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Isolation and Characterization of Oviduct-specific Glycoproteins from Ampulla and Isthmus Parts of Cyclic and Acyclic Buffalo for Studying Differential Microenvironment

Shubhra Singh · Shiv Prasad · H. P. Gupta · Sumit Singhal · Atul K. Gupta · Anil Kumar

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Abstract The present study characterized the glycoproteins synthesized by buffalo oviduct. Scanning electron microscopy analyses of the ampullary and isthmic segments of cyclic and acyclic buffaloes showed ultrastructural variations in ciliated and nonciliated cells. Mucosal proteins were extracted by scrapping of different segments of oviduct and, after centrifugation, the remainder tissues were subjected to establish primary cell culture system of oviduct epithelial cells and conditioned media were prepared. Time- and concentration-dependent effects of trypsinization on the establishment of primary monolayer culture showed that 0.25% trypsin for 1–2 min at 37 °C were the optimal conditions. Total protein content in oviductal tissues and conditioned media was quantified and analyzed by SDS-PAGE which showed marked variation in different segments of the oviduct. Western blot analysis revealed five major oviduct-specific glycoproteins (OGPs) in cyclic oviduct (ampulla and isthmus) with *Mw* 180, 95, 75, 66 and 35 kDa in the oviduct extract and two glycoproteins with *Mw* 95 and 66 kDa in conditioned media. However, in acyclic oviduct (ampulla and isthmus), three glycoproteins were immunostained with *Mw* 180, 95 and 66 kDa in the oviduct extract and one glycoprotein with *Mw* 66 kDa in conditioned media. Indirect Enzyme-Linked Immuno Sorbent Assay (ELISA) results showed significant differences of OGPs in different segments of cyclic and acyclic buffaloes and, thus, indicative of segmental variation in the synthesis and secretion of glycoproteins. Oviductal extract secretes more amounts of OGPs as compared to the conditioned medium. The role of these OGPs may be defined and exploited for influencing the fertilization process and/or subsequent embryonic development.

Keywords Oviduct · Cell culture · Trypsinization · Viability · Oviduct-specific glycoproteins (OGPs) · Microenvironment

S. Singh · A. K. Gupta · A. Kumar (✉)
Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar 263145, Uttarakhand, India
e-mail: anilkumar.mbge@gmail.com

S. Prasad · H. P. Gupta · S. Singhal
Department of Animal Reproduction, Gynaecology & Obstetrics, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar 263145, Uttarakhand, India

Introduction

Animal reproduction, specifically buffalo reproduction, has to play an important role to develop faster and more efficient means of fulfilling the necessities of man. Buffalo (*Bubalus bubalis*) has been an integral part of livestock agriculture in Asia for over 500 years. However, despite its great importance in the economic sector, buffalo remains relatively neglected. The reproductive efficiency also remains poor mainly due to delayed puberty, poor expression of estrus, summer anoestrus, long postpartum service period and low conception rate. One of the most important issues negatively affecting bovine reproduction is early embryonic mortality, which may account for up to 40% of reproductive losses [1]. The oviduct is the first site of contact with the early embryo and has the potential to contribute important factors that affect fertility.

Successfully established in vitro fertilization procedures might distract from the fact that the oviduct provides an optimal microenvironment for gamete maturation, fertilization and early embryonic development. Indeed, the oviduct has long been regarded merely as a passage way that may be circumvented. However, it is now clearly evident that the oviduct is an active organ that maintains and modulates the fluidic milieu for sperm capacitation, fertilization and early embryonic development [2–4]. These complex functions are dependent on activities of epithelia that line the deep longitudinal mucosal folds [5]. The ultrastructure of the bovine oviductal epithelium has been thoroughly characterized by electron microscopic analyses [6]. The epithelium has been shown to consist of two cell types: ciliated cells and nonciliated secretory cells, displaying cyclic changes in their morphology [7, 8]. Ciliated cells are involved in oocyte transport and possibly in the regulation of spermatozoa progression [9], whereas nonciliated cells actively secrete protein factors. The role played by these factors is largely unknown. However, an increasing number of studies have emphasized their possible involvement in early embryonic development [10]. These cells and their functions are controlled by the ovarian steroids: estrogen and progesterone. During the follicular phase of development, estrogen is essential for differentiation of the oviduct epithelium and development and maturation of secretory cells concomitant with production of macromolecules. Although oviductal fluid is composed predominantly of plasma derivatives [11], it also contains nonserum macromolecules that are synthesized and secreted by the oviductal epithelium under ovarian steroid regulation. Oviductal secretory glycoproteins have been identified and characterized in mice [12], hamsters [13, 14], rabbits [15], sheep [10, 16] and baboons [17]. Studies in pigs [18] and humans [19] also suggest that their oviducts produce unique macromolecules that are not of serum origin. The biological significance of these secretory glycoproteins is unknown. Studies in mice [20], hamsters [21], sheep [10] and baboons [22] indicate that the antigens characterized in each of these species are present within the oviduct during the postovulatory phase of the estrous cycle and that they do become associated with the zonae pellucidae and/or the perivitelline spaces of oviductal ova and embryos. Thus, it may be that in mammals, generally, the oviductal epithelium synthesizes and secretes glycoproteins, which form an important component of the luminal fluids at the time of ovulation and fertilization.

In the last decade, it has been recognized that the oviduct and its secretions play roles in various reproductive events. Detection and analyses of de novo synthesized and secreted proteins from the oviduct epithelia have demonstrated a temporal and spatial distribution of macromolecules and differences in protein distribution among species. Despite the lack of indisputable evidence, it is, nevertheless, widely thought that the oviduct microenvironment exerts an active functional role in some of the early events of embryonic development [23]. In particular, the protein fraction seems to play an active role, especially since it binds to the

zona pellucida. This is particularly evident in the rabbit where a 60 μm thick layer is deposited within 48 h after ovulation [24], and it has been shown also in mouse [12], pig [25] and hamster [13, 14]. In the mouse, one protein is also found in the perivitelline space, thus, providing a specialized microenvironment during fertilization and early embryogenesis [26]. An in vitro study by Satoh et al. (1994) [27] has shown the existence of two embryogenesis-stimulating activities in a medium conditioned by oviduct cells. Oviduct-secreted factors have also been shown to influence sperm capacitation [28] and to maintain sperm motility [29].

