Relative expression of oxytocin receptor gene in buffalo endometrium in late luteal phase and pregnancy stages

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Abstract

Molecular level information related to buffalo (Bubalus bubalis) reproduction and related genes is not present at appropriate level. If such exploration is made in the form of comparison between expression of genes is made between non-pregnant and pregnant phase, it may be helpful to aid manipulate the reproduction. Hence, the present study was carried out to reveal mRNA quantitative real time expression of oxytocin receptor (OTR) mRNA. IFN-τ is considered as the substance of maternal recognition of pregnancy and shut down the probable mechanisms which lead to luteolysis. Such mechanism includes shutting down of OTR. Therefore, relative expression of OTR was studied in endometrial tissue of three groups. The groups were non-pregnant late luteal phase, pregnancy stage I (pregnancy of <42 days) and pregnancy stage II (>42 days of pregnancy). With designed primer and GAPDH as house-keeping gene, relative mRNA expression was measured in Real-time PCR. After statistical analysis of results, the gene found to be expressed in all three stages with non-significant difference.

Introduction

In Bovines, 60–70% of pregnancy losses take place in first three weeks of the pregnancy, the period of pregnancy recognition (Sreenan & Diskin 1983). Simultaneously, many factors have been found to be involved in pregnancy success, viz communication established between embryonic and maternal system, cessation of luteal regression and responsible mechanisms, the immune response favourable for pregnancy and preparation of the uterus for implantation (Wang & Dey 2006). Failure of manner of coordination between mechanisms behind the phenomenon of pregnancy even may lead to termination of pregnancy. In cattle, corpus luteum (CL) of pregnancy produces more Prostaglandin E2 (PGE2) (Weems et al. 1998) and less oxytocin (OT) (Wathes et al. 1984) than the CL of the cycle. Prostaglandin F2α (PGF2α), a luteolysin in buffalo as in cattle (Skarzynski et al. 2003) and sheep (Jenkin 1991), is responsible for luteal regression and commencement of next oestrous cycle (McCracken et al. 2012) which is opposite to pregnancy. During luteal regression, episodic pulses of OT secretion become united to the release of PGF2α following synthesis of endometrial OT receptors (OTRs). When pregnancy get established, a signal is sent by conceptus itself in form of interferon-τ (IFN-τ) which ensures maintenance of CL and continued progesterone (P4) production that is must for sustaining pregnancy (Sakumoto et al. 2014). In both sheep and cattle, IFN-τ prevents up-regulation of OTR expression in the endometrial luminal epithelium and superficial glandular epithelium (Dorniak et al. 2012) as well as development of the pulsatile pattern of PGF2α release needed to attain luteolysis (Wathes & Lamming 1994). It can be seen that in order to prevent embryonic loss and to withstand the pregnancy, the comparative expression of single gene may contribute which is also found in case of expression of OTR. Hence, the importance of relative expression of OTR is noticeable during oestrous cycle and pregnancy. By keeping all these facts, the present study was directed to study relative expression of OTR in endometrial tissue of non-pregnant cyclic (late luteal phase) and pregnant animals. Preceding to the follicular phase in cyclic animals, late luteal phase lies, which is common in pregnant and cyclic animals. While in pregnancy stages, two different stages were chosen namely, pregnancy stage I (<42 days of pregnancy) and pregnancy stage II (>42 days of pregnancy). The intention was to reveal the relative expression of OTR between three chosen physiological conditions of reproduction in buffalo.

Material and methods

Experimental animals

Apparently healthy female buffalo reproductive tracts (n = 6 for each group) were procured from the abattoir located in Bareilly, Uttar Pradesh, India immediately after slaughter under aseptic conditions and transported to laboratory on ice. External and internal characteristics of the CL, endometrium and cervix were examined (Arosh et al. 2002) for non-pregnant late
luteal stage. For predicting the stage of pregnancy, pregnant samples were brought and processed immediately. The foetus with foetal membrane was carefully removed and on the basis of crown rump/crown vertebral rump length, approximately days of pregnancy was decided (Assis Neto et al. 2010).

**Collection of tissue samples**

Uteri were dissected from the surrounding tissues, washed with DEPC-treated phosphate buffered saline, and cut open on their longitudinal axis along the greater curvature. Approximately 100 mg endometrium tissue was collected in 1 ml of RNA later and kept at −20°C until use.

**RNA isolation and first strand cDNA synthesis**

Total RNA isolated from all stored endometrial samples with QIAGEN RNeasy plus mini kit followed by checking the quality and concentration of isolated RNA by spectrophotometric and electrophoretic analysis. After DNase treatment (Fermentas), approximately 2 μg of RNA was reverse transcribed to first strand cDNA by high-capacity RNA-to-cDNA kit (Applied Biosystems by Life Technologies, USA).

**Primer designing and standardization**

Gene specific pairs of primers were designed for OTR and GAPDH gene from IDT. The detail of primers is given in Table 1. Prepared cDNA was then standardized to decide annealing temperature (Table 1) with PCR followed by checking of amplified product on 2% gel electrophoresis.

