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Effect of buffalo PDC-109 on caudal epididymal spermatozoa of bubaline species

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ABSTRACT

A study was conducted to assess the effect of buffalo seminal PDC-109 on its cauda epididymal spermatozoa. PDC-109 protein was purified by applying two-step chromatography procedures and included into epididymal spermatozoa. Epididymal ejaculates were splited into four groups as Gr 1: Control (without PDC), Gr 2: 20 µg/mL PDC-109, Gr 3: 40 µg/mL PDC-109 and Gr 4: 80 µg/mL PDC-109. Semen quality parameters (SQPs) and *in vitro* fertility assay were evaluated. Significant improvement in post-thaw SQPs was observed in Gr 2 than in Gr 3, however, Gr 1 had significantly higher value than other groups. It is concluded that PDC-109 has dose dependent effect as increased dose causes detrimental effect in buffalo cauda epididymal sperm.

Keywords: Buffalo, Caudal epididymal spermatozoa, *In vitro* capacitation, *In vitro* fertilization, PDC-109

Bovine seminal plasma proteins (BSPs) have beneficial and/or detrimental effects on sperm. BSPs partly originate from blood plasma and partly synthesized by testes, epididymis, vas deferens and seminal vesicles (Manjunath *et al.* 2008). BSPs modulate spermatozoa fertilizing ability (Batova *et al.* 1993). Interaction of PDC-109 with sperm plasma membranes results in cholesterol efflux (Srivastava *et al.* 2012). Srivastava *et al.* (2013) reduced PDC-109 concentration with use anti-PDC-109 in bovine species. There was no systematic study on effect of buffalo PDC-109 on its cauda epididymal spermatozoa. Therefore, the study was designed to assess the effect of buffalo PDC-109 on its cauda epididymal spermatozoa during cryopreservation.

MATERIALS AND METHODS

Adult healthy Murrah breeding bulls (n=4), 4–6 years were maintained at GPC, ICAR-Indian Veterinary Research Institute, Izatnagar, were used for semen collection and isolation and purification of PDC-109. Ejaculates were collected in morning by standard AV method. The study consisted of two phases; in first phase, ejaculates were collected, isolated, purified the PDC-109 and in second phase, isolated and purified PDC-109 were included in epididymal ejaculates and studied the SQPs and *in vitro* zona binding ability of the sperm.

Ejaculates were centrifuged at 5°C at 4,000 g for 20 min to separate the seminal plasma for PDC-109 isolation and purification. It was again subsequently centrifuged at 10,000 g for 60 min at 5°C to get clear the seminal plasma and stored at –80°C. Heparin-binding proteins and PDC-109 were isolated and purified as per the method described by Gasset *et al.* (1997) with minor modifications. The isolated protein was identified against protein molecular weight markers on SDS-PAGE (Laemmli 1970) and confirmed by western blot (Towbin *et al.* 1979).

Healthy adult buffalo testes (n=4) which were collected and utilized for collection of caudal epididymal spermatozoa from slaughter house, Bareilly, Uttar Pradesh, India. Epididymal semen sample was centrifuged at 150 g for 10 min at 37°C. Spermatozoa pellet was reconstituted with Tris buffer to 5 mL. Cauda epididymal suspension with motility of 70% and more were selected for study. Extended epididymal suspension was evaluated with the SQPs and cholesterol content in spermatozoa. Experimental groups were grouped into four groups as Gr 1: spermatozoa in EYTG diluter without PDC-109 (Control), Gr 2: Tris-Fructose-Citric acid (TFC) buffer with 20 µg/mL PDC-109, Gr 3: TFC buffer with 40 µg/mL PDC-109 and Gr 4: TFC buffer with 80 µg/mL PDC-109. All groups were incubated for a period of 20 min at 37°C and the final dilution was 60×10^6 spermatozoa. Initial incubation of epididymal sperm with seminal proteins for a period of 10 min was done to allow PDC-109 to interact with spermatozoa. Extended samples were filled in 0.5 mL French midi straws and sealed with poly vinyl alcohol powder.

Diluted semen samples of control and treatment groups

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were cooled, frozen and stored with standard procedures. SQPs such as sperm forward progressive motility (Salisbury *et al.* 1985), viability with fluorochromes Hoechst 33258 (Harrison and Vickers 1990), acrosome integrity by Fluorescein isothiocyanate labelled lectin + propidium iodide, FITC-PSA+PI (Sukardi *et al.* 1997), plasma membrane integrity (hypo-osmotic swelling test, HOST; Jeyendran *et al.* 1984), cholesterol content of spermatozoa by cholesterol estimation kit, *in vitro* capacitation by Chlortetracycline (CTC) assay (Fraser *et al.* 1995) and *in vitro* zona binding assay (Fazeli *et al.* 1993) were estimated.

