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Effect of hypotaurine and cysteine on sperm cytological parameters of cooled and post thaw boer goat semen

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ABSTRACT

The purpose of this study was to determine the influence of antioxidant additives (hypotaurine and cysteine) in different concentrations to the cryopreserving media on the semen cytological parameters pre freezing and post thawing (motility, membrane integrity, morphology, acrosome integrity and viability).The experiment was done on 30 ejaculates collected by artificial vagina method from 5 boer goat bucks during April to May 2011. After collection, ejaculates qualifying standard criteria were pooled. Pooled ejaculates were washed for seminal plasma removal and then diluted in medium based on Tris in which antioxidants were added in various concentrations (hypotaurine 5, 10 and 20mM; cysteine 5, 10 and 20mM) or without antioxidants (control). The diluted semen was cooled at 4ºC, filled in 0.25ml French straws and then stored in liquid nitrogen. The results showed that semen quality did not differ ($P < 0.05$) in terms of morphology and acrosome integrity with antioxidants supplementation after cooling. Hypotaurine and cysteine significantly improved the characteristics of boer goat semen motility, membrane integrity, morphology, acrosome integrity and viability after cryopreservation. Addition of hypotaurine at 10mM and cysteine at 5mM concentration leads maximum improvement in liquid and frozen boer goat sperm cytological characteristics.

Introduction

Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material by means of artificial insemination (Bucak et al., 2009). However procedures related with cryopreservation of semen for artificial Insemination produces cold shock and oxidative attack on the sperm membrane, which reduces the post thaw quality of semen for AI (Evans, 1988; Maxwell and Watson, 1996). The plasma membrane of mammalian spermatozoa presents high concentrations of polyunsaturated fatty acids, which make it susceptible to ROS-induced peroxidative damage, especially following cryopreservation, with a subsequent loss of sperm functions (Aitken et al., 1989; Lenzi et al., 2002).

Mammalian semen contains a variety of antioxidants, such as reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), superoxide dismutase (SOD), taurine and hypotaurine, as a defense functioning mechanism against peroxidative damage (Alvarez and Storey, 1989; Beconi et al., 1991; Zini et al., 1993; Bilodeau et al., 2000). This antioxidant capacity in spermatozoa may be insufficient to prevent LPO and maintain sperm functions during the freeze-thawing process (Aurich et al., 1997; Storey, 1997). In goat species furthermore, removing the seminal plasma made spermatozoa more vulnerable due to the decreasing the concentration of natural antioxidant components of semen. Therefore it is more imperative to restore the normal level of antioxidants in semen by adding them in extenders while processing semen for cryopreservation. It also overcomes the detrimental effects of ROS (Donoghue and Donoghue, 1997).

The inclusion of antioxidants in the cryopreservation media has improved the quality of semen against ROS-induced damage (Sariozkan, 2000). Various antioxidants have been used for the purpose. Hypotaurine and cysteine are also from them. 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 a 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 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

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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 speciesi.e., ram (Uysaland Bucak, 2007) goat semen (Bucak and Uysal, 2008), bull semen (Bilodeau, et al., 2001) and boar (Szczesniak-Fabianczyk, 2006).

Supplementation of cryopreservation media with these antioxidants did not give similar results in all environmental conditions. The response of different breeds to the antioxidants supplementation is also different. Therefore, this study was aimed to assess the effect of hypotaurine and cysteine for boer goat cryopreservation in the tropical conditions of Malaysia.

Materials and Methods

Animals

The animals (3-4 year-old) boer goat bucks were used. These animals were kept at the arRaudhah Bio-Tech Farm SdnBhdKampungBunga Raya 48050 Kuang, Selangor, Malaysia, as semen donor for AI purpose since last two years. They were maintained under uniform feeding, housing and lighting conditions. The routine feeding program of the farm was also applied for experimental animals.

