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Effect of Semen Collection in Tris Extender Supplemented with Hypotaurine and Cysteine on Characteristics of Cooled and Post Thaw Boer Goat Spermatozoa

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Abstract: The objective of this study was to investigate whether pre freezing and post thawing (motility, morphology, viability, acrosome integrity and membrane integrity). Boer goat spermatozoa are affected by collecting in an Tris citric acid fructose extender supplemented with hypotaurine or cysteine. Semen was collected from four bucks into tubes containing 0 mL extender (Contro I), 2 mL extender without supplementation of antioxidants (Control II), 2 mL extender supplemented with hypotaurine 10 mM and 2 mL extender with cysteine 5 mm. No significant ($p>0.05$) improvement was observed by adding extender into collection tubes either supplemented or not with antioxidants before freezing and after freeze thawing. While a slight improvement was observed in motility, membrane integrity and acrosome integrity when 2 mL Tris citric acid fructose extender supplemented with either hypotaurine or cysteine. In conclusion, addition of extender in collection tubes either supplemented or not with antioxidants is not beneficial for Boer goat semen cryopreservation.

Key words: Goat, sperm, hypotaurine, cysteine, cryopreservation, Malaysia

INTRODUCTION

Sperm cryopreservation involves several steps such as collection, centrifugation, dilution, cooling, freezing and thawing (Luvoni, 2006; Nathanailides *et al*., 2011; Wang *et al*., 2011). Each of these steps can cause sperm damage which impairs normal sperm function and fertilizing potential. Mammalian sperm membranes incorporate many unsaturated fatty acids and are susceptible to Lipid Peroxidation (LPO) in the presence of Reactive Oxygen Species (ROS) leading to decreased sperm quality (Lenzi *et al*., 2002; Bucak *et al*., 2007).

In routine semen handling procedures for Artificial Insemination (AI) the sperm which are created in a relatively hypoxic environment (Mann, 1964) are exposed during and after collection to the relatively hyperoxic environment in the Artificial Vagina (AV) (Mann and Lutwak-Mann, 1981). The cells exposed to such environments are likely to be damaged by oxygen and its free radicals (Max, 1992), the spermatozoa so exposed may damage by those free radicals. There are natural trapping systems such as tocopherol, ascorbic acid, glutathione and catalase in most mammalian cells which protect them from such damage (Vandemark *et al*., 1949; Max, 1992). However, caprine semen is deficient in such substances which make it more prone to the damage caused by free radicals than other cells (Salamon and Maxwell, 1995). Semen collection is generally conducted using artificial vagina with empty semen collecting tubes and in routine cryopreservation protocols antioxidants are applied after semen collection when the semen exposed to atmospheric oxygen and could had sustained free radical damage. Therefore, ejaculated spermatozoa collected in this way may be subjected to harmful modification in their function in motility and fertilizing capacity at exposure to aerobic conditions. Researchers postulated that ejaculated spermatozoa exposed to aerobic conditions with insufficient antioxidants concentration in seminal plasma are subjected to harmful modification in their functions.

Ejaculated spermatozoa employed for liquid or frozen storage collected in a way that minimize the effect of oxidative stress due to free radicals by collecting them in a tube containing extender supplemented with antioxidants may possibly enhance sperm motility and survival for preservation. Thus, collection of semen in a small volume of medium contains antioxidants might

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protect the cells from free radical damage and improve the activity and integrity of the cells. It was considered that antioxidants if applied as soon as semen is exposed to atmospheric oxygen might provide more protection from peroxidation to the spermatozoa.

Addition of antioxidants in extenders aimed to reduce the oxidative stress and prevent the cold shock and cryo injury to spermatozoa. Thus, present study was carried out to investigate addition of hypotaurine and cysteine at the time of semen collection to protect sperm cells from damage caused by free radicals and reduce the oxidative stress.

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. The experimental animals were subjected to the same feeding program of the farm. Animals were fed twice daily in order to achieve a predetermined feed intake of 2.5% body weight (on dry matter basis) per goat per day. Water was available *ad libitum*.