**Real-time PCR**

The expression study was carried out by quantitative Real-time PCR (qRT-PCR) (Step One Plus by Applied Biosystems®, USA) by using standardized gene specific primers (Table 1) in triplicate. The amplification was carried out in 10 µL reaction volume using Fast SYBR Green qPCR Master mix and following thermal profile of the same. Negative and positive controls were included for the qPCR assay. For each sample, a dissociation curve was generated after completion of amplification and analysed in comparison to negative and positive controls, to determine specificity of PCR reaction.

**Quantification of gene expression and statistical analysis**

*C* values of gene were normalized with that of GAPDH to obtain $\Delta C_T$ followed by calculation of $\Delta\Delta C_T$ by keeping non-pregnant late luteal stage as calibrator. Fold change was calculated (Livak & Schmittgen 2001) and Log2 fold change was presented graphically. GraphPad PRISM v.5.0 software (GraphPad Software, Inc., San Diego, CA) was used to perform the statistical analysis. Kruskal–Wallis non parametric test was performed to find the significant differences between $\Delta C_T$ of groups. The confidence level was set at 95%. Dunn’s multiple comparison test was chosen for the pairwise comparisons. Significance threshold was set at *p*-value <.05.

## Results

### Sample collection

Total 18 samples, *n* = 6 from each group were collected. In pregnancy stages, samples from pregnancy stage I were in range of 28–38 days and pregnancy stage II samples were from 48–56 days of pregnancy which was decided on the basis of foetal crown rump/crown vertebral rump length measurement.

**Relative expression of OTR mRNA in endometrium during oestrous cycle and pregnancy**

The amplification plot of targeted region and their dissociation curve is given in Figures 1 and 2. A single dissociation curve peak indicates absence of any non-specific amplification at annealing temperature used in analysis. qRT-PCR analysis did not reveal significant differences in OTR gene expression in buffalo endometrium between non-pregnant stage, pregnant stage I and pregnant stage II. Relative expression of targeted gene between selected groups (*p* > .05) is shown in Figure 3. The gene has been found up-regulated in pregnant stages but with non-significant difference.

### Discussion

During oestrous cycle and among different phases, the OTR mRNA expression is found highest prior and a few days after the luteolysis, that is, around 17 days of previous cycle up to 5 days of the next cycle (Jenner et al. 1991; Robinson et al. 2001). During rest of the oestrous cycle, it remains at a comparatively lower level and even undetectable between days 10 and 15 in the epithelium (Robinson et al. 2001). The stage taken in this study as a control for comparison is the late luteal phase of oestrous cycle, that is, from 11 to 16 days of oestrous cycle and is the period in which OTR are at the lowest concentration. OT creates its effect positively on PGF$_{2\alpha}$ and its pulsatile release suggesting its role in luteolysis (Spencer & Bazer 1996). The mRNA expression has been found down regulated in the window of maternal recognition of pregnancy in various species in vivo and in vitro (Salamonsen & Findlay 1990; Raw et al. 1995; Sharp et al. 1997; Kimmins & MacLaren 2001; Bazer et al. 2008). At the same time, in bovine endometrial

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**Table 1.** Sequence of designed primers of OTR and GAPDH.

| Gene  | Primer sequence                      | Amplicon length | Annealing temperature |
|-------|--------------------------------------|-----------------|-----------------------|
| OTR   | F: 5′-CCTGGATCTACATGTCTTCAC-3′        | 134 bp          | 60°C                  |
|       | R: 5′-GTGGGCCAGGTTGCTCCTTT-3′         |                 |                       |
| GAPDH | F: 5′-TGACCCCCCTCATTGACCCCTCTTC-3′   | 143 bp          | 60°C                  |
|       | R: 5′-GATCTCGCTCTCCTGGAAGATG-3′       |                 |                       |

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epithelial cell line, it has been also demonstrated that OT under the effect of IFN-τ, suppressed PGF$_{2\alpha}$ accumulation without modulating OTR expression (Krishnaswamy et al. 2009) suggesting the presence of alternative or supportive action of OT on the PGF$_{2\alpha}$ and luteolysis along with mediation through OTR. In ewes, it has been hypothesized that the inhibitory action of IFN-τ on OTR up-regulation is achieved indirectly by inhibiting the up-regulation of oestrogen receptor α (Lamming et al. 1995, Spencer et al. 1995).

In the current study, the samples from early pregnancy are ranging approximately from 28 to 38 days, which includes post-maternal recognition stage along with lowered IFN-τ concentration. This may be the reason for not getting the significant OTR down-regulation. Simultaneously, being hormone of pregnancy, P4 remains elevated in pregnancy. This may have effects on the conformation of receptor as illustrated in one
study that P4 can influence the cholesterol in cell membrane and resulting in conformational changes in OTR, resulting inhibition of OT action (Gimpl & Fahrenholz 2002). In conclusion, the results of OTR relative expression showed non-significant difference which is different than that of earlier reported in bovines. It may suggest the presence of alternative or supportive action of OT on luteolysis along with OTR.

Disclosure statement
No potential conflict of interest was reported by the authors.

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