Semen Collection, evaluation

The ejaculates (06samples per buck) were collected twice week by artificial vagina during April to May 2011. Immediately after collection each ejaculate was immersed into water bath at 37ºC prior evaluation. The semen samples were evaluated as follows: Volume: Ejaculate volume was determined by collecting semen into a graduated tube. Mass activity: The mass activity was evaluated in a drop of semen without coverslip under low magnification(40X) phase contrast microscope. Sperm Motility: Progressive linear motility was assessed by standard subjective ranking method. A wet mount of diluted semen (2.9% sodium citrate + drop of semen) was prepared by placing a 5 µl drop of fresh semen under cover slip at magnification of about 200X under phase contrast microscope. At least 200 spermatozoa, selected randomly from minimum of four microscopic fields were observed for percent motile spermatozoa and percent spermatozoa with straightforward progression. The results were expressed in % motility. Sperm motility estimations were performed in 4 different microscopic fields for each semen sample. The mean of the 4 successive estimations were recorded as the final motility score. The activity and motility % was recorded on warm stage maintaining the temperature about 37ºC. Sperm concentration: The concentration of spermatozoa was determined using a haemocytometer. The total number of spermatozoa per ejaculate were calculated by multiplication of the semen volume with sperm concentration. Sperm viability: 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 was 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. Acrosome Integrity: The percentage of acrosome integrity (with normal apical ridges) were determined by evaluation of sperm smears stained with nigrosineoisin 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. Membrane Integrity: The Hypoosmotic Swelling Test (HOST) was used for membrane integrity as it is the complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. The assay were performed in 100 mOsm/kg hypoosmotic (9 g fructose + 4.9 g sodium citrate+ one liter of distilled water solution (Revell and Mrode 1994). 30 µl of semen and 300 µl of above solution was mixed and incubated (37 °C) for one hour; 0.2 ml of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated at 400X magnification under the phase-contrast microscope. A total of 200 spermatozoa were counted in at least 5 different microscopic fields. The percentages of sperm with swollen and curled tails then recorded.

Semen dilution and freezing

After collection and evaluation, ejaculates qualifying the standard criteria as 1-2ml volume with concentration of greater than $2.5x10^9$ sperm /ml having >75%progressive motility and >85% of sperms with normal morphology were processed for cryopreservation. A tris extender was used as the base extender.The ejaculates were diluted with tris extender without egg yolk at 1:1 and centrifuged at 1500x g for 3 minutes to remove the seminal plasma. The supernatant were discarded and sperm rich fraction were split in to 07 part according to experimental design and mixed with extenders containing Hypotaurine (5, 10 and 20mM), Cysteine (5, 10 20mM) and without antioxidant (control) at 37ºC. Composition of the treatment extenders is given in the Table 1. Diluted semen sample with antioxidants were cooled to 4ºC in 2 hours. Each extended semen sample was diluted with freezing extender. Final concentration was adjusted to $120X10^6$ sperm/straw. Filling and sealing of the straws was done by automatic filling and sealing machine. The straw were equilibrated in a horizontal position in cold cabinet for 30 minutes and then placed in contact with Liquid Nitrogen LN_2 vapor 3 cm above the surface of LN_2 for 10 minutes in an expandable polystyrene box, then immersed into liquid nitrogen for storage.

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

Statistical Analysis

The effect of various concentrations of hypotaurine and cysteine on sperm quality parameters was analyzed by one-way analysis of variance (ANOVA) using PROC GLM of SAS 9.1version.All data were compared across treatment groups. Means significantly that are different 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 ± S.E.M.

Results

The effects of different concentrations of hypotaurine and cysteine on sperm characteristics pre freezing are presented in Table 2 and 3. The antioxidant hypotaurine at 10 mM caused increase in sperm motility, membrane integrity, morphology acrosome integrity and viability in comparison to other groups. For the cysteine groups, supplementation with 5mM produces

better results as compared to 10mM and 20mM concentration and control group. Slight improvements were observed in morphology and acrosome integrity of spermatozoa with the supplementation but no significant difference has been detected in both parameters between the groups.

The effect of various concentrations of hypotaurine and cysteine on post thaw sperm characteristics is set out in Table 3and 4. Hypotaurine at 10mM and cysteine at 5mM showed more positive effect than other concentrations of hypotaurine and cysteine and control group during freezing thawing process.

Discussion

The antioxidantare present in the reproductive tract fluids of animals(Meizel et al., (1980) and have been found to protect liquid and frozen stored semen against lipid peroxidation and loss of sperm motility during incubation (Alvarez and Story, 1983).Hypotaurine, present in mammalian sperm, is essential for sperm functions, such as capacitation, motility, fertilizing ability and early embryonic development (Meizel et al., 1980; Boatman et al., 1990). In present study, Hypotaurine, when applied at a dose of 10mM, improved sperm motility, morphology and functional membrane integrity (HOST), acrosome integrity and viability during liquid and frozen thaw semen of boer goat. These result agrees with studies carried out in rabbit (Alvarez and Storey, 1983), hamster (Boatman et al., 1990) and human (Donnelly et al., 2000) semen, where improvement was observed in the presence of hypotaurine, during sperm liquid storage or in the unfrozen state. Chen, et al., (1993) reported that extenders supplemented with hypotaurine significantly improve the motility of frozen bull sperm.

