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Identification of a decapacitation factor in goat seminal plasma*

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ABSTRACT

The seminal plasma is complex mixture of the secretions of the testis, epididymis and the accessory glands. The secretory proteins of the epididymal fluid as well as those of the accessory glands appear along with spermatozoa as semen at the time of ejaculation. Some of these proteins coat the sperm surface and prevent the premature activation of the spermatozoa in the female reproductive tract. The activation of spermatozoa, which requires the removal of sperm-coating proteins, is necessitated for the entry of the spermatozoa into the oocyte at the site of fertilization. The adsorption of radiolabelled seminal plasma proteins on the sperm surface showed an absence of high molecular weight proteins, thus suggesting the reorganization of the sperm surface proteins during the process of capacitation. The effect of lyophilized goat seminal plasma proteins revealed an inhibitory effect on the capacitation after an initial preincubation of 3.5 h. The absorption of radiolabelled seminal plasma proteins on the sperm surface showed an absence of high molecular weight proteins, thus suggesting the reorganization of the sperm surface proteins during the process of capacitation. The amount of protein for a 50% decrease in the acrosome reaction as compared to the control in a dose-dependent response, ID_{50} is 2.45 mg. The factor is removed by the help of ultracentrifugation for 6 h and the pellet obtained showed an inhibitory effect at an initial preincubation of 3.5 h and the value of ID_{50} determined is 1.25 mg. An apparent mechanism of capacitation involves the rearrangement of sperm surface proteins and removal of the factor by ultracentrifugation shows that it is a high molecular weight protein.

Key words: Accessory glands, Capacitation, Decapacitation, Decapacitation factor (DF), Seminal plasma, Sperm surface proteins

Seminal plasma, a complex mixture of secretions originating from the testis, the epididymis and male accessory glands (e.g. prostate, seminal vesicle and Cowper's glands), contains polypeptides, and some of these bind to the surface of the spermatozoa during epididymal transit and ejaculation. The spermatozoa possess limited biosynthetic capabilities, hence it appears that interaction of spermatozoa with surrounding fluid could play a major role in conferring new properties to sperm membrane.

Chang (1957) showed that ability of the capacitated spermatozoa to fertilize eggs in rabbit is inhibited when these sperms are mixed with homologous or heterologous seminal plasma. A decapacitation factor (DF) is present in the seminal plasma, which inhibits fertilization. Bedford and Chang (1962) subjected the seminal plasma to high-speed centrifugation so as to remove it from the supernatant, and it was described to be a high molecular weight factor. The reversible decapacitation activity of rabbit seminal plasma is associated with a glycoprotein of 1.15 kDa molecular weights (Reyes *et al.* 1975). A re-examination of molecular weight properties of acrosome stabilizing factor (ASF) showed that ASF consists of 2 forms with molecular weight Mr. 259 and 129 kDa (Thomas *et al.* 1986). Vadnais and Roberts (2010) reported that heparin-binding proteins in seminal plasma consisting of 3 spermadhesins, AQN-3, AQN-1, AWN and a BSP protein pB1, inhibited *in vitro* capacitation (in capacitating conditions) and cooling induced capacitation-like changes. Novak *et al.* (1986) studied the abundance of cysteine-rich secretory protein 3 (CRISP3), which was positively related to first cycle conception rate. Further, clustering and SP1 in seminal plasma together with sperm citrate synthase were also predictive of fertility and hence proteins within sperm and seminal plasma were identified that could serve as biomarkers of semen quality and fertility in stallions. This type of information has not been well investigated as such in farm animals with regard to the coating/uncoating of the proteins that affect the process of capacitation and further fertilization as well. The present study was undertaken to understand the mechanism of capacitation and establishing the presence of a decapacitation factor in seminal plasma of goat as very scanty information is available in such species.

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MATERIALS AND METHODS

Collection of semen: Semen was collected from the healthy bucks maintained at the cattle yard of National Dairy Research Institute by using artificial vagina (41°C) in a pre-warmed vessel at 35°C. Ejaculates of good quality showing high motility (at least 3– on a 5– point scale) were pooled. Sperm counts were made in duplicate using a haemocytometer and sperm motility assessed by examining a uniform drop of semen.