Analysis of variance (ANOVA) was performed using a generalized linear model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001). Differences with values of $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

SQPs of caudal epididymis are presented in Table 1. The SQPs (Table 2) and cholesterol content (Table 3) of sperm were significantly ($p<0.05$) reduced in post thawed than in pre-freezed semen. These SQPs were significantly ($p<0.05$) higher Gr 1 followed by Gr 2, 3 and 4. CTC assay was done to understand the effect of PDC-109 on capacitation status. The per cent of spermatozoa showing pattern F (Non-capacitated) was significantly ($p<0.05$) higher in Gr 1 whereas spermatozoa showing CTC pattern B (capacitated) and AR (acrosome reacted) were significantly ($p<0.05$) higher in Gr 2 followed by Gr 3 and 4 in the post-thaw stage. Cholesterol content of spermatozoa was reduced significantly ($p<0.05$) in post thawed stage as compared to pre-freeze stage and significantly ($p<0.05$) higher in Gr I followed by Gr 2, 3 and 4. *In vitro* zona binding assay revealed that the zona binding index was significantly ($p<0.05$) higher in treatment group (Gr 2, 20 µg/mL PDC-109) as compared to untreated control group (Table 4).

An important observation was that high concentration of PDC-109 (80 µg/mL) treated sperm had significantly lower motility than other groups. The results indicate that PDC-109 stimulates spermatozoa motility as dose dependent with higher concentrations being deleterious (Arangasamy 2003, Kumar 2005, Harshan *et al.* 2009).

Table 1. Sperm functional parameters of cauda epididymal spermatozoa at fresh stage (n=4)

Concentration ($\times10^9$)	Motility (%)	Viability (%)	Acrosomal integrity (%)	Plasma membrane integrity (%)	Cholesterol (µg/10^8 sperm)
5.10±0.66	81.25±1.25	87.00±0.82	83.50±0.96	81.25±0.48	25.00±0.50

Table 2. Effect of PDC-109 on forward progressive motility, viability and acrosomal integrity of cauda epididymal spermatozoa at pre-freeze and post-thaw stage (n=4)

Group	Progressive motility (%)		Viability (%)		Acrosomal Integrity (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Gr 1	72.50 ± 1.44^{a}	52.50 ± 1.44^{bA}	84.00 ± 0.58^{aA}	64.75 ± 1.97^{bA}	81.50 ± 1.71^{a}	66.25 ± 2.63^{bA}
Gr 2	73.75 ± 1.25^{a}	43.75 ± 2.39^{bB}	83.25 ± 1.11^{aAB}	53.00 ± 0.91^{bB}	80.75 ± 2.46^{a}	57.25 ± 2.39^{bB}
Gr 3	71.25 ± 1.25^{a}	37.50 ± 1.44^{bC}	83.00 ± 0.41^{aAB}	41.75 ± 1.25^{bC}	78.75 ± 1.80^{a}	53.50 ± 2.10^{bB}
Gr 4	70.00 ± 0.00^{a}	25.00 ± 2.04^{bD}	81.25 ± 0.48^{aB}	30.75 ± 1.11^{bD}	77.50 ± 1.55^{a}	50.25 ± 1.49^{bB}

Means bearing different superscripts (a, b) in the rows differ significantly ($P<0.001$). Means bearing different superscripts (A, B, C, D) in a columns differ significantly ($P<0.05$). Gr 1, Control (without PDC-109); Gr 2, 20 µg/ml PDC-109; Gr 3, 40 µg/ml PDC-109 and Gr 4, 80 µg/ml PDC-109.

Table 3. Effect of PDC-109 on plasma membrane integrity and cholesterol content of spermatozoa of cauda epididymal spermatozoa at pre-freeze and post-thaw stage (n=4)

Group	Plasma membrane integrity (%)		Cholesterol (µg/10^8 sperm)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Gr 1	71.75 ± 0.85^{aB}	60.75 ± 1.60^{bA}	22.75 ± 0.65^{aA}	18.87 ± 0.22^{bA}
Gr 2	75.75 ± 1.55^{aA}	45.00 ± 0.71^{bB}	18.93 ± 0.30^{aB}	15.45 ± 0.32^{bB}
Gr 3	73.25 ± 0.75^{aAB}	36.00 ± 0.71^{bC}	15.68 ± 0.43^{aC}	13.54 ± 0.26^{bC}
Gr 4	72.25 ± 0.48^{aB}	29.75 ± 0.63^{bD}	12.93 ± 0.22^{aD}	10.66 ± 0.12^{bD}

Means bearing different superscripts (a, b) in the rows differ significantly ($P<0.001$). Means bearing different superscripts (A, B, C, D) in a columns differ significantly ($P<0.05$). Gr 1, Control (without PDC-109); Gr 2, 20 µg/ml PDC-109; Gr 3, 40 µg/ml PDC-109 and Gr 4, 80 µg/ml PDC-109.

