Effects of prostasomes on functional parameters of fresh and cryopreserved-thawed spermatozoa of crossbred Karan Fries (KF) bulls

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ABSTRACT
Prostasomes are extracellular vesicles that fuse with sperms thereby improving its functional parameters. Present study aimed to isolate and characterise prostasomes from semen of KF bulls, and to investigate prostasomes effects on functional parameters of KF bull spermatozoa. Isolated prostasomes were characterized with respect to the binding of FTTC-conjugated CD 26 antibodies, as well as protein, cholesterol and phospholipids content. Subsequently, effects of prostasomes supplementation (1 mg/ml) were investigated on ROS production, Ca²⁺ signalling, mitochondrial membrane potential and acrosome integrity of fresh and cryopreserved-thawed spermatozoa. Isolated prostasomes were immunostained positively. Prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids. When sperm samples were supplemented with prostasomes, ROS production decreased, while all other functional parameters improved.

Key words: Bull, Prostasomes, Sperm functional parameters.

INTRODUCTION

Prostate is a prominent accessory sex gland present in male domestic mammals. Spermatozoa expelled in prostatic fluid shows better motility and viability (Ronquist and Brody, 1985). Prostate secretory acinar cells release prostasomes into seminal plasma (Ronquist and Hedström, 1977). Prostasomes fuse with sperms and improve several sperm functional parameters, such as antioxidiant activity (Saez et al., 1998), Ca²⁺ signalling (Burden et al., 2006), viability (Kravets et al., 2000) and acrosome integrity (Pons-Rejraji et al., 2011). Most of the literature is available about human seminal prostasomes, which suggests a possibility that prostasomes may play similar roles in bulls.

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Isolation of prostasomes from semen: Prostasomes were isolated from seminal plasma according to the protocol of Siciliano et al. (2008). Briefly, semen was centrifuged for 10 min at 800 g. Supernatant was centrifuged at 10,000 g for 20 min to pellet cell debris. Supernatant obtained was ultracentrifuged for 2 h at 100,000 g to pellet prostasomes. Pellet representing prostasomes fraction was resuspended in Tris-HCl buffer (30 mM, pH 7.6), made isotonic with 130 mM NaCl. Suspensions were purified by Sephadex G-200 (GE Healthcare, Uppsal, Sweden) chromatography, to separate prostasomes from an amorphous substance at 6 mL/h, and 2 mL fractions were collected. The eluant was the isotonic Tris-HCl buffer, and the eluate was monitored at 260 and 280 nm. Those fractions with elevated absorbance at 260/280 nm were collected (Ronquist et al., 2013) and analysed for aminopeptidase activity, a marker enzyme for prostasomes (Laurell et al., 1982). Those fractions with relatively high aminopeptidase activity were pooled and ultracentrifuged at 100,000 g for 2 h. The pellet representing the prostasomes was resuspended in the isotonic Tris-HCl buffer.

MATERIALS AND METHODS
Selection of animals and procurement of semen: Present study was conducted at ICAR-NDRI, Karnal, Haryana. For isolation of prostasomes, ejaculates (mass activity ≥++++) were obtained from KF bulls (n=6), and 20 mL of pooled seminal plasma were processed. To study the effects of prostasomes on the sperm functional parameters, fresh and cryopreserved semen samples (n=9; three samples from each bull) from KF bulls (age= 23-37 months; body weight= 305.5-410.8 kg) were obtained from Artificial Breeding Research Center, ICAR-NDRI.

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Characterisation of prostasomes

Immunostaining of prostasomes with FITC-conjugated CD 26 antibodies: For immunostaining of prostasomes, 0.2% gelatin was coated over the slide and dried overnight. Then the prostasomes pellet was smeared over the gelatin layer and dried off. It was flooded with the 4% formaldehyde solution and kept for 20 minutes. It was drained off and washed three times with ice cold PBS. Before immunostaining, blocking of non-specific binding sites was done with blocking buffer (3% BSA in PBS) for one hour at room temperature. Immunostaining of prostasomes with 1:50 dilutions of CD 26 antibody (antibody in 3% BSA; orb13855, Biorbyt Ltd.) was performed in darkness and left undisturbed at 37°C for one hour. It was washed with PBS avoiding the direct exposure to light. A coverslip was applied over it and quickly examined using fluorescent microscope.

Estimation of cholesterol and phospholipids content in prostasomes: Purified prostasomes were analysed for their phospholipids and cholesterol content. Phospholipids and cholesterol content was estimated by using phospholipid assay kit (Sigma-Aldrich; MAK122) and EnzyChrom™AF cholesterol assay kit (BioAssay Systems; E2CH-100) respectively.