Iodination of seminal plasma proteins: The procedure involved the adsorption of the lyophilized crude goat seminal plasma proteins (CGSP) radio-labelled seminal plasma proteins on the surface of the epididymal spermatozoa as described in our earlier experiment (Kaur and Sharma 2009).

Capacitation of goat spermatozoa: The loose sperm pellet obtained on centrifugation of goat semen was processed for capacitation (Anand *et al.* 1989). Aliquots containing 0.5 ml of the sperm suspension were incubated at 37°C in a CO_2 incubator. The samples were examined at regular intervals for motility and acrosome morphology. Acrosome reaction was induced after the 4 h incubation period by adding 10 mM $CaCl_2$ (final concentration) and incubating for 10 min at 37°C. A small aliquot was taken to assess the sperm motility and % acrosome reaction determined by the triple-stain method (Talbot and Chacon 1981). The slides were rinsed with distilled water, air-dried and viewed at 100×. About 200 to 300 cells per slide were counted and the % acrosome reaction before addition of $CaCl_2$ and after addition of $CaCl_2$ (after induction of acrosome reaction) was calculated as follows:

$$\% \text{ Acrosome reaction (AR\%)} = \frac{\text{Total number of acrosme reacted cell} \times 100}{\text{Total number of cell counted}}$$

Adsorption of iodinated seminal plasma proteins on surface of epididymal spermatozoa and capacitated spermatozoa: 4×10^7 cells/ml were incubated with the iodinated seminal plasma for 10 min as described by Kaur and Sharma (2009). The pellet was washed in the TBS buffer for 2 to 3 times to remove the non-specifically bound iodinated seminal plasma proteins. The surface proteins of the sperm pellet were extracted in 100 µl of 2% SDS for 30 min and later centrifuged at 20 000 × g for 20 min.

Similarly, the capacitated cells were recovered by centrifugation at 800 × g for 10 min and washed twice with TBS and aliquots of 4×10^7 capacitated cells were incubated with radiolabelled seminal plasma proteins for 20 min. Added an equal volume of the sample buffer (2×) to both the supernatants and heated for 10 min at 100°C to carry out SDS-PAGE by the method of Laemelli (1970). After the completion of electrophoresis, the gel was sliced using 1.5 mm thick gel slicer in the respective lanes and radioactivity of the gel slices was determined in the γ -counter.

Effect of the lyophilized CGSP proteins on the capacitation of goat spermatozoa: In vitro synchronous goat acrosome reaction assay was employed to monitor the effects of CGSP proteins on the sperm acrosome reaction assay as done by Drisdel *et al.* (1996).

$$\% \text{ AR inhibition} = \frac{(100 - \Delta AR\%) \times 100}{\text{Positive control acrosome reaction}}$$

Determination of ID_{50} of the lyophilized CGSP proteins: The amount of protein required to cause an approximate 50% decrease in acrosome reaction as compared to the control was determined by the dose-dependent response. The lyophilized CGSP proteins was added to the capacitation medium containing goat spermatozoa at a concentration of 5×10^7 cells/ml after 3.5 h of incubation.

Ultracentrifugation of the lyophilized CGSP proteins for removal of the high molecular weight factors: The lyophilized CGSP proteins were suspended in 50 mM PBS (pH 7.4) and were spun at 105,000 ×g in ultracentrifuge using the fixed angle rotor type 55.2 Ti. Initially the centrifugations were carried out for various durations to standardize the time during which the sediment formed on centrifugation contains the inhibitory activity.

First, the suspension was centrifuged for 2 h and an aliquot of 10 ml from upper layer of the supernatant was withdrawn. The aliquot drawn was then concentrated in the millipore concentrator with a 10 kD cut off. The supernatant was then stored at –20°C. The pellet was then resuspended and again centrifuged for 4 h and an aliquot was removed from the upper supernatant. The pellet was resuspended and recentrifuged again. This procedure was repeated after 6 and 10 h. The inhibitory effect of the supernatant collected after the centrifugations were carried out for 2 h (Sup-DF_1), 4 h (Sup-DF_2), 6 h (Sup-DF_3) and 10 h (Sup-DF_4), was studied on the capacitation of the goat spermatozoa.

Determination of ID_{50} of ultracentrifuged pellets: The amount of protein required to cause an approximate 50% decrease in acrosome reaction as compared to control was determined by dose-dependent response. The ultracentrifuged pellet was added to capacitation medium containing goat spermatozoa at a concentration of 5×10^7 cells/ml after 3.5 h of capacitation.

RESULTS AND DISCUSSION

The study confirmed significant adsorption of seminal plasma proteins on the surface of goat epididymal spermatozoa and capacitated spermatozoa. The results corroborated with studies carried out by Metz *et al.* (1990). The results obtained (Fig.1) on binding studies on uncapacitated i.e., epididymal spermatozoa exposed to seminal plasma proteins, revealed that proteins of Mr 158, 38.8, 25.1, 15.8, 12.9, 12.0, 11.2, 10.5, 9.6, 8.9, 8.3 and 7.5 kDa were readily adsorbed on epididymal sperm surface whereas protein of Mr 27 kDa were not adsorbed on sperm

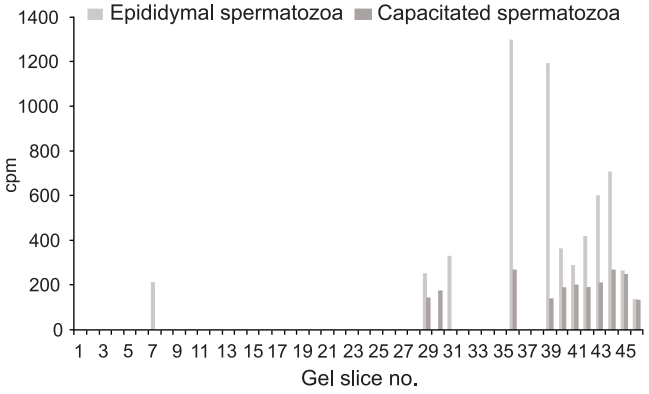

Fig. 1. Binding/adsorption of the iodinated seminal plasma proteins on the surface of the uncapacitated and capacitated spermatozoa.

Table 1. Binding/adsorption of the Iodinated seminal plasma proteins on the surface of the uncapacitated and capacitated spermatozoa

Gel slice no.	Radioactivity of the protein bands adsorbed on the surface of epididymal spermatozoa	Radioactivity of the protein bands adsorbed on the surface of capacitated spermatozoa	Molecular weight of proteins adsorbed on the sperm surface (Mr.)
7	213	-	158
29	252	143	38.8
30	-	175	27.
31	328	-	25.1
36	1289	267	15.8
39	1189	141	12.9
40	364	190	12.0
41	289	199	11.2
42	419	193	10.5
43	599	213	9.6
44	706	268	8.9
45	262	248	8.3
46	137	134	7.5

surface. In capacitated spermatozoa, binding studies showed disappearance of protein of Mr 158 and 25.1 kDa whereas an additional protein of Mr. 27 kDa was identified along with those identified on the surface of epididymal spermatozoa (Table 1). The findings thus suggested that seminal plasma proteins are readily adsorbed on sperm surface and during the process of capacitation there is a rearrangement/relocalization of some of the proteins along with disappearance of a high molecular weight protein. It could be that during process of capacitation the binding sites on the sperm surface get modified. Capacitation is broadly defined as the total of all changes in sperm that enables them to penetrate zona pellucida and enter vitellus. Kaul *et al.* (2000) reported that capacitated sperm surface proteins lack high molecular weight proteins. These observations

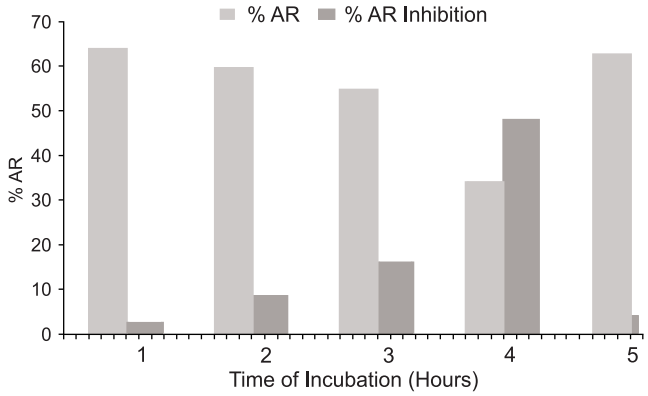

Fig. 2. Effect fo the lyophilised CGSP proteins on the capacitation of goat spermatozoa.

