EXPRESSION PROFILING OF CANDIDATE EMBRYOTROPHIC GENES OF BUFFALO OVIDUCT DURING DIFFERENT STAGES OF OESTROUS CYCLE

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ABSTRACT

The aim of the present study was to identify the gene expression profile of three candidate genes namely heat shock protein 70, oviductal glycoprotein and osteopontin in buffalo oviduct during different stages of the oestrous cycle. Slaughter house derived buffalo oviducts were categorized as belonging to early, mid and late phase of the oestrous cycle based on the morphological appearance of CL and progesterone concentration. The relative abundance of each of the candidate gene transcript was quantified by real time RT-PCR. Transcripts of all the three candidate genes were found to be expressed in OEC throughout the oestrous cycle. The results of the present study demonstrate that gene expression in the buffalo oviduct is clearly regulated during the oestrous cycle, as these candidate genes play a crucial role in several reproductive processes. It was found that Hsp 70 gene was significantly upregulated in OEC belonging to the early and late phases when compared to mid phases of the cycle. The expression of OVGP mRNA was found to be maximum during the early phase and it remained low during the mid and late phases while expression of OPN mRNA was maximum during the early phase and declined sequentially during the mid and late phase of oestrus cycle.

Keywords: *Bubalus bubalis*, buffaloes, gene expression, buffalo oviduct, oestrous cycle, quantitative PCR

INTRODUCTION

The oviduct plays a key role in crucial reproductive events that include gamete maturation, gamete transport, sperm capacitation, fertilization, and early embryonic development. The oviduct equips itself to perform these specialized functions by undergoing marked physiological and anatomical changes during the oestrous cycle. At the molecular level, these changes are recognized as variations in the expression of few genes or proteins, some of

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which are important for fertility and reproductive outcomes. Differential expression pattern of genes of the oviduct were earlier analyzed by adopting molecular techniques like cDNA hybridization (Meikle *et al*., 2001), semi quantitative RT-PCR (Lok *et al*., 2002; Briton-Jones *et al*., 2004), real time PCR (Gabler *et al*., 2003; Kenngott *et al*., 2011) and microarray technology (Bauersachs *et al*., 2003; 2004) in several species. However, to our knowledge, such studies on oviductal transcriptome profiling are scarce in bubaline species.

Although a number of genes were documented to be differentially expressed until now, few like the transcripts of heat shock protein 70, oviduct specific glycoprotein and osteopontin are of specific interest, as these are embryotrophic, undergo remarkable changes and are reflected by oviductal biosynthetic activity. Heat shock proteins (Hsps) are ubiquitously present molecular chaperons that protect the gametes and embryos from a variety of stress. Alteration in the Hsp 70 gene expression levels is considered as an embryotrophic mechanism to preserve gamete competence, thermotolerance to spermatozoa and developing embryos (Hansen, 2004). Unique to the oviduct is the oviduct specific glycoprotein (OVGP) that is conserved among species. This protein is found to bind with the surface of oocytes and spermatozoa (Verhage *et al*., 1998) and initiate a signalling cascade during the process of fertilization and cell proliferation of early embryos thereby improving cleavage rates (Pradeep *et al*., 2011). Osteopontin (OPN) is another glycoprotein present in three different isoforms in the oviductal secretions and seminal plasma. This calcium-binding protein plays an important role in fertilization by being involved in the acrosome reaction (Goncalves *et al*., 2009) and increases cell-extra cellular matrix communication required for embryo development through interaction with integrins (Gabler *et al*., 2003).

Research on gene expression patterns at different stages of the oestrous cycle in buffaloes are very important for understanding the regulatory processes occurring in the oviduct and would throw light on developing appropriate *in vitro* resources required for application of assisted reproductive technologies to improve the reproductive performance of this species.

The objective of this study is to investigate differences in expression of embryotrophic candidate genes like Hsp 70, OVGP and OPN at mRNA level in the buffalo oviduct during different stages of the oestrous cycle.

MATERIALS AND METHODS

Determination of the stages of oestrous cycle

Buffalo oviducts obtained from slaughterhouse were categorized into three groups namely, early, mid and late phases of the oestrous cycle based on the morphology of the ovary and blood progesterone concentrations. Key features of the gross morphology of corpus luteum for grouping of oviducts under different phases adopted by Ireland *et al*. (1980); Wijayagunawardane *et al*. (1996) were followed and are shown in Table 1.