Effect of prostasomes on sperm functional parameters: To investigate the effect of prostasomes on sperm functional parameters, 20×10⁶ spermatozoa from fresh and cryopreserved-thawed semen were obtained in 1 mL of sp-TALP media, and subsequently supplemented with prostasomes (1 mg/mL). The ratio of prostasomes protein to sperm protein, obtained as 2:1, was found optimum to conduct this investigation. For every experiment, a control was kept, which contained 20×10⁶ spermatozoa in 1 mL of sp-TALP without prostasomes. To adjust the sperm concentration to 20×10⁶ per mL of sp-TALP, one mL each of fresh semen (mass activity ≥ four) and cryopreserved-thawed semen were subjected to swim up procedure. Motile spermatozoa were obtained and concentration was adjusted to 20×10⁶ in one mL of sp-TALP.

To analyze effect of prostasomes on ROS production, prostasomes supplemented and control sperms were incubated in sp-TALP for one hour at 37 °C and 5% CO₂. Thereafter, ROS concentration in the sp-TALP was estimated at zero, 20, 40, and 60 min of incubation by using ROS estimation ELISA kit (MBS029376).

Prostasomes effect on Ca²⁺ ion signalling was investigated after 20 min incubation of prostasomes supplemented and control samples at 37 °C and 5% CO₂. The analysis was based on the Ca²⁺ ion signalling kit protocol (FLUOFORTE® Calcium Assay Kit; ENZ-51016). Detection of Ca²⁺ ion signalling in the supplemented and control samples was performed 20 min post incubation by continuous monitoring of the fluorescent signals, emitted because of calcium signalling, at a time span of about 24.2 seconds for ten cycles.

Effects of prostasomes on mitochondrial membrane potential and acrosomal integrity of spermatozoa were determined before and after one hour of incubation with prostasomes at 37 °C and 5% CO₂. Mitochondrial membrane potential was determined according to the kit protocol (MitoPT™ JC-1 100 Test Kit–924). Acrosomal integrity was analyzed as per the procedure of Chowdhury et al. (2014).

Statistical analysis: Effects of prostasomes supplementation as well as time of incubation on ROS production by spermatozoa, calcium signalling, mitochondrial membrane potential and acrosome integrity of spermatozoa was analyzed by one-way ANOVA and Duncan’s multiple comparison test) using SPSS, USA (16.00).

RESULTS AND DISCUSSION

Characterization of the prostasomes: Isolated prostasomes were initially characterised by positive binding of FITC-conjugated CD26 antibodies with prostasomal membrane (Fig1). Subsequently, chemical composition of isolated prostasomes was analysed to characterise the prostasomes. Prostasomes showed higher proportion of both protein (2.44±0.06 g%) and cholesterol (21.99±0.17 mg%) as compared to phospholipids (11.67±0.27 mg%). Present findings were in agreement with Minelli et al. (1998), who reported the presence of CD26 surface antigen on stallion seminal prostasome-like vesicles. CD26 also characterizes human prostasomes (Carlsson et al., 2006). A high protein concentration and high cholesterol to phospholipids ratio (1.88:1) in KF seminal prostasomes was in agreement with Piehl et al. (2006), who reported that cholesterol: phospholipids ratios in boar, human and equine prostasomes were 1.8, 2.0 and 1.7 respectively. High cholesterol concentration in prostasomes was in corroborations with Pons-Rejraji et al. (2011), who reported that prostasomes are cholesterol rich vesicles.

Fig 1: Fluorescent micrograph of binding of FITC-conjugated CD 26 antibodies with the prostasomes membrane (indicated by arrows).
Effect of prostasomes supplementation on sperm functional parameters

ROS production in incubation medium: ROS production was significantly higher during incubation of cryopreserved-thawed sperms compared to fresh sperms. Reduction in ROS production was noted during incubation of both fresh and cryopreserved-thawed sperms with prostasomes. Overall response in case of fresh sperms was significantly better as compared to cryopreserved-thawed sperms (Table 1), as the decline in ROS concentration in case of fresh supplemented sperms initiated as early as 20 min of incubation, which continued until 60 min. However, in case of cryopreserved-thawed supplemented sperms, it reduced significantly from zero to 20 min and thereafter it decreased insignificantly.

Excessive ROS originating from immature sperm (Fisher and Aitken, 1997) can infiltrate into seminal plasma, impairing normal spermatozoa functions by peroxidation of unsaturated fatty acids in their membrane. High sensitivity of spermatozoa to this peroxidative damage is due to relatively higher content of unsaturated fatty acids in their plasma membranes (Alvarez and Storey, 1989).

