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Term placenta shows methylation independent down regulation of *Cyp19* gene in animals with retained fetal membranes

Sandeep Ghai [a], Rachna Monga [a], T.K. Mohanty [b], M.S. Chauhan [c], Dheer Singh [a,*]

[a] Molecular Endocrinology Laboratory, Animal Biochemistry Division, National Dairy Research Institute, Karnal-132001, Haryana, India
[b] Livestock Production and Management Division, National Dairy Research Institute, Karnal-132001, Haryana, India
[c] Animal Biotechnology Centre, National Dairy Research Institute, Karnal-132001, Haryana, India

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ABSTRACT

Retention of fetal membranes (RFM) is the major post-partum disorder in dairy cattle. *Cyp19* gene encodes the aromatase enzyme responsible for catalyzing the rate limiting step in estrogen biosynthesis, an important hormone for placental maturation and expulsion. The present study was aimed for comparative analysis of *Cyp19* gene expression and its epigenetic regulation in placental cotyledons of animals with and without RFM. Significantly lower expression of *Cyp19* gene was found in placental samples of RFM affected animals in comparison to normal animals. Methylation analysis of 5 CpG dinucleotides of placenta specific *Cyp19* gene promoter I.1 and proximal promoter, PII showed hypo-methylation of both PI.1 and PII in term placenta of normal and diseased animals. In conclusion, a mechanism other than promoter methylation is responsible for decreased aromatase expression in placental cotyledons of animals suffering from RFM.

1. Introduction

Post parturition reproductive diseases are a serious problem in dairy cattle. These diseases not only lower productivity and fertility but they also bring economic loss. It is therefore, important to detect post-partum reproductive disorders as early as possible, and to develop prophylactic measures to prevent these disorders. One of the most important post-partum diseases in dairy cattle is retention of fetal membranes (RFM) (Stephen, 2008; El-Wishy, 2007). In cattle, fetal membranes are expelled within 6–12 h after calving. The failure to push out all or a part of the placenta from the uterus within 12 h of calving leads to RFM (Drillich et al., 2006). In dairy cattle, 4–11% of spontaneous calvings have been reported to result in RFM (Hashem and Hussein, 2009) which is comparatively higher in buffalo (Arthur et al., 1989; Laven and Peters, 1996; Ahmed et al., 2009). A wide variation (2.89–12.23%) has been reported in buffaloes with a maximum at the fifth parity (30%) and associated with malnutrition (Choudhury et al., 1993; Gupta et al., 1999). Surprisingly, 54% of Iraqi buffaloes studied developed RFM (Azawi et al., 2008) while the incidence of RFM in buffalo in India is 21% (Satya pal, 2003).

A failure in the separation of cotyledonary villi from the crypts of the maternal caruncles results in RFM (Weithril, 1965). The etiology of this disorder however, is yet to be completely understood. Complex metabolic disturbances during the pre-partum period have been proposed to be the probable reason of RFM (Michal et al., 2006). It has been suggested that a surge in estrogen concentration in bovine maternal blood (Robertson, 1974; Peterson et al., 1975; Hoffmann et al., 1976; Hunter et al., 1977) and placenta (Veenhuizen et al., 1960; Inaba et al., 1983) before parturition is important in normal parturition and placental expulsion. It is generally recognized that estrogens play an important role in the maturation process of the placentomes (Grunert et al., 1989). Cows and buffaloes with RFM have been observed to possess higher levels of progesterone and lower levels of estradiol 17-β compared to normal animals (Matton et al., 1987; Thomas et al., 1992; Kankofer et al., 1996; Hashem and Hussein, 2009; Ali et al., 2009). Moreover, it has been shown that in cattle and goats, low levels of estrogens and the reduction or absence of the estrogen peak near delivery are associated with abortion, dystocia and placental retention (Engeland et al., 1999; Zhang et al., 1999a,b). Therefore, down-regulation of estradiol is supposed to be an important factor responsible for RFM.

The Cytochrome P450 aromatase enzyme encoded by the *Cyp19* gene has been found to play an important role in converting progesterone to estrogen in the placenta (Flint et al., 1975; Mason et al., 1989; Nelson et al., 1996). A change in the estradiol levels in animals affected with RFM thus might be due to the change in expression levels of *Cyp19* gene. However, there is no report of the expression status of *Cyp19* gene in RFM- affected farm animals (cattle and buffalo). *Cyp19* gene has been found to be down regulated by the promoter switching phenomenon during

* Corresponding author. Tel.: +91 184 2259135; fax: +91 184 2250042.
E-mail addresses: dheer@ndri.res.in, drdheer.singh@gmail.com (D. Singh).

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folliculogenesis in cattle and buffalo (Vanselow et al., 2005; Sharma et al., 2009). Methylation of the proximal promoter region is partly responsible for this promoter switching in ovary (Vanselow et al., 2005; Fürbass et al., 2008) and differential promoter usage in placenta (Vanselow et al., 2008). Recently, we found that DNA methylation is also involved in stage specific *Cyp19* gene expression in buffalo placenta (Ghai et al., 2010). DNA methylation patterns are also found to change in response to environmental stimuli such as diet or toxins due to which the epigenome seems to be most susceptible during early *in utero* development. Aberrant DNA methylation changes have been detected in several diseases, particularly cancer where genome-wide hypo-methylation coincides with gene-specific hypermethylation (Tost, 2009). Therefore, the role of promoter methylation linked down regulation of the *Cyp19* gene in animals with RFM cannot be ignored.