Limited information is available concerning oviduct-specific secretory molecules in the buffalo, an economically important animal. The goal of the present study is to establish the primary cell culture system of buffalo oviduct epithelial cells and further investigate, identify and characterize oviduct-specific glycoproteins (OGPs) synthesized by the different regions of cyclic and acyclic buffaloes in order to study the differential microenvironment in different parts of oviduct. The study also investigates the time- and concentration-dependent effect of trypsinization on buffalo oviduct epithelial cells used for primary cell culture to compare the protein microenvironment in oviductal extracts and conditioned media. The present study, for the first time, clearly indicates the use of trypsin for establishment of buffalo oviduct epithelial cell culture and the study of differential microenvironment.

Material and Methods

Collection and Processing of Buffalo Oviduct

Buffalo oviducts were collected as (1) ipsilateral to the ovary containing corpus luteum (n=50, cyclic) and (2) the side, ovary not containing corpus luteum (n=50, acyclic) from a nearby slaughterhouse. These were transported to the laboratory on ice at 4 °C in 50-ml tubes of phosphate–buffered saline (PBS). The individual oviduct was cleaned of surrounding tissues, washed in PBS (pH 7.4) three times and then (disinfected) rapidly in 70% ethanol and grouped into four categories: 1) cyclic ampulla (Ca), 2) cyclic isthmus (Ci), 3) acyclic ampulla (Aa) and 4) acyclic isthmus (Ai). All the procedures took place within 3 h following the death of the buffalo.

Scanning Electron Microscopy

The oviductal segments were processed for scanning electron microscopy as described earlier [30]. Oviducts of the cyclic and acyclic buffaloes were used in this experiment. The oviducts were removed, trimmed free of fat and extraneous tissue and cut to separate the ampulla and isthmus. Strips of tissue were cut into small pieces of 2–3 mm along the borders of the fimbrial folds and the luminal ridges of the ampulla and isthmus. These tissues were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h. After washing with phosphate buffer, they were postfixed in 1% osmium tetroxide in the same buffer for 2 h and then dehydrated in a series of graded ethanol alcohol (30%, 50%, 70% 80%, 90% and absolute ethanol alcohol) and dried in the critical point dryer machine (CPD). Fixation and washing were carried out on ice or at 4 °C. The tissue specimens were mounted on stubs with conductive carbon tape and coated with a 20-nm layer of gold in ion sputterer (JEOL, Tokyo, Japan). The specimens were examined with a JSM-6610LV scanning electron microscope (JEOL) operated at 10 kV. All photographs were taken in the secondary electron mode with the beam incident to the surface of the tissue.

Isolation of Protein from Oviductal Tissue

Oviducts were scraped gently along their length with a glass microscope slide to extrude the mucosal tissue in 10 ml of PBS. The proteins were extracted from mucosal tissue in PBS with gentle stirring for 12 h at 4 °C. Afterwards, they were centrifuged at 3,000*g* for 30 min to remove the cellular debris and at 25,000*g* for 20 min to discard the insoluble particles. The supernatant containing soluble proteins was stored at −20 °C in aliquots of 1 ml until further use.

Preparation of Single-Cell Suspension of Oviduct Epithelial Cells

The mucosa were extruded by mechanical pressure with a glass slide along the oviduct. The mucosal tissue containing epithelial cells was washed with 6 ml of Tissue Culture Medium 199 (TCM-199). To remove the cell's clumps epithelial cells were treated with different concentrations of trypsin as 0.25%, 0.50%, 1.0% and 1.5% and for different time periods separately as 1, 2, 5, 10 and 20 min. The trypsinization effect was neutralized with TCM-199 supplemented with 10% fetal bovine serum (FBS). The cells were collected after centrifugation at 1,000 rpm for 5 min and washed with TCM-199 only for viability assays.

Determination of Cell Count and Viability

Cell viability was determined by trypan blue exclusion assay using a hemocytometer. Cells were incubated with 0.4% w/v trypan blue solution for 2–3 min at room temperature (RT). The percentage cell survival for each treatment group was determined by counting the dead cells and the live cells. The number of viable cells was calculated by dividing the number of living cells by the total number of cells in percentage.

Development of In Vitro Primary Cell Culture System of Buffalo Oviduct Epithelial Cells

After optimization of concentration and time of trypsin treatment, epithelial cells from the ampulla and isthmus of the oviduct were isolated and cultured according to Eyestone and First (1989) [31] with modification. Briefly, the mucosal tissue containing epithelial cells was washed with 12 ml of TCM-199 without antibiotics containing 10% heat-treated FBS by centrifugation at 200*g* for 5 min each. Supernatant was removed and cells were treated with trypsin (0.25%) for 1 min and mixed properly. Trypsin action was stopped by adding TCM-199+10% FBS and centrifuged at 200*g* for 5 min. Cells were further washed three times in TCM-199+10% FBS medium by centrifugation at 200*g* for 5 min. Epithelial cells were recovered after passive sedimentation. The cellular suspension was diluted 50 times in culture medium and incubated at 39 °C in a humidified atmosphere of 5% CO_2 in air. The medium was renewed every 2 days until the cells reached confluency (1 week).

Preparation of Conditioned Medium Using Serum-free Medium of Primary Culture

The primary epithelial cell culture used for the preparation of conditioned media was cultivated in 25-cm^2 flasks. About five flasks (5 ml/25 cm^2 flasks) could be prepared from one oviduct tube. After cells had reached confluency, monolayers were washed three times within 24 h with serum-free TCM-199 before conditioning. Conditioned medium was collected every 48 h, centrifuged at 500×*g* for 10 min at 20 °C to remove cells and cellular

fragments and stored at 4 °C. After three harvests (6 days) from the same monolayer, the three collections of media were pooled and stored at −80 °C in aliquots of 1 ml.

