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 4°C, 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.

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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 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 (Sarozkan, 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 (Pasanter-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 cystine (Bucak et al., 2008). Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post thawed sperm motility in many species, e.g., ram (Uysal and 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 boar 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 arRa’udhah 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 (06 samples 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 37°C 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 37°C. 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-nigrosine 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. 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 hypossotonic (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 2.5x10⁹ 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 the different extenders. The semen samples were diluted with the extenders containing hypotaurine (5, 10 and 20mM), Cysteine (5, 10 20mM) and without antioxidant (control) at 37°C. Composition of the treatment extenders are given in the Table 1. Diluted semen sample with antioxidants were cooled to 4°C in 2 hours. Each extended semen sample was diluted with freezing extender. Final concentration was adjusted to 120X10⁶ 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 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 9.1 version. 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 3 and 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 antioxidant are 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 spermatological 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 spermatozoa, e.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 5M during cooling and freezing-thawing processes.

**Acknowledgements**

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**References**


Table 1. Composition of extender

<table>
<thead>
<tr>
<th>Extender components</th>
<th>Tris Egg Yolk + Hypotaurine 5mM</th>
<th>Tris Egg Yolk+Hypotaurine 10mM</th>
<th>Tris Egg Yolk+Hypotaurine 20mM</th>
<th>Tris Egg Yolk+Cystiene 5mM</th>
<th>Tris Egg Yolk+Cystiene 10mM</th>
<th>Tris Egg Yolk+Cystiene 20mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (g)</td>
<td>2.422</td>
<td>2.422</td>
<td>2.422</td>
<td>2.422</td>
<td>2.422</td>
<td>2.422</td>
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<tr>
<td>Citric acid (g)</td>
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<td>1.34</td>
<td>1.34</td>
<td>1.34</td>
<td>1.34</td>
<td>1.34</td>
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<tr>
<td>Fructose (g)</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypotaurine (mM)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
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<td>0</td>
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<tr>
<td>Cysteine (mM)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
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<tr>
<td>Distilled water(ml)</td>
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<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
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<tr>
<td>Glycerol(ml)</td>
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<td>0.06</td>
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<tr>
<td>Streptomycin (g)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
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<tr>
<td>Egg Yolk (ml)</td>
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<td>20</td>
<td>20</td>
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</tbody>
</table>


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 of hypotaurine in cooled semen

<table>
<thead>
<tr>
<th>Extender</th>
<th>Motility</th>
<th>Membrane Integrity</th>
<th>Morphology</th>
<th>Acrosome integrity</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Egg Yolk (TEY)</td>
<td>68.7±0.30</td>
<td>57.2±0.61</td>
<td>85.5±0.63</td>
<td>63.4±0.45</td>
<td>85.8±0.32</td>
</tr>
<tr>
<td>Tris Egg Yolk 0.5mM Hypotaurine</td>
<td>69.5±0.34</td>
<td>58.0±0.39</td>
<td>85.8±0.48</td>
<td>63.9±0.75</td>
<td>87.8±0.48</td>
</tr>
<tr>
<td>Tris Egg Yolk 1.0mM Hypotaurine</td>
<td>70.7±0.76</td>
<td>59.6±0.60</td>
<td>86.5±0.52</td>
<td>64.7±0.39</td>
<td>88.2±0.64</td>
</tr>
<tr>
<td>Tris Egg Yolk 2.0mM Hypotaurine</td>
<td>70.2±0.38</td>
<td>59.4±0.45</td>
<td>85.9±0.43</td>
<td>64.2±0.66</td>
<td>87.8±0.51</td>
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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 of cysteine in cooled semen

<table>
<thead>
<tr>
<th>Extender</th>
<th>Motility</th>
<th>Membrane Integrity</th>
<th>Morphology</th>
<th>Acrosome integrity</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Egg Yolk (TEY)</td>
<td>68.7±0.30</td>
<td>57.2±0.61</td>
<td>85.5±0.63</td>
<td>63.4±0.45</td>
<td>85.8±0.32</td>
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<tr>
<td>Tris Egg Yolk 0.5mM Cysteine</td>
<td>70.8±0.59</td>
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<td>86.2±0.32</td>
<td>64.6±0.68</td>
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<td>58.9±1.16</td>
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<td>Tris Egg Yolk 2.0mM Cysteine</td>
<td>69.4±0.42</td>
<td>58.3±0.26</td>
<td>85.8±0.32</td>
<td>63.9±0.43</td>
<td>87.3±0.44</td>
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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 yolk extender supplemented with various concentration of hypotaurine in post thaw semen

<table>
<thead>
<tr>
<th>Extender</th>
<th>Motility</th>
<th>Membrane Integrity</th>
<th>Morphology</th>
<th>Acrosome integrity</th>
<th>Viability</th>
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</thead>
<tbody>
<tr>
<td>Tris Egg Yolk (TEY)</td>
<td>60.3±0.47</td>
<td>52.3±0.66</td>
<td>84.1±0.52</td>
<td>57.2±0.41</td>
<td>84.6±0.33</td>
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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 extender supplemented with various concentration of cysteine in post thaw semen

<table>
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<th>Extender</th>
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<th>Acrosome integrity</th>
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<tr>
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<td>58.1±0.43</td>
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</tr>
<tr>
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<td>85.4±0.33</td>
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N=10, Values with different superscripts within column differ significantly at P<0.05; ns No significant difference.