Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(5): 232-237

Received: 28-07-2019
Accepted: 30-08-2019

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Bacillus megaterium in vitro growth inhibition and sperm motility and viability improvement in karan fries (Tharparkar × Holstein Friesian) and Sahiwal bulls by prostasomes supplementation

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Abstract
Prostasomes are extracellular vesicles released by prostate gland during ejaculation. They can fuse with sperms, and improve different sperm functional parameters. Present study aimed to investigate prostasomes effects on *in vitro* growth of *Bacillus megaterium* bacteria, as well as, on motility and viability of Karan Fries and Sahiwal bulls' spermatozoa. Bacteria was incubated with prostasomes for overnight in Luria-Bertani broth, and subsequently inoculated into culture plates from the broth. Spermatozoa was obtained by swim up technique from fresh and cryopreserved-thawed semen of Karan Fries and Sahiwal bulls and incubated with prostasomes (1mg/ml). Prostasomes supplementation revealed inhibitory effect on *Bacillus megaterium* growth, and beneficial effect on sperm motility and viability in case of fresh and cryopreserved-thawed Karan Fries and Sahiwal bulls' spermatozoa. Present investigation suggests prostasomes supplementation can be advantageous towards the improvement of quality of fresh and cryopreserved semen.

Keywords: Prostasomes, bacteria, inhibition, spermatozoa, motility, viability

Introduction
Since transcription and translation processes do not occur within the spermatozoa, therefore, largely, the spermatozoa are dependent on their extracellular environment for their protection against oxidative stress, acrosome integrity and motility, and perhaps for many other of its functional parameters [1]. Seminal plasma is a nutritive and protective medium for the sperms [2] in which sperms are suspended at the time of ejaculation, and it is secreted by epididymis and accessory sex glands of male [3]. Several studies reported that supplementation of seminal plasma to the spermatozoa enhances the functional parameters of spermatozoa [4]. Additionally, in the past few decades, several studies reported that extracellular vesicles secreted by the male reproductive tract are involved in the process of sperm maturation and improvement of sperm functional parameters [5].

One of the accessory sex glands present in all male domestic mammals is prostate gland, which secretes prostatic fluid at the time of ejaculation. Prostatic fluid was reported to improve the motility of spermatozoa expelled during ejaculation [6]. The secretory acinar cells of the prostate gland were reported to release prostasomes along with soluble proteins and other molecules, into the seminal plasma [5]. Prostasomes are exosome like vesicles secreted into the extracellular environment of spermatozoa at the time of ejaculation [6], and fuse with the spermatozoa under suitable conditions [7] that leads to the transfer of membrane components and genetic material to spermatozoa, improving its fertilizing ability [8]. Prostasomes-sperm fusion also improves several sperm functional parameters, such as antioxidant activity [9-10], Ca^{2+} signalling [10-11], viability [12] and acrosome integrity [10-13]. Most of the literature is available about human seminal prostasomes, which suggests a possibility that prostasomes may play similar roles in bulls. A growth inhibitory effect on the bacteria was also reported by the human seminal prostasomes supplementation [14].

Present investigation was undertaken to study the effects of isolated bull prostasomes on the *in vitro* growth of *Bacillus megaterium* bacteria. In addition to this, effects of the bull prostasomes on the motility and viability of spermatozoa obtained from fresh and cryopreserved-thawed semen of Karan Fries (KF) and Sahiwal bulls was also analysed.

Materials and Methods

Selection of animals and procurement of semen

Present study was conducted at ICAR-NDRI, Karnal, and Haryana. To study the effects of prostasomes on the sperm functional parameters, fresh and cryopreserved semen samples (n=9 samples; three samples from each of three bulls) from KF and Sahiwal bulls (age= 23-37 months; body weight= 305.5-410.8 kg) were obtained from Artificial Breeding Research Centre, ICAR-NDRI.

Isolation of prostasomes from semen and its characterization

For isolation of prostasomes, ejaculates (mass activity≥+++) were obtained from the bulls, and 20 mL of seminal plasma were pooled and processed. Prostasomes were isolated from seminal plasma according to the previously reported protocol [7]. 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. Prostasomes were further purified by Sephadex G-200 (GE Healthcare, Uppsala, 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 [15] and analysed for amino peptidase activity, a marker enzyme for prostasomes [16]. Those fractions with relatively high amino peptidase 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. Isolated prostasomes were characterized by immunostaining of prostasomes with FITC-conjugated CD 26 antibodies [10], and based on their cholesterol and phospholipids content [10].