Semen extenders: All chemicals were of reagent grade purchased from Sigma-Aldrich, St. Louis, MO. The Tris buffer consisted of 250 mM Tris, 88.5 mM citric acid, 69.38 mM fructose and antibiotic (80000 IU penicillin and 100000 μg streptomycin mL^{-1}) was used in collection tubes and washing solution while 18% (v/v) egg yolk were added in the buffer and used as cooling extender. Tris egg yolk freezing extender consisted glycerol 8% (v/v) (Liu *et al*., 1998).

In present study, two antioxidants (hypotaurine 10 mM and cysteine 5 mM) were tested in collection tubes for cryopreservation of Boer goat semen. They were included in basic extender Tris citric acid fructose extender without egg yolk and glycerol when used in the collection tubes and washing solution.

Semen collection and processing for cryopreservation: A total 32 ejaculates (two ejaculates from each buck for each method of collection) were used in this study. In the previous experiments the semen collected into empty tubes from all four bucks was high quality (average volume 0.75 mL, motility >75%, concentration $>2.5\times10^9$ sperm mL^{-1} and normal morphology >85%). Semen was collected into empty tubes (Control I) or tubes containing 2 mL Tris citric acid extender without egg yolk supplemented with 0% antioxidants (Control II), tubes containing 2 mL Tris citric acid extender without egg yolk supplemented with 10 mM hypotaurine and tubes containing 2 mL Tris citric acid without egg yolk extender supplemented with 5 mM cysteine. The extender with and without antioxidant supplementation were added into the graduated tubes.

The graduated tubes were fixed in Artificial Vagina (AV) and temperature of diluent was maintained by covering the AV in leather cover. Semen was collected by artificial vagina twice a week. Immediately after collection each ejaculate was immersed into water bath at 37°C prior evaluation. Ejaculate volume was determined by collecting semen into a graduated tube (subtracting 2 mL from total volume of collection in case when 2 mL extender added in the collection tubes). Aliquots of the collected semen were evaluated for motility.

At least 200 spermatozoa, selected randomly from minimum of four microscopic fields were observed for percent motile spermatozoa and percent spermatozoa with straight forward progression. The results were expressed in percentage of the motility. The mean of the 4 successive estimations were recorded as the final motility score. The motility percentage was recorded on a slides warmer maintaining at 37°C. The rest of the semen was diluted with Tris extender with their respective antioxidants without egg yolk centrifuged (Sigma 2-16 P, Sartorius) at 1500×g for 3 min to remove the seminal plasma.

The supernatant were discarded and sperm rich fraction was mixed with cooling extenders at 37°C. Extended semen was placed in cooling chamber at 4°C for 2.5 h. Cooled semen was diluted with freezing extender and maintained for another 30 min. Final concentration was adjusted at 120×10^6 sperm/straw. Straws were filled and sealed by automatic filling and sealing machine (MRSI-CE, IMV, France). The straws were equilibrated in a horizontal position in cold cabinet for 30 min. After equilibration, straws were placed in contact with liquid nitrogen vapors approximately 3 cm above the surface of liquid nitrogen for 10 min in an expandable polystyrene box. Then the straws were immersed in liquid nitrogen for storage.

Immediately after cooling, semen samples were evaluated for motility, membrane integrity, acrosome integrity, morphology and viability. Post thaw examination was performed 24 h after freezing where four straws of each frozen semen were thawed at 37°C for 30 sec and pooled prior to evaluation.

Sperm motility: The sperm motility was estimated subjectively by preparing a wet mount of diluted cooled semen by placing a 5 μL drop of semen under coverslip at magnification of about 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.

Sperm viability: The sperm viability of the samples was assessed by means of the nigrosin-eosin 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 spermatozoa under the phase-contrast microscope at 1000× magnification. 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.