Gel Electrophoresis of Proteins Isolated from Oviductal Extracts and Conditioned Media

The conditioned media were centrifuged to eliminate the few contaminating cells and concentrated by using "DNA–Protein concentrator (Eppendorf)". Protein concentration of all samples was determined by the method of Lowry (1951) [32] using bovine serum albumin (BSA) as standard. After quantification, oviductal tissue protein (Ca, Ci, Aa and Ai) and conditioned media protein (CaM, CiM, AaM and AiM) were analyzed by using SDS-PAGE electrophoresis on 10% acrylamide gels [33].

Detection of Oviduct-specific Glycoproteins (OGPs) by Western Blot Analysis

Immediately after electrophoresis, proteins present in oviductal extract and conditioned media were transferred onto nitrocellulose membrane at 90 mA constant current for 3 h using a semidry assembly according to the procedure of Towbin et.al. (1979) [34]. After blotting, the membranes were washed in blocking solution (Tris–NaCl buffer pH 8.0, skimmed milk powder 5% (w/v)) for 2 h at 4 °C. Oviductin (goat polyclonal IgG) from Santa Cruz Biotechnology, Inc. was used as primary antibody to detect OGPs in the oviductal extracts and conditioned media. The membranes were incubated for 2 h at RT with the primary antibody (1:1,000 dilutions). After washing with the wash buffer (Tris–NaCl buffer pH 8.0, Tween-20), the membranes were incubated for 1 h with rabbit antigoat IgG-alkaline phosphatase (ALP) conjugate diluted (1:1,000) in PBS, pH 7.4 and detected using 5-bromo, 4-chloro, 3-indolyl phosphate and nitroblue tetrazolium.

Glycoprotein Staining

Oviduct OGPs in the oviductal extracts and conditioned media were stained by using the "Thermo Scientific Pierce Glycoprotein Staining Kit" which detects glycoprotein sugar moieties in polyacrylamide gels and on nitrocellulose membranes. When treated with oxidizing reagent (periodic acid), glycols present in glycoproteins are oxidized to aldehydes. After completing the procedure, the glycols are stained, yielding magenta bands with a light pink or colorless background.

Quantitative Analysis of OGPs by Enzyme-Linked Immuno Sorbent Assay (ELISA)

OGP levels in the oviductal extracts and conditioned media were quantified by indirect ELISA. The optimum concentration of the antibody and the dilution of the antigen (oviduct extract) to be used in ELISA were detected by checker board titration. Then 96-well microtiter plate was coated with 100 ng of the oviductal extracts and conditioned media proteins in 0.05 M carbonate–biscarbonate buffer (pH 9.4) and then blocked with 5% skimmed milk in PBS (pH 7.2) for 2 h at RT. After washing the plate with 0.5% skimmed milk in PBS, 100 μl of primary antibody (Oviductin, 1:5,000 dilutions) were added to the wells and incubated for 2 h at RT. After washing the wells thrice, 100 μl of ALP-conjugated secondary antibody (rabbit antigoat IgG-ALP conjugate, 1:5,000 dilutions) was added to each well and incubated for 1 h at RT. Wells were washed three times and then the enzyme reaction was carried out at RT for 30 min with p-nitrophenyl sulphate sodium salt as a substrate. The chemiluminescence of each well was measured by ELISA reader at 405 nm.

Results and Discussion

Scanning Electron Microscopy

The observation under scanning electron microscopy showed that the oviduct epithelial cells of buffalo were composed of two different cell types, ciliated and nonciliated cells. The oviduct epithelial cells at the acyclic phase showed numerous ciliated cells. The cilia consistently projected over the apex of the nonciliated cells. At high magnification (2,200×), the nonciliated cells showed spherical shape with numerous short microvilli (Fig. 1a). At the cyclic phase, the numbers of ciliated cells were decreased, whereas the numbers of nonciliated cells were increased (Fig. 1b). At high magnification (2,200×), the apical surfaces of the nonciliated cells showed round shape with numerous small microvilli. Therefore, this study has revealed that the oviducts of the buffalo undergo a cyclic change in ciliation during the cyclic and acyclic stages. In particular, the ampulla is extensively ciliated during the acyclic phase, while large numbers of cilia are hidden during the cyclic phase. The epithelium of the isthmus showed similar changes but to a lesser extent (Fig. 2). Similar cyclic changes have been observed in the pig-tailed monkey [35], the Chinese Meishan pig [30] and the cow [8]. During acyclic phase in the ampulla and isthmus, large numbers of high ciliated cells correspond to the function of transporting ovulated oocytes. In the cyclic phase, there are a number of nonciliated cells as small rounded spheres with short microvilli at the apical surface corresponding to their secretary function required for the nutritional support for the embryonic development [36]. It has been suggested that the regular cycle of ciliated and nonciliated cells of the epithelial cells in the mammalian oviduct depends upon the levels of circulating estrogen and progesterone [37].

