Storage of Nili-Ravi Buffalo (Bubalus bubalis) Semen in Skim Milk Extender Supplemented with Ascorbic Acid and α-Tocopherol

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Abstract.- We investigated the effect of ascorbic acid and α-tocopherol supplementation in skim milk extender on the preservability of buffalo bull spermatozoa stored at 5°C. For this purpose, semen samples were collected from Nili-Ravi buffalo (Bubalus bubalis) bulls (n = 3) and diluted at 37°C with skim milk extender containing ascorbic acid (0.5mM) or α-tocopherol (1.0mM) or without any supplement (Control). The sperm concentration in the extender was adjusted at 10 x 10^6 motile spermatozoa per ml. Diluted semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for five days. Semen quality assays for sperm motility, plasma membrane integrity, normal apical ridge and abnormalities were performed at 1st, 3rd and 5th day of storage. Percentage of sperm motility was lower (P < 0.05) in extender containing α-tocopherol as compared to ascorbic acid and control at 1st and 3rd day of storage. However, it did not differ at 5th day of storage. There was no difference (P > 0.05) in percentage of plasma membrane integrity and normal apical ridge of buffalo bull spermatozoa for five days of storage. Moreover, sperm abnormalities (head, mid piece and tail) remained similar (P > 0.05) in all experimental extenders for five days to storage. It is concluded that ascorbic acid and α-tocopherol addition in skim milk did not improve the semen quality of Nili-Ravi buffalo bull spermatozoa stored at 5°C for five days.

Key words: Buffalo bull semen, antioxidant, glutathione, liquid storage.

INTRODUCTION

Sperm cells have a high content of unsaturated fatty acids in their membranes and lack a significant cytoplasmic component containing antioxidants (Andrabi et al., 2009). Therefore, it makes spermatozoa more susceptible to lipid peroxidation by reactive oxygen species molecules (ROS; Storey, 1997). During freezing and storage of spermatozoa, exposure to oxygen and light radiation accelerate the production of ROS molecules and lipid peroxidation of sperm plasma membrane (Andrabi et al., 2008).

It is well established that chilling of buffalo semen resulted in deceased semen quality which is highly associated with decreased antioxidant activity and higher ROS production (El-Sissy et al., 2007; Kumaresan et al., 2005, 2006). Moreover, buffalo bull spermatozoa are more susceptible to oxidative damage as compared to cattle bull spermatozoa (Nair et al., 2006; Kumaresan et al., 2005, 2006). It is believed that this difference is due to higher contents of polyunsaturated phospholipids present in plasma membrane of buffalo bull spermatozoa (Sansone et al., 2000). Freezing process accelerate the production of ROS molecules which may decrease the viability of buffalo bull spermatozoa during storage (Kumaresan et al., 2005, 2006; Garg et al., 2008). Therefore, supplementation of antioxidants in semen extender is required to decrease the ROS-mediated damages to buffalo spermatozoa.

Ascorbic acid and α-tocopherol are naturally occurring antioxidants in buffalo semen, to protect the spermatozoa from oxidative damage (Sansone et al., 2000). However, the indigenous antioxidant system to protect the spermatozoa integrity from ROS during freezing is insufficient (Baumber et al., 2005; Sreejith et al., 2006; Nichi et al., 2006). It was observed that supplementation of ascorbic acid and α-tocopherol in semen extender improved the quality of cryopreserved Nili-Ravi buffalo semen.
(Andrabi et al., 2008). We hypothesized that supplementation of ascorbic acid and α-tocopherol in skim milk extender may improve the semen quality of Nili-Ravi buffalo bull spermatozoa stored at 5°C.

Therefore, present experiment was designed to investigate the effect of ascorbic acid and α-tocopherol supplementation in skim milk extender on the semen quality (motility, plasma membrane integrity and morphology) of Nili-Ravi buffalo bull spermatozoa stored at 5°C.

**MATERIALS AND METHODS**

Skim milk (SKIMZ®; CANDIA) 10% (w/v) was used as stock extender for preparation of experimental extenders. Experimental extenders were prepared by adding ascorbic acid 0.5mM and α-tocopherol acetate 1.0mM in extenders, extender without supplement served as control. Antibiotics (Streptomycin sulphate @ 1 mg/ml and Benzyl Penicillin @ 1000 IU/ml) were added in each extender at room temperature.

Two consecutive ejaculates were collected from three Nili-Ravi buffalo bulls maintained at Semen Production Unit, Qadirabad, Sahiwal, Pakistan with artificial vagina for three weeks (replicates). Collected semen was immediately transferred to the laboratory for initial evaluation. Sperm motility was assessed using phase contrast microscope at 37 °C and 400X. Sperm concentration was determined by Neubauer haemocytometer. Qualifying semen ejaculates were pooled from three bulls having motility > 60% and split into three aliquots for further processing. Three aliquots were diluted at 37°C with skim milk extender containing ascorbic acid (0.5mM) or α-tocopherol acetate (1.0mM) or without any supplement (control). The diluted semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for five days.

Plasma membrane integrity of buffalo bull spermatozoa was assessed by hypo-osmotic swelling assay (HOS). The HOS solution contained sodium citrate 0.735g and fructose 1.351g dissolved in 100ml distilled water. A semen sample (50µl) was mixed with 500µl pre-warmed (37°C) HOS solution and incubated at 37°C for 30-40 min. After incubation, a drop of semen sample was evaluated with phase contrast microscope at 400X. One hundred spermatozoa were observed and the percentage of cells with curled tails (intact plasma membrane) was recorded as HOS positive (Akhter et al., 2008). Sperm morphology (acrosome, head, mid piece and tail) was assessed by fixing 500 µl of semen samples with 50µl of 1% formal citrate. A drop of semen sample was studied using phase contrast microscope at 1000X. One hundred spermatozoa were studied to determine the percentage of intact acrosomes, head, mid piece and tail abnormalities (Andrabi et al., 2008).