Antibacterial effect of prostasomes

It was performed as per the previously reported protocol [14]. Briefly, *Bacillus megaterium* cultures were obtained from National Collection of Dairy Cultures, Division of Dairy Microbiology, ICAR-NDRI, and Karnal. Then they were inoculated in two of the yeast extract-free Luria-Bertani broth (LB, 1% Bactotryptone, 0.5% NaCl, pH 7.5). One of the two broths was supplemented with prostasomes (1mg/mL), marked as test. Another one was supplemented with BSA (1mg/mL), marked as positive control. A third LB broth, not inoculated with the bacteria, was also prepared marked as negative control. All the broths were cultured overnight by incubating them at 37 ˚C with continuous shaking. After overnight incubation, about 40 µL portions of prostasomes supplemented, BSA supplemented and uninoculated broths were plated on modified LB culture plates, marked as prostasome plate (test plate), BSA plate (positive control) and uninoculated plate (negative control). From the prostasomes supplemented and BSA supplemented broths, approximately $1X10^5$ colony forming units (CFU)/mL were transferred on culture plates. The culture plates were incubated for overnight at 37 ˚C. Next day the culture plates were observed for the growth of the bacteria. Negative control was kept to observe for any unwanted contamination of the broth media.

Effect of prostasomes on sperm motility and viability

To investigate the effect of prostasomes on sperm motility and viability, $20x10^6$ spermatozoa from fresh and cryopreserved-thawed semen of KF and Sahiwal bulls were obtained in 1 mL of sp-TALP media. During study, prostasomes were supplemented at a dose rate of 1 mg/mL of media. 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 $20x10^6$ spermatozoa in 1 mL of media without prostasomes. To adjust the sperm concentration to $20x10^6$ per mL of sp-TALP, one mL each of fresh semen (mass activity ≥ +++) and cryopreserved-thawed semen was subjected to swim up procedure. Motile spermatozoa were obtained and concentration was adjusted to $20x10^6$ in one mL of sp-TALP.

Promotive effect of prostasomes on sperm progressive motility

Effects of the prostasomes on sperm motility was analysed in accordance with a previous method [17-18]. Initially, the sperms were immobilized. To immobilize the spermatozoa, $20x10^6$ motile spermatozoa in one mL of sp-TALP, obtained through swim up procedure, was subjected to centrifugation at 800g for 10 minutes. The supernatant was removed as completely as possible. The spermatozoa, present in the pellet, were resuspended in a 50 mM Tris-HCl buffer, containing 138 mM NaCl and 1 mM $MgCl_2$ (pH 7.3), and washed three times at 800 g for 15 min with this buffer. Attempts were made to remove the supernatant buffer-solution as completely as possible after each centrifugation. Thereafter, the spermatozoa were again resuspended in this buffer solution. Following these procedures, the spermatozoa showed either weak or no forward motility.

After this, one of the immobilized samples from both fresh and cryopreserved semen was supplemented with prostasomes (marked as "test") and another one was not supplemented (marked as "control"). These were then incubated in CO_2 incubator at 37 ˚C for 1 h with 5% CO_2. After incubation, sperm motility was evaluated by recording the number of sperm passages over a fixed line in Neubauer chamber (a border line of secondary square of RBC counting chamber) of haemocytometer for 1 min.

Effect of prostasomes on sperm viability

To analyse the effect of prostasomes on sperm viability, prostasomes supplemented (test) and control sperms were incubated in sp-TALP for one hour at 37 ˚C with 5% CO_2. For determination of viability of spermatozoa, two microliters of sp-TALP containing sperms and 20 µL of eosin-nigrosine stain were mixed properly on a clean grease free pre warmed (37 ºC) glass slide. Thereafter, 5 µL of the mixture was drawn on another pre warmed (37 ºC) glass slide and was further observed for viable colourless cells. On an average 200 spermatozoa were counted under oil immersion objective (1000x) fields and the percentage of live spermatozoa was determined.

