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Research Article

Effect of Heparin and Gelatin Binding Buffalo Seminal Plasma Proteins on Sperm-Egg Binding Assay

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ABSTRACT

Effect of heparin and gelatin binding buffalo seminal plasma proteins were assessed on fertility status of buffalo cauda spermatozoa by sperm-zona pellucida binding test. The results showed difference between protein treated and control groups in the relative number of sperm cells bound to the zona pellucida of a batch of *in vitro* matured buffalo oocytes. These differences suggest a relationship between the sperm-zona pellucida binding capacity and the fertility related seminal plasma proteins.

Key words: Sperm-egg binding assay, Seminal plasma, Heparin, Gelatin, Proteins, Buffalo.

A simple and reliable laboratory test to predict the fertility of breeding bulls has long been required for the bovine artificial insemination industry. Unfortunately no such test is available and attempts to develop such a test have met with little success. Sperm morphology and motility, the number of sperm per insemination, the percentage of acrosome reacted sperms *in vitro* have been extensively evaluated as an indication of the ability of sperm to fertilize an egg. However, they are of limited value in predicting fertility (Graham and Pace, 1970; Salisbury *et al.*, 1978). Some *in vitro* techniques have been designed to assess the fertility of semen, one of these tests is the homologous system based on sperm-zona pellucida binding which can be applied to predict the outcome of *in vitro* fertilization in human (Burkman *et al.*, 1988), bull (Fazeli *et al.*, 1993), stallion (Fazeli *et al.*, 1995b) and boar (Fazeli *et al.*, 1995a) semen. Evidence suggests that seminal plasma, which is a complex mixture of secretions from testis, epididymis and accessory glands, contains factors that modulate the fertilizing ability of sperm (Amann and Griel, 1974; Henault *et al.*, 1995). Recent studies have shown that proteins from bovine seminal plasma (BSP) may modulate the properties of sperm (Killian, 1992; Bellin *et al.*,1994). Killian *et al.* (1993) have recorded that two proteins (26 kDa and 55 kDa) predominate in higher fertility bulls and two proteins with different isoelectric point(pI) 16 kDa, pI 4.1; 16 kDa, pI 6.7 predominate in lower fertility bulls (Bellin *et al.*, 1994). Another group of seminal plasma proteins associated with fertility have been isolated on the basis of their heparin-binding property (i.e. five classes of heparin-binding proteins (HBPs) with molecular weight ranging from 14-31kDa) Nass *et al.* (1990).The major protein fraction of bovine seminal plasma was represented by three acidic proteins, designated as BSP-A1/-A2, BSP-A3 and BSP-30 kDa (collectively called BSP proteins). The BSP-A1/-A2, BSP-A3 has a molecular weight of 15-16 kDa, whereas BSP-30 kDa protein has a molecular weight of 28-30 kDa (Manjunath, 1984: Desnoyers *et al.*, 1994). There have been few studies of fertility related proteins in the seminal plasma of buffaloes. The aim of the present study was to investigate the effect of heparin-binding and gelatin-binding buffalo seminal plasma proteins on sperm - zona pellucida binding assay.

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MATERIALS AND METHODS

Collection of epididymal spermatozoa: Fifteen animals have been used for spermatozoa collection (Abattoir materials testes along with epididymides were collected and used for cauda epididymal sperms) and fifteen pools were tested. The abattoir materials were taken to laboratory in a thermos flask containing normal saline at 30-35°C. After washing thoroughly the excess tissues were trimmed and the cauda

epididymis was isolated from testes. Convoluted tubules were exposed by several incisions with a Bard Parker blade. The sperm-in fluid from the tubules was collected in a petri dish and mixed with 10 ml of modified capacitation medium (BO (Brackett and Oliphant, 1975)). Spermatozoa were collected the same day from two or three epididymides of different bulls, mixed and used for a single test. The collected sperm cells were washed twice with capacitation medium by centrifugation at 2000rpm for 10 minutes. The sperm motility was assessed by phase-contrast microscopy (x400). The sperm pellets were resuspended with capacitation medium containing bovine serum albumin 6 mg/ml and heparin 10 µg/ml. Sperm concentrations were made upto 1 x 10^8 sperm cells/ml and 1 ml aliquots were made (total 3 aliquots). One aliquot was kept as a control and in other two aliquots gelatin-binding (GB) and heparin-binding (HB) buffalo seminal plasma proteins were added at 40 µg/ml, respectively as per the method described by Arangasamy *et al.* (2005). These aliquots were placed under mineral oil in microfuge tubes and incubated at 37°C in an atmosphere containing 5% Co_2 for upto 6 hours. Gelatin and heparin binding proteins were isolated with gelatin and heparin sepharose affinity column chromatography from buffalo bull seminal plasma (*bubalus bubalis*) as per the method described by Arangasamy *et al.* (2005). Five ejaculates each from fourteen bulls which are maintained by the two A.I. centres was used for seminal plasma collection. In the earlier other experiments six concentrations of gelatin and heparin proteins from 10 µg to 60 µg were used. Out of these 40 µg gave better results and as per our experimental design 40 µg was used in this present test (Arangasamy and Singh, 2007).

Collection of buffalo oocytes and in vitro maturation: 485 ovaries were collected from local abattoir immediately after slaughter and transported to the laboratory in sterile normal saline solution (NSS 0.85%) at 30-35°C supplemented with 50 IU penicillin G/ml and 50 µg streptomycin/ml. The ovaries were washed several times with sterile NSS, then exposed to 70% ethanol for 30-60 seconds and finally washed in fresh NSS. Follicular fluid from surface follicles (>2 mm) of buffalo ovaries were collected by aspiration method with an 18 gauge needle attached to a 10 ml syringe and poured into a large square searching petri dish containing oocyte collection medium (OCM (Modified Dulbecco's phosphate buffer saline)). Morphologically culturable oocytes, i.e., those having compact, multilayered, cumulus oocyte complexes (COCs) and evenly granulated cytoplasm, were selected under a stereomicroscope and transferred to another petri dish containing OCM, followed by 6 washings in oocyte washing media and finally 4 washings in maturation medium. The COCs were cultured in 50-100 µl (10-12 oocytes/drop) droplets of maturation medium (Tissue Culture Medium-199 supplemented with 10 % Fetal calf serum (FCS) + 5 µg/ml Follicle stimulating hormone) under paraffin oil in petri dishes. They were then incubated at 39°C in a moist atmosphere containing 5% CO_2 for upto 24 hours. After 24 hours of maturation, the maturation stages were assessed as per their expanded cumulus cell mass (interruption between the adjacent layers of cumulus cells as well as between the cumulus cells) in stereomicroscope.

Evaluation of matured oocytes, In vitro insemination and statistical analysis: After 24 hours of maturation, cumulus cells surrounding the oocytes were removed by gentle pipetting with capillary tube in fertilization BO medium and then washed several times in fertilization BO medium. After washing, the oocytes were placed in 10µl of fertilization BO medium (10-12 oocytes). The 40-45 µl of capacitated sperm suspension containing GB protein 40 µg/ml, HB protein 40 µg/ml and control (without protein), respectively, were added to 5-10 µl BO medium containing oocytes and then co-incubated at 39°C and 5% CO_2 in humidified air for 6 hrs. At least 20 oocytes were used to estimate the sperm-zona pellucida binding of each group. Two hours after sperm addition, the sperm cells which were loosely attached to the zona pellucida were removed and fresh fertilization medium was added to the droplets. After 6 hours of incubation, the oocytes were washed and placed on a grease-free glass slide, and a coverslip was applied. The number of sperm cells bound per egg was scored under phase-contrast microscope at 400 x magnification. The sperm count was made by dividing the entire surface of egg into four parts, and one part was counted and the count multiplied by four. The experiments were designed to express the sperm-zona pellucida binding capacity of a particular protein treated groups in terms of relative

number of sperms bound to the zona pellucida as compared to control group. Two-way analysis of variance was used for statistical analysis of data. The terms used in the model were difference between the number of spermatozoa bound to zona pellucida of control and protein treated groups.

RESULTS AND DISCUSSION

The recovery of oocytes from follicular aspiration is shown in Table 1. A total of 359 oocytes were retrieved by aspiration of 2-8 mm diameter follicles that appeared on the surface of 485 buffalo ovaries. Their population comprised of 244 (67.97%) culturable and 129 non-culturable oocytes. The total number of oocytes and the number of culturable oocytes recovered per ovary were 0.74 and 0.50, respectively (Table 1). A total number of 244 oocytes were cultured for 24 hours and their maturation status was assessed by cumulus cell expansion (Fig.1). The proportion of oocytes considered to be matured based on cumulus cell expansion was 52% (128/244) (Table 1). The results of the sperm-zona pellucida binding assays have been presented in Table 2.& Fig.2. The mean (SE) number of sperms bound to the zona pellucida of matured oocytes were higher in heparin-binding protein treated semen samples (354.65(26.43)) followed by gelatin binding protein (338.55(27.04)) and control (296.85(29.10)). No significant difference was observed between the protein-treated and control groups. However, the number of sperm bound to the zona pellucida was higher in heparin-binding protein treated samples, followed by gelatin-binding protein treated samples.

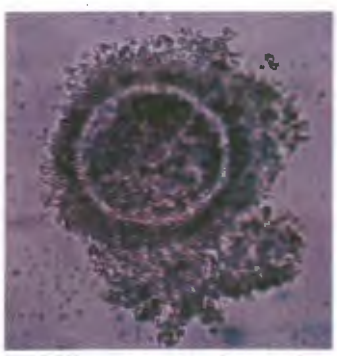
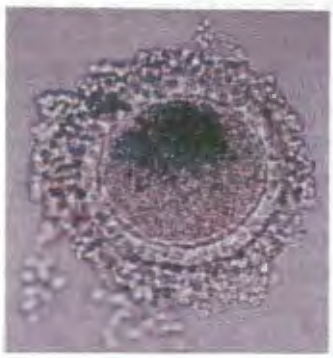


Fig 1.a. Buffalo cumulus-oocyte complex (immature oocyte) (Phase contrast x 200).



b. Buffalo cumulus-oocyte complex exhibiting cumulus expansion at 24 hrs of *in vitro* maturation (Phase contrast x 200)

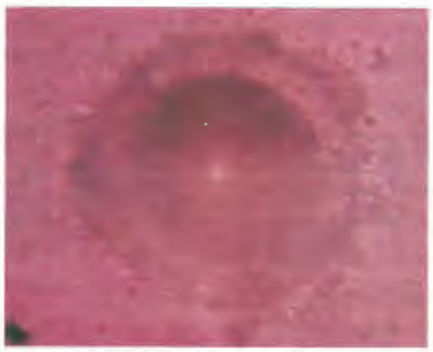


Fig 2. Microphotograph of capacitated spermatozoa bound to the Zona pellucida of a matured oocyte (Phase contrast x 400

In buffaloes, the reported average oocyte yield per ovary has varied between 0.4 (Totey *et al.*, 1992) and 1.76 (Samad *et al.*, 1998). The average number of culturable oocytes obtained in this study (67.97%) was slightly higher than those described by Totey *et al.* (1992) and lower as recorded earlier by Samad *et al.* (1998) and Sahoo *et al.* (1998). The variation in the oocytes recovery may be attributed primarily to the yielding capacity of an ovary, which is mainly dependent upon the presence of antral follicles and the different methodology employed for recovery of oocytes. Various factors, such as the age of the animal at slaughter, its nutritional status, its breed and the physiological conditions of the genital tract also play an important role in oocyte recovery. In cattle, the number of oocytes of acceptable quality is around 8-12 per ovary (Gordon, 1994). Unlike cattle, the

total as well as culturable oocytes obtained per ovary is low in buffaloes (Totey *et al.*,1992), which might be due to lower turnover rate of follicles and higher rate of loss of the developing follicles due to follicular atresia in buffaloes (Agarwal and Tomar, 1998).The low oocyte recovery in buffalo in comparison to cattle in general is due to lower reserve (average number 11,384) of primordial follicles in buffaloes than cattle (average number 50,000) (Danell, 1987; Settergren, 1987). Besides, the slaughter of unproductive and subfertile buffaloes (Totey *et al.*, 1992) and high incidence of atresia (80-90%) in abattoir-recovered ovaries (Ocampo *et al.*, 1994) are other possible reasons for reduced oocyte yield.

Table 1 : Oocytes recovery by aspiration from buffalo ovaries for *in vitro* maturation

Attributes	Total number
Ovaries	485
Oocytes recovered	359
(oocytes/ovary)	(0.74)
Culturable oocytes (%)	244 (67.97%)
Culturable oocytes used for maturation	244
Oocytes matured (%)	128 (52%)

Table 2 : Effect of buffalo seminal plasma proteins on sperm-zona pellucida binding assay

Proteins	No. of oocytes	No. of Sperms bound per egg (Mean±SE)
HB. Protein	20	354.65±26.43
GBProtein	20	338.55±27.04
Control	20	296.85±29.10

HB = Heparin-binding protein
GB = Gelatin-binding protein

Cumulus cell expansion has been postulated to be important in achieving complete oocyte maturation since it was correlated to the fertilization rate and developmental potential in ovine oocytes (Staigmiller and Moor, 1984). In the present study about 52% oocytes were considered to be matured after 24 hours of *in vitro* culture supplemented with 10 % FCS based on cumulus-cell expansion. However, in the present study, the percentage of mature oocytes was lower than that recorded by Ghosh (2001). This variation might be due to techniques employed by different technical personnel, the time lapse between collection and onset of culture time of oocytes. It was evident from the microscopical examination that there was interruption between the adjacent layer of cumulus cells as well as between the cumulus cells and oocytes. Assays based on the ability of spermatozoa to bind to homologous or heterologous zona pellucida have been developed as diagnostic tests in several animal species. The results of the zona pellucida binding assay can be presented as the total number of spermatozoa bound to the zona pellucida (Zhang *et al.*, 1998). The present study recorded more sperm bound to the matured oocyte zona pellucida than other reports available so far (Fazeli *et al.*, 1993, 1997). The number of sperm bound to the zona pellucida were higher in the heparin-binding protein treated samples followed by the gelatin- binding protein treated samples. However, no significant difference was observed between the protein treated samples and control. The observed results in the present study were higher than those recorded earlier in cattle (Fazeli *et al.*, 1993, 1997). These variation might be due to the kind of semen (Cauda epididymal, ejaculated, frozen thawed semen), the concentration of sperm cells, the type of oocytes (matured or immatured), media used and temperature in the incubator. Fazeli *et al.* (1993, 1997), used immatured oocytes for spermatozoa binding assay. The matured oocytes have been used in the present study might be the reason, more sperm bound to oocytes were recorded. The most likely source of variation was the batches of oocytes obtained from the abattoir, the quality of the oocytes and their nuclear maturational stage (Oehninger *et al.*, 1991) which influences the number of spermatozoa that will bind to the zona pellucida. The discrepancy in the rate of sperm binding might be due to variation in fertilizing frequencies *in vitro* Sahoo *et al.* (1998). Though the difference between the protein treated and control samples were non-significant, the number of sperm bound to the zona pellucida were higher in the proteins treated group than the control group. Thereby, the present observation provides an indication of these proteins' role on sperm-egg binding assay.

In conclusion, in the present study a number of proteins (7 in GB proteins, molecular weight ranging from 13 to 61 kDa, and 8 in HB proteins, molecular weight ranging from 13 to 71 kDa) were pooled and used for sperm-egg binding assay. It is possible that these proteins might behave differently when used singly. While scanning the literature no such attempts have been made so far, for studying the seminal plasma protein effects on sperm-egg binding as far as buffaloes are concerned. However, there is a need to study these proteins individually and test their effects on sperm-egg binding assay.

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