Preparation of Single-cell Suspension of Oviduct Epithelial Cells

Epithelial cells isolated from oviduct were in clumps, but for development of primary cell culture system, it should be in single cell-suspension. For this, we used trypsin. It is an essential reagent for routine cell culture work. In the cultivation of mammalian cells, it has been extensively used to obtain single cells from tissues and organs [38]. It has also been widely used for the subcultivation and scale-up of several cell lines to detach cells from

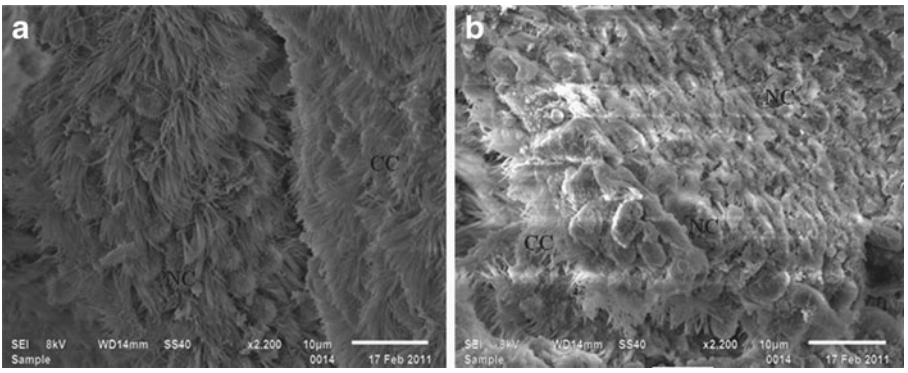

Fig. 1 Ultrastructural aspects observed using SEM of buffalo oviduct. **a** Acyclic ampulla showing the numerous ciliated cells (CC) and lesser nonciliated cells (NC) in spherical shape with short microvilli on the apical surfaces. **b** Cyclic ampulla showing the numerous nonciliated cells (NC) and lesser number of ciliated cells (CC). Observations done at high magnification (2,200×)

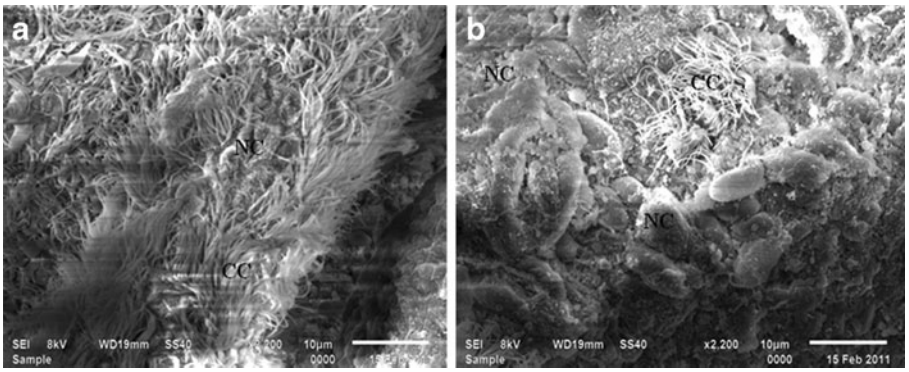

Fig. 2 Ultrastructural aspects observed using SEM of buffalo oviduct. **a** Acyclic isthmus showing the numerous ciliated cells (CC) and lesser non-ciliated cells (NC) in spherical shape with short microvilli on the apical surfaces. **b** Cyclic isthmus showing the numerous non-ciliated cells (NC) and lesser number of ciliated cells (CC). Observations done at high magnification (2,200×)

either static or carrier surfaces [39]. It may damage the cell membrane in contact of cells during long trypsinization. However, there is no specific report on time-dependent and concentration-dependent effects of trypsinization on bovine oviduct epithelial cells. In the present study, the time- and concentration-dependent effects of trypsinization on bovine oviduct epithelial cells were investigated. The effect of trypsin was investigated by testing increasing concentration of trypsin (0.25% to 1.5%) on bovine oviduct epithelial cells at five time points (1, 2, 5, 10 and 20 min), whereas 1 min was considered as control. Viability was decreased from 5 min and continued up to 20 min in our experiments. Cell viability after trypsinization with 0.25% to 1.5% trypsin for 1 to 20 min was quantified by trypan blue exclusion assay (Fig. 3). The results showed that trypsin decreased the proliferation of bovine oviduct epithelial cells depending on the concentration and exposure time of trypsinization.

After increasing the concentration of trypsin from 0.25% to 1.5%, the cell membranes were strongly affected, and the percentages of viable cells reduced to 93% and 63%, respectively, were detected by trypan blue exclusion assay (Table 1 and Fig. 4). The same trends were obtained after increasing the time of trypsinization for 10 and 20 min as the

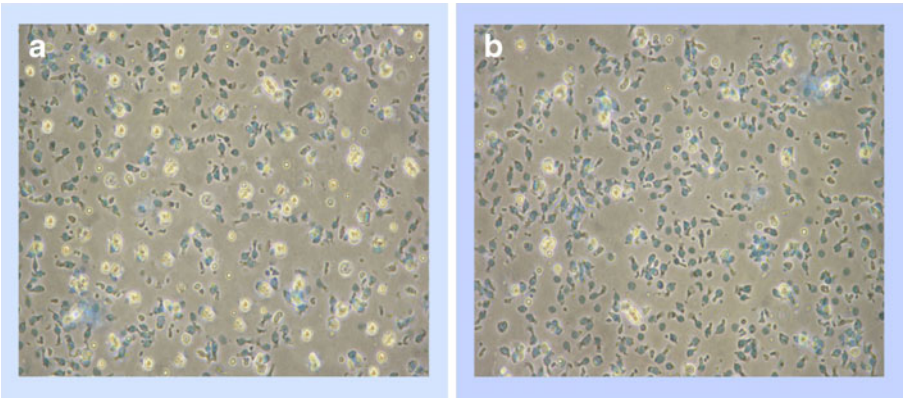

Fig. 3 Trypan blue exclusion assay for cell viability (viable cells have a clear cytoplasm, whereas nonviable cells have a blue cytoplasm) **a** after 1-min trypsinization and **b** after 10-min trypsinization

Table 1 Effect of increasing concentration of trypsinization on cell viability of oviduct epithelial cells of buffalo

The number of viable cells was calculated by dividing the number of living cells with the total number of cells in percentage

Concentration of trypsin (%)	No. of viable cells	Total no. of cells	% viability
0.25%	3.25×10^6	3.40×10^6	93.75%
0.50%	2.90×10^6	3.35×10^6	86.56%
1.0%	1.95×10^6	2.75×10^6	70.90%
1.5%	1.65×10^6	2.60×10^6	63.46%

percentage of cell viability reduced to 81% and 72%, respectively (Table 2 and Fig. 5). The findings suggest that a short trypsinization should be used in cell culture area to maintain membrane integrity of the cells. Some other studies also reported the trypsin-induced changes in cultured keratinocytes [40] and endothelial cells [41] which support our results.