Statistical analysis

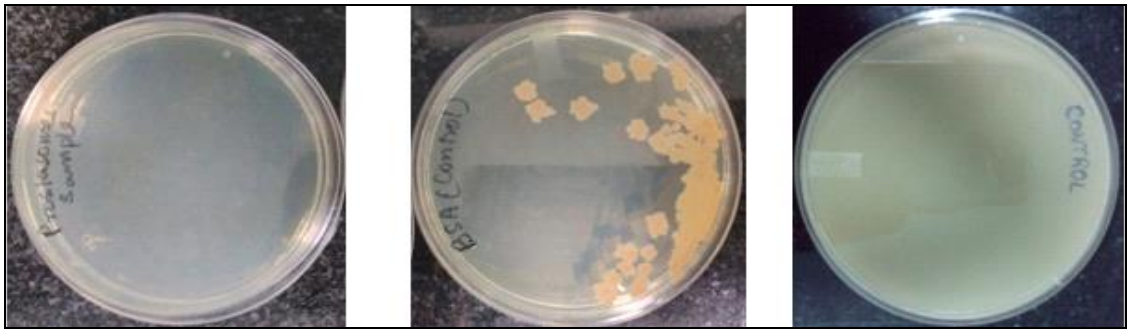
Promotive effect of prostasomes on progressive motility of immobilized spermatozoa was compared to control using student's t test. The effects of prostasomes supplementation on percent viability were analysed by one-way ANOVA (Bonferroni's multiple comparison tests) using SPSS, USA (16.00).

Results

Antibacterial effect of prostasomes

Growth media containing prostasomes (1mg/ml) inhibited the

growth of *Bacillus megaterium* while a satisfactory growth was seen on the growth media containing bovine serum albumin (positive control). The negative control plate did not exhibit bacterial growth (Figure 1).



A B C

Fig 1: Antibacterial effect of prostasomes (A) Test: Growth media supplemented with prostasomes; no bacterial growth was observed; (B) Positive control: Growth media containing BSA; clear bacterial growth was observed (C) Negative control: Growth media neither supplemented with prostasomes, nor inoculated with the bacteria; no bacterial growth was observed.

Promotive effect of prostasomes on progressive motility of spermatozoa

Present study revealed that prostasomes significantly ($P<0.01$) promoted the recovery of motile spermatozoa *in vitro* as compared to the control in both the breeds. The average recovery of motile spermatozoa in case of KF and Sahiwal fresh semen supplemented with prostasomes were 49.5±0.79 and 49.83±0.83 passages per min respectively, while the respective values in case of KF and Sahiwal cryopreserved semen were 33.22±1.03 and 34.05±0.49 passages per min. Thus, the recovery of motile spermatozoa in treatment groups was significantly higher ($P<0.01$) as compared to control groups. The recovery of motile spermatozoa was more prominent in case of fresh semen of KF (2.47%) and Sahiwal bulls (2.49%) as compared to the cryopreserved semen of the KF (1.65%) and Sahiwal bulls (1.70%). The average values have been given in Table 1.

Table 1: Promotive effect of prostasomes on progressive motility of spermatozoa (passages per min) of fresh and cryopreserved semen of KF and Sahiwal bulls

Semen type	Treatment	Mean ± S. E.	Percent motility
KF Cryopreserved Semen	Supplemented	33.22±1.03[a]	1.65%
	Control	1.42±0.34[b]	0.07%
KF Fresh Semen	Supplemented	49.5±0.79[a]	2.47%
	Control	7.27±0.48[b]	0.36%
Sahiwal Cryopreserved Semen	Supplemented	34.05±0.49[a]	1.70%
	Control	1.91±0.45[b]	0.09%
Sahiwal Fresh Semen	Supplemented	49.83±0.83[a]	2.49%
	Control	6.5±0.29[b]	0.32%

[A, B] vary significantly ($P<0.05$) within column, for each semen type.

Effects of prostasomes on sperm viability

In the present study, sperm viability after incubation was found to be significantly higher ($P<0.05$) in supplemented samples as compared to control. In case of KF cryopreserved supplemented samples, percent viability decreased non-significantly ($p>0.05$) from 79.11±1.40% to 60.88±0.78% after 1h of incubation, but in case of control samples it decreased significantly ($P<0.01$) from 79.55±1.41% to 41.77±1.26%. Similarly, sperm viability decreased non significantly during incubation in case of KF fresh (89.66±1.46% vs.76.66±1.91%) and Sahiwal cryopreserved (81.22±0.57% vs. 72.44±0.85%) supplemented samples as compared to the significant reduction in sperm viability in KF fresh (88.66±1.44% vs. 66.44±4.18%) and Sahiwal cryopreserved (80.66±0.68% vs. 53.33±0.79%) control samples. But the decrease of sperm viability in case of Sahiwal fresh semen supplemented (91.88±1.17% vs. 81.33±1.06%) and control samples (90.0±1.10% vs. 81.60±1.10%) was not significantly different ($p>0.05$). The average values have been given in Table 2.

Table 2: Prostasomes effect on percent sperm viability obtained from fresh and cryopreserved semen of KF and Sahiwal bulls.

Semen type	Treatment	Incubation Period	Mean ± S.E.
KF Cryopreserved Semen	Supplemented	Before	79.11±1.40[b]
		After	60.88±0.78[b]
	Control	Before	79.55±1.41[b]
		After	41.77±1.26[a]
KF Fresh Semen	Supplemented	Before	89.66±1.46[b]
		After	76.66±1.91[b]
	Control	Before	88.66±1.44[b]
		After	66.44±4.18[a]
Sahiwal Cryopreserved Semen	Supplemented	Before	81.22±0.57[b]
		After	72.44±0.85[b]

	Control	Before	80.66±0.68[b]
		After	53.33±0.79[a]
Sahiwal Fresh Semen	Supplemented	Before	91.88±1.17[a]
		After	81.33±1.06[a]
	Control	Before	90.0±1.10[a]
		After	81.60±1.10[a]

[A, B] vary significantly within column for each semen type.

Discussion

Antibacterial effect of prostasomes

In the present study, growth media containing prostasomes (1mg/ml) inhibited the growth of *Bacillus megaterium*, while a satisfactory growth was seen on the growth media containing bovine serum albumin. The present findings were in agreement with earlier reports that a sufficient concentration of human prostasomes was able to inhibit the growth of *Bacillus megaterium* in their cultures [14-19].

The antibacterial activity of prostasomes was attributed to its effect on bacterial membrane. It has been demonstrated that prostasomes contain large amounts of chromogranin B [20]. A C-terminal fragment of chromogranin B is known to have potent antibacterial activity. These peptides exert antibacterial activity due to their ability to form channels through the bacterial wall and membranes [21]. The prostasomes were also reported to cause bacterial membrane deformation, which resulted in the formation of membrane cavities. A complete pore formation in the bacterial membrane resulted in the abolition of action potential across the cell membrane of the bacteria [14-19]. It also led to loss of electrochemical and chemical gradients across the bacterial membranes [14]. Therefore, those conditions, which were necessary to the bacterial survival, got lost resulting in bacterial cell death.

Effect of prostasomes on motility and viability of spermatozoa

The prostasomes vesicles are enriched with different proteins and divalent cations such as calcium, zinc, and magnesium. Studies with prostasomes reported the presence of 139 different proteins [22], while other studies have reported the presence of 440 different proteins and divalent cations. About one third of these identified proteins have been found as prostasomal enzymes [23]. Since, it has been shown that prostasomes have ability to fuse with sperms [5], so they can exchange their contents with the sperms. This clearly leads to the conclusion that they have the ability to affect many of the sperm functional parameters. This is further supported by the work done with human prostasomes, which are shown to be involved in several biological functions including antioxidant capacity [9], and interactions with sperm to promote forward motility [24]. Such findings were also reported in studies with equine prostasome [25].