Table 4. Effect of PDC-109 on chlortetracycline staining pattern and zona binding index of spermatozoa of cauda epididymal spermatozoa at post-thaw stage

Group	Chlortetracycline staining pattern (%)			Zona binding index
	F pattern	B pattern	AR pattern	
Gr 1	61.25 ± 1.49^{aC}	27.25 ± 1.25^{bB}	11.5 ± 0.65^{bA}	242.3 ± 4.62^{b}
Gr 2	45.00 ± 1.22^{bC}	37.5 ± 0.65^{aB}	17.5 ± 0.65^{aA}	264.7 ± 7.24^{a}

Means bearing different superscripts (a, b) in the rows differ significantly (P<0.001). Means bearing different superscripts (A, B, C, D) in a columns differ significantly (P<0.05). Gr 1, Control (without PDC-109); Gr 2, 20 µg/ml PDC-109; Gr 3, 40 µg/ml PDC-109 and Gr 4, 80 µg/ml PDC-109.

Sperm viability was significantly reduced in high concentration PDC-109 treated group. Similar pattern were reported by Arangasamy (2003), Harshan *et al.* (2009) and Kumar (2005) in their study that treatment of buffalo cauda epididymal spermatozoa with buffalo HBPs. Way *et al.* (2000) reported that exposure of bovine spermatozoa to accessory sex gland fluid accelerated cell death and concluded that rapid removal of spermatozoa from seminal plasma was critical for maximal viability. Reduction in protein could either be the cause of increased susceptibility of membrane damage as a result of associated lipid efflux leading to cryodamage which is mirrored by decreased spermatozoa viability in present study.

At post thaw stage, PDC-109 treated groups had higher number of acrosome reacted spermatozoa than in control group. Similar pattern was reported by Arangasamy (2003), Harshan *et al.* (2009) and Kumar (2005) after treatment of buffalo cauda epididymal spermatozoa with buffalo HBPs. BSP proteins initiate cholesterol efflux when epididymal spermatozoa are exposed to them; later the membrane bound protein interacts with heparin and high-density lipoproteins (HDL). The results at pre-freeze and post thaw stage for acrosomal reaction (AR) are in agreement with Roncoletta *et al.* (2006) suggested that BSP proteins would stabilize the sperm membrane in first step (decapacitating factor) and prevent a premature AR.

The role of PDC-109 explains the finding of Singh *et al.* (2014) that addition of seminal plasma to the sperm induces hyperactivation and acrosome reaction in *in vitro.* Addition of HBP to epididymal sperm induced heparin stimulated acrosome reactions (Miller *et al.* 1990). Epididymal sperm incubated with crude seminal plasma proteins for 20 min prior to incubation with heparin were able to respond to lysophosphatidyl choline and undergo the acrosome reaction with maximum level at 60 µg per mL of crude BSP proteins and were 2.9 times of control value (Therien *et al.* 1997).

The mechanism involved in sperm capacitation and the acrosome reaction by BSP proteins (PDC-109) has been proposed to be due to cholesterol and phospholipid efflux from the sperm membrane leading to a decrease in the molar ratio of cholesterol to phospholipids in the spermatozoa plasma membrane (Srivastava *et al.* 2012) or BSP proteins might be involved in modification of the composition of the sperm membrane lipids that occur during capacitation and acrosome reaction (Desnoyers and Manjunath 1992). Similar pattern of results on HOST were reported by Arangasamy (2003), Harshan *et al.* (2009) and Kumar (2005). Harshan *et al.* (2009) reported that HOS response in the entire PDC-109 treated group was significantly lower than the spermatozoa of control group at post-thaw stage. The reduction in HOS response was to be dose dependent with the highest dose showing the greatest reduction. The influence of PDC-109 on cauda epididymal spermatozoa HOS response as a whole could also be due to stabilizing effect of PDC-109 during the initial exposure of the spermatozoa at first step followed by participation in modifications of the membrane necessary for the acrosome reaction to proceed in a latter step (Roncoletta *et al.* 2006). Also, in case of buffalo spermatozoa more amount of phosphatidylcholine may facilitate more binding of PDC-109 which leads to cholesterol efflux during freeze-thaw rendering spermatozoa plasma membrane to cryoinjury/ cryodamage.