ROS concentration was significantly higher (P<0.05) in cryopreserved than fresh sperm samples. Present observations were in corroboration with Kadirve et al. (2014), who reported a significant increase in lipid peroxidation in the frozen-thawed spermatozoa, as compared to fresh spermatozoa in buffalo bull. Similar findings were reported by studies in bull (Rajoriya et al., 2013) and boar (Kim et al., 2011).

Prostasomes supplementation significantly reduced ROS production, which was not observed in control. It may be due to the sperm membrane stabilizing effects of prostasomes (Saez et al., 1998). A transfer of cholesterol from prostasomes to spermatozoa has been proposed as a mechanism that stabilizes sperm membrane and reduces its lipid peroxidation (Cross and Mahasreshti, 1997). However, a slight reduction in ROS production observed in control might be due to the antioxidant activity of EDTA present in sp-TALP, as EDTA prevents cellular lipid peroxidation (González-Cuevas et al., 2011).

Calcium signalling within spermatozoa: Prostasomes supplementation increased the intensity of calcium signalling within both fresh and cryopreserved-thawed sperms as depicted by significantly higher (p<0.01) calcium signalling in prostasomes supplemented as compared to control sperms (Fig 2). Calcium signalling was significantly higher in fresh spermatozoa as compared to cryopreserved-thawed spermatozoa. However, it was noteworthy that prostasomes supplementation significantly increased (p<0.05) the intensity of calcium signalling in cryopreserved-thawed spermatozoa as compared to control-fresh spermatozoa.

![Calcium signalling](image)

**Table 1**: Effect of prostasomes supplementation on *in vitro* ROS production (IU/ml) by fresh and cryopreserved KF sperms.

<table>
<thead>
<tr>
<th>semen type</th>
<th>treatment</th>
<th>time (min)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>supplemented</td>
<td></td>
<td>17.47±1.14(^{\text{aw}})</td>
<td>6.82±0.48(^{\text{aw}})</td>
<td>4.52±0.24(^{\text{aw}})</td>
<td>3.29±0.19(^{\text{aw}})</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>17.91±0.98(^{\text{aw}})</td>
<td>16.80±0.79(^{\text{as}})</td>
<td>15.85±0.81(^{\text{as}})</td>
<td>15.82±0.77(^{\text{as}})</td>
</tr>
<tr>
<td>Cryopreserved-thawed</td>
<td>supplemented</td>
<td></td>
<td>48.91±2.16(^{\text{as}})</td>
<td>26.45±1.57(^{\text{as}})</td>
<td>20.50±1.19(^{\text{as}})</td>
<td>18.33±0.31(^{\text{as}})</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>49.43±2.21(^{\text{as}})</td>
<td>46.52±2.08(^{\text{as}})</td>
<td>43.96±2.11(^{\text{as}})</td>
<td>42.67±2.31(^{\text{as}})</td>
</tr>
</tbody>
</table>

\(^{\text{aw}}\) vary significantly (P<0.05) within rows; \(^{\text{as}}\) vary significantly (P<0.05) within columns.
Table 2: Effect of prostasomes supplementation on the mitochondrial membrane potential of the spermatozoa.

<table>
<thead>
<tr>
<th>Semen Type</th>
<th>Treatment</th>
<th>Incubation Period</th>
<th>Mean±SEM values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreserved-thawed</td>
<td>Supplemented</td>
<td>Before</td>
<td>29244.1±1146.5b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>26513.3±1228.5b</td>
</tr>
<tr>
<td>Control</td>
<td>Before</td>
<td>29034.6±1228.5b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>11131.8±664.2b</td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Supplemented</td>
<td>Before</td>
<td>66095.4±3711.2d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>54168.1±4517.9d</td>
</tr>
<tr>
<td>Control</td>
<td>Before</td>
<td>65534.1±3601.4d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>22042.8±2864.6d</td>
<td></td>
</tr>
</tbody>
</table>

a,b,c,d vary significantly (P<0.05) within column.

Table 3: Effect of prostasomes supplementation on acrosome integrity of the spermatozoa.

<table>
<thead>
<tr>
<th>Semen Type</th>
<th>Treatment</th>
<th>Incubation Period</th>
<th>Mean±SEM values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreserved-thawed</td>
<td>Supplemented</td>
<td>Before</td>
<td>84.88±1.82c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>82.33±1.11c</td>
</tr>
<tr>
<td>Control</td>
<td>Before</td>
<td>84.22±1.45c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>65.55±1.47c</td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Supplemented</td>
<td>Before</td>
<td>96.0±0.55d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>85.55±1.08d</td>
</tr>
<tr>
<td>Control</td>
<td>Before</td>
<td>94.66±0.33d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>73.33±0.97d</td>
<td></td>
</tr>
</tbody>
</table>

a,b,c,d vary significantly (P<0.05) within column.