Samples were considered to be derived from non-cycling animals when the ovaries seemed to be inactive with none of the visible structures like follicle or corpus luteum at any stage of development or regression. Further, blood collected from the slaughtered animals was used for progesterone hormonal estimation using radio immunoassay.

Collection of oviductal epithelial cells

Oviducts ipsilateral to the ovary at different stages of the oestrous cycle were ligated both at the infundibular and utero-tubal ends, trimmed off the surrounding tissues and washed several times with physiological saline. Oviductal epithelial cells (OEC) were harvested by gentle stripping of the oviducts into petri dish containing phosphate buffer saline (PBS) as per the procedure of Velazquez *et al.* (2010). After few washes in PBS, OECs were obtained by pelleting the cells at 3,000 rpm for 15 minutes.

Reverse transcription

RNA was extracted from harvested OEC using Trizol Reagent as per manufacturer's instructions. Reverse transcription was carried out using a commercial cDNA synthesis kit. Briefly, 1 µl of random hexamer primer, 10 µl of RNA extract, and 9 µl of nuclease free water were incubated at 65°C for 5 minutes and snap chilled. Further 4 µl of Reaction buffer, 2 µl of 10 mM dNTP, 1 µl of RNase Inhibitor and 1 µl of M-MuLV reverse Transcriptase enzyme were added to the mixture and incubated for 5 minutes at 25°C, followed by 60 minutes at 42°C. The reaction was terminated by heating at 70°C for 5 minutes, cooled on ice and stored at -20°C.

Quantitative PCR for the candidate embryotrophic genes

Relative quantification of candidate embryotrophic genes namely Hsp 70, OVGP and OPN genes was performed using the SYBR green technology. The reaction mix comprised of 5 µl of SYBR Green mastermix (2x), 1.0 µl of 10 pmol forward primer, 1.0 µl of 10 pmol reverse primer,(Hsp 70 FP-5'-AACAAGATCACCATCACCAACG-3', RP-5'-TCCTTCTCCGCCAAGGTGTTG-3', OVGP FP-5'-GGGAAAGGTTCGTCAGTTCA-3', RP-5'-CATACGCTTTCTGGACGACA-3', OPN FP-5'-GCAAATCAGAAGTGTGATAGA C-3', RP-5'-CCAAGCCCAACATATGAGTT-3' with accession numbers AM_174550, XM_611787 and NW_ 255516 respectively) 1.0 µl of cDNA and 2.0 µl of nuclease free water. In the negative control 1 µl of nuclease free water was added instead of template. The cycling conditions for quantitative PCR included an initial denaturation of 94°C for 3 minutes, followed by 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by default melting curve analysis with denaturation and annealing steps alone. Ct values of each candidate gene were calculated by the real plex software and normalized against the housekeeping β-actin gene (FP-5'-CTT CCT GGG CAT GGA GT- 3', RP-5 -TTG CTG ATC CAC ATC TGC T-3') of *Bubalus bubalis.* Changes in expression of candidate genes at different stages of the oestrous cycle were calculated with respect to those values of OEC from inactive ovary using the $2^{-\Delta\Delta ct}$ method described by Livak and Schmittgen (2001).

Statistical analysis

The statistical analysis for mRNA expression was performed on fold change values from quantitative real time PCR analyses normalized to corresponding β-actin. Values reported are means ± SEM of six sets of biological replicates in each group. All data were normally distributed and underwent equal variance testing. Model parameters included candidate genes (Hsp 70, OVGP and OPN and stages of the oestrous cycle (Early, mid and late phase). The main effects of each parameter and the interactions between the parameters were determined. The experiments were analyzed with the general linear model of SPSS 10.0 for Windows. Multiple means were compared

by one way ANOVA and when a significant effect was obtained the difference between means was determined by a Duncan multiple range test. A *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSIONS

Buffaloes play a prominent role in rural livestock production particularly in Asia where more than 97% of the population is present out of the estimated 185.29 million head. The riverine type of *Bubalus bubalis* is more favoured in India and Pakistan owing to its high milk yield. Despite advancements in buffalo breeding and farm management practices along with increasing implementation of newly developed reproductive techniques in buffaloes, the production performance is still low. Reproductive efficiency is known to be the primary factor affecting productivity in female buffalo but is found to be greatly hampered by late attainment of puberty, seasonality of calving, long postpartum anestrum and subsequent calving interval (Danell, 1987). Earlier studies have been undertaken to understand the influence of ovary and ovarian hormones on buffalo oestrous cycle and pregnancy. It is worth mentioning that oviduct is equally an important niche for orchestration of the maternal - embryo dialogues for a successful pregnancy. The oviducts undergo significant morphological and functional changes during different stages of the oestrous cycle, thus making itself a conducive depot and conduit for the gametes and embryo. Keeping the above facts in view, the present study is designed to focus on better understanding of the embryotrophic role of Hsp 70, OVGP and OPN by analyzing their differential expression pattern in buffalo oviduct during different stages of the oestrous cycle.

Correlation of the stages of oestrous cycle with the morphology of corpus luteum and progesterone concentration

Oviducts collected from buffaloes were categorized as belonging to the early, mid and late phases initially by correlating the appearance of the CL as mentioned in Table 1. However, the accuracy of estimating the stages of the oestrous cycle was further confirmed by correlating with additional parameters like the weight of the CL, ratio of the wet weight of CL to ovary and plasma progesterone concentrations and are presented in Table 2.

The values represented in the table reveal that the concentration of blood progesterone was maximum during the mid phase, followed next by the late phase, and minimum during the early phase. As per the categorization norms followed, the mid phase of oestrous cycle in our study is nothing but the luteal phase and the late phase coincides with the follicular phase. The observed trend in rise and fall of progesterone concentrations for staging of oestrous cycle was in agreement with the patterns documented by Killian *et al*. (1989); Jazayeri *et al*. (2010) during the luteal and follicular phases respectively. As reported by Wijayagunawardane *et al*. (1996), it is more meaningful to construe that comparatively higher progesterone levels are maintained during the mid stage of the oestrous cycle due to the presence of a greater sized functional CL than those with a regressing CL present at the late phase and near the time of ovulation. Our categorization of staging of oestrous cycle based on progesterone concentrations was further justified by Bennett *et al*. (1988) who recorded a typical pattern of progesterone decline near the time of ovulation when the CL is small and just emerging.

Detection of gene expression of candidate genes in buffalo oviduct during various phases of the oestrous cycle

The basal gene expression of three embryotrophic candidate genes - Hsp 70, OVGP and OPN in the oviduct was determined by conventional RT-PCR before analyzing their differential expression pattern. Amplicons of Hsp 70, OVGP, OPN and β-actin specific primers yielded products of 275 bp, 240 bp, 290 bp and 283 bp respectively. Thus, it was found that transcripts of all the three candidate genes were found to be expressed in buffalo oviduct during early, mid and late phases of the oestrous cycle. No bands were observed in non-reverse transcribed controls indicating that the observed signals were not obtained by the amplification of genomic DNA.

Further, the abundance of mRNA transcripts of the each of the candidate gene normalized to the values of β-actin during early, mid, and late phase of the oestrous cycle analyzed using quantitative PCR were discussed below.

Expression profiling of Hsp 70 gene in buffalo oviduct

Comparative analysis of the expression of Hsp 70 mRNA during different phases of the oestrous cycle represented in Figure 1 revealed that Hsp 70 mRNA was expressed maximally during the late phase (88-fold change) followed by a gradual decline during the early phase (77-fold change) and lowest in the mid phase (56-fold change) of the oestrous cycle, when compared to the expression levels of Hsp 70 in an inactive ovary (one fold change).

This is in agreement with the findings of Bauerssachs *et al*. (2004) wherein a 6.8-fold upregulation of Hsp 70 transcripts was documented during late phase that approaches estrum when compared to mid phase or diestrum in bovines. Similarly, Mariani *et al*. (2000) has shown that Hsp 70 expression was upregulated during late phase in the ampullary region of the rat oviducts. However, Muthukumar *et al*. (2014) reported that the expression level of Hsp 70 analyzed by immuno blotting during mid phase was higher, but it should be noted that the target organ chosen was the the cervico-vagina of buffalo and not the oviducts.

