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Differential expression of ten candidate genes regulating prostaglandin action in reproductive tissues of buffalo during estrous cycle and pregnancy

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ABSTRACT

Prostaglandins (PGs) are the key mediators of several female reproductive functions, including luteolysis, ovulation, fertilization, implantation, pregnancy, and parturition. The present study was conducted in buffalo endometrial and luteal tissues between nonpregnant and two stages of pregnancy (29–38 days of pregnancy, 48–56 days of pregnancy) tissue samples. The genes involved from synthesis upto receptor level effect of PGs ($PGF_{2\alpha}$ and PGE_2) were studied for their relative mRNA expression. We have collected the endometrial and luteal tissues from slaughtered animals and confirmed the stages by external examination and crown vertebral rump length measurement of the foetus. The mRNA expression of COX-2 and PGFS genes revealed high significant rise in the transcript at pregnancy stage I as compared to the late luteal phase of nonpregnant. However, EP2 and EP3 genes were highly upregulated in pregnancy stage II. The expression of PLA2G4A and PGT genes showed difference in their transcripts in pregnancy, however, the difference was nonsignificant as compared to the nonpregnant stage. The findings emerged from this study also suggested the strict regulation at COX-2 mRNA level than at synthase enzyme's level. Among the four subtypes of EP gene, we have observed highly significant expression difference in EP2 followed by EP3 after implantation.

1. Introduction

As far as the milk production of buffalo is concerned, among the Asian countries, India alone contributes 95% of buffalo milk and about 63% of total world buffalo milk [23]. To get the maximum return from livestock farming, optimum fertility of animals is necessary to be looked upon as production is indirectly related with reproductive traits. In the dairy cow, it has been shown that a negative genetic correlation exists between milk production and reproduction traits. Selection is based mainly on milk production for many years which may be responsible for most of the decline in fertility in this species. Use of a more balanced index and of genomic selection will allow us to inverse this negative trend [10]. It is estimated that around 18–40% of cattle and buffaloes are culled per annum due to infertility or sterility in India [25]. Fertility is considered as a multi-factorial, low heritable trait and its deterioration has been caused by the complex interactions of a network of genetic, environmental and managemental factors, making it difficult to conclude the exact reason for such decline [63]. Moreover, since 1980s, the early embryonic mortality has been considered as the major cause for repeat breeding in animals [34]. Understanding reproductive biology deeply and that to regarding different reproductive phases may open the gateways to manipulate the reproduction in beneficial way.

Pregnancy should be considered as the most important to be targeted to understand its phenomenon at molecular level. Endometrium plays an important role in the elongation of conceptus as well as implantation and nourishment of the developing embryo. The elongating conceptus secretes copious amounts of interferon-τ

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(IFN-τ), which acts on the uterine luminal epithelium to suppress the transcription of estrogen receptor α (ESR1) and oxytocin receptor (OTR) genes [11] thereby inhibiting luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) resulting in pregnancy maintenance.

PGs are involved in several female reproductive functions, including luteolysis, ovulation, fertilization, implantation, pregnancy, and parturition [36]. Improper production and action of PGs lead to multiple deficiencies in the reproductive processes [33]. Mechanism of PG synthesis, metabolism and cell signaling is complex and is regulated through several enzymatic pathways. $PGF_{2\alpha}$ is well-known for its role of luteolysis, whereas another PG, PGE_2 has an important role in the maintenance of pregnancy. PGE_2 is weakly luteotropic due to its ability to stimulate progesterone production from luteal steroidogenic cells [1,28,64], mitogenesis, angiogenesis, anti-apoptosis and vasodialation [2,3]. One school of thoughts postulate that IFN-τ is proposed to alter the ratio of PGE_2:$PGF_{2\alpha}$ in the favour of former [40]. PG biosynthesis is dependent on arachidonic acid release from membrane phospholipids catalyzed by phospholipase A2 (PLA2) enzymes [14]. The first step of PG production is catalyzed by the enzyme cyclo-oxygenase (COX), which converts arachidonic acid to the unstable PGG_2 and finally is converted to PGH_2 by peroxides [42]. PGES and PGFS are terminal PG synthases, which exhibit tissue specific distribution and convert PGH_2 into PGE_2 and $PGF_{2\alpha}$, respectively [54]. After synthesis, PGs need to get released out of the endometrial cell and enter the blood circulation to reach the ovarian tissue for their action. Prostaglandin transporter (PGT) [9] mediates both efflux and influx of $PGF_{2\alpha}$ and PGE_2 [8,44,45]. The ultimate action of these PGs is produced by binding to their respective receptors. Prostaglandin E receptor (EP) protein encoded by the gene PTGER/EP is a member of the G-protein coupled receptor family having four subtypes found in the bovine [2] and ovine [31]. Prostaglandin $F_{2\alpha}$ receptor (FP) encoded by the gene PTGFR/FP is also a member of the G-protein coupled receptor family. These receptors are subjected to modulations at various reproductive stages by hormonal and other stimuli.