The endoplasmic reticulum has been demonstrated as primary Ca\(^{2+}\) storage organelle in somatic cells, but mature sperms have no ER (Suarez, 2008), indicating that there must be other organelles which spermatozoa may use as Ca\(^{2+}\) stores. Prostasomes store Ca\(^{2+}\) (Ek et al., 2002). Prostasomes-spermatozoa fusion exhibited a proportional rise of Ca\(^{2+}\) concentration within spermatozoa, which reached maximal values after 20 min of prostasomes sperm mixing (Palmerini et al., 1999).

Mitochondrial membrane potential (\(\Delta \Psi_m\)) of spermatozoa: \(\Delta \Psi_m\) indicates sperm viability. In prostasomes supplemented cryopreserved-thawed sperms, the decrease in \(\Delta \Psi_m\) from 29244.1±1146.5 to 26513.3±1228.5 RFU after one hour of incubation was not significant (p>0.05) (Table 2), but significantly (p<0.01) declined from 29034.6±1228.5 to 11131.8±664.2 RFU in control sperms. Similarly, in fresh sperms, though reduction in \(\Delta \Psi_m\) was observed in both prostasomes supplemented and control sperms, the reduction in control sperms (66095.4±3711.2 vs. 54168.1±4517.9) was significantly more as compared to supplemented sperms (65534.1±3601.4 vs. 22042.8±2864.6). Moreover, \(\Delta \Psi_m\) in fresh sperms was significantly higher as compared to control.

Since prostasomes harbour many different kinds of enzyme systems, small signaling molecules and neuroendocrine markers, they may play a role in regulating sperm viability and functions (Kravets et al., 2000). \(\Delta \Psi_m\) was significantly (p<0.01) higher in fresh sperms as compared to cryopreserved-thawed sperms. When compared to control, post-incubation \(\Delta \Psi_m\) was significantly higher in prostasomes supplemented fresh and cryopreserved-thawed sperms. Present findings were in corroboration with Wang et al. (2001), who observed an intensely positive immunostaining on the sperm mid-piece with anti-prostasomes antibodies, and proposed that prostasomes binding activate the mitochondrial function within spermatozoa.

Acrosome integrity of spermatozoa: In prostasomes supplemented cryopreserved-thawed sperms, decrease in percent acrosome integrity from 84.88±1.82 to 82.33±1.11 % after one hour of incubation was not significant (p>0.05). However, acrosome integrity decreased significantly (p<0.01) from 84.22±1.45 to 65.55±1.47 % after one hour of incubation in control sperms (Table 3). Similar type of results was obtained in prostasomes supplemented fresh sperms, in which the decrease in percent acrosome integrity (96.0±0.55 vs. 85.55±1.08%) after one hour of incubation was significantly less (p<0.05) than that of control (94.66±0.33 vs. 73.33±0.97%).

In the present study, acrosome integrity of spermatozoa in fresh sperms was significantly higher (P<0.01) than cryopreserved sperms. It was in agreement with Kadirve et al. (2014), who reported a significant decrease in acrosome integrity of frozen-thawed spermatozoa as compared to fresh spermatozoa in buffalo bulls. When compared to prostasomes supplemented cryopreserved-thawed sperms, reduction in acrosome integrity after 1 h of incubation was significantly greater (P<0.01) in control sperms.

An efflux of sperm membrane cholesterol leads to capacitation. Seminal plasma inhibits capacitation. As prostasomes are rich in cholesterol, they are probably responsible for this inhibitory activity (Pons-Rejraji et al.,...
2011). Thus, prostasomes play a protective role for ejaculated spermatozoa by preventing premature capacitation, and thereby promoting acrosome integrity.

CONCLUSION

Based on the results of present investigation, it can be concluded that prostasomes in bull seminal plasma perform different physiological functions like antioxidant activity, improvement of functional parameters of spermatozoa viz. calcium signaling, mitochondrial membrane potential, viability and acrosome integrity. Thus, in the light of present conclusion, it can be stated that prostasomes enhance the quality of semen and functional parameters of spermatozoa. Further studies to improve the quality of cryopreserved semen and effectiveness of assisted reproductive technologies by incorporating prostasomes are warranted.

REFERENCES


