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Suppression of COX-2 mRNA abundance in *in vitro* cultured goat (*Capra hircus)* endometrial cells by RNA interference and effect on PGF2-α and PGE2 concentrations

Arul Suresh[a], I.J. Reddy[b,*], Ashish Mishra[b], S. Mondal[b]

[a] Animal Physiology Division, ICAR-National Dairy Research Institute, SRS, Bengaluru, Karnataka 560030, India
[b] Animal Physiology Division, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560030, India

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ABSTRACT

Cyclooxygenase-2 (COX-2) has important functions in the synthesis and release of endometrial prostaglandin F2α (PGF2α). Excessive production of COX-2 leads to an increase in endometrial PGF2α synthesis and subsequently causes luteolysis and early embryonic mortality. The aim of this study was to investigate in goats the effects of COX-2 small interference RNA (siRNA) on COX-2 mRNA abundance and the secretion of PGF2α and PGE2 in goat endometrial cells. Endometrial cells isolated from goat uteri were cultured at 38.5 °C and 5% CO_2. The cells were treated with different concentrations (0, 10, 25, 50, 100, 250, 500, 750 and 1000 nM per well) of three different COX-2 siRNAs at confluency for 24 h. At 24 h post culture, COX-2 mRNA abundance was quantified using qPCR and PGF2α and PGE2 concentrations were quantified in the culture medium. There was a lesser relative abundance of COX-2 mRNA in endometrial cells at 100 to 1000 nM siRNA. The greatest extent of abundance suppression, however, was observed with 1000 nM siRNA. Transfection of COX-2 siRNA (1000 nM) to endometrial cells suppressed the COX-2 mRNA abundance by 77%, 82%, and 84% with siRNA 1, 2, 3, respectively. Furthermore, with COX-2 siRNA transfected cells, there was a lesser ($P < 0.05$) PGF2α concentration than in cells not transfected, whereas PGE_2 secretion was not affected. The results of the study provide evidence that COX-2 siRNA used in this study suppresses COX-2 mRNA abundance and PGF2α secretion but there was no association between PGE2 concentrations and COX-2 mRNA abundance in goat endometrial epithelial cells.

1. Introduction

Prostaglandins (PGs) have important functions in ovulation, recognition of pregnancy, luteolysis, implantation, decidualization and parturition in ruminants (Poyser, 1983; Dubois et al., 1998). The production of PGs is primarily catalysed by two rate limiting enzymes [i.e., cyclooxygenases -1 and -2 (COX-1 and COX-2)], referred also to as prostaglandin endoperoxidase H synthases -1 and -2 (PGHS-1 and PGHS-2), respectively. The PGHS-1 and PGHS-2 compounds are responsible for the conversion of arachidonic acid into PGG_2 and PGG_2 to PGH_2, respectively. The downstream enzymes in the pathway, prostaglandin E synthase (PGES) and prostaglandin F synthase (PGFS), catalyse the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively. There are functions of $PGF_{2\alpha}$ as the luteolytic agent (Bazer et al., 1994) that has important regulatory actions during the estrous cycle in ruminants. In contrast, PGE_2 functions to inhibit spontaneous regression of the corpus luteum (CL; Magness et al., 1981). One function of PGE_2 is to enhance endometrial

* Corresponding author.
E-mail address: reddyij123@gmail.com (I.J. Reddy).