In cell culture, trypsin solutions are widely used without determination of whether their application could have any deleterious effects while preparing the single cell suspension from normal tissues. In this study, it was demonstrated that trypsinization with 0.25% trypsin for 1–2 min at 37 °C was the best method for obtaining single cell from oviductal tissues for monolayer culture. Previous data suggest that prolonged exposure of cells to active trypsin might damage cell viability [42]. It can trigger the metabolic changes by interaction with the cellular membrane which induces cellular death [43]. Our results also confirmed the gradual decrease in bovine oviduct epithelial cell viability with the increasing time and concentration of trypsinization. In another study, it has been reported that some epitopes of the cell membrane were particularly sensitive to trypsin and were lost after trypsinization [44]. Therefore, the effect of trypsin is very important for starting a new experiment including its exposure which may differ with manufacturers and which is usually not mentioned throughout scientific literature and, in particular, buffaloes. To our best knowledge, time-dependent and concentration-dependent effects of trypsinization on bovine oviduct epithelial cell viability have not been demonstrated by any of the researchers.

Primary Cultures in Serum-containing Medium

In this study, a simple procedure was employed for the isolation of epithelial cells from bovine oviducts. Dissociation of cells was carried out by mechanical scrapping of the

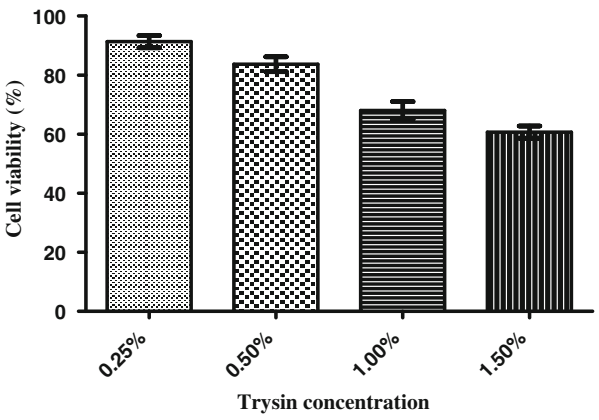

Fig. 4 Concentration-dependent effect of trypsinization on cell viability

Table 2 Effect of different trypsinization times on cell viability of oviduct epithelial cells of buffalo

Time (min) for trypsinization	No. of viable cells	Total no. of cells	% viability
1 min	2.25×10^6	2.45×10^6	91.83%
2 min	1.90×10^6	2.15×10^6	88.37%
5 min	2.10×10^6	2.40×10^6	87.50%
10 min	2.45×10^6	3.00×10^6	81.66%
20 min	1.75×10^6	2.40×10^6	72.91%

The numbers of viable cells were calculated by dividing the number of living cells with the total number of cells in percentage

oviduct as described by Eyestone and First (1989) [31]. This procedure avoids the use of proteolytic enzymes that might alter cell surface integrity. This cell isolation process provided a large number of small aggregates and some isolated cells so as to obtain single isolated cells; trypsin was used as 0.25% for 1 min. Two different cell types were obtained by this technique: secretory cells and ciliary cells. Cell cultures were initiated in TCM-199 medium containing 10% FBS without addition of antibiotics. Viability of the cells was well preserved (93%) as determined by trypan blue exclusion after trypsinization, and 1 h after inoculation, numerous ciliary movements were observed. After 1 day of culture, some cells were attached to the bottom of the flasks and formed small monolayers and others were reassociated to form organoids with numerous ciliary cells at the periphery. These cell clusters exhibited rapid motility in the culture medium. One day later, when the medium was first renewed, the monolayers were extended and some organoid structures were attached to the culture support. The ciliary activity was still present. After 4 days of culture, the attached organoids were spreading on the bottom-forming monolayers. Finally, after 6 days, the cultures had reached confluency, and in the monolayers, only two cell populations were observed: a population of large polygonal cells containing some vacuoles surrounded by a second population of smaller cells exhibiting ciliary movement (Fig. 6).

Oviduct Cell Monolayer in Serum-free Medium

Serum requirements of oviduct cells during primary culture were examined. As previously observed, confluency was never achieved when serum was omitted indicating that seric components are needed for cell attachment and proliferation [45, 46]. However, our results demonstrate that oviduct cells can be further cultured for another 1 week in serum-free medium after l week proliferation and monolayer formation in the presence of FBS. Cultured