Cysteine are precursor of intracellular glutathione biosynthesis, it penetrate cell membrane easily and protects sperm cell from toxic oxygen metabolites causing lipid peroxidation of sperm plasma membrane under invitro conditions (Meister and Tate, 1976). In present study, addition of cysteine exhibited positive impact on cooled and frozen sperm parameters such as motility, membrane integrity, morphology, acrosome integrity, and viability. Furthermore, 5mM cysteine gives better results in cooled and frozen semen when comparing with other concentrations. These findings are in agreement with Funahashi and Sano (2005) who reported that a semen extender with 5mM cysteine improved the viability and membrane integrity of boar semen cell during liquid storage.Similarly Anghel et al., (2010) reported improvement in sperm cytological characteristics of frozen goat semen when tris extender supplemented with 5mM concentration of cysteine. However Uysal and Bucak (2007) reported best post thawing spermatlogical indicators at 10 mM concentrations in ram semen. Studies performed in bull (Bilodeau et al., 2000), ram (Uysal and Bucak 2007) also showed beneficial effects of addition of cysteine in extenders..

The axosoma and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility (Garner and Hafez, 1993). Cryopreservation can induce axonemal and mitochondrial damage, resulting in the deterioration of sperm motility and morphological-functional integrity. Based on the current results, it can be hypothesized that antioxidants showed cryoprotective influence on the integrity of the axosoma and mitochondria, improving post-thawed sperm motility, morphological integrity over the head, middle and tail pieces.

In conclusion, findings emerging from this study demonstrated that supplementation of semen diluent with antioxidants hypotaurine and cysteine exert beneficial effects on the quality of the frozen-thaw boer goat semen. Hypotaurine and cysteine supplementation resulted in higher overall efficiency of cooled and frozen boer goat semen. They improves the important aspects of spermatozoae.g., motility, membrane integrity, morphology, acrosome integrity and viability when compare with control. The maximum better quality was observed when tris egg yolk extender was supplemented with hypotaurine 10mM and cysteine 5mMduring cooling and freezing-thawing processes.

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References

Aitken,R. J., Clarkson,J. S., and S. Fishel.,1989.Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of Reproduction*, 41:183–197.

Alvarez, J.G., and Storey,B.T., 1983.Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biol. Reprod. 29: 548-555.

Alvarez, J.G., and Storey, B.T., 1989.Role of glutathione peroxidase in protectingmammalian spermatozoa from loss of motility caused by spontaneous lipidperoxidation. Gamete Res. 23: 77–90.

Anghel, A., Zamfirescu, S., Dragomir, C., Nadolu, D., Elena, S., and Florica, B., 2010. The effect of antioxidants on cytological parameters of cryopreserved buck semen. Romanian Biotechnology letters 15: 26-32.

Aurich, J.E., Schonherr, U., Hoppe, H., and Aurich, C., 1997. Effect of antioxidants onmotility and membrane integrity of chilled-stored stallion semen.Theriogenology 48: 185–192.

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

Beconi, M.T., Affranchino, M.A., Schang, L.M., and Beorlegui, N.B., 1991. Influence ofantioxidants on SOD activity in bovine sperm. Biochem. Int. 23: 545–553.

Bilodeau, J.F., Chatterjee, S., Sirard, M.A., and Gagnon, C., 2000. Levels of antioxidantdefenses are decreased in bovine spermatozoa after a cycle of freezing andthawing. Mol. Reprod. Dev. 55: 282–288.

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: 275-286.

Boatman, D.E., Bavister, B.D., and Cruz, E., 1990. Addition of hypotaurine can reactivate immotile golden hamster spermatozoa. J. Androl. 11:66-72.

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

Bucak,M. N.,Ateşşahin, A., and Yüce,A., 2008.Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research*, 75:128–134.

Bucak,M. N.,Tuncer,P. B., Sariözkan,S., and Ulutaş,P. A.,2009. Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Ruminant Research*, 81:13–17.

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

Donnelly, E.T., McClure, N., and Lewis, S.E., 2000. Glutathione and hypotaurine in vitro:effects on human sperm motility, DNA integrity and production of reactiveoxygen species. Mutagenesis 15: 61–68.