Sperm morphology: Morphologically normal spermatozoa were assessed using nigrosin-eosin stain (Evans and Maxwell, 1987).

Acrosome integrity: 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.

Membrane integrity: 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 100 m Osm hypo-osmotic solution containing 9.0 g fructose, 4.9 g sodium citrate at 37°C for 60 min. 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. When exposed to the hypoosmotic solutions, biochemically-active spermatozoa increase their volume in order to establish equiblirium between the fluid compartment within the spermatozoa and the extracellular environment. Swelling causes changes in both cell size and shape that can be evaluated using a phase contrast microscope. The percentage of sperm with swollen and curled tails were then recorded (Revel and Mrode, 1994; Buckett *et al*., 1997).

Statistical analysis: A statistical analysis was performed with the SAS statistical software (9.1 Version, SAS Institute, Cary, NC, USA). The effects of the antioxidants as a fixed effect on all variables was analyzed according to the following equation (PROC GLM):

$$Y_{ij} = \mu + D_i + \xi_{ij}$$

Where:

Y_{ij} = Dependent variable
μ = Overall mean
D_i = Effect of i antioxidant (hypotaurine and cysteine)
ξ_{ij} = Residual error

Differences among antioxidants were determined using the Least Significant Difference (LSD) test. A level of significance below 0.05 was considered significant. Results were expressed as mean±SEM.

RESULTS

Table 1 shows the percentages of motility, membrane integrity, morphology, acrosome integrity and viability in cooled Boer goat spermatozoa collected into empty tubes or tubes containing Tris citric acid fructose extender and Tris citric acid fructose extender either supplemented with hypotaurine or cysteine. No significant ($p>0.05$) improvement was observed by adding extender into collection tubes either supplemented with antioxidants before freezing. However, a slight improvement was observed in motility percentage, membrane integrity, acrosome integrity, morphology and viability when 2 mL Tris citric acid fructose extender was supplemented with either hypotaurine or cysteine.

Table 1: Mean (±SEM) percentages of motility, membrane integrity, acrosome integrity and viability in pre freezing Boer goat spermatozoa collected into empty tubes and tubes containing Tris extender supplemented with and without hypotaurine and cysteine

	Percentage^{NS}				
Antioxidants treatment	Motility	Membrane integrity	Morphology	Acrosome integrity	Viability
0.0 mL Tris extender (Control I)	70.00±2.89	63.75±1.65	84.00±0.71	63.75±1.11	86.25±0.25
2.0 mL Tris extender without antioxidants (Control II)	70.00±2.94	64.75±0.85	84.75±1.11	63.25±1.03	87.75±0.48
2.0 mL Tris extender with 10 mM hypotaurine	71.25±2.32	65.50±1.89	86.75±0.48	64.50±1.32	88.75±0.85
2.0 mL Tris extender with 5 mM cysteine	70.00±1.83	65.25±2.50	85.75±0.85	63.75±0.85	88.25±0.85

NS: Not Significant

Table 2: Mean (±SEM) percentages of motility, membrane integrity, acrosome integrity and viability in post thawing Boer goat spermatozoa collected into empty tubes and tubes containing Tris extender supplemented with and without hypotaurine and cysteine

	Percentage[NS]				
Antioxidants treatment	Motility	Membrane integrity	Morphology	Acrosome integrity	Viability
0.0 mL Tris extender (Control I)	64.25±0.85	61.75±1.11	83.00±0.91	59.25±0.48	85.00±0.82
2 mL Tris extender without antioxidants (Control II)	65.00±1.29	62.00±1.29	83.75±0.75	60.00±1.29	85.75±0.85
2 mL Tris extender with 10 mM hypotaurine	65.75±1.11	62.25±1.65	85.00±0.41	60.75±1.11	86.00±0.91
2 mL Tris extender with 5 mM cysteine	65.25±1.49	62.25±1.55	84.25±0.63	60.50±1.55	85.75±1.31

NS: Not Significant

Data for the effect of extender in collection tubes either supplemented with antioxidants or not after freezing are shown in Table 2. Non-significant differences were observed in motility, membrane integrity, acrosome integrity and viability of post thaw Boer goat spermatozoa amongst the treatments. While motility, membrane integrity, acrosome integrity and viability was slightly higher in samples collected in extender than the empty tubes.