Promotive effect of prostasomes on progressive motility of spermatozoa

Sperm motility is one of the most important factors in the judgment of semen quality. In the present investigation, the recovery of *in vitro* motility of the immobilized spermatozoa was significantly ($P<0.01$) affected by prostasomes supplementation to different types of semen. Present findings were in agreement with the observations of Stegmayr and Ronquist [17], who recorded the sperm progressive motility value of recovered spermatozoa as 56 passages per min., on incubation of immobilized spermatozoa in the prostasomes supplemented swim up medium. Wang *et al.* [26] also found the motility stimulating effect of prostasomes and reported that the addition of prostasomes increased the proportion of motile spermatozoa from about 15% to 50-70%, thus there was an increase in the progressive motility of spermatozoa by about 35 to 55%. Present findings were also in agreement with the observation that presence of prostasomes in swim-up media significantly increased the revival of motile spermatozoa, and the percentage of spermatozoa presenting progressive motility until 1 h of incubation [27]. After the period of incubation, the sperm motility was found to be diminished, most probably because of loss of fuel in sperms [26]. Additionally, the supplementation of the swim up medium with prostasomes increased the recovery of freeze-thawed and motile spermatozoa [18]. These observations were in corroboration with the findings in the present investigation, in which an increase of sperm progressive motility over 1 h of incubation by about 18-23% and 7% in case of prostasomes supplemented cryopreserved and fresh semen respectively over unsupplemented samples was observed. The reasons of proportionately lesser increase in the progressive motility of the fresh-supplemented sperm samples compared to cryopreserved-supplemented sperm samples might be better ATP reserves and less exposure to stress in case of fresh semen samples as compared to cryopreserved semen samples [28], and hence, the better recovery of fresh-control sperms as compared to cryopreserved-control sperms.

The reason proposed behind the improvement of sperm motility by prostasomes supplementation is that prostasomes modulate the concentrations of divalent cations in and around the spermatozoa and augment spermatozoa with calcium signalling proteins that in turn enhances the flagellar motility [29]. Recent work done with prostasomes demonstrated that prostasomes contain distinct subsets of proteins that might be linked to adenosine triphosphate (ATP) synthesis. This in turn might be of importance in the role of prostasomes as a supporting source of ATP synthesis [15]. Additionally, prostasomes also stimulate a prostasomal ATPase activity [30], which in turn may enhance the energy availability to spermatozoa. These reports support the role of prostasomes in promotion of sperm motility.

Moreover, another justification of motility stimulating effect of prostasomes could be a prostasomes-sperm cell close contact due to which sperm membrane properties, such as membrane permeability to Ca^{2+} and H^{+}, might be changed. Another potent factor is vasoactive intestinal peptide present in the prostasomes [20], which is a known stimulator of adenylate cyclase activity. Adenylate cyclase then synthesizes the second messenger cyclic AMP (cAMP) from ATP. Indeed, cAMP has a potent well demonstrated effect in the initiation and maintenance of sperm motility [31]. Elevated cAMP levels also lead to protein kinase activation that in turn leads to enhanced phosphorylation of multiple endogenous proteins [32], one of them is axokinin (an axonemal protein), which is associated with this activation of mammalian sperm towards sperm motility.

Effect of prostasomes on viability of spermatozoa

Since prostasomes harbour many different kinds of enzyme

systems, small signalling molecules and neuroendocrine markers, this suggests that these vesicles play a complex role in regulating sperm viability and function [12]. After incubating sperms with prostasomes, immunostaining of spermatozoa was performed, which revealed that the whole sperm cell was stained and the stain was most intense on the midpiece [26]. This indicated that prostasomes could adhere to the sperm cell and it is more intense on mid piece. Since mid-piece is the region where the mitochondria are located, it is possible that prostasomes may activate the mitochondrial function. In this way, prostasomes supplementation may prevent the reduction of sperm viability during incubation.

Reports indicated that spermatozoa from abnormal semen samples showed a significantly lower mitochondrial membrane potential (ΔΨm) as compared to spermatozoa from normal semen samples [33]. Low-activity sperm display the decrease of mitochondrial membrane potential as an apoptotic feature [34]. The damage to the ΔΨm is a landmark event of early cell apoptosis, and it is thought to be one of the earliest events in the course of the apoptosis cascade [35]. This suggests that ΔΨm may be an indicator of sperm cell viability in a semen sample.

Conclusion

Based on the results of present investigation, it can be concluded that prostasomes in bull seminal plasma perform different physiological functions. Prostasomes can have antibacterial activity as revealed by their growth inhibiting effect on *Bacillus megaterium* bacteria. Prostasomes supplementation increased the motility of spermatozoa and also prevented the significant reduction in sperm viability. 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 necessary.

Acknowledgement

Funding for research work from Indian Council of Agricultural Research (ICAR), New Delhi and Director, NDRI is highly acknowledged. The authors are also thankful to Dr. Pradip Behare, Scientist, DM Division, for his support to accomplish this investigation.

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