The reasons for the high levels of Hsp 70 during late phase of the oestrous cycle can be regarded as multifaceted. Hsp 70 is found to act as a repressor of steroid receptors and modulate the function of steroid hormones. Thus Salvetti *et al*. (2008) reported an increase in the levels of Hsp 70 in bovine oviducts during proestrum or the late phase when there was downregulation of oestrogen receptors. Another reason attributable to the high levels of Hsp 70 mRNA during the late phase is the rise in body temperature with concomitant increase in temperature of the reproductive organs that is a common feature in farm animals prior to estrum and until ovulation. It is likely that Hsp 70 is thus upregulated during this heat stressed conditions as seen in the findings of our present study.

Expression profiling of OVGP gene in buffalo oviduct

The relative abundance of OVGP transcripts in OEC during different stages of the oestrous cycle is represented in Figure 2. Expression of OVGP gene was found to be upregulated up to 124-fold during the early phase of the oestrous cycle, while it was down regulated up to 13-fold and 12-fold during the mid and late phases respectively, when compared to the oviduct from a non-cycling or anoestrus animal. The expression of OVGP is specific to the oviduct and its synthesis is controlled by steroids. While oestradiol exerts

a positive effect on OVGP expression in the follicular and periovulatory phase, its effect is markedly reduced in the luteal phase when the progesterone level is elevated thus supporting the hormonal regulation of OVGP expression in oviducts reported by Buhi *et al.* (1996); Verhage *et al*. (1998). Thus, could be the reason for the upregulation of OVGP transcripts observed by us in the oviducts belonging to the early phase and is further substantiated by the temporal association studies carried out by Sutton *et al*. (1984) in sheep and Boice *et al*. (1990) in cows, that revealed the influence of the oestrogen concentration on the expression of OVGP. However, the expression of OVGP did not differ significantly between the mid and late phases of the oestrous cycle. The expression profile of OVGP demonstrated in our findings is similar to those of Lok *et al*. (2002) in human and Baeursachs *et al*. (2003) in bovine who recorded 2.7-fold upregulation in early phase when compared to mid phase suggesting the potential role of OVGP during gamete maturation, fertilization and early embryonic events.

In contrast, findings of McBride *et al*. (2004) revealed no significant alterations in the expression of OVGP throughout the oestrous cycle in golden hamster. In another report by Hachen *et al*. (2012), the expression of OVGP was found to decrease in the freshly ovulated stage, lowest in the mid phase and upregulated 100-fold in the early and late follicular stage in felines. However, these changes in mRNA expression could be attributed to variation between species especially those with long oestrous cycle.

Expression profiling of OPN gene in buffalo oviduct

Quantitative PCR analysis of OPN transcripts in buffalo oviduct presented in Figure 3 revealed that OPN was differentially expressed with 128.15-fold, 84.74-fold and 17.51-fold change during early, mid and late phases of the oestrous cycle respectively. This significantly higher expression level of OPN mRNA observed immediately after ovulation during the early phase of the oestrous cycle in buffalo oviduct is in concurrence with the findings of Gabler *et al*. (2003) wherein OPN transcripts of bovine OEC tended to be higher during early phase and lower during late phase. Gabler *et al*. (2003) reported that integrins follow an oestrogen dependant maximal expression in the bovine oviduct before ovulation than the late luteal mid phase. In turn, more osteopontin could be bound by integrins near ovulation as it is seen in this study than during the inactive late phase of the cycle.

Although osteopontin is expressed in buffalo OEC throughout the oestrous cycle, it could be seen that the OPN gene expression decreased significantly during the follicular phase and remained high during the luteal stage. This is quite expected as demonstrated by higher expression of OPN gene during the peri implantation period by Garlow *et al*. (2002) in pigs and Joyce *et al*. (2005) in goats substantiating that OPN is involved in embryonic implantation and placentation.

CONCLUSION

Thus, it could be seen that the chosen candidate genes are differentially expressed during the different stages of the oestrous cycle and are regulated by the dominant steroid of the respective phase, thus explaining the significant role of these oviduct-expressed genes in early embryo development.

Table 1. Staging of the oestrous cycle based on the characteristics of corpus luteum.

