of acrosomal changes, extracellular release of ALT and AST of yak semen in Table 2.

The overall total sperm motility, live sperm and HOST-reacted sperm of yak semen extended in Tris extender differed significantly ($P<0.05$) between additives and between stages. Critical difference test revealed that total sperm motility, live sperm and HOST-reacted sperm were significantly ($P<0.05$) higher in extender containing additives than in control. The difference between taurine, trehalose and vitamin E was not significant for sperm motility and HOST-reacted sperm, but per cent live sperm was significantly ($P<0.05$) higher for taurine than for trehalose and vitamin E. The per cent sperm motility, live sperm and HOST-reacted sperm dropped significantly ($P<0.05$) from fresh semen to equilibrated semen and from equilibrated semen to frozen semen.

The total incidence of acrosomal changes differed significantly between additives, between stages and due to

Table 1. Per cent sperm motility, live spermatozoa and HOST-reacted sperm (mean*±SE) of yak semen in tris extender containing three additives at different stages of processing and freezing

Stage	Additives											
	% Sperm motility				% Live spermatozoa				% HOST-reacted sperm			
	Taurine	Trehalose	Vit E	Control	Taurine	Trehalose	Vit E	Control	Taurine	Trehalose	Vit E	Control
Fresh semen	$81.50^{a}\pm$ 0.89	$81.50^{a}\pm$ 0.89	$81.50^{a}\pm$ 0.89	$81.50^{a}\pm$ 0.89	$87.90^{a}\pm$ 0.76	$87.90^{a}\pm$ 0.76	$87.90^{a}\pm$ 0.76	$87.90^{a}\pm$ 0.76	$79.25^{a}\pm$ 0.64	$79.25^{a}\pm$ 0.64	$79.25^{a}\pm$ 0.64	$79.25^{a}\pm$ 0.64
After equilibration	$73.75^{b}\pm$ 1.14	$72.00^{b}\pm$ 1.28	$71.70^{b}\pm$ 1.22	$68.50^{b}\pm$ 1.48	$81.10^{b}\pm$ 0.95	$78.75^{b}\pm$ 1.16	$78.20^{b}\pm$ 1.06	$76.55^{b}\pm$ 1.06	$72.70^{b}\pm$ 1.10	$70.85^{b}\pm$ 1.03	$70.00^{b}\pm$ 1.27	$67.65^{b}\pm$ 1.07
After freezing	$62.75^{c}\pm$ 1.38	$60.50^{c}\pm$ 1.49	$60.20^{c}\pm$ 1.64	$56.25^{c}\pm$ 1.25	$71.15^{c}\pm$ 1.09	$68.30^{c}\pm$ 0.86	$68.20^{c}\pm$ 1.12	$65.10^{c}\pm$ 0.95	$62.00^{c}\pm$ 1.32	$59.10^{c}\pm$ 1.24	$60.15^{c}\pm$ 1.34	$57.15^{c}\pm$ 1.06
Overall	$72.67^{A}\pm$ 1.20	$71.33^{A}\pm$ 1.32	$71.1^{A}\pm$ 1.35	$68.75^{B}\pm$ 1.48	$80.05^{A}\pm$ 1.04	$78.32^{B}\pm$ 1.17	$78.10^{B}\pm$ 1.19	$76.52^{C}\pm$ 1.32	$71.32^{A}\pm$ 1.10	$69.70^{A}\pm$ 1.22	$69.80^{A}\pm$ 1.20	$68.02^{B}\pm$ 1.29

$^{a, b, c}$Means bearing different letter superscripts differ significantly ($P<0.05$) within column; $^{A, B, C}$means bearing different letter superscripts differ significantly ($P<0.05$) within row.

Table 2. Per cent total incidence of acrosomal changes, extra cellular release of alanine transferase (ALT) and aspartate transferase (AST) (mean*±SE) of yak semen in tris extender containing 3 additives at different stages of processing and freezing