The data are presented as means ± (SD). Effects of treatments on different semen quality parameters were analyzed by using Analysis of Variance (ANOVA). When the F–ratio was found significant (P<0.05), LSD test was used, to compare the treatment means (MINITAB® Release 12.22, 1998).

**RESULTS**

**Motility of buffalo bull spermatozoa**

The data on motility of buffalo bull spermatozoa in three experimental extenders are presented in Figure 1. Percentage of motility was observed lower (P < 0.05) in extender containing α-tocopherol as compared to ascorbic acid and control at 1st and 3rd day of storage at 5°C, respectively. However, sperm motility in all experimental extenders did not differ (P>0.05) at 5th day of storage at 5°C.

**Plasma membrane integrity of buffalo bull spermatozoa**

The data on plasma membrane integrity of buffalo bull spermatozoa in three experimental extenders are presented in Figure 2. Percentage of buffalo bull spermatozoa with intact plasma membrane remained similar (P > 0.05) in extender containing ascorbic acid, α-tocopherol and control at 1st, 3rd and 5th days of storage at 5°C.

**Normal apical ridge of buffalo bull spermatozoa**

The data on normal apical ridge of buffalo bull spermatozoa in three experimental extenders are presented in Figure 3. Percentage of buffalo bull spermatozoa with normal apical ridge remained similar (P > 0.05) in extender containing ascorbic acid, α-tocopherol and control at 1st, 3rd and 5th days of storage at 5°C.
spermatozoa with intact acrosomes did not differ (P>0.05) in extender containing ascorbic acid, \(\alpha\)-tocopherol and control at 1\(^{st}\), 3\(^{rd}\) and 5\(^{th}\) days of storage at 5°C.

Experimental extenders are given in Table I. Percentage of abnormalities (head, mid piece and tail) of buffalo bull spermatozoa remained similar (P > 0.05) in all three experimental extender for five days of storage at 5°C.

### Table I.- Effect of ascorbic acid (Vitamin C; 0.5mM) and \(\alpha\)-tocopherol (Vitamin E; 1.0mM) supplementation in skim milk extender on abnormalities (Mean ± SD: n=3; head, mid piece and tail) of buffalo bull spermatozoa at 1\(^{st}\), 3\(^{rd}\) and 5\(^{th}\) day of storage at 5°C.

<table>
<thead>
<tr>
<th>Day of storage</th>
<th>Extender</th>
<th>Head</th>
<th>Mid piece</th>
<th>Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skim milk</td>
<td>1.00±0.0</td>
<td>0.33±0.6</td>
<td>2.33±1.2</td>
</tr>
<tr>
<td></td>
<td>Skim milk + vitamin C</td>
<td>1.00±1.0</td>
<td>1.00±0.0</td>
<td>2.67±1.2</td>
</tr>
<tr>
<td></td>
<td>Skim milk + vitamin E</td>
<td>0.67±0.6</td>
<td>0.67±0.6</td>
<td>3.33±0.6</td>
</tr>
</tbody>
</table>

Means within rows did not differ (P>0.05).

Sperm abnormalities of buffalo bull spermatozoa

The data on the abnormalities (head, mid piece and tail) of buffalo bull spermatozoa in three experimental extenders are given in Table I. Percentage of abnormalities (head, mid piece and tail) of buffalo bull spermatozoa remained similar (P > 0.05) in all three experimental extender for five days of storage at 5°C.
DISCUSSION

Freezing process accelerated the ROS production and reduced anti-oxidative activity which resulted in decreased motility, plasma membrane integrity and intact acrosomes of buffalo bull spermatozoa (El-Sissy et al., 2007). In our study, motility of liquid preserved buffalo bull spermatozoa did not differ in extender containing ascorbic acid and α-tocopherol as compared to control. Contrary to our findings, higher sperm motility of Murrah buffalo bull spermatozoa was observed in tris-egg yolk and milk egg yolk extender (Raina et al., 2002). Similarly, in a recent study (Andrabi et al., 2008) on Nili-Ravi buffalo semen higher post thaw sperm motility, plasma membrane integrity and normal apical ridge was reported after the addition of ascorbic acid and α-tocopherol in tris-citric acid extender. It is pertinent to mention that supplementation of vitamin C and E in milk based extenders failed to improve the preservability of bovine semen (Beconi et al., 1993; Foote et al., 2002). Similarly, catalase supplementation in milk extender at 5°C in egg yolk extender found non beneficial for bovine spermatozoa (Foote, 1962). It is believed that supplementation of antioxidants in milk based extender did not improve semen quality because of a naturally occurring antioxidant casein in milk which alter the requirement of extra antioxidant supplementation (Foote et al., 2002). Reactive oxygen species molecules at physiological levels are essential for the spermatozoa, and it was observed that higher concentration of Vitamin C and E may result in impairment of semen quality (Andrabi et al., 2008) and decreases the success of fertility of bull semen in vitro (Dalvit et al., 1998).

Assessment of sperm abnormalities is one of the commonest methods to assess the viability of buffalo bull spermatozoa (Sajjad et al., 2007). In our study, sperm head, mid piece and tail abnormalities did not differ in all the three experimental extenders. It is noteworthy to mention that semen processing does not increase the proportion of buffalo bull spermatozoa with head, mid piece and tail abnormalities (Akhter et al., 2008).

It is concluded that ascorbic acid and α-tocopherol addition in skim milk did not improve the sperm motility, plasma membrane integrity and morphology of Nili-Ravi buffalo bull spermatozoa stored at 5°C for five days.

REFERENCES


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