Donoghue, A.M., andDonoghue, D.J., 1997. Effects of water- and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. Poultry Sci. 76: 1440-1445.

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

Evans, G., 1988. Current topics in artificial insemination of sheep. Aust. J. Biol. Sci. 4:103–116.

Feilman, J.H., Green, T.R., and Eicher, A.U., 1987. The oxidation of hypotaurine to taurine:bis-aminoethyl-alpha-disulfone, a metabolic intermediate in mammalian tissuespermatozoa. 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 ReprodFertil 77: 271-280.

Funahashi, H., and Sano, T., 2005. Selected antioxidants improve the function of extended boar semen stored at 10ºC. Theriogenology, 63: 1605-1616.

Garner, D.L., and Hafez, E.S.E., 1993. Spermatozoa and seminal plasma. In: Hafez, E.S.E.(Ed.), Reproduction in Farm Animals. Lea &Febier, Philadelphia, pp. 167–182.

Guerin,P., Guillaud, J., and Menezo, Y., 1995. Hypotaurine in spermatozoa and genetal 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., Pursel V.G., Gerrits, R.J., and Thomas, C.H. 1972.Free amino acids composition of porcine seminal, epididymal and seminal vesicals fluids. J AnimSci34: 430-434.

Lenzi, A., Gandini, L., Lombardo, F., Picardo, M., Maresca, V., and Panfili, E., 2002.Polyunsaturated fatty acids of germ cell membranes, glutathione andglutathione-dependent enzyme-PHGPx: from basic to clinic. Contraception65: 301–304.

Maxwell, W.M.C., and Watson, P.F., 1996. Recent progress in the preservation of ram semen.Anim. Reprod. Sci. 42: 55–65.

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

Meizel, S., Liu, C.W., Working, P.K., and Mrsny, R.J.,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 mammmals. Dev. Growth Differ. 22: 483-494.

Pasantes-Morales, H.,and Fellman, J.H., 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; p. 105-117.

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

Sariozkan, S., Bucak, M.N., Tuncer, P.B., Ulutas, P.A., and Bilgen, A., 2000. The nfluence of cysteine and taurine on microscopic-oxidative stress parametters and fertilizing ability of bull semen following cryopreservation. Cryobiology 2: 134-138.

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., Bochenek, M., Smorag, Z., Silvestre, M.A., 2006. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatinestructure.ReprodBiol 3: 81-87.

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. *ActaVeterinaria Brno*, 76: 383–390.

Van der Horst , C.J. G., and Grooten H. J.G., 1966. The occurance of hypotaurine and other sulpher containing amino acids in seminal plasma and spermatozoa of boar, bull and dog.BiochimBiophysActa 117: 495-497.

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

Zini, A., de Lamirande, E., and Gagnon, C., 1993. Reactive oxygen species in semen ofinfertile patients: levels of superoxide dismutase- and catalase-like activities inseminal plasma and spermatozoa. Int. J. Androl. 16: 183–188.

Table 1.Composition of extender

Extender components	Cooling Extender							Freezing Extender
	Tris Egg Yolk control	Tris Egg Yolk + Hypotaurine 5mM	Tris Egg Yolk+ Hypotaurine 10mM	Tris Egg Yolk+Hypotaurine 20mM	Tris Egg Yolk+Cystiene 5mM	Tris Egg Yolk+Cystiene 10mM	Tris Egg Yolk+Cystiene 20mM	
Tris (g)	2.422	2.422	2.422	2.422	2.422	2.422	2.422	2.422
Citric acid (g)	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.34
Fructose (g)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hypotaurine (mM)	0	5	10	20	0	0	0	0
Cysteine (mM)	0	0	0	0	5	10	20	0
Distilled water(ml)	80	80	80	80	80	80	80	73
Glycerol(ml)	0	0	0	0	0	0	0	07
Pencilline (g)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Streptomycine (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Egg Yolk (ml)	20	20	20	20	20	20	20	20

Table – 2 Mean (± S.E.M) percentage of motility, membrane integrity, morphology, acrosome integrity and viability in tris citrate egg yolk extenders supplemented with various concentration ofhypotaurine in cooled semen