Cholesterol contents were significantly higher in Gr 1 than in other groups. Cholesterol content decreased significantly and sequentially with an increase in PDC concentration at both the stages. Thérien *et al.* (1998) reported cholesterol efflux of 19.1% after 8 h of incubation of bovine cauda epididymal spermatozoa with PDC-109 at the concentration of 120 µg per mL. Numerous studies have shown that cholesterol alters the bulk biophysical properties of biological membranes. PDC-109 in the presence of phospholipids causes strong efflux of cholesterol from sperm membrane (Muller *et al.* 2002) and is time and concentration dependent manner (Therien *et al.* 1998). Treatment of cauda epididymal spermatozoa of buffalo with PDC-109 led to increased cryoinjury (Harshan *et al.* 2009). At higher concentration of PDC-109 and/or longer exposure, more cholesterol and phospholipids are removed, decrease spermatozoa resistance to cold shock and freezing (Srivastava *et al.* 2012)). Lower cholesterol content in PDC treated groups is indicative of increased cryoinjury or cryo-damage during cryopreservation.

At post thaw stage, it was observed that spermatozoa of the treated Gr II (20 µg/mL PDC-109) had higher number of CTC pattern B (capacitated) and pattern AR (acrosome reacted) than the spermatozoa of control group. Harshan *et al.* (2009) reported a decrease in number of non-capacitated spermatozoa in PDC-109 treated groups than in the control

at varying incubation period. This effect reached a maximum level at 20 µg/mL of PDC-109 (2.2 folds higher than control). It was observed that increase in number of capacitated spermatozoa at pre-freeze stage has negative effect on conception rate (Roncoletta *et al.* 2006). Chauhan *et al.* (1997) reported that heparin treatment (10 µg/mL) enhanced the capacitation rate of buffalo spermatozoa and decreased the percentage of acrosome intact, non-capacitated (pattern F) spermatozoa. Florman and First (1988) observed that exposure of spermatozoa to seminal plasma in *in vitro,* increased the percentage of capacitated spermatozoa. Therefore, partially or fully cryopreserved spermatozoa demonstrate capacitation-like behaviour revealed by a greater proportion of CTC fluorescent and their ability to undergo the AR or fertilize oocytes in *in vitro* (Rajoriya *et al.* 2020). This cryocapacitation is thought to be partly responsible for the reduced fertility of frozen-thawed bull semen. Cryocapacitation involves a different regulatory mechanism of protein tyrosine phosphorylation than heparin (Rajoriya *et al.* 2020).

BSP proteins might be involved in modification of the composition of the sperm membrane lipids that occur during capacitation and acrosome reaction (Desnoyers and Manjunath 1992). Lower cholesterol efflux, i.e. higher cholesterol in control group might be the reason of lower capacitation and AR in the respective group as compared to PDC-109 treated group. Removal of about 30% cholesterol from sperm membranes did not induce any capacitation and AR. A release of about 50–65% cholesterol was required to induce capacitation and acrosome reaction (Iborra *et al.* 2000). Therien *et al.* (1997) reported maximum capacitated and acrosome reacted spermatozoa when PDC-109 was added at the rate of 40 µg/mL of semen. Harshan *et al.* (2009) reported an increase in the number of capacitated and acrosome reacted post-thaw spermatozoa in PDC-109 treated group than in the control at varying incubation periods. These findings suggest that PDC-109 might be responsible for capacitation and acrosome reaction of spermatozoa. The findings of the present study is suggestive of increase in per cent of spermatozoa showing pattern B (capacitation) and pattern AR (acrosome reacted) may be due to prolonged exposure of PDC-109 present media causing more cholesterol and phospholipids efflux leads to capacitation and further acrosomal reaction.

A significant difference was observed in between the 20 µg/mL PDC-109 treated group and control with respect to binding index (BI). Arangasamy (2003) reported non-significant difference in 40 µg/mL HBPs treated and control group, however, comparable with the findings of Harshan *et al.* (2009) who reported 40 µg per mL PDC-109 treated and control group. It is observed that both PDC-109 aid in bringing about hyperactivated motility of spermatozoa. It is required for sperm penetration of the oocyte zona pellucida during fertilization and is induced by an increase in flagellar Ca^{++} (Marquez and Suarez 2006). This could be the reason for increased sperm binding to ova in the Group II (20 µg/mL PDC-109) compared to control group.

From this study, it can be concluded that PDC-109 has effective as dose dependent manner as increased dose causes detrimental effect in buffalo sperm. Further study needs to be taken as PDC-109 less than 20 µg/mL to optimize the correct dosage to improve SQPs and minimize cryoinjury to buffalo spermatozoa upon storage at ultra-low temperatures.

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