Characteristics (Appearance of corpus luteum)	Stages of the oestrous cycle		
	Early	Mid	Late
External	Red, recently ovulated, point of rupture not covered by epithelium	Apex of corpus luteum reddish to orange, point of rupture covered over	Light yellow to white, corpus lutuem almost embedded in the ovary
Internal	Red, cells loosely organised, occasionally filled with blood	Red or dark brown at the apex only, remainder of corpus luteum is orange, small cavity in the centre	Orange to yellow, slightly gritty
Vasculature on the surface of corpus luteum	Not visible	Limited to periphery. In some, covers the apex of the corpus luteum	Not visible (no bleeding)
Follicles (medium to large sized)	Absent	May be present	Present

Table 2. Concentrations of progesterone (ng/ml) and wet weights of corpus luteum during different stages of oestrous cycle.

Stage of the oestrous cycle	Progesterone (ng/ml)	Weight of corpusluteum (g)	Ratio of weight of corpus luteum to that of the ovary (g)
Early	$1.34^{a}\pm0.21$	$1.40^{b}\pm0.22$	$0.25^{b}\pm0.02$
Mid	$3.26^{c}\pm0.79$	$2.68^{c}\pm0.28$	$0.54^{c}\pm0.04$
Late	$2.22^{b}\pm0.44$	$0.23^{a}\pm0.04$	$0.06^{a}\pm0.01$

Values are expressed as mean ± SEM.

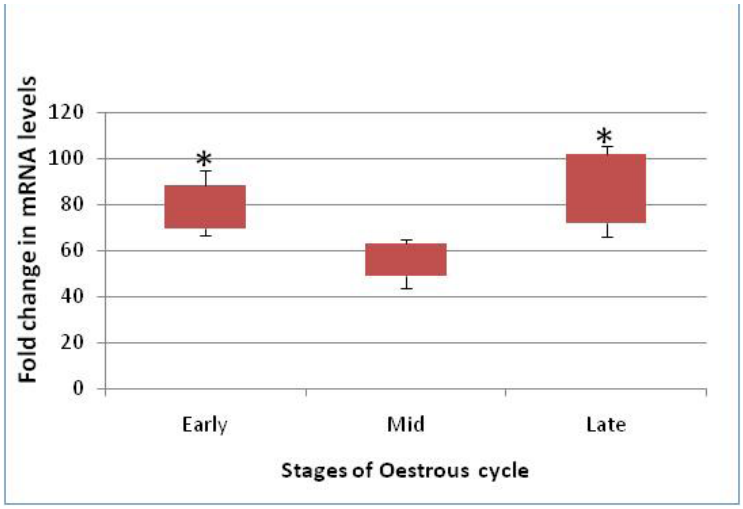

Figure 1. Expression of Hsp 70 mRNA in buffalo oviducts during different stages of the oestrous cycle.

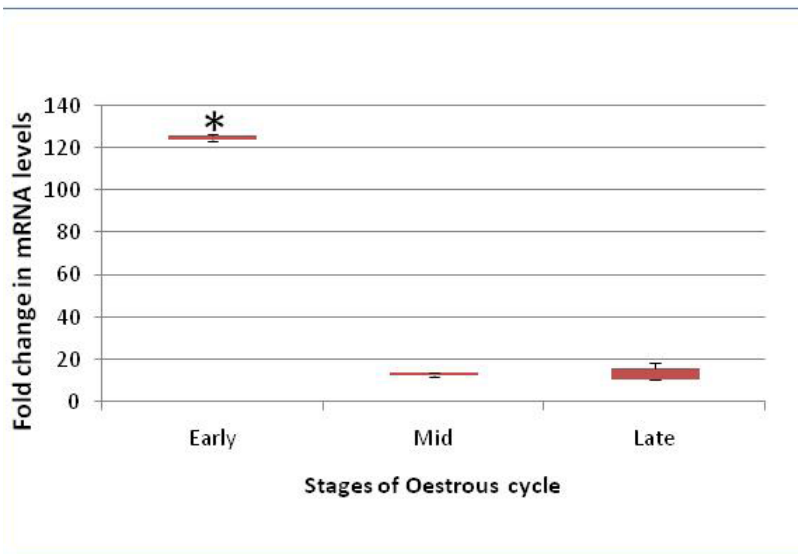

Figure 2. Expression of OVGP mRNA in buffalo oviducts during different stages of the oestrous cycle.

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