DISCUSSION

Cryopreservation causes extensive chemical and physical damages to sperm membranes which are attributed to alterations in the transition from the lipid phase, increases in lipid peroxidation of the membrane induced by Reactive Oxygen Species (ROS) and mechanical stress on cell membranes due to osmotic stress and temperature changes (Alvarez and Storey, 1992; Mayers, 2005).

It is well documented that ROS, formed by the univalent reduction of oxygen (Bilodeau *et al*., 2000; Misra and Fridorich, 1972) are responsible for maintenance of physiological processes in sperm, e.g., capacitation and the acrosome reaction (O'Flaherty *et al*., 1999). However, large quantities of ROS decreased the viability of cryopreserved sperm, giving rise to lipid peroxidation due to the large amount of polyunsaturated fatty acids present in the sperm plasma membrane (Taylor, 2001).

Normally there is a balance between radical generating and scavenging system. However, high generation of Reactive Oxygen Species (ROS) by sperm processing (collection, centrifugations, composition and dilution ratio of washing, cooling and freezing extenders and thawing) accompanied by low scavenging and antioxidant levels in serum, seminal plasma induce a state of oxidative stress (Anghel *et al*., 2010).

Mammalian semen contains many enzymatic (Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-PX), Catalase (CAT) and Superoxide Dismutase (SOD)) and non enzymatic (ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine and hypotaurine (Saleh and Agarwal, 2002) antioxidants.

The antioxidants present in the seminal plasma are the most important form of the protection available to spermatozoa against ROS (Sikka, 2004). They provide defense mechanism through three levels of protection, namely prevention, interception and repair (Anghel *et al*., 2010).

This antioxidant capacity in sperm cell however, may be insufficient in preventing oxidative stress during centrifugation, cooling and freezing or thawing processes. The main causes of sperm damage during cryopreservation are classified as cold-shock (Watson, 2000), osmotic stress (Watson, 2000) and oxidative stress (Aitken and Krause, 2001; Sikka, 2004; Agarwal *et al*., 2003). Mammalian spermatozoa are harmed by hydrogen peroxide and lipid peroxidation (Brzezinska-Slebodzinska *et al*., 1995) that shortening its life span *in vivo* and affecting the preservation of semen for AI (Alvarez and Storey, 1983).

All cellular components including lipids, protein, nucleic acid and sugars are potential targets of oxidative stress. The extent of oxidative stress induced damage depends on the nature and amount of ROS involved and on the duration of ROS exposure and extra cellular factors such as temperature, oxygen concentration and composition of surrounding environment (ion, protein and ROS scavengers) (Anghel *et al*., 2010).

Presence of high concentration of polyunsaturated fatty acid within the lipid fractions necessitate the presence of an efficient antioxidant system to protect against per oxidative damage and possible associated sperm dysfunction (Aitken *et al*., 2004). Approaches to increase the percentage of viable post-thaw cells have included various extenders (O'Hara *et al*., 2010) refrigeration times (Paulenz *et al*., 2002; Menchaca *et al*., 2005), dilution rates (Kasimanickam *et al*., 2007), glycerol concentrations (El-Alamy and Foot, 2001), equilibration times (Herold *et al*., 2006) and inclusion of antioxidants (Bucak *et al*., 2009).

It has been reported by many studies (Sariozkan *et al*., 2009; Memon *et al*., 2011) that addition of antioxidants improved the quality of semen against ROS-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 lipidperoxidation. Hypotaurine is a precursor of taurine which exists in mammalian sperm including men (Van der Horst and Grooten, 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 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 *et al.*, 2006).