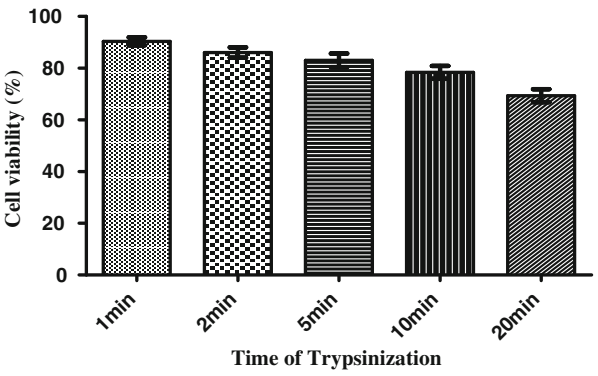

Fig. 5 Time-dependent effect of trypsinization on cell viability

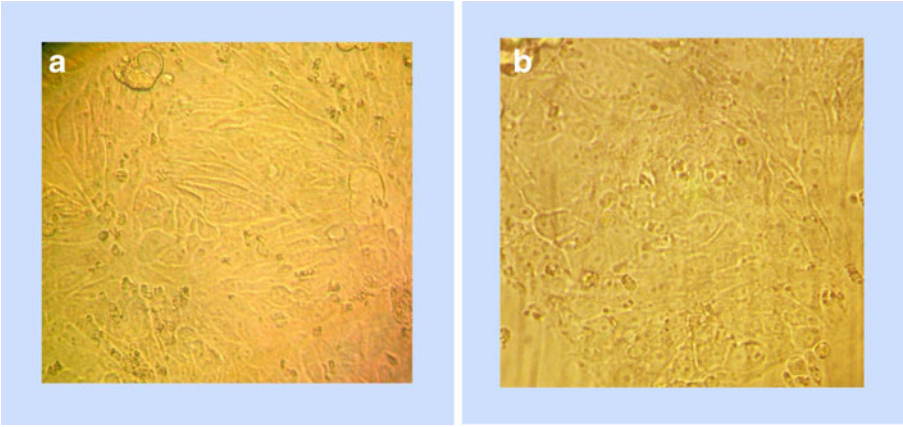

Fig. 6 Phase contrast microscopy of buffalo oviduct epithelial cells in monolayer culture (7 days). **a** Ampulla. **b** Isthmus

bovine oviductal epithelial cells showed a wide variety of secretory activities, and these secretory factors may influence early embryonic development or sperm function. For the study of these factors, oviductal secretory proteins in serum-free medium were quantified using the Lowry method and analyzed by SDS-PAGE electrophoresis.

Gel Electrophoresis of Proteins Isolated from Oviductal Tissue and Conditioned Medium

Several studies have demonstrated that bovine oviduct cells secrete specific proteins in monolayer [47] or explant culture [48–50]. However, it is not known whether oviduct cells maintain this activity when cultured in the absence of serum because the synthesis of some proteins by differentiated oviduct cells is under hormonal control [48, 51].

We extracted the proteins from oviductal cells using a mild processing and recovered approximately 5.2 mg/ml of proteins from ampulla of cyclic oviduct tissue which is almost double than the total amount of proteins present in ampulla of acyclic oviduct (2.5 mg/ml) but the isthmus part of oviduct does not show any significant difference between cyclic and acyclic oviducts (Table 3). In conditioned media of monolayer from different parts of oviduct recovered after 6 days of culture, protein content is very low as compared to oviduct extract as shown in Table 4. Total protein was found to be maximum in cyclic ampulla (31.2 mg/segment) and minimum in acyclic isthmus (7.2 mg/segment). Thus, it clearly indicates differential expressions of proteins in different parts of buffalo oviduct are contributing in differential microenvironment. The major protein in oviduct epithelial cells had a relative molecular mass of 66 kDa and apparently was BSA (Fig. 7) as it was also reported

Table 3 Determination of protein contents in different segments of oviduct

Samples of different tissues	Protein concentration (mg/ml of sample)	Total protein/ segment (mg)
Cyclic ampulla (Ca)	5.2	31.2
Cyclic isthmus (Ci)	1.5	9.0
Acyclic ampulla (Aa)	2.5	15.0
Acyclic isthmus (Ai)	1.2	7.2

Table 4 Determination of protein contents in conditioned media of segments of oviduct

Conditioned media of monolayer from different parts of oviduct	Protein concentration (mg/ml)
Cyclic ampulla media (CaM)	0.76
Cyclic isthmus media (CiM)	0.44
Acyclic ampulla media (AaM)	0.28
Acyclic isthmus media (AiM)	0.22

by Gerena and Killian (1990) [52] in cows. To corroborate the fact that these proteins originate from the oviduct, we compared the protein pattern of oviduct extracts with the proteins present in the conditioned medium of oviductal cells cultured for 6 days without serum. In total, more than 22 bands were visualized after one-dimensional SDS-PAGE and Coomassie blue staining of oviduct extraction of ampulla, out of which only 13 bands were observed in the conditioned media. Major bands had approximate molecular masses of 35, 40, 43, 47, 56, 60, 66, 82, 95, 120, 150,180 and 220 kDa. Other parts of the oviduct also had more number of bands in oviductal tissue protein than conditioned medium as described in Table 5. The differences concerning the SDS-PAGE results on extracted proteins and proteins secreted in conditioned media may be due to the presence of cytosolic protein in oviduct extracts or due to the low protein secretion in a medium without serum. There are three proteins with *Mw* 220,190 and 35 kDa which was only present in cyclic buffalo and was absent from acyclic buffalo (indicated by an arrow in Fig. 7). This may be due to the presence of different steroids in different cycle stages which affect the secretion of different proteins.

Detection of Oviduct-specific Glycoproteins (OGPs) by Western Blot Analysis

In order to eliminate the contamination of the electrophoretical pattern by serum minor proteins, Western blotting was done using Oviductin (goat polyclonal IgG) against OGPs.

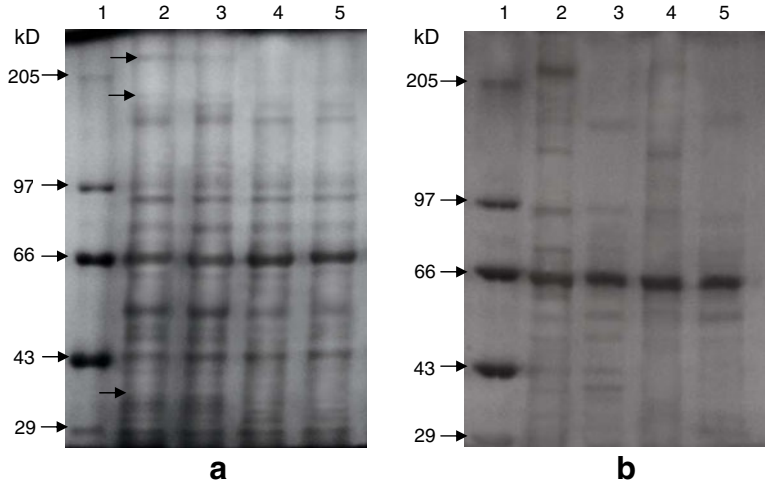

Fig. 7 One-dimensional SDS-PAGE separation of **a** *lane 1* high range molecular marker, *lanes 2 to 5* oviductal tissue protein samples Ca, Ci, Aa and Ai and **b** *lane 1* high range molecular marker, *lanes 2 to 5* conditioned media proteins samples (Ca(M), Ci(M), Aa(M) and Ai(M))

