

Effect of stallion seminal plasma proteins on *in-vitro* capacitation of equine spermatozoa

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ABSTRACT

This study was undertaken to see the capacitation response with the group of heparin (HBPs) and gelatin binding (GBPs) stallion seminal plasma proteins. Frozen thawed stallion spermatozoa were incubated with heparin (10 µg/ml) in the presence of HBPs and GBPs @ 20, 40 and 60 µg/ml. The percentage of capacitated sperm was evaluated after the induction of acrosome reaction with progesterone (100 µg/ml) from 0 and 4 h. Significant differences were observed in the percentage of acrosome reacted spermatozoa at 4 h incubation between HBPs treated and control groups. The GBPs treated group showed non significant difference. The highest value of acrosome reacted sperm was seen in HBPs 40 µg, and in GBPs 60 µg. It can be concluded that the addition of HBPs enhanced the capacitation responses, and best dose level was 40 µg of protein in HBPs groups for induction of acrosome reaction.

Key words: Acrosome reaction, Gelatin, Heparin, *In-vitro* capacitation, Proteins, Seminal plasma, Stallion, Spermatozoa

Success rates of *in-vitro* fertilization using equine gametes remain poor. The ability to induce the acrosome reaction, *in vitro*, has proven to be a useful tool to evaluate sperm function, as well as being a monitor of an essential step for *in-vitro* fertilization (Meyers *et al.* 1996, Graham 1996). Bovine seminal plasma (BSP) (GBP) proteins in the sperm membrane influence sperm fertilizing ability and the success of cryopreservation process (Nauc and Manjunath 2000). In cattle (Miller *et al.* 1990), horse (Varner *et al.* 1993) and buffalo (Arangasamy 2003), heparin binds to ejaculated sperm and modulates the capacitation process, thereby, enhancing the ability of sperm to undergo the zona pellucida-induced acrosome reaction (Florman and Babcock 1991). HSP-1 and HSP-2 (recently, renamed SP-1 and SP-2; Ekhlasi-Hundrieser *et al.* 2005) are the most abundant proteins in equine seminal plasma, accounting for 70–80% of the total proteins. They belong to the short seminal Fn-2 type proteins (Ekhlasi-Hundrieser *et al.* 2005) and are the equine orthologs to the major bovine heparin-binding proteins (BSP), which are involved in early fertilization steps (capacitation) (Manjunath and Therien 2002). Hence present study was undertaken to assess the effect of HBPs and GB proteins from stallion seminal plasma on *in-vitro* capacitation and acrosome reaction with frozen stallion spermatozoa.

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MATERIALS AND METHODS

Capacitation medium and chemicals: The sperm capacitation medium used in this study was a modified Brackett and Oliphant (BO) (1975) medium. All other chemicals used were of analytical grade and were obtained from commercial or registered suppliers. The effect of group of heparin binding proteins (HBPs 5) molecular weight ranging from 17 to 83 kDa (results not shown) and gelatin binding proteins (GBPs 4) molecular weight ranging from 18 to 83 kDa, were seen on capacitation response of spermatozoa.

Sperm culture: Six tests were conducted. Frozen Marwari stallion spermatozoa were thawed at 37°C and the collected sperm cells were washed twice with capacitation medium (by centrifugation at 2000 rpm for 10 min). The sperm motility was assessed with phase contrast microscope. The sperm pellets were resuspended with capacitation medium containing BSA (6 mg/ml) and heparin (10 µg/ml). Sperm concentrations were adjusted to 1×10^8 sperm cells/ml and seven 1 ml aliquots were prepared. One aliquot was kept as control, while to the remaining aliquots either HB proteins or GB proteins (Arangasamy 2003) were added @ 20, 40 and 60 µg/ml. These aliquots were placed under mineral oil in eppendorf tubes in 5% CO₂ at 37°C up to 4 h.

Induction of acrosome reaction with progesterone and sperm staining: Capacitation status was assessed by inducing acrosome reaction (AR) at 0 h and at the end of 4 h.