In routine extension and freezing procedures for semen cryopreservation of buck, antioxidants added into either cooling, freezing or in both media after washing. It was reported by several researchers that addition of synthetic antioxidants prevents the adverse effect of peroxide (Salmon and Maxwell, 1995). However, results reported by these researchers are not similar. For example Donnelly *et al.* (2000) reported that addition of antioxidants (hypotaurine) did not improve motility, DNA integrity of human frozen thaw spermatozoa. Similarly no improvement was observed by Funahashi and Sano (2005) in the survival rate of boar sperm preserved at 10°C with antioxidants. Martins-Bessa *et al.* (2007) also did not see any improvement in post thaw motility when they added antioxidants in the extenders. Contrary to these results hypotaurine successfully improve post thaw viability on frozen thaw rabbit spermatozoa (Alvarez and Storey, 1983). Malo *et al.* (2010) reported higher percentages of viability and acrosome integrity when 10 mM cysteine was added in freezing extender. Ijaz *et al.* (2009) reported better quality in terms of motility, membrane integrity, viability and acrosome integrity when butylated hydroxytoluene antioxidant added into the cooling extender. Chen *et al.* (1993) observed improvement in post thaw motility when antioxidant added in the second component of extender the one containing glycerol. Variability in the results could be because the time of application of antioxidants and medium in which they were applied.

Semen collection is generally conducted using artificial vagina with empty semen collecting tubes and in routine cryopreservation protocols antioxidants are applied after semen collection when the semen exposed to atmospheric oxygen it could had sustained free radical damage. Therefore, ejaculated spermatozoa collected in this way may be subjected to harmful modification in their function in motility and fertilizing capacity at exposure to aerobic conditions. Therefore, in the present study a small volume of medium contains antioxidants were added in the collection tubes with the aim to protect the spermatozoa from free radical damage and improve the activity and integrity of the sperm cells. Hypothesis of the present study was that if antioxidants applied as soon as semen is exposed to atmospheric oxygen might provide more protection from peroxidation to the spermatozoa.

In the present study, it was observed that small volume extender either supplemented with antioxidants or not did not provide better results. This further supported by the result obtained by Yamashiro *et al.* (2006) who observed that small volume of extender for semen collection did not provided better results. De Pauw *et al.* (2003) stated that beneficial effect of collecting bovine spermatozoa with egg yolk Tris extender could not be replaced by collecting semen in egg yolk free diluent. This is in agreement with the findings that egg yolk free extender in collection tubes is not beneficial for goat spermatozoa. The protective mechanism of egg yolk against the detrimental components of seminal plasma seems to rely on phospholipids and low density lipoprotein components which protect spermatozoa (Shanon and Curson, 1983).

However, in caprine species presence of bulbourethral gland secretions in semen limited the application of egg yolk extender in collection tubes. Studies (Roy, 1957; Iritani and Nishikawa, 1963) reported that diluents containing egg yolk were detrimental effect on goat spermatozoa. This is being due to the fact that buck seminal plasma contains an enzyme secreted by the bulbourethral glands which in the presence of egg yolk by hydrolysis, leads to the formation of lysophosphatidylcholines which are toxic to sperm (Leboeuf *et al.*, 2000). Therefore, protocols for extension of goat semen includes the additional step of removing

the seminal plasma by washing when using egg yolk (Naing *et al*., 2010). Otherwise processing the semen for extension in an egg yolk free extender or extender contains low egg yolk concentration. De Pauw *et al*. (2003) implicates that the motility apparatus was not protecting when semen will be collected with extender. They further stated that only the membrane integrity of spermatozoa collected with extender was protected without a corresponding protection of the regulatory mechanisms responsible for motility. It also agrees with the present finding that in present study a small volume of extender either supplemented with antioxidants or not was not improving the characteristics of liquid stored or post thaw goat spermatozoa.

CONCLUSION

It can be concluded from the present study that addition of extender in collection tubes either supplemented or non-supplemented with antioxidants is not beneficial for Boer goat semen cryopreservation.

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