Table 5 Total number of bands in different regions of cyclic and acyclic buffalo from oviductal tissue and conditioned media confirmed by SDS-PAGE analysis

Source	Oviductal tissue	Conditioned media	Common bands	Molecular weight (kDa) of common bands
Cyclic ampulla	22	13	13	35, 40, 43, 47, 56, 60, 66, 82, 95, 120, 150,180, 220
Cyclic isthmus	22	10	10	35, 40, 43, 47, 56, 60, 66, 82, 95,180
Acyclic ampulla	19	9	9	35, 47, 56, 66, 82, 95,150, 180, 220
Acyclic isthmus	19	7	7	35, 47, 56, 66, 82, 95,180

Oviduct extract and conditioned medium proteins from different regions of the cyclic and acyclic buffalo oviducts were separated by SDS–PAGE, transferred to nitrocellulose membranes and treated with Oviductin. Several proteins with different molecular weights were detected in SDS-PAGE of oviductal extract and conditioned medium. However, after incubation of these proteins with Oviductin, these bands were abolished and only OGPs were detected. Oviductal extract have more number of OGPs as compared to conditioned medium.

Western blot analysis revealed five major OGPs in cyclic oviduct (ampulla and isthmus) with *Mw* 180, 95, 75, 66 and 35 kDa in the oviduct extract and two glycoproteins with *Mw* 95 and 66 kDa in conditioned media. However, in acyclic oviduct (ampulla and isthmus), three glycoproteins were immunostained with *Mw* 180, 95 and 66 kDa in the oviduct extract and one glycoprotein with *Mw* 66 kDa in conditioned media (Fig. 8). The differences concerning the Western blot results on extracted proteins and proteins secreted in conditioned medium may be due to the presence of cytosolic proteins in oviduct extracts or due to the low protein secretion in a medium without serum.

There are similarities between the buffalo oviductal proteins and those described in other mammalian species, but molecular weight varies. The molecular masses of other OGPs, those identified in cow (*Mw* 97) [49], mice (*Mw* 215) [12], hamsters (*Mw* 200–250) [14, 53],

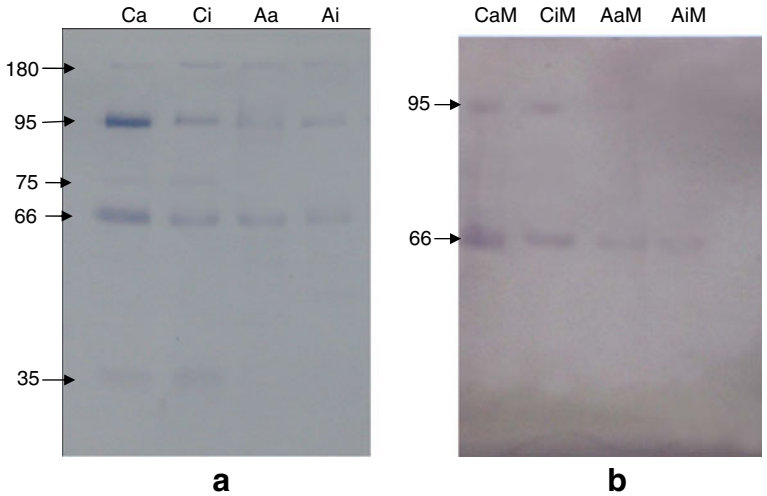

Fig. 8 Detection of oviduct-specific glycoproteins by Western blot analysis of **a** oviductal tissue proteins (Ca, Ci, Aa and Ai) and **b** conditioned media proteins (Ca(M), Ci(M), Aa(M) and Ai(M))

rabbits (*Mw* 71) [54], pigs (*Mw* 115), [18], sheep (*Mw* 90–92) [10, 16], baboons (*Mw* 100–130) [17] and humans (*Mw* 130 [19]; *Mw* 540 [55]) are within the ranges described here. In our study, a glycoprotein (*Mw* 180) was found new and was not reported yet in buffalo oviduct.

This report adds to the definition of the role of identified mammalian oviductal glycoproteins. To date, the biological function(s) of these molecules is unknown. Specific oviductal antigens may affect the final maturational processes that sperm undergo prior to fertilization. A component of oviductal fluids, probably a glycosaminoglycan, is reported to positively affect bovine sperm capacitation and the acrosome reaction [56]. Oviductal secretory macromolecules have also been shown to associate with the zonae pellucidae and/or the perivitelline spaces of oviductal ova and embryos [21, 57].

Glycoprotein Staining

OGPs in the oviductal extracts and conditioned media were stained by using the "Glycoprotein Staining Kit" which detects glycoprotein sugar moieties in polyacrylamide gels and on nitrocellulose membranes (Fig. 9). Glycoprotein staining showed that all the proteins detected by Western blotting were of glycoproteins so it confirmed the presence of OGPs in oviduct extract and conditioned medium of different parts of cyclic and acyclic buffalo oviducts. Sutton et al. (1986) [16] also found periodic acid-Schiff stained glycoprotein in sheep oviduct.