Progesterone 100 µg was added to each aliquot and incubated for an additional 15 min. After incubation, sperm smears were prepared, stained and assessed for AR. A thin smear of semen or spermatozoa on a glass slide was dried in air and fixed in methanol at 37°C for 10 min. The slide was kept in Giemsa stain for 1 h and washed under tap-water, air dried and examined under phase contrast microscope. Interpretation was made as per the spermatozoa-staining pattern. Acrosome intact: deeply stained purple acrosomal contents; and acrosome reacted: Without acrosomal staining (or) irregular acrosomal staining.

Statistical analysis: The data were analysed as per the standard procedure (Snedecor and Cochran 1989). The student's t - test was carried out to compare the protein effects between protein treated and control groups. All values are expressed as mean $\pm$ SE.

RESULTS AND DISCUSSION

Higher percentage of acrosome-reacted sperm was observed in HBPs-treated group followed by GBPs (Table 1). At 0 h incubation, only in the control group, the per cent of acrosome reacted sperms were conducted. During 4 h incubation, per cent of acrosome reacted sperm differed ($P < 0.05$) between the HBPs-treated and control groups. Among the concentration used, 40 µg showed highly significant ($P < 0.01$) difference. The GBPs-treated group showed non-significant difference, however the values were on higher side than the control group.

The investigation of *in-vitro* induced acrosome reaction in stallion spermatozoa may provide an indication of fertility. Our results showed that overall effect of seminal plasma proteins on acrosome reaction was similar for both proteins but significant capacitation response was exhibited with HBPs-treated group than control group at 4 h; this finding is similar with the earlier report by Lindholmer (1974). The present results indicate that seminal plasma proteins had dose dependent effect on induction of acrosome reaction. This might be due to the basic nature of both proteins being similar,

Table 1. Effect of (per cent mean $\pm$ SE) seminal plasma proteins on induction of acrosome reaction during *in-vitro* capacitation (0–4 h) of stallion spermatozoa

Protein	0 hr	4 h	
		HBP	GBP
Concentration (µg)			
20	-	20.50 $\pm$ 1.28*	19.79 $\pm$ 1.63
40	-	22.13 $\pm$ 1.27**	18.68 $\pm$ 1.59
60	-	21.55 $\pm$ 1.66*	20.54 $\pm$ 1.63
Control	4.86 $\pm$ 0.60	16.31 $\pm$ 1.02	16.31 $\pm$ 1.02

Means bearing different superscripts in a column differ between protein treated and controls ($P < 0.05$)*, ($P < 0.01$)**; n, 6: indicates replicates, HBP, heparin binding proteins, GBP, gelatin binding proteins.

i.e. binding capacity, biological role on sperm cell. This finding was in accordance with the earlier observations in bovine species (Chandonnet *et al.* 1990, Manjunath *et al.* 1994). Previous reports on stallion semen indicates that calcium ionophore A 23187 has been more widely used for *in-vitro* capacitation of equine spermatozoa (Blue *et al.* 1987). Incubation with heparin (100 µg/ml) for 4 h or with calcium ionophore (1 or 10 µmol/l) for 60 min resulted in a maximal increase in the percentage of acrosome reacted spermatozoa (Christensen *et al.* 1996). The addition of vesicular fluid to capacitation medium enhances the acrosome reaction (Agarwal and Vanha Pertulla 1987). The proportion of seminal plasma at $\leq 5\%$, sperm are able to maintain more desirable motility compared to samples containing a higher proportion of seminal plasma (10–30%), during both cooled storage and cryopreservation (Alghamdi *et al.* 2002, Kareskoski and Katila 2008). The present findings in HBPs group are higher than those of earlier reports in bovine sperm cells and in buffalo ejaculated sperm (Sa *et al.* 2001). GBPs group also showed higher acrosome reacted sperm per cent than control, and the effect was concentration dependent, in concurrence with the earlier report of Therein *et al.* (1995). The exact mechanism involved in sperm capacitation and the acrosome reaction has been proposed to involve a decrease in the molar ratio of cholesterol to phospholipids in the sperm plasma membrane as one of the early steps (Langalis *et al.* 1988). 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). Present study provides the evidence to indicate beneficial effect of HBPs and GBPs on acrosome reaction and capacitation. However, there is a need to study these proteins individually and their modulating action on sperm acrosome reaction and capacitation *in vitro* and finally to correlate the same with fertility.

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