Extender	Sperm parameters				
	Motility	Membrane Integrity	Morphology ns	Acrosome integrity ns	Viability
Tris Egg Yolk (TEY)	68.7±0.30^{b}	57.2±0.61^{c}	85.5±0.63^{a}	63.4±0.45^{a}	85.8±0.32^{b}
Tris Egg Yolk 05mM Hypotaurine	69.5±0.34^{ab}	58.0±0.39^{bc}	85.8±0.48^{a}	63.9±0.75^{a}	87.8±0.48^{a}
Tris Egg Yolk 10mM Hypotaurine	70.7±0.76^{a}	59.6±0.60^{a}	86.5±0.52^{a}	64.7±0.89^{a}	88.2±0.64^{a}
Tris Egg Yolk 20mM Hypotaurine	70.2±0.38^{a}	59.4±0.45^{ab}	85.9±0.43^{a}	64.2±0.66^{a}	87.8±0.51^{a}

N=10, Values with different superscripts within column differ significantly at P<0.05; ^{ns} No significant difference.

Table – 3 Mean (± S.E.M) percentage of motility, membrane integrity,morphology, acrosome integrity and viability in tris citrate egg yolk extenders supplemented with various concentration ofcysteine in cooled semen

Extender	Sperm parameters				
	Motility	Membrane Integrity	Morphology ns	Acrosome integrity ns	Viability
Tris Egg Yolk (TEY)	68.7±0.30^{b}	57.2±0.61^{a}	85.5±0.63^{a}	63.4±0.45^{a}	85.8±0.32^{b}
Tris Egg Yolk 05mM Cysteine	70.8±0.59^{a}	59.4±0.47^{a}	86.2±0.32^{a}	64.6±0.68^{a}	88.0±0.39^{a}
Tris Egg Yolk 10mM Cysteine	69.8±0.46^{ab}	58.9±1.16^{a}	85.8±0.29^{a}	63.9±0.56^{a}	87.2±0.57^{a}
Tris Egg Yolk 20mM Cysteine	69.4±0.42^{b}	58.3±0.26^{a}	85.8±0.32^{a}	63.9±0.43^{a}	87.3±0.44^{a}

N=10, Values with different superscripts within column differ significantly at P<0.05; ^{ns} No significant difference.

Table – 4 Mean (± S.E.M) percentage of motility, membrane integrity,morphology, acrosome integrity and viability in tris citrate egg yolkextender supplemented with various concentration ofhypotaurinein post thaw semen

Extender	Sperm parameters				
	Motility	Membrane Integrity	Morphology	Acrosome integrity	Viability
Tris Egg Yolk (TEY)	60.3±0.47^{b}	52.3±0.66^{b}	84.1±0.52^{c}	57.2±0.41^{b}	84.6±0.33^{a}
Tris Egg Yolk 05mM Hypotaurine	64.2±0.80^{a}	52.3±0.53^{b}	85.7±0.42^{ab}	58.7±0.51^{a}	85.2±0.48^{a}
Tris Egg Yolk 10mM Hypotaurine	66.0±0.63^{a}	55.5±0.65^{a}	86.5±0.50^{a}	59.6±0.58^{a}	85.5±0.54^{a}
Tris Egg Yolk 20mM Hypotaurine	64.4±1.13^{a}	52.9±0.60^{b}	85.0±0.36^{bc}	59.0±0.39^{a}	85.0±0.53^{a}

N=10, Values with different superscripts within column differ significantly at P<0.05; ^{ns} No significant difference.

Table – 5 Mean (± S.E.M) percentage of motility, membrane integrity,morphology, acrosome integrity and viability in tris citrate egg yolk extenders supplemented with various concentration of cysteine in post thaw semen

Extender	Sperm parameters				
	Motility	Membrane Integrity	Morphology	Acrosome integrity	Viability
Tris Egg Yolk (TEY)	60.3±0.47^{b}	52.3±0.66^{b}	84.1±0.52^{b}	57.2±0.41^{b}	84.6±0.33^{a}
Tris Egg Yolk 05mM Cysteine	64.7±0.44^{a}	54.0±0.33^{a}	85.8±0.44^{a}	59.2±0.62^{a}	85.5±0.54^{a}
Tris Egg Yolk 10mM Cysteine	63.9±0.65^{a}	52.9±0.34^{ab}	85.9±0.31^{a}	58.1±0.43^{a}	85.4±0.49^{a}
Tris Egg Yolk 20mM Cysteine	60.4±0.58^{b}	52.4±0.65^{b}	85.4±0.33^{a}	58.1±0.52^{a}	85.2±0.35^{a}

N=10, Values with different superscripts within column differ significantly at P<0.05; ^{ns} No significant difference.