Quantitative Analysis of OGPs by Enzyme-Linked Immuno Sorbent Assay (ELISA)

In order to detect the OGPs' semiquantitative variations among the different segments of oviduct of cyclic and acyclic buffaloes, indirect ELISA was performed using Oviductin (polyclonal antibody) specific to OGPs obtained from Santa Cruz Biotechnology, Inc. Results from ELISA studies indicate significant differences between the ampulla and isthmus parts of cyclic and acyclic buffaloes in synthesis and secretion of glycoproteins. Oviductal extract contains more amounts of OGPs as compared to conditioned medium (Fig. 10 and Fig. 11). In cyclic buffalo, the ampulla secretes greater amounts of OGPs than

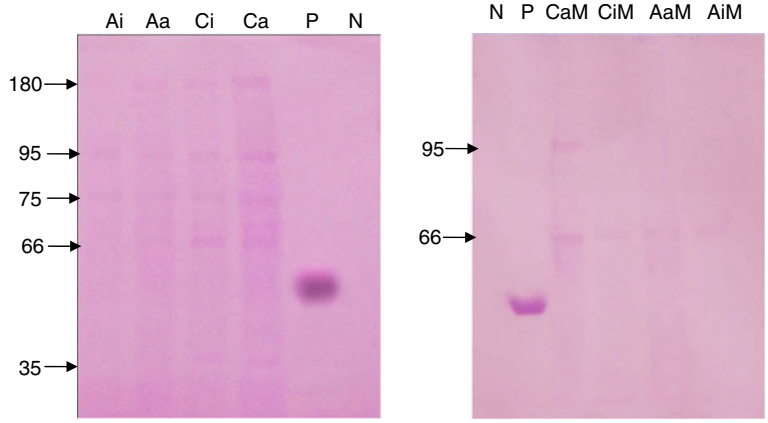

Fig. 9 Detection of oviduct-specific glycoproteins by glycoprotein staining of oviductal tissue proteins (Ca, Ci, Aa and Ai) and conditioned media proteins (CaM, CiM, AaM and AiM). *P* positive control, *N* negative control

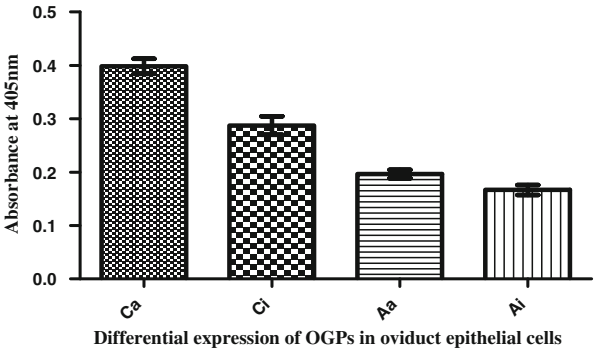

Fig. 10 Differential expression of oviduct-specific glycoproteins in oviductal tissue proteins (Ca, Ci, Aa and Ai) by using ELISA

does the isthmus in both oviductal extract and conditioned media. The same trends were obtained for acyclic buffaloes but at a lower concentration. Hyde and Black (1986) [58] also demonstrated that the estrogen-induced sulfated oviductal glycoprotein (SOG) is produced by both the ampulla and the isthmus, but the ampulla secretes more SOG. Synthesis and secretion of proteins are also greater for the ampulla compared to the isthmus in gilts [18].

Knowledge of the microenvironment provided by the oviduct at ovulation, fertilization and during the early cleavage stages of embryonic development increases our ability to understand both actual and potential interactions that occur in the oviduct and between oviduct proteins and gametes and embryos. In the present study, we examined the luminal surface of the ampulla and isthmus parts of oviduct from cyclic and acyclic buffaloes by scanning electron microscopy and identified various regional variations and cyclic changes in the epithelial cells. The regional differences may reflect regional variations in sensitivity to circulating ovarian steroid hormones. Besides this, a method has been described to obtain the monolayer of oviduct epithelial cells of buffalo using different parts of the oviduct and preparation of conditioned media. Oviductal extracts and conditioned media were analyzed by SDS-PAGE, and it was found that oviductal extracts have more number of proteins as compared with conditioned media. The extract and media were also used to identify the potential OGPs in different regions of cyclic and acyclic buffaloes by Western blot and ELISA. These glycoproteins were involved in providing microenvironment for embryonic development. Our study indicates that quantities of OGPs were highest in the ampulla of cyclic buffalo and decreased in acyclic buffalo, thus, providing differential microenvironment in different parts of cyclic and acyclic buffaloes. This was due to estrogen which was

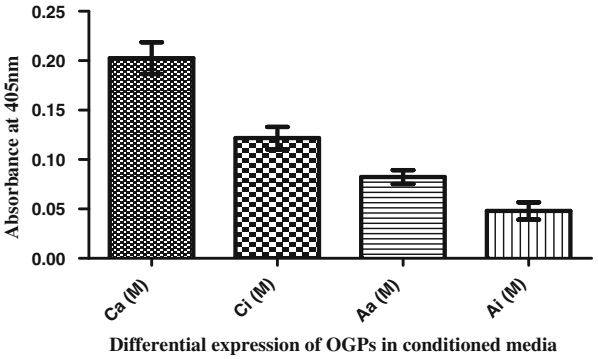

Fig. 11 Differential expression of oviduct-specific glycoproteins in conditioned media proteins (Ca(M), Ci(M), Aa(M) and Ai (M)) by using ELISA

prominently present in cyclic stages. Abe et al. (1998) [59] also indicate that the synthesis and secretion of OGPs occur in response to estrogen stimulation in hamster. The specific synthesis of proteins by the buffalo oviduct suggests that these proteins affect or contribute to a microenvironment facilitating or essential for fertilization and early cleavage stage preimplantation development. Further studies are necessary to determine the functional role of these proteins in fertilization and early embryonic development at the level of the oviduct or gametes.

Collectively, this study and other previous reports clearly demonstrate that the mammalian oviduct synthesizes and releases unique glycoproteins. These glycoproteins may significantly affect the fertilization process and/or subsequent embryonic development. Elucidation of the biochemical nature and biological function of the oviductal antigens would provide further knowledge of the reproductive process. Such information, particularly in buffaloes, may prove useful in developing improved culture media for embryo development and cryopreservation that would be beneficial for the embryo transfer technology and scientists investigating the genetic regulation of embryonic development.

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