Stage	Antioxidants											
	Total incidence of acrosomal changes (%)				ALT (IU/l)				AST (IU/l)			
	Taurine	Trehalose	Vit E	Control	Taurine	Trehalose	Vit E	Control	Taurine	Trehalose	Vit E	Control
Fresh semen	$3.60^{a}\pm 0.27$	$3.60^{a}\pm 0.27$	$3.60^{a}\pm 0.27$	$3.60^{a}\pm 0.27$	–	–	–	–	–	–	–	–
After equilibration	$6.53^{b}\pm 0.24$	$7.20^{b}\pm 0.37$	$7.53^{b}\pm 0.35$	$8.87^{b}\pm 0.16$	$5.70^{a}\pm 0.67$	$6.17^{a}\pm 0.95$	$6.41^{a}\pm 0.49$	$7.6\,8^{a}\pm 0.51$	$5.01^{a}\pm 0.59$	$7.05^{a}\pm 0.54$	$7.04^{a}\pm 0.62$	$8.20^{a}\pm 0.76$
After freezing	$10.40^{c}\pm 0.41$	$12.07^{c}\pm 0.45$	$11.60^{c}\pm 0.46$	$14.33^{c}\pm 0.41$	$13.10^{b}\pm 0.96$	$13.73^{b}\pm 0.84$	$14.67^{b}\pm 0.63$	$16.24^{b}\pm 1.08$	$11.12^{b}\pm 0.90$	$13.97^{b}\pm 1.02$	$14.90^{b}\pm 0.86$	$17.81^{b}\pm 0.60$
Overall	$6.84^{C}\pm 0.46$	$7.62^{B}\pm 0.56$	$7.58^{B}\pm 0.53$	$8.93^{A}\pm 0.68$	$9.40^{B}\pm 1.02$	$9.95^{B}\pm 1.06$	$10.54^{AB}\pm 1.02$	$12.00^{A}\pm 1.12$	$8.06^{C}\pm 0.87$	$10.51^{B}\pm 0.97$	$10.97^{B}\pm 1.04$	$13.01^{A}\pm 1.20$

[a, b, c]Means bearing different letter superscripts differ significantly (P<0.05) within column; [A, B, C]means bearing different letter superscripts differ significantly (P<0.05) within row.

interaction (P<0.01). Critical difference test showed that incidences of total acrosomal changes were significantly (P<0.05) lower in extender containing additives than that in control group. The incidences of entirely lost acrosome and total acrosomal changes were significantly (P<0.05) lower in extender containing taurine than that containing trehalose and vitamin E.

The extracellular release of ALT and AST in yak semen differed significantly (P<0.05) between additives and between stages. The critical difference test showed that the extracellular release of ALT was significantly (P<0.05) lower in extender containing taurine and trehalose than that in control while the extracellular release of AST in the extender containing taurine was significantly (P<0.05) lower than that of trehalose, vitamin E and control.

The process of cryopreservation, viz. cooling, freezing and thawing cause both physical and chemical stresses on sperm membranes (Chatterjee *et al.* 2001) and also exert an oxidative stress (Salvador *et al.* 2006). Reactive oxygen species (ROS) or free radicals are produced during cooling, freezing and thawing of semen of bull, ram, buck and boar (Bilodeau *et al.* 2001, Funahasi and Sano 2005, Bucak *et al*. 2008, Anghel *et al*. 2010). These free radicals act on the phospholipids of cell membrane leading to lipid peroxidation, sperm damage and acrosomal disintegration (Kim and Parthasarathy 1998). Mammalian sperm cells are particularly susceptible to lipid peroxidation due to the fairly low activity of enzymatic anti-oxidative system and because cellular and intracellular sperm membranes are rich in polyunsaturated fatty acids that are easily susceptible to free radical-induced peroxidative damage. Unlike somatic cells, that rely on cytoplasmic enzymes, such as catalase, superoxide dismutase and glutathione peroxidase, for their antioxidant defence, spermatozoa are almost devoid of most of their cytoplasm and hence lose this protection (Hsieh *et al*. 2006). Lipid peroxidation triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, structural damage to DNA and cell death. Antioxidants partially ameliorate the negative effects of ROS produced during cryopreservation by acting against the free radicals and scavenging them to protect the cells from sub-lethal damage.

In the present study, antioxidants namely Taurine, Trehalose and vitamine E were incorporated in Tris extender and showed significant effects in improving the quality of frozen yak semen at different stages of processing. Concentrations of Taurine 50 mM, Trehalose 100 mM and vitamin E 2 mM were reported to be better for improvement of post-thaw quality of semen of different species (Uysal and Bucak 2007, Mazumdar *et al.* 2012). However, several workers reported that influence of antioxidants, viz. taurine, trehalose and vitamin E on quality of frozen semen depended on concentration of these antioxidants (Kishor *et al.* 2011, Kumar and Afreja 2012).

In the present study, percentage of sperm motility, live sperm and HOST-reacted sperm in yak semen were found to be significantly (P<0.05) higher in Tris extender added with Taurine, Trehalose or vitamin E than in that added with no additive (control), but the difference between Taurine, Trehalose and vitamin E was non-significant except for percentage of live sperm. The percentage of live sperm was significantly (P<0.05) higher for Taurine than for trehalose and vitamin E. Similar observation of better post thaw quality of semen in Tris extender containing Taurine, Trehalose and vitamin E than that in control was reported in bulls (Kishor *et al.* 2011), buffalo bull (Kumar and Atreja 2012, Mazumder*et al.* 2012) and buck (Anghel *et al.* 2009) semen. Kishor *et al.* (2011) reported that both after equilibration and after freezing sperm motility, viability and HOST-reactive spermatozoa were significantly (P<0.05) higher in Tris extender supplemented with Taurine than with catalase, ascorbic acid or no additive. Kumar and Atreja (2012) recommended Taurine (50 mM) and Trehalose (100 mM) in Tris-based-egg yolk extender for significant (P<0.05) improvement in post-thaw sperm motility and live sperm per cent in buffalo semen. Significantly (P<0.05)

higher percentage of live sperm recorded with Taurine than with Trehalose in yak semen is in conformity with that of Mazumder *et al*. (2012), who reported that post-thaw sperm motility and intact acrosome were significantly (P<0.05) higher in Tris extender supplemented with Taurine than that with Trehalose. Several other workers reported in different species of animals that addition of additives in the extender had no significant effect or had adverse effect on some post thaw parameters of semen (Atessahin *et al.* 2008, Asadpour *et al.*2011). The discrepancies in the effect of different additives on the post-thaw quality of semen might be due to species variation, the difference in the concentration of additives, and their interaction with other constituents of the extender used in different studies.

The present finding of lower incidences of acrosomal changes in the extender containing additives than that in control was in agreement with that reported by Swain *et al.* (2009) in bull semen, Badr *et al*. (2010) and Kumar and Atreja (2012) in buffalo semen, and Khalili *et al.* (2009) in buck semen. Kumar and Atreja (2012) recommended Taurine (50 mM) or Trehalose (100 mM) and Badr *et al*. (2010) recommended Trehalose (100 mM) in Tris-based-egg yolk extender for significant (P<0.05) improvement in post-thaw acrosomal integrity in buffalo semen over control group. The findings of the present study revealed that incorporation of taurine (50 mM) in Tris extender resulted in lower incidence of acrosomal changes. Significantly (P<0.05) higher percentage of intact acrosome after freezing semen in Tris extender containing 100 mM was also reported by Aisen *et al.* (2002) in ram semen and Khalili *et al.* (2009) in buck semen. However, other workers reported that incorporation of additives in the extender had no significant effect on post-thaw acrosomal integrity of spermatozoa (Bucak *et al*. 2007, Atessahin *et al.*2008). The discrepancies in the effect of different additives on the post thaw quality of semen might be due to the difference in the concentration of additives in the extender and species of animals used in different studies.

The extracellular release of ALT was significantly (P<0.05) lower in extender containing taurine or trehalose than that containing no additive. The difference between Taurine, Trehalose and vitamin E was not significant. The extender incorporated with vitamin E recorded apparently lower values of ALT activity than that of control and apparently higher values than that of Taurine and Trehalose. The extracellular release of AST was significantly (P<0.05) lower in extender containing Taurine than that containing Trehalose, vitamin E or no additive and the values for Trehalose and vitamin E were significantly (P<0.05) lower than that for control.

The findings of the present study revealed that addition of Taurine, Trehalose and vitamin E in Tris extender significantly improved the post-thaw quality of yak semen and 50 mM of taurine in Tris extender could be effectively used to obtain better quality of frozen yak semen. This will further improve the semen quality of cryopreserved yak semen and finally the overall fertility.

REFERENCES

Aisen E G, Medina V H and Venturino A. 2002. Cryopreservation and post thaw fertility of ram semen frozen in different trehalose concentration. *Theriogenology* **57**: 1801–08.

Anghel A, Zamfirescu S, Dragomir C, Nadolu D, Elena S and Florica B. 2010. The effects of antioxidants on the cytological parameters of cryopreserved buck semen. *Romanian Biotechnological Letters* **15**: 26–32.

Anghel A H, Zamfirescu S and Coprean D. 2009. Influence of vitamin E on microscopic and oxidative parameters of frozen-thawed ram semen. *Lucrari Stiintifice* **54**: 261–65.

Asadpour R, Jafari R and Tayefi-Nasrabadi H. 2011. Influence of added vitamin C and vitamin E on frozen-thawed bovine sperm cryopreserved in citrate and tris based extender. *Veterinary Research Forum* **2**: 37–44.

Atessahin A, Bucak M N, Tuncer P B and Kizil M. 2008. Effects of antioxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze-thawing process. *Small Ruminant Research* **77**: 38–44.

Badr M R, Mary G, El-Malak A and Hassan M H. 2010. Effect of trehalose on cryopreservation, oxidative stress and DNA integrity of buffalo semen. *Journal of Reproduction and Fertility* **1**: 50–57.

