Antioxidants improve the semen quality following cryopreservation in Indian yak bulls

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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 hydroxytoluene, 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
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 1000x 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>
<thead>
<tr>
<th>Stage</th>
<th>% Sperm motility</th>
<th>% Live spermatozoa</th>
<th>% HOST-reacted sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Taurine Trehalose Vit E Control</td>
<td>Taurine Trehalose Vit E Control</td>
<td>Taurine Trehalose Vit E Control</td>
</tr>
<tr>
<td>Fresh semen</td>
<td>81.50± 81.50± 81.50± 81.50±</td>
<td>81.50± 81.50± 81.50± 81.50±</td>
<td>81.50± 81.50± 81.50± 81.50±</td>
</tr>
<tr>
<td>After equilibration</td>
<td>73.75± 72.00± 71.70± 68.50±</td>
<td>81.10± 78.75± 78.20± 76.59±</td>
<td>72.70± 70.85± 70.00± 67.65±</td>
</tr>
<tr>
<td>After freezing</td>
<td>62.75± 60.50± 60.20± 56.25±</td>
<td>71.15± 68.30± 68.20± 65.10±</td>
<td>62.00± 59.10± 60.15± 57.15±</td>
</tr>
<tr>
<td>Overall</td>
<td>72.67± 71.33± 71.1± 68.75±</td>
<td>80.05± 78.32± 78.10± 76.52±</td>
<td>71.32± 69.70± 69.80± 68.02±</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Stage</th>
<th>Total incidence of acrosomal changes (%)</th>
<th>ALT (IU/l)</th>
<th>AST (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Taurine</td>
<td>Trehalose</td>
</tr>
<tr>
<td></td>
<td>Fresh semen</td>
<td>0.27±0.30</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>After equilibration</td>
<td>0.24±0.37</td>
<td>0.67±0.05</td>
<td>0.95±0.49</td>
</tr>
<tr>
<td></td>
<td>After freezing</td>
<td>0.41±0.45</td>
<td>0.96±0.84</td>
<td>0.84±0.63</td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>0.46±0.56</td>
<td>0.96±0.53</td>
<td>0.53±0.68</td>
</tr>
</tbody>
</table>

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 vitamin 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 Atreja 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 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.

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