Table 2. Effect of the lyophilized CGSP proteins on the capacitation of goat spermatozoa

Time of incubation when CGSP is added (2.5 mg/ml)	Δ %AR	% AR inhibition
Control	65.3 (5)	–
0 h	63.5 (5)	2.8
1 h	59.5 (5)	8.9
2 h	54.8[a] (5)	16.1
3 h	34.0[b] (5)	47.9
4 h	62.5 (5)	4.3

The mean significant difference is 3.34. a, Significantly different from control ($P < 0.05$; Dunnett's multiple comparison test);b, significantly different from control ($P < 0.01$, Dunnett's multiple comparison test). Number of samples is given in bracket.

corroborated our results, that in the process of capacitation seminal plasma proteins play an important role. Though changes take place at the surface, proteins are derived from seminal plasma and coating-uncoating of seminal plasma proteins is an important event in the whole process of capacitation.

The factor under investigation did not exert its inhibitory effect in goat (Table 2) when added at beginning of the capacitation period but when added after an incubation of minimum 2 h it started exerting its inhibitory effect, and is quite significant when added after 3 h of incubation in capacitation medium. The study of *in-vitro* synchronous assay revealed that the control sample had 65.3% acrosome reaction determined by the triple stain method (Fig. 2) as compared to the samples which had the decapacitation factor added at a concentration of 2.5 mg/ml. The decapacitation factor exerted an inhibitory effect on capacitation at 0, 1, 2, 3 and 4h the % acrosome reaction is 63.5, 59.5, 54.8, 34 and 62.5 respectively. Similar studies carried out in rabbit seminal plasma has shown inhibitory effects on the acrosome reaction as well as the fertilizing ability of the spermatozoa. The finding indicated that after 3h (at a period of 4 h), the changes in % acrosome reaction was reversed back to almost the value

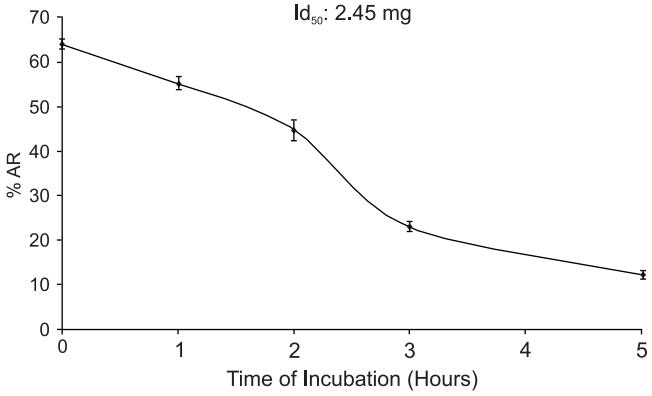

Fig. 3. Determination of ID_{50} of the lyophilised CGSP proteins.

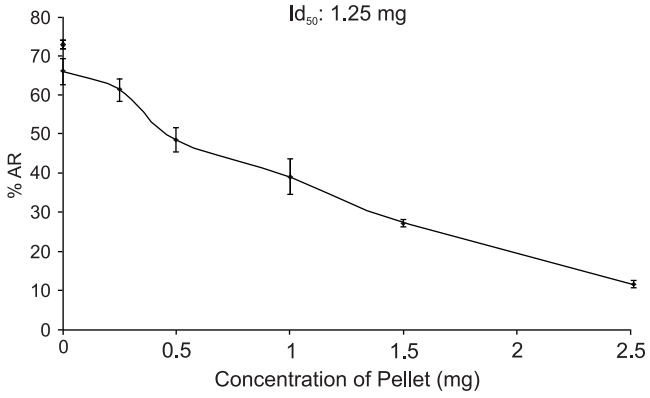

Fig. 4. Determination of the ID_{50} of the ultracentrifuged pellet

Table 3. Assessment of the time when exactly the CGSP proteins started exerting their inhibitor effect (CGSP added at a concentration of 2.5 mg/ml)

CGSP addition after initiation of capacitation (h)	Δ %AR	% AR inhibition
Control (no addition)	65.8 (5)	–
2.5 h	48.5 [a] (5)	26.7
3.0 h	32.5[b] (5)	50.6
3.5 h	29.3[b,c] (5)	55.5

The mean significant difference is 1.79. a, Significantly different from control ($P < 0.05$; Dunnett's multiple comparison test); b, significantly different from control ($P < 0.01$, Dunnett's multiple comparison test); C, nonsignificant from the value of 3.0 h; number of samples given in bracket.