Ball B A and Vo A. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. *Journal of Andrology* **22**: 1061–69.

Bansal A K and Bilaspuri G S. 2011. Impact oxidative stress and antioxidants on semen functions. *Veterinary Medicine International* doi: 10.4061/2011/686137. 1–7.

Bilodeau J F, Blanchette S, Gagnon I C and Sirard M A. 2001. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* **56**: 275–86.

Borah B K D. 2013. 'Cryo-preservation of semen and artificial insemination in yak (*Poephagus grunniens* L.).' Ph D Thesis. Assam agricultural University, Jorhat, India

Bucak M N, Atessahin, A and Yuce A. 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research* **75**: 128–34.

Bucak M N, Atessahin A, Varisli O, Yuce A, Tekin N and Akcay A. 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology* **67**: 1060–67.

Chatterjee S, de Lamirande E and Gagnon C. 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. *Molecular Reproduction and Development* **60**: 498–506.

Deori S, Das P J, Paul V, Hussain M, Medhi D, Bam J, Bhattacharya D and Deb S M. 2016. Cryopreservation of semen for *ex situ* conservation of Yak (*Poephagus grunniens* L.). International Conference Global Biodiversity, Climate Change and Sustainable Development, Itanagar, Oct. 15–18, Pp. 109.

Deori S. 2017. The effect of freezing on cryosurvival of yak sperm. *SAARC Journal of Agriculture* **15**: 215–18.

Donoghue A M and Donoghue D J. 1997. Effects of water and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poultry Science* **76**: 1440–55.

Foote R H. 1970. Influence of extender, extension rate on glycerolating technique on fertility of frozen bull semen. *Journal of Dairy Science* **53**: 1478–82.

Funahashi H and Sano T. 2005. Select antioxidants improve the

function of extended boar semen stored at 10°C. *Theriogenology* **63**: 1605–16.

Hsieh Y Y, Chang C C and Lin C S. 2006. Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *International Journal of Biological Science* **2**: 23–39.

Jeyendran R S, Van-der-Ven H H, Perez-Pelaez M, Crabo B G and Zaneveld L J. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.

Khalili B, Farshad A, Zamiri M J, Rashidi A and Fazeli P. 2009. Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. *Asian-Australasian Journal of Animal Sciences* **22**: 1614–19.

Kim J G and Parthasarathy S. 1998. Oxidation and spermatozoa. *Reproduction and Endocrinology* **16**: 235–39.

Kishor A, Raina V S, Mohanty T K, Gupta A K, Bishist R, Singh M and Rao T K S. 2011. Evaluation of antioxidants for preservation of cattle semen. *Indian Veterinary Journal* **88**: 37–39.

Kumar R and Atreja S K. 2012. Effect of incorporation of additives in Tris-based egg yolk extender on buffalo (*Bubalus bubalis*) sperm tyrosine phosphorylation during cryopreservation. *Reproduction in Domestic Animal* **47**: 485–90.

Mazumdar H, Deka B C, Sinha S, Biswas R K, Das G C, Sarmah B K and Das P K. 2012. *Effect of additives on quality of frozen Swamp buffalo semen*, pp. 191–92. National symposium on addressing animal Reproductive Stresses Through Biotechnological tools and XXVIII Annual Convention of ISSAR, Guwahati, Nov. 21–23.

Pena F J, Johannisson A, Wallgren M and Rodriguez-Martinez H. 2003. Antioxidant supplementation *in vitro* improves boar semen motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science* **78**: 85–98.

Reddy N S S, Mohanarao G J and Atreja S K. 2010. Effects of adding taurine and trehalose to a tris based egg yolk extender on buffalo (*Bubalus bubalis*) sperm quality following cryopreservation. *Animal Reproduction Science* **119**: 183–90.

Roca J, Rodriguez M J, Gil M A, Carvajal G, Gracia E M, Cuello C, Vazquez J M and Martinez E A. 2005. Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *Journal of Andrology* **26**: 15–24.

Salvador I, Yaniz J, Viudes De Castro M P and Gomez E A. 2006. Effect of solid storage on caprine semen conservation at 5°C. *Theriogenology* **64**: 252–60.

Satorre M M, Breininger E, Beconi M T and Beorlegui N B. 2007. Alpha-tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm. *Theriogenology* **68**: 958–65.

Swain D K, Kudu A K, Rath A and Mohapatra A P K. 2009. Effect of addition of antioxidants on post-thaw semen quality of cryopreserved bull sperm. *Indian Journal of Animal Sciences* **79**: 470–72.

Uysal O and Bucak M N. 2007. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Veterinaria Brno* **76**: 383–90.

Watson P F. 1975. Use of a giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Veterinary Record* **97**: 12–15.