The decrease in estradiol levels in placental cotyledons of animals affected with RFM may probably be due to the down regulation of *Cyp19* gene which in turn might be regulated by promoter methylation. In view of the above points, the present study was focused on determining the *Cyp19* gene expression in normal and RFM affected buffalo which is major milk yielding animal in India and contributes more than 60% of country's milk production. Further studies were done to understand the relationship between *Cyp19* gene expression and promoter methylation levels in buffaloes affected with RFM.

2. Materials and methods

2.1. Collection of placental tissues

Placenta of the animals which expelled their fetal membranes naturally within 12 h of parturition was considered as normal placenta. The animals which did not expel their fetal membranes naturally but the placenta that was ejected manually after oxytocin injection was considered as retained placenta. Tissue samples ($n = 3$) were collected from NDRI cattle yard immediately after expulsion. The samples were washed with chilled normal saline containing antibiotics to remove contamination and blood and then brought to the laboratory as early as possible (10–15 min). The samples to be used for RNA isolation were immediately kept in RNA*later*® (Qiagen, GmbH, Germany) till further processing.

2.2. Separation of placental cotyledons and isolation of DNA and RNA

The buffalo's placental cotyledons were excised and removed with the help of sterilized scissors and forceps. The appropriate amount of tissue (100 mg) required for DNA and RNA isolation was chopped into pieces and homogenized into DNAzol® and TRIzol® (Life Technologies (India) Pvt. Ltd.), respectively. Total RNA was isolated by modified TRIzol® method (Chomczynski and Sacchi, 1987) and DNA was isolated using DNAzol® reagent following manufacturer's instructions. The DNA and RNA were quantified spectrophotometrically and the respective integrities were evaluated by normal (0.7%) and denaturing (1.5%) agarose gel electrophoresis.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The cDNA was synthesized using a Fermentas First strand cDNA synthesis kit (Fermentas, Germany). The reaction mixture contained 2 μg of total RNA, 1 μl of random hexamers (0.2 μg/μl) and DEPC treated water up to 11 μl. The contents were incubated at 65 °C for 10 min followed by 2 min incubation at room temperature. The reagents added subsequently were: 4 μl of 5× reaction buffer (250 mM Tris–HCl, pH 8.3; 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 μl of RNase inhibitor (20 IU), 2 μl of dNTP mix (10 mM), 2 μl of M-MuLV reverse transcriptase (200 IU) to a final volume of 20 μl. The contents were incubated at 25 °C for 10 min, 42 °C for 30 min and 95 °C for 3 min.

The cDNA was amplified with gene specific primers (Table 1) in a reaction mixture containing 2 μl of RT product, 0.2 μM primers (gene-specific forward and reverse primers), 1× PCR buffer [10 mM Tris HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin], 0.2 mM dNTP mix, 1 U *Taq* polymerase (1 U/μl) made to 50 μl with nuclease free water. The amplification was done in a thermocycler under two different cycle conditions. For *Cyp19* gene expression, two-step PCR was performed as nested PCR. For the first amplification, 2 μl of cDNA was used as template, for the second amplification, 5 μl of PCR product from the first reaction was used. PCR reactions were performed by incubating the contents at 94 °C for 2 min, followed by 32 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and the final extension of 4 min at 72 °C. The GAPDH was used as a housekeeping gene.

2.4. Real-time PCR

For mRNA expression analysis by quantitative real-time PCR, SYBR green dye-based expression assays were performed for *Cyp19* gene in normal and retained placental tissues. The reaction mixture containing 2× SYBR® Universal PCR Master Mix (Fermentas), 200 nM gene specific primers (CYPF and CYPR), Table 1 and 2 μl of cDNA to a final volume of 10 μl was incubated in the M J MiniCycler (BioRad). The reaction conditions used were 94 °C for 2 min, followed by 32 cycles of 94 °C for 20 s, 57 °C for 15 s, 72 °C for 15 s. and the final extension of 4 min at 72 °C. GAPDH was used as internal control. *Cyp19* and GAPDH expressions were determined by measuring PCR product fluorescence compared to cycle number to determine CT values. Relative *Cyp19* expression for each tissue sample was calculated using the formula:

$$\Delta CT = CT_{Cyp19} - CT_{GAPDH}$$

The fold change in *Cyp19* gene expression ($\Delta Cyp19$) between normal and RFM animals was calculated with the formula

$$(\Delta Cyp19) = 2^{-(\Delta CT_{Normal} \Delta CT_{RFM})}$$

2.5. Bisulfite modification of DNA

Genomic DNA (1 μg) isolated from placental cotyledons of animals with normal and retained fetal membrane was bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA). The procedure is based on the method developed by Frommer et al. (1992). It is based on the principle that treatment of single stranded DNA with sodium bisulfite under acidic conditions changes all the cytosines in the DNA to uracil while 5′-methyl cytosines resist this change. Amplification of the bisulfite-treated DNA by PCR followed by sequencing reveals the positions of 5-methylcytosine in the gene. Bisulfite-treated DNA was eluted in 10 μl volumes of elution buffer with 4 μl used for each PCR. The frequency of methylation at 5 individual CpG sites within the 363-bp and 340-bp region of respective PI.1, the distal promoter responsible for placenta specific *Cyp19* gene expression and PII, the proximal promoter responsible for ovary specific *Cyp19* gene expression was assessed using bisulfite-specific sequencing. Bisulfite sequencing PCR (BSP) primer pairs specific for modified DNAs were designed to contain no CpG sites. The Methyl Primer Express software® v1.0 (Applied Biosystems) was used to design primers specific for BSP (Table 2) and positions of the CpG residues to be analyzed were also located using the same.