Table 4. Standardization of the time required for the removal of the inhibitory factor by high speed centrifugation

Duration of centrifugation (h)	Δ AR %	% AR inhibition
0	66.3 (5)	–
2 (Sup-DF_1)	13.5[a] (5)	79.60
4 (Sup-DF_2)	41.5 [a] (5)	37.80
6 (Sup-DF_3)	55.5 [a] (5)	16.29
10 (Sup-DF_4)	59.3[b] (5)	10.56
6 h (pellet obtained on centrifugation)	36.5 [a] (5)	44.90

The mean significant difference is 1.04. a, significantly different from control ($P < 0.01$; Dunnett's multiple comparison test); b, non-ignificant from the value obtained after 6 h of centrifugation; number of samples given in bracket.

similar to that of the control. The % acrosome reaction inhibition is 2.8, 8.9, 16.1, 47.9 and 4.3. The statistical analysis by Dunnett's multiple comparison test confirmed that the mean significant difference is 3.34 and the values of % inhibition were significant after a pre-incubation of 2h. It was significantly different from control at $P<0.05$ whereas at 3 hr it was significantly different from control at $P<0.01$. The ID_{50} (the concentration exhibiting 50% of inhibition as compared to the control) determination studies demonstrate that the lyophilized CGSP proteins have an inhibitory effect at 2.45 mg/ml (Fig. 3)

The total duration of incubation for capacitation is 4 h and to study mechanism of action of the factor under investigation, determination of the time duration of pre-incubation in the capacitation medium was carried out. The findings revealed (Table 2) that the factor exerted its effect most significantly after a pre-incubation in the capacitation medium for 3.5 h. The values of % acrosome reaction (Table 3) are 65.8 in the control and 48.5, 32.5 and 29.3 when the lyophilized CGSP proteins are added at a concentration of 2.5 mg/ml after a pre-incubation of 2.5, 3.0 and 3.5 h. The mean significant difference is 1.79 and most significant inhibitory effect is at addition of the CGSP proteins after 3.5 h, significantly different from control ($P < 0.01$, Dunnett's multiple comparison test).

Bedford and Chang (1970) demonstrated that the high-molecular weight decapacitation factor can be removed by ultracentrifugation. The duration for centrifugation was standardized by taking an aliquot of 10 ml from the centrifuged sample and replacing by same volume of buffer. The aim was to determine retention of the decapacitation factor in supernatant and time taken by it settle down as the pellet. As the duration of centrifugation increased the amount of the factor present in the supernatant decreased (Table 4). The Δ AR% is 66.3% at the beginning of centrifugation process. It increased after the CGSP suspension was centrifuged. A reverse trend was observed in case of % acrosome reaction inhibition. The results revealed that removal of factor from supernatant is correlated with the decrease in % acrosome reaction inhibition, 79.6 after 2 h, 37.8 after 4 h, 16.29 after 6 h and 10.56 after 10 h of centrifugation. The findings indicated that duration of 6 h is sufficient to remove the factor centrifugation and even after centrifugation for 10 h the factor cannot be completely removed from the supernatant.

The pellet obtained after long hours of centrifugation may

Table 5. Effect of the ultracentrifuged pellet on the capacitation of goat spermatozoa (total time of centrifugation: 6 h)

Addition of ultracentrifuged pellet on initiation of capacitation (h)	Δ % AR	% AR inhibition
Control (no addition)	66.0 (5)	–
2.0 h	56.0 (5)	15.0
2.5 h	52.5 (5)	20.1
3.0 h	44.5 (5)	32.6
3.5 h	33.8[a] (5)	48.8

The mean significant difference is 9.16. a, significantly different from control ($P < 0.05$; Dunnett's multiple comparison test); number of samples given in bracket.

be having the decapacitation factor and hence the inhibitory effect of the pellet was also studied. From Table 4 it is evident that the ultracentrifuged pellet is also following the same trend of inhibitory effect as that of the lyophilized CGSP. The % acrosome reaction inhibition is significant at addition of the pellet after a pre-incubation of 3.5 h in the capacitation period. The ID_{50} of the ultracentrifuged pellet was 1.25 mg/ml (Fig. 4).