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vascular permeability at the sites of blastocyst apposition and decidualization (Kennedy and Lukash, 1982; Keys and Kennedy, 1990) and PGE_2 also has a primary function in blastocyst hatching (Baskar et al., 1981) and implantation (Holmes and Gordashko, 1980). The importance of PGs has been confirmed in mice where targeted disruption of COX-1 (Langenbach et al., 1995) or COX-2 genes (Morham et al., 1995) induced multiple dysfunctions in female reproductive processes. In most mammalian species, $PGF_{2\alpha}$ and PGE_2 are universally important in the regulation of endometrial function (Narumiya and FitzGerald, 2001). Relatively little is known about the biosynthetic pathways leading to the formation of PGE_2 and $PGF_{2\alpha}$. Results from studies with COX-1 and COX-2 knockout mice provided evidence that COX-2, but not COX-1, is important for normal ovulation, implantation, and decidualization. The expression of the COX-2 gene is modulated in response to various stimuli and rate of gene transcription is highly correlated with PG production during luteolysis and maternal recognition of pregnancy (Burns et al., 1997; Charpigny et al., 1997). The extent of COX-2 effects on the regulation of PGF2α, and luteolytic pulse releases in goats may differ from those of other ruminants (McCracken et al., 1999; Weems et al., 2006; Niswender et al., 2000; Dávila, 2017). Results indicate that the functions of COX-2 are inhibited by aspirin and indomethacin, which inhibit both actions of COX-1 and COX-2 (Mitchell et al., 1994; Bala et al., 2008). A different research approach, however, is required to specifically inhibit the abundance of COX-2 mRNA. An experimental approach that would suppress the abundance of COX-2 RNA by utilizing interference (RNAi) may be effective for gaining a thorough understanding of prostaglandin production regulation by COX-2. Post-transcriptional gene silencing using RNAi has been an efficient and promising approach to study gene function. Since its discovery in studies with C. *elegans* (Fire et al., 1998), the RNAi approach has recently emerged as a highly effective technique in functional genomics studies for silencing gene expression in a highly specific way by the addition of double stranded RNA (Caplen et al., 2001; Kobayashi et al., 2007). Results of earlier studies provided evidence that RNAi is a valid and efficient method to inhibit COX2 gene expression and $PGF_{2\alpha}$ synthesis in cumulous granulosa cells of cattle (Kobayashi et al., 2007). There, however, is no information concerning the use of RNAi for studying gene functions in relation to the regulation of prostaglandin production in goats. It, therefore, was hypothesised that siRNA could be applied to decrease PGF2α and PGE2 transcript abundance in endometrial cells of goats.

2. Materials and methods

2.1. SiRNA design

The mRNA sequence of goat COX-2 was determined in NCBI (Ac. No. JN 793538). To select an efficient interfering RNA sequence and effective dose to knock down COX- 2 gene expression, three siRNAs (siRNA1, siRNA2 and siRNA3; Table 1) targeting different regions of the caprine COX- 2 mRNA with different siRNA concentrations were evaluated. Three specific siRNA for COX-2 were designed using the siRNA design technique (siRNA Target Designer, Promega, USA) based on the mRNA sequence (XM_593336.3) while siRNA was obtained using chemical synthesis procedures (Sigma, USA (Table 1).

2.2. Isolation of endometrial epithelial cells

Goat uteri were collected at a local slaughter house and transported to the laboratory on ice. The stages of estrous cycles were classified according to colour, vasculature size and CL consistency (Miranda-Moura et al., 2010). Uteri from the mid-estrous cycle were used for the isolation of epithelial cells. The uterine lumen was washed three times with sterile Ca^{2+} and Mg^{2++} - free Hanks Balanced Salt Solution (HBSS) supplemented with 100 IU/ml gentamycin and 0.1% BSA. There was 1 to 3 ml of sterile HBSS containing 0.3% trypsin infused into the uterine lumen. The ends of the horn ipsilateral to CL were tied to retain the trypsin solution for solubilizing the epithelial cells that were isolated by incubation at 37 °C for 60 min. The cell suspension obtained from the digestion was filtered through a 70 μM strainer to remove un-dissociated tissue fragments. The filtrate was washed three times with HBSS supplemented with gentamycin and 0.1% BSA by centrifugation at 600 x *g* for 10 min. The cell density of cultured endometrial cells was quantified using an automated cell counter. Briefly, 0.5% trypan blue was added to 0.1 ml of the cells and the number of stained cells in relation to total cells was quantified. Cells which were unstained with trypan blue were viable while blue- stained cells were considered to be non-viable. Cell viability was more than 95% at the time of seeding.

2.3. Determination of the concentration of COX-2 siRNA to suppress COX-2 mRNA abundance

To determine the concentration of COX-2 siRNA to be used for transfection to reduce COX-2 mRNA abundance, three different

Table 1
Locations and sequences of three different siRNAs for caprine COX-2 mRNA and siRNA sequences for targeting caprine COX-2.

Sense/Antisense	siRNA design	Start on Target	Target sequence
s	CCAUUUGGCUGCGGGAACAdTdT	866	CCATTTGGCTGCGGGAACA
a	UGUUCCCