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RESEARCH ARTICLE

Prostasomes as interlocutor for zygotic epigenome health: role of prostasome secretome in maintenance of zygotic epigenome

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Abstract: Prostasomes as transgenerational epigenetic toolbox has not been established, its role in transferring small RNA, lncRNA as paternal messages is yet to be explored. The present study was undertaken to establish role of prostasomes in modulating testicular and vaginal environmental cues such as protein and RNA messages through to zygote and thereby acting as interlocutor of paternal genome. The results from the study found out prostasomes fusion with spermatozoa takes place at the time of maturation and capacitation wherein it causes downregulation of ROS (Protein and mRNA) in terms of decreased NADPH and NOS activity significantly ($p<0.01$), it was also found to modulate mitochondrial membrane potential significantly ($p<0.01$). Prostasome supplementation lead to increased survival of spermatozoa in sp-TALP medium and was found to be superior to cryopreserved and fresh semen when used in routine in vitro fertilization (IVF) experiment. The study further envisages to establish prostasomes as repository of novel biomarkers for infertility in male and female and as diagnostic tool in in vitro fertilization and somatic cell nuclear transfer protocol and thereby establishing prostasomes as one of the fundamental vesicles influencing transgenerational transfer of RNA and protein to zygotic genome thereby influencing zygotic genome health.

Keywords: Zygotic genome, ROS, transgenerational, prostasome, small RNA, Secretome.

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Introduction

Prostasomes are extracellular vesicles that fuse with sperm cells in the acidic environment of the vagina thus modifying the composition of the spermatozoan membranes. (Kravets et al. 2000) reported that prostasomes harbor numerous enzyme systems, many small signaling molecules and neuroendocrine markers, thus these vesicles may play a complex role in regulation of sperm viability and in facilitation of the fertilization process. Besides, the prostasomes have been implicated in several other reproductive functions, such as the improvement of sperm motility, capacitation, and acrosome integrity and acrosome reaction of sperm as well as coagulation and liquefaction of seminal fluid in humans (Wasylewska and Wasylewski, 2007). Role of mitochondrial DNA (mtDNA) methylation and demethylation with respect to sperm health is still unknown. The genome health of healthy spermatozoon is maintained by seminal plasma and its secreted vesicles through its crosstalk with mtDNA by its secretome comprising of ncRNA, eRNA and other putative RNA enzymes which contributes to mitochondrial genome and seminal plasma exosomes (Epididymosomes and Prostasomes). The role of ncRNA, tRNA and other RNA enzymes has not been elucidated *vis –a-vis* zygotic genome health. There is dearth of literature w.r.t. RNA enzymes both de novo in spermatozoa and Extracellular Vesicles (EV, Prostasomes and Epididymosomes), low mitochondrial membrane potential and high ROS production have been detected in spermatozoa from infertile patients (Wang X et al. 2003) only one report has been noted regarding long range genome silencing in spermatozoa (Bohacek J et al. 2020). Epididymosomes (vesicles that fuse with sperm during epididymal transit) carry RNA payloads matching those of mature sperm and can deliver RNAs to immature sperm in vitro (Nejabati, HR et al. 2021) the role of Prostasomes which contributes to about 20% of total RNA and small RNA content of spermatozoon has not been studied.

The present study was undertaken to establish role of prostasomes in modulating spermatozoa health both morphologically and biochemically. The study envisaged to study how prostasome which fuses with spermatozoa transfer small RNA (ncRNA, eRNA, lncRNA) to spermatozoa and how ROS affects these small RNA to be transferred to spermatozoa and

then to zygote impinging on "zygotic genome health" after fertilization. The study found out that supplementation of Prostasome during maturation of spermatozoa significantly reduced ROS which might affect mitochondrial DNA (mtDNA) mtDNA as a loosely packaged structure, therefore, it is more easily damaged by ROS than the nuclear genome (Yakes F.M and Van Houten B 1997). Point mutations, rearrangement and/or decreased content of mtDNA are all features correlated with sperm dysfunctions and infertility (Luo S.M et al. 2013, Rosati A.J., et al. 2020). Conversely, a low mtDNA copy number has been suggested as an indicator of good-quality sperm (May-Panloup P et al. 2003); thus, its manipulation may be a powerful therapeutic strategy to decrease aging-associated mtDNA mutations (Jiang M et al. 2017). Interestingly, supplementation of prostasomes in sp-TALP improved maturation of spermatozoa and had favorable impact on motility, ATP production, ROS production. It reduced ROS production in vitro significantly ($P<0.001$), the effect of reduced ROS production on epigenetic processes are not fully described in spermatozoa the field of epigenome health is unexplored in spermatozoa.

The present study was undertaken to establish that prostasomes which carry payload of small RNA and other discrete RNA enzymes are the factors which modulate spermatozoan epigenome and envisages to elucidate prostasome secretome and its potential role in spermatozoon reprogramming thereby elucidating role of prostasome secretome in paternal epigenome mediated transgenerational transfer and metabolic heredity.

Material and methods

Sephadex G-200 was purchased from GE Healthcare, Uppsala, Sweden. FITC-conjugated CD26 antibody (orb13855) and Caveolin-1 antibody (orb15247) were purchased from Biorbyt Ltd., 5 Orwell Furlong, Cowley Road, Cambridge, Cambridgeshire, CB4 0WY, United Kingdom. Cholesterol Assay Kit (E2CH-100) was purchased from BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA. Phospholipid assay kit (MAK122) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Bovine Reactive Oxygen Species (ROS) ELISA Kit (Cat. No: MBS029376) was purchased from MyBioSource, San Diego, California, United States. Protein Estimation Kit by Biuret method, 250 reactions (KT19) was obtained from Geneilabs.

Isolation of the Prostasomes

Prostasomes were isolated from semen of both Karan Fries and Sahiwal bulls according to the protocol of (Siciliano et al. 2008) with slight modifications. Briefly, eight to ten ejaculates (mass activity > or = +++) were collected from Sahiwal and KF bulls (n=6 from each breed) and 20 mL of seminal plasma were pooled together separately and processed soon for prostasomes isolation. Semen was centrifuged for 10 min at 1,000 g in order to separate spermatozoa and possible other cells from the seminal plasma, and further ultracentrifuged at 10,000 g for 20 min to pellet possible cells and cell debris. The supernatant was subsequently subjected to another ultracentrifugation for 2 h at 100,000 g to pellet the prostasomes. The prostasomes were resuspended in a Tris-HCl buffer (30 mM, pH 7.6) made isotonic with 130 mM NaCl. The suspensions were purified by Sephadex G-200 (GE Healthcare, Uppsala, Sweden) chromatography, to separate them 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 absorbances at 260/280 nm were collected 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 100,000g for 2h. The pellet representing the prostasomes was resuspended in the isotonic Tris-HCl buffer and adjusted to a protein concentration of 0, 0.5, 1, 1.5, and 2 mg/mL using a Protein Assay kit (Biuret method-based kit), for standardization of sperm protein (20×10^6 sperms) to prostasome protein ratio isolated prostasomes were subjected to chemical composition evaluation. Isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both Karan Fries and Sahiwal bull semen (Table 1). The variations in individual constituent of the prostasomes were not significant ($P>0.05$) during different seasons in both Karan Fries as well as Sahiwal bulls.

Estimation of ROS and its effect on motility

Standardization of dose dependent response of prostasomes on sperm motility

To standardize the dose of prostasomes to be used for assessing sperm motility, 20 million immobilized sperms of both Karan Fries and Sahiwal bulls were incubated with different concentrations of prostasomes viz. 0, 0.5, 1, 1.5 and 2 mg/ml in sp-TALP for 1h at 37ÚC, 5% CO_2 in CO_2 incubator. After incubation, Neubauer's chamber of hemocytometer was charged with 0.1 µL of the above sample containing around 2000 spermatozoa. Number of motile sperms passing across a border line of RBC counting chamber was counted for 1 min. Both Sahiwal and Karan Fries sperms showed a dose dependent change in motility. Sperm motility increased significantly ($P<0.01$) up to 1 mg/ml of prostasomes followed by a non-significant ($P>0.05$) elevation up to 2mg/ml

Table 1 Chemical composition of prostasomes isolated from pooled seminal plasma of Karan Fries and Sahiwal bulls

Composition Breed	Protein (g%)	Cholesterol (mg%)	Phospholipids (mg%)
Karan Fries	2.44±0.06	21.99±0.17	11.67±0.27
Sahiwal	2.85±0.05	24.51±0.32	12.66±0.29

prostasomes concentration (Table 2). Since the sperm motility did not vary significantly (P>0.05) from 1 to 2 mg/ml concentration of prostasomes, 1mg/ml concentration of prostasomes was selected for further experiments. The optimum sperm protein: prostasome protein ratio, obtained as 1:2 (the combination of $20x10^6$ sperms/mL and prostasomes protein concentration 1mg/ml) was subsequently used to study the functional parameters of spermatozoa

Acrosomal Integrity Test by Giemsa's Stain

Staining was carried out as per the procedure described by (Chowdhury et al. 2014). The stock Giemsa's stain was prepared as per manufacturers' protocol. Briefly, to study the effect of prostasomes on acrosome integrity, the spermatozoa and prostasomes were incubated together in sp-TALP media. Initially, motile sperms were obtained from fresh semen by swim up technique. Their concentration was adjusted to $20x10^6$ sperms/mL in one mL of sp-TALP medium. Cryopreserved semen ($20x10^6$ sperms in 0.25 mL of extended semen) was added to 0.75 mL of sp-TALP. The test (prostasomes supplemented) and control (not supplemented with prostasomes) samples were incubated for 1 hour in CO_2 incubator with 5% CO_2 at 38ÏC. Then, 3 µL of Sorenson Phosphate Buffer (SPB) was put into clean grease free pre-warmed slide, to which 30 µL of sp-TALP from test and control samples (before and after incubation) was mixed and a smear was drawn and air dried. The slides were put into 5% formaldehyde solution for fixing at 37 ÚC for 30 min (Campbell et al. 1960). The slides were removed and washed and was counted for acrosomal integrity (membrane fragility and phospholipid) by staining with working geimsa solution under oil immersion (100x).

Estimation of Cholesterol Content in Prostasomes

Purified prostasomes were used for estimation of their cholesterol content. Cholesterol content was estimated by using ELISA kit (E2CH-100) procured from BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA. The range of linear detection was 1 to 100 mg/dL cholesterol. Briefly, 50 µL-diluted standards were transferred into wells of a clear 96-wells plate. 50 µL diluted prostasome samples were transferred in separate wells. For each reaction well, 55 µL Assay Buffer was mixed with 1 µL Enzyme Mix and 1 µL Dye Reagent. 50 µL of this Working Reagent was added to each standard and sample well. Plate was tapped to mix it well. The plate was incubated for 30 min at room temperature. The O.D. values were measured at 570 nm using micro scan MS-5608A plate reader. Standard curve was obtained by plotting the absorbance (vertical axis) of the standards against their concentration (horizontal axis) using 4- parameter logistic regression. The concentration of the samples was determined from the plotted standard curve.

Estimation of Phospholipids Content in Prostasomes

Purified prostasomes were used for the estimation of their phospholipids content. Phospholipids content was estimated by using Phospholipid assay kit (MAK122), which was purchased from Sigma-Aldrich, St. Louis, MO, USA. The range of linear detection was 3–200 mM. Briefly, the standards were prepared by adding 24 µL of the 2 mM Phosphatidylcholine standard to 216 µL of water to prepare a 200 µM standard working solution. Thereafter, 0, 30, 60, and 100 µL of the 200 µM standard working solution was transferred into tubes. Water was added to each tube to bring the volume to 100 µL, generating 0 (blank), 60, 120, and 200 µM standards. Transfer 20 µL of standards into separate wells of 96 well plate. The samples were estimated in 20 µL aliquots of each sample into two separate wells of a 96 wells plate. Thereafter, 80 µL of the appropriate Reaction Mix was required for each reaction (well). Reaction Mixes were allowed to equilibrate to room temperature. 80 µL of the appropriate reaction mix was added to each well and mixed well. Then, the plate was incubated for 30 min at room temperature in darkness. The absorbance of the samples and standards was measured at 570 nm.

Prostasomes supplementation and its effect on the concentration of reactive oxygen species (ROS)

Fresh semen (mass activity > or = +++) was initially subjected to swim up procedure. Motile spermatozoa were obtained by swim up procedure and concentration was adjusted to $20x10^6$ in one mL of sp-TALP media. Cryopreserved straws ($20x10^6$ sperms per straw) were directly used in the study. Entire content of a straw (0.25 mL) was added to 0.75 mL of sp-TALP (so that final volume was 1mL). The prostasomes supplemented and control sperm samples obtained from various semen types were incubated in

Table 2 Effect of different concentrations of prostasomes on motility of immobilised sperms of Sahiwal and KF bulls

Prostasomes (mg/ml)	Sahiwal		Karan Fries	
	No. of sperms motile	% Motility	No. of sperms motile	% Motility
0	5.0 ± 1.5^a	0.25	6.0 ± 0.6^a	0.30
0.5	24.0 ± 3.1^b	1.20	23.3 ± 3.2^b	1.16
1	45.7 ± 1.8^c	2.25	43.3 ± 1.9^c	2.16
1.5	49.7 ± 1.5^c	2.48	49.7 ± 0.9^c	2.48
2	52.0 ± 1.2^c	2.60	51.3 ± 1.5^c	2.56

Mean ± S.E. values with different superscripts differ significantly (P<0.05) within a group.

sp-TALP for one hour at 37 ÚC and 5% CO_2. ROS concentration in the sp-TALP was estimated at zero, 20, 40, and 60 minutes by ROS estimation ELISA kit (Cat. No: MBS029376) as per manufacturers protocol. The sensitivity of this kit was 5.0 IU/mL. The detection range of this kit was 31.2 - 1000 IU/mL

Mitochondrial membrane potential of spermatozoa (Anti apoptotic effect of prostasomes)

It was carried out in terms of mitochondrial membrane potential of spermatozoa. Motile sperms were obtained from fresh semen by swim up technique. The concentration was adjusted to 20x 10^6 sperms/mL in 1 mL of sp-TALP medium. Cryopreserved semen (20x10^6 sperms in 0.25 mL of extended semen) was added to 0.75 mL of sp-TALP. The test (prostasomes supplemented) and control (not supplemented with prostasomes) samples were incubated for 1 h in CO_2 incubator with 5% CO_2 at 37ÚC. Mitochondrial membrane potential of spermatozoa was determined before and after incubation with prostasomes. Mitochondrial membrane potential was determined according to the kit protocol (Mitochondrial Permeability Transition Detection Kits, MitoPT™ JC-1 100 Test Kit – catalog no. 924).

Statistical analysis

Comparison of different semen parameter values of semen samples having similar mass activity was performed by univariate multiple analysis of variance (Bonferroni's multiple comparison test). Effect of different concentrations of prostasomes on motility of immobilised sperms of Sahiwal and KF bulls was analyzed by one-way ANOVA (Bonferroni's multiple comparison test). The effects of prostasomes supplementation as well as time of incubation on ROS production by spermatozoa, ATP concentration, calcium signaling, mitochondrial membrane potential, acrosome integrity, percent viability, and matrix metalloproteinase activity of spermatozoa was analyzed by one-way ANOVA (Bonferroni's multiple comparison test). Promotive effect of prostasomes on progressive motility of immobilized spermatozoa was compared to control using student's t test. Differences were considered significant at least at level $P<0.05$. SPSS 16.0 software was used for the statistical analysis. Microsoft excel worksheet was used for the preparation of graphs.

Results and discussion

Isolation of Prostasomes

Isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both KF and Sahiwal bull semen. The variations in individual constituent of the prostasomes were not significant ($P>0.05$) during different seasons in both Karan Fries as well as Sahiwal bulls (suppl table 1a, b) the prostasomes isolated conformed to molecular composition and lipid composition as per (Frenette G et al. 2002), and was established for first time in KF and Sahiwal bulls at National Dairy Research Institute (NDRI), Karnal, India.

Characterization of the Prostasomes

Prostasomes are microvesicles, with their membranes containing Caveolin-1 and CD26 antigens (the surface markers), and exhibit antibacterial and antioxidant properties. These features of prostasomes were used in the present study for their characterization.

Identification of surface markers (Caveolin-1 and CD26)

FITC-conjugated Caveolin-1 and CD26 antibodies binding with prostasomes were used for identification of Caveolin-1 and CD26 antigens on prostasomes in this study. Prostasomes immunostained positively for FITC-conjugated Caveolin-1

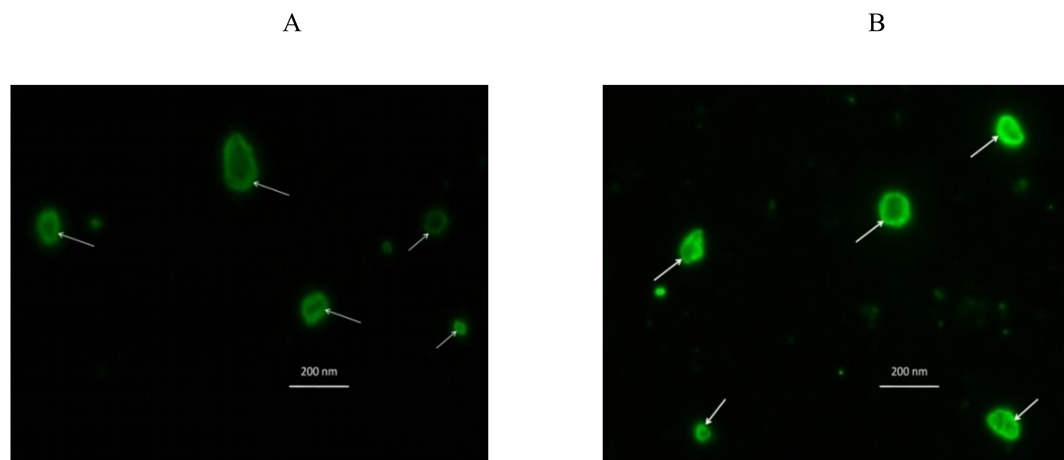

Fig. 1 Fluorescent micrograph of binding of (A) FITC-conjugated Caveolin-1 antibodies (B) FITC-conjugated CD 26 antibodies with the prostasomes membrane (indicated by arrows).

antibodies and FITC-conjugated CD 26 antibodies as depicted in Figure 1.

Effect of prostasomes on progressive motility of immobilized spermatozoa

Unpaired t-test analysis revealed that prostasomes significantly (P<0.01) promoted the recovery of motile spermatozoa in swim up media as compared to the control in both the breeds. The average recovery of motile spermatozoa in case of Karan Fries and Sahiwal fresh semen supplemented with prostasomes were 49.5±0.79 and 49.83±0.83 motility per min respectively, while the respective values in case of Karan Fries and Sahiwal cryopreserved semen were 33.22±1.03 and 34.05±0.49 motility per min. Thus, the recovery of motile spermatozoa in treatment group was more prominent in case of fresh semen of Karan Fries (2.47%) and Sahiwal bulls (2.49%) as compared to the cryopreserved semen of the Karan Fries (1.65%) and Sahiwal bulls (1.70%). The average values have been given in (Table 3) and depicted in (**Fig. 2)** Seminal plasma is a mixture of secretions from testes, epididymis and other accessory sex glands and its composition varies among animal species. For example, human and mouse seminal plasma contains secretions mainly from seminal vesicles (70–80% of the volume), and less from the prostate (20%), epididymides – testes (5%) and bulbourethral

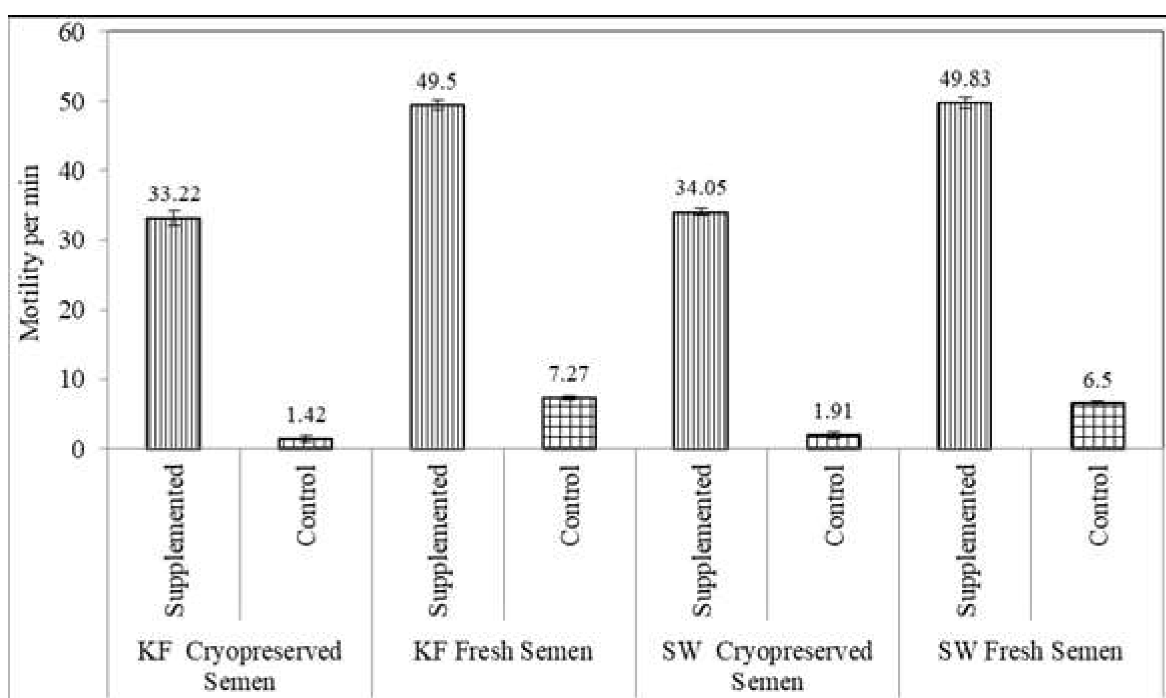

Fig. **2** Promotive effect of prostasomes on progressive motility of immobilised spermatozoa obtained from fresh and cryopreserved semen of Karan Fries (KF) and Sahiwal (SW) bulls

Table 3 Promotive effect of prostasomes on progressive motility of immobilised spermatozoa (motility per min) of fresh and cryopreserved semen of Karan Fries and Sahiwal bulls

Semen type	Treatment	Progressive Motility	
		Mean ± S.E.	Percent motility
Karan Fries Cryopreserved Semen	Supplemented	33.22±1.03^{a}	1.65
	Control	1.42±0.34^{b}	0.07
Karan Fries 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

For each semen type, values having different superscripts (small letters) vary significantly (P<0.01) within columns.

glands (<1%) (McGraw et al. 2015). Isolated prostasomes were subjected to chemical composition evaluation, the isolated prostasomes showed higher proportion of both protein and cholesterol as compared to phospholipids, in both KF and Sahiwal bull semen. The variations in individual constituent of the prostasomes were not significant (P>0.05).

Prostasomes supplementation and its effect on the concentration of reactive oxygen species (ROS)

ROS production was highest (P<0.01) in hot humid followed by hot dry and winter months respectively in Karan Fries as well as Sahiwal bulls in both cryopreserved and fresh semen samples. When the semen was supplemented with prostasomes (1mg/ml) and incubated for 60 min, the ROS concentration decreased significantly (P<0.01) with increase in incubation time. The decline initiated as early as 20min (P<0.01) in all three seasons in both the breeds. In KF cryopreserved semen the concentration averaged 48.91±2.16 IU/ml (0 min), 26.45±1.57 IU/ml (20 min), 20.50±1.19 IU/ml (40 min), and 18.33±0.31 IU/ml at 60 min (Table 4 and Figure 3). Similar type of response, but of significantly (P<0.01) lower magnitude, was seen in fresh semen obtained from Karan Fries bulls (Table 4 and Fig. 3). The values averaged 17.47±1.14 IU/ml (within 0 min), 6.82±0.48 IU/ml (20 min), 4.52±0.24 IU/ml (40 min), and 3.29±0.19 IU/ml (60 min).

The univariate multiple ANOVA revealed that ROS production was significantly (P<0.01) reduced by different factors namely breed, season, type of semen, prostasomes treatment, and time of incubation. It was significantly higher in prostasome supplemented KF (P<0.01) as compared to prostasome supplemented Sahiwal semen. The concentration was significantly elevated (P<0.01) in cryopreserved than fresh semen in both the breeds. The highest (P<0.01) concentration was observed in hot humid followed by hot dry and winter months. Incubation of sperms with prostasomes significantly lowered the ROS production (P<0.01), and by the end of 1h, the concentration was found to be lowest in both the breeds. The decline in ROS production was high in fresh semen of both breeds the ROS measured the production of nitric oxide (NOS) and nascent oxygen (0_2^-) produced during the time period of 0-60 min. The ROS was assayed by ELISA utilizing Kit (Cat. No: MBS029376)

Table 4 Effect of Prostasomes on *in vitro* ROS production (IU/ml) by Karan Fries sperms cryopreserved during different seasons

Season	Treatment	Time (min)			
		0	20	40	60
Hot dry	Supplemented	50.24 ± 3.17^{bA}	26.01 ± 0.56^{aA}	19.46 ± 0.43^{aA}	18.17 ± 0.59^{aA}
	Control	49.78 ± 2.92^{aA}	46.52 ± 2.70^{aB}	43.81 ± 2.39^{aB}	42.78 ± 2.69^{aB}
Hot humid	Supplemented	54.24 ± 2.44^{bA}	31.01 ± 3.19^{aA}	23.14 ± 3.42^{aA}	18.90 ± 0.53^{aA}
	Control	55.41 ± 3.02^{aA}	52.52 ± 2.78^{aB}	50.24 ± 2.59^{aB}	49.44 ± 3.14^{aB}
Winter	Supplemented	42.24 ± 1.67^{bA}	22.36 ± 0.64^{aA}	18.89 ± 0.19^{aA}	17.93 ± 0.48^{aA}
	Control	43.11 ± 1.78^{bA}	40.52 ± 1.02^{abB}	37.81 ± 1.43^{abB}	35.78 ± 0.78^{aB}
Overall Mean	Supplemented	48.91 ± 2.16^{bA}	26.45 ± 1.57^{aA}	20.50 ± 1.19^{aA}	18.33 ± 0.31^{aA}
	Control	49.43 ± 2.21^{aA}	46.52 ± 2.08^{aB}	43.96 ± 2.11^{aB}	42.67 ± 2.31^{aB}

For each season, values having different superscripts (small letters) vary significantly (P<0.01) within rows, while values having different superscripts (capital letters) vary significantly (P<0.01) within columns.

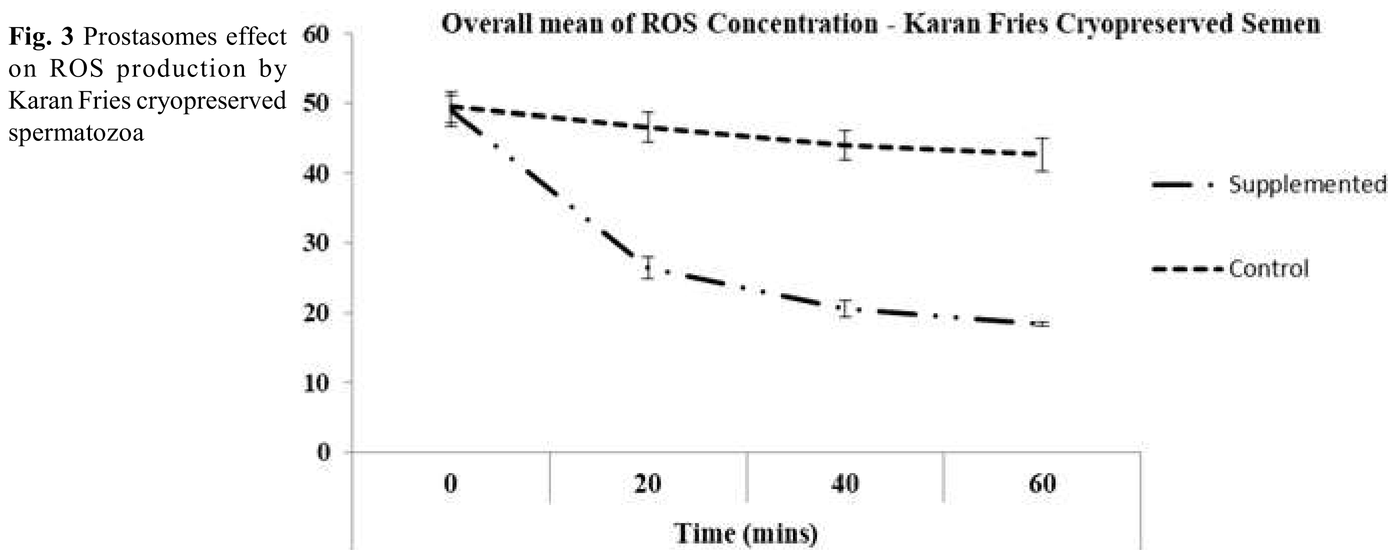

Fig. 3 Prostasomes effect on ROS production by Karan Fries cryopreserved spermatozoa

as per manufacturers instruction. The significant drop in ROS production (Table 5, Fig 4) is due to lowered NADPH levels both in cryopreserved and Fresh semen, the ROS decline is predictive of spermatozoa health and further zygote health.

The ROS decline noticed in fresh and cryopreserved semen may be selected for insemination and in vitro fertilization (IVF) as the prostasome was used in FERT-TALP (in vitro fertilization media) was shown to promote maturation of spermatozoa and leads to significant release of pronuclei in in vitro fertilized oocyte providing evidence that prostasomes are major vehicles for intergenerational transfer of RNA to spermatozoa. The reduced ROS levels were due to scavenging of nascent oxygen species (0_2^-) by electron transport chain through reduction of NADPH, present study is in corroboration with (Saez et al. 2000) who reported decreased in ROS production of seminal PMN cells and seminal plasma. The prostasome fuses with spermatozoa membrane forming spermatozoal seath surrounding mtDNA and tail piece the mechanism through which prostasome delivers the payload (small RNA and Protein) to spermatozoa is still unclear. The role of this nascent RNA and small RNA pool in prostasomes and epididymosmes are yet to defined, the mode of fusion of EV's especially prostasomes to spermatozoa and thereafter delivery of small RNA's needs to be addressed. The decline in ROS found after supplementation with Prostasome may be an indicator of epigenome health of spermatozoa can also influence zygotic health.

Effect of prostasomes on acrosome integrity of spermatozoa

To evaluate the effects of prostasomes on acrosome integrity, both fresh (mass activity > or = +++) as well as cryopreserved sperms ($20x10^6$) were incubated with prostasomes (1mg/ml) and without prostasomes for 1 h in an atmosphere of 5% CO_2 and 38°C temperature. Thereafter, acrosomal status of the spermatozoa was assessed by Giemsa's staining method. The results are expressed in (Table 6) and (Fig. 5).

In case of prostasomes supplemented Karan Fries cryopreserved semen sample, the decrease in mean percent acrosome integrity

Table 5 Effect of Prostasomes on *in vitro* ROS production (IU/ml) by Karan Fries sperms obtained from fresh semen during different seasons

Season	Treatment	Time (mins)			
		0	20	40	60
Hot dry	Supplemented	17.74±0.77^{bA}	6.95±0.55^{aA}	4.78±0.13^{aA}	3.60±0.17^{aA}
	Control	18.31±1.03^{aA}	17.05±0.95^{aB}	16.89±1.21^{aB}	16.46±1.35^{aB}
Hot humid	Supplemented	20.94±0.56^{cA}	8.17±0.43^{bA}	5.07±0.35^{aA}	3.67±0.14^{aA}
	Control	20.78±0.64^{bA}	18.60±0.63^{abB}	17.46±0.62^{aB}	17.56±0.35^{aB}
Winter	Supplemented	13.75±1.29^{bA}	5.48±0.71^{aA}	3.71±0.15^{aA}	2.60±0.17^{aA}
	Control	14.64±0.71^{aA}	14.07±0.63^{aB}	13.33±0.89^{aB}	13.46±0.86^{aB}
Overall Mean	Supplemented	17.47±1.14^{cA}	6.82±0.48^{bA}	4.52±0.24^{abA}	3.29±0.19^{aA}
	Control	17.91±0.98^{aA}	16.80±0.79^{aB}	15.85±0.81^{aB}	15.82±0.77^{aB}

For each season, values having different superscripts (small letters) vary significantly (P<0.01) within rows, while values having different superscripts (capital letters) vary significantly (P<0.01) within columns.

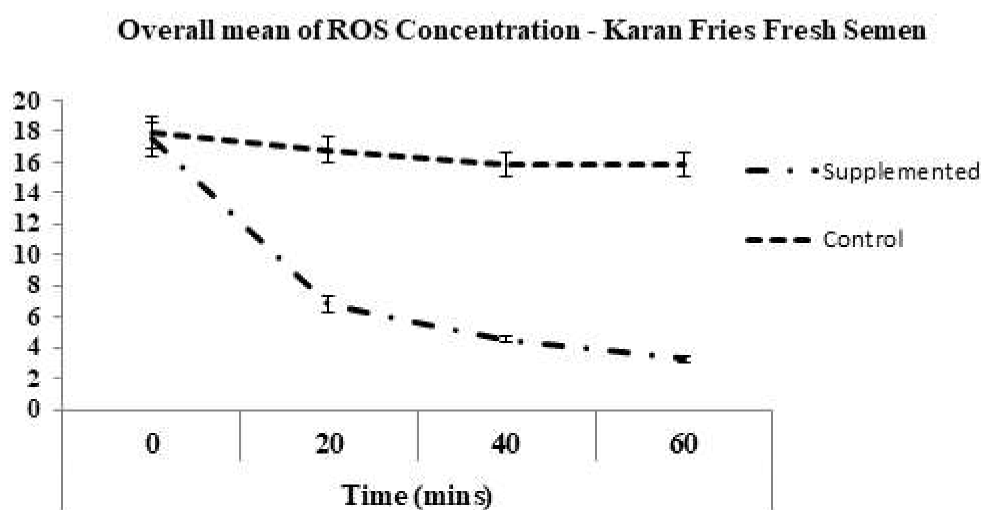

Fig. 4 Prostasomes effect on ROS production by Karan Fries fresh spermatozoa

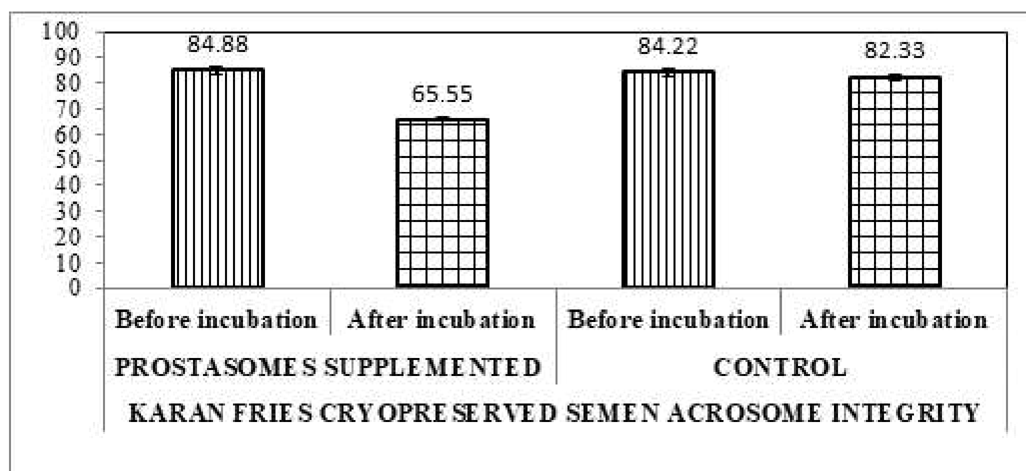

Fig. 5 Prostasomes effect on acrosome integrity of spermatozoa obtained from cryopreserved semen of KF bulls

Table 6 Mean ± SE values of acrosome integrity in KF cryopreserved and fresh semen

Season	Treatment	Cryopreserved		Fresh	
		Incubation Period			
		Before	After	Before	After
Hot dry	Supplemented	85.00±2.30^{bA}	81.0±0.50^{bA}	97.66±0.88^{bA}	85.33±2.02^{bA}
	Control	84.0±2.08^{bA}	64.33±1.45^{aB}	91.00±0.59^{bA}	73.66±2.33^{aB}
Hot Humid	Supplemented	79.33±1.76^{bA}	73.66±0.88^{bA}	95.66±0.33^{bA}	88.33±0.66^{bA}
	Control	80.33±1.45^{bA}	61.66±1.41^{aB}	90.00±0.55^{bA}	73.66±0.85^{aB}
Winter	Supplemented	90.33±1.20^{bA}	86.0±0.57^{bA}	94.66±0.66^{bA}	83.00±1.52^{bA}
	Control	88.33±1.66^{bA}	70.66±0.66^{aB}	91.00±0.60^{bA}	72.66±2.18^{aB}
Overall Mean	Supplemented	84.88± 1.82^{bA}	82.33± 1.11^{bA}	96.0± 0.55^{bA}	85.55± 1.08^{bA}
	Control	84.22± 1.45^{bA}	65.55± 1.47^{aB}	91.66± 0.33^{bA}	73.33± 0.97^{aB}

For each season, values having different superscripts (small letters) vary significantly (P<0.01) within rows, while values having different superscripts (capital letters) vary significantly (P<0.01) within columns.

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 1h of incubation in control semen samples (incubated with swim up media only). Similar type of results was obtained in case of KF fresh semen, and Sahiwal semen both cryopreserved and fresh as depicted. The present study found out that the destabilization of acrosomal membrane had cholesterol efflux during capacitation when in vagina and its phospholipid showed decline from 0hr through to 60min as was the trend for ROS and motility, thus establishing that prostasomes and other EV's plays a role in both capacitation and acrosome reaction, the study is in consonance with Soderquist et al. 1996. Secretome from the epididymis and accessory sex glands contain energy substrates, ions, proteins, RNAs and lipids, among others components, which could be found freely dissolved and in solution or encapsulated in extracellular vesicles or exosomes (Vojtech et al. 2014). Therefore, it seems obvious to think that the exosomes released by epididymis and accessory sex glands could play a role in the communication between seminal plasma and sperm. There is increasing evidence showing that exosomes detected in different body fluids participate in intercellular communication, through the selective incorporation of their cargo into the target cell (Keerthikumar et al. 2016). This functional involvement has been proposed for exosomes released by the epididymides (epididymosomes), which are capable of modifying the lipid composition of the sperm membrane, thus contributing to the acquisition of sperm motility potential (Sullivan and Saez 2013). The present study contributes on the shortcomings of keerthikumar et al. 2016 and Sullivan and Saez 2013 by finding that prostasomes may be one of the significant partners contributing to seminal plasma its small RNA component which causes decreased NOS concentration and thereby favoring zygotic genome activation (ZGA). Additionally, many studies have shown the role of prostate-derived exosomes (prostasomes) in the stimulation of sperm motility while avoiding at the same time premature capacitation and spontaneous acrosome reaction (Aalberts et al. 2014), the present study is in consonance with Aalberts et al. 2014 with regards to acrosome reaction and capacitation and found that both the parameters were

upregulated with shortened time for capacitation in KF and sahiwal breeds. Of note, exosomes contained in seminal plasma have been reported to increase the expression of immune- and inflammatory response-related genes in porcine endometrial tissue, reaching similar levels as those observed in the endometrium from naturally mated pigs (Bai et al. 2018). This suggests that communication between seminal plasma and the female reproductive tract could also be through the interaction with seminal exosomes.

Role of Prostasomes as interlocutor of Zygotic Genome Health

Prostasomes deposits and transfer RNA payload to spermatozoa during its transit from seminiferous tubule through to epididymis and prostate they deposit DNA, RNA and proteins to spermatozoa which may be transgenerationally transferred, it has been reported that spermatozoa inherits tRNA Fragments (Sharma U et al. 2016), spiRNA, small RNA, lncRNA, ncRNA forming sperm transcriptome, it remains unresolved as to how environmental cues from testicular, oviductal, vaginal, epididymis and prostate is transferred through to spermatozoa and then to zygote modulating spermatozoan and zygotic genome, whether this is epigenetically regulated by deposition of histone ubiquitins (H2Aub) molecules and coordinated by epigenetic marks for histone demethylase and DNA demethylase as early as 2 cell zygote at the before start of minor ZGA in both mouse and buffalo (*Bubalus bubalis*). The study proposes to further on the findings by establishing role of Paternal environment in transgenerational transfer of spermatozoal DNA as broader scope of embryonic genome health.

The present study establishes prostasome as interlocutor of epigenetic transfer of proteins and small RNA to spermatozoa as a phenomenon associated with transgenerational transfer of testicular epigenetic modifications. It also emphasizes on spermatozoon environmental cues like ROS and mtDNA modification especially small RNA mediated enhancer like control of zygotic genome as major mechanism governing zygotic genome health with Prostasomes as one of the fundamental vesicles influencing transgenerational transfer of RNA and protein to zygotic genome thereby influencing zygotic genome health.

Conclusions

Prostasomes have been identified as extracellular vesicular bodies which has effect on spermatozoon maturation. Its role in maintaining embryo epigenome health has not been studied. The present study establishes prostasomes as major extracellular vesicle body affecting spermatozoon fertilizing ability through transfer of small RNA molecules or "gemmules" thereby contributing to fertilizing ability, transgenerational transfer of epigenome and genomic molecules to zygote thereby acting as interlocutor of zygotic genome health. The prostasomes and to some extent epididymosomes maintains zygotic genome health through modulation of spermatozoan genomic and epigenomic payload thereby maintaining spermatozoon fertilizing ability and further influencing zygotic genome health. The study demonstrates that prostasomes when supplemented in sp-TALP medium was superior to normal fresh semen and cryopreserved semen in routine in vitro fertilization (IVF) experiment as they transfer "gemmules" or RNA transcripts which influences the zyogotic (zygotic) health via epigenomic transfer of RNA transcript transgenerationally indicating towards the future scope about utilizing prostasomes for augmenting and increasing the efficacy of IVF and SCNT methodologies especially as the prostosomes and to some extent epididymosomes in combination can be used in IVF and SCNT protocols to generate high quality, superior blastocysts, zygote and fetus as they influence the zygotic genome health. The prostasomes as the extra vesicular organelles loaded with RNA transcript (spiRNA, eRNA, tRNA, lncRNA) has not been studied to modulate the epitranscriptome of zygote, with the advent of scRNAseq and Hi-C technologies the role of these payloads in modulating "zygotic genome health" in routine IVF and SCNT technologies will be defined. We have previously defined the role of GLUT1 and HSP70.1 in regulating Zygotic Genome Activation (ZGA) in buffalo (*Bubalus bubalis*) in routine IVF, The role of prostasomes in regulating ZGA and paternal genome contribution to zygotic genome activation and zygotic genome health has to be elucidated. The potential of combination of Prostasome and epididymosome as supplement in sp-TALP and SCNT protocols has to be defined as paternal genome unification is highly error prone and subjected to intense methylation and RNA enzymes processing it would be of interest to define and identify the biomarkers form Prostasomes which contribute to zygotic genome health.

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