The studies established presence of a factor in goat seminal plasma which is precipitated by ultracentrifugation and that inhibits the Ca^{2+} induced acrosome reaction of goat spermatozoa pre-incubated in capacitation media for 3.5 h in which the acrosome reaction can be induced in controls. Sperm coating and uncoating by the proteins is associated with the processes of decapacitation and capacitation respectively (Nixon *et al.* 2006, Bi *et al.* 2009). The results obtained by the binding studies are in correlation with results carried out by us in the earlier experiments suggesting that there is a re-arrangement of the proteins on the sperm surface. As there is no difference in the binding of the low Mr proteins further insight into the role of these proteins is required. The disappearance of the high Mr protein from the surface of the capacitated spermatozoa suggested that the protein could be a decapacitation factor, which is either being removed or modified during the process of the capacitation of goat spermatozoa.

When the sperm reaches the female reproductive tract, it is activated by so-called capacitation factors that initiate a delicate reorientation and modification of molecules within the plasma membrane in equine spermatozoa (Gadella *et al.* 2001). Both ASF and AF-1 have the similar biological activity. Presently, it is not clearly known whether effect of ASF is directly on capacitation (and ultimately on acrosome reaction) or on the acrosome reaction (Thomas *et al.* 1986). Similarly, AF-1 does not show any inhibitory activity when the oocytes rather than the spermatozoa are treated with AF-1 (Reddy *et al.* 1979). Thus, if their effect is on the capacitation then the term decapacitation factor would be more appropriate than either ASF (acrosome stabilizing factor) or AF-1 (anti-fertility factor-1).

Drisdel *et al.* (1996) reported that an acrosomal reaction inhibitory glycoprotein (ARIG) of Mr 74 000 is capable of blocking sperm exocytosis induced by several signal transduction agonists. It was further established that ARIG inhibits the acrosome reaction not when added at the start of the capacitation period but when added after 2.5 h of capacitation suggesting that it is either removed or inactivated during the capacitation process. Our studies corroborated the results of Drisdel *et al.* (1996), wherein the decapacitation factor starts exerting its inhibitory effect towards the end of the incubation period but not in the beginning. Fraser *et al.* (1990) identified an anionic surface-associated factor, having Mr 40 000 which inhibits the fertilizing ability in a reversible manner. It can be removed by gentle centrifugation and added back to the capacitated spermatozoa. Nixon *et al.* (2006) identified 4 identical proteins and one of them, phosphotidylethanolamine binding protein 1 (PBP1), is the primary candidate for the decapacitation factor. Similarly, Bi *et al.* (2009) identified an isoform of phospholipid zeta-1 (PLCZ1) localized on the acrosome and is a physiological inhibitor of PLC, which is an intrinsic DF in the sperm to prevent premature capacitation and acrosome reaction.

Ye Bi *et al.* (2009) identified NYD-SP27 (an isoform of phospholipase C Zeta 1 (PLCZ1)) as a physiological inhibitor of PLC that acts as an intrinsic decapacitation factor in sperm to prevent premature capacitation and acrosome reaction. Novak *et al.* (2010) established that the fertility index and farrowing rate tended to be positively correlated ($P < 0.10$) with a 25-kDa protein, identified as glutathione peroxidase (GPX5), an antioxidant enzyme that may protect sperm membranes from oxidative damage. The significance of these candidate proteins needs further investigation as markers of fertility in boars. Capacitation like changes may be induced by the cooling and the thawing process wherein the seminal plasma proteins may also play a role in preventing sperm damage (Vadnais and Roberts 2010, Leahy and Gadella 2011).

Our results established the fact that there is an acrosome reaction inhibitory factor present in the goat seminal plasma. The factor present does not exert its inhibitory effect when added at beginning of the capacitation period but when added after an incubation of minimum 2 h it starts exerting its inhibitory effect and is quite significant when added after 3.5 h of incubation in the capacitation medium. Similar studies have shown inhibitory effects on the acrosome reaction as well as fertilizing ability of spermatozoa. Further studies are required to purify the protein to homogeneity and understand the role of this factor in capacitation of the goat spermatozoa. The results thus suggested the presence of a high molecular protein which plays a role as decapacitation factor when epididymal spermatozoa are exposed to the seminal plasma proteins at the time of ejaculation so as to prevent the premature capacitation prior to fertilization.

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