Therefore, the comparative study of mRNA expression of genes related to PG synthesis, transport and effector pathway in different reproductive stages may elucidate the regulation of PG signaling in buffalo reproduction. In view of the above facts, the study was conducted to study the relative expression of the genes involved in PG synthesis and transport (PLA2G4A, COX-2, PGFS, PGES and PGT in endometrium) and for action (FP and EP in luteal tissue) in nonpregnant and pregnant buffalo reproductive tissues.

2. Material and methods

2.1. Experimental material and grouping of samples

Apparently healthy female Murrah buffalo reproductive tracts were brought from the abattoir located in Bareilly, Uttar Pradesh, India immediately after slaughter under aseptic conditions and transported to the laboratory on ice. From the total number of samples (n = 18), six were from late luteal stage of estrous cycle which were confirmed on the basis of their colour, vasculature, size and consistency of corpus luteum [4,59,65] and 12 samples were from pregnancy stages which were further subdivided into two stages on the basis of measurement of crown vertebral length of foetus [7,19,41]. Among pregnancy samples, five belonged to pregnancy stage I (29–38 days of pregnancy) and seven samples were from pregnancy stage II (48–56 days of pregnancy).

2.2. Collection of tissues for mRNA expression study and IFN-τ ELISA

Uteri were dissected, washed with DEPC-treated ice cold physiological saline, and cut open on their longitudinal axis along the greater curvature. The intercaruncular endometrial tissue from nonpregnant and gravid uterus along with luteal tissues were collected, washed with ice cold physiological saline and stored in RNAlater at −20 °C.

For Bovine IFN-τ ELISA, approximately 200–300 mg of uterine endometrial tissue was collected and immediately frozen by immersing in liquid nitrogen and stored at −80 °C.IFN-τ measurement in collected tissue samples was carried out by Bovine IFN-τ ELISA kit (CUSABIO, Code: CSB-E16948B, US, Sensitivity: 1.56 pg/ml) as per manufacturer's protocol. The assay employs the quantitative sandwich enzyme immunoassay technique. A standard curve was obtained by plotting standard's OD values in the abscissa and concentrations in the ordinate. The concentration of test samples were obtained by extrapolating the OD values against the concentrations.

2.3. Total RNA isolation and first strand cDNA synthesis

Total RNA was isolated from the endometrium and luteal tissues using the RNeasy Plus Mini Kit (74106, QIAGEN) according to manufacturer's instruction. The isolated RNA samples were treated with DNA free DNase treatment and removal reagents (EN0521, Fermentas Inc.). The quality and quantity of RNA was determined by gel electrophoresis and Nanodrop spectrophotometer at 260 and 280 nm wavelengths. First strand cDNA was reverse transcribed from approximately 2 μg total RNA using oligo (dT) primer with High Capacity RNA-to-cDNA Kit (Applied Biosystems 4387406) in a final volume of 20 μL according to manufacturer's instruction. The resulting cDNA was diluted at 10 ng/μL for quantitative reverse transcription-PCR (qRT-PCR) analysis.

2.4. Gene specific primers for qRT-PCR and optimization of PCR reaction

Ten sets of gene specific primers (PLA2G4A, COX-2, PGES, PGFS, PGT, FP, EP1, EP2, EP3 and EP4) were designed using Integrated DNA Technologies (IDT). The details of primers are given in Table 1. *GoTaq* master mix (2×) was used for PCR reaction and was carried out in a volume of 25 μL in a 250 μL PCR tube to validate the primers. In brief, PCR was carried out in a thermocycler (Applied Biosystems, USA) and the protocol was standardized at different annealing temperatures and time combinations for amplification of a specific product. Amplified products were checked by agarose gel electrophoresis and annealing temperature was decided.

2.5. Quantitative real time PCR (qRT-PCR)

The expression studies were carried out by qRT-PCR (StepOnePlus™ Applied Biosystems, USA) in 10 μL volume reaction using Fast SYBR Green qRT-PCR Master mix (Applied Biosystems Inc.) with the following cycle profile: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 58–60 °C for 30 s. The detection of single product was verified by dissociation curve analysis. To confirm that genomic DNA contamination was not contributing to specific cDNA amplification, random samples were analyzed in the absence of AMV reverse transcriptase. Nontemplate control was always included in each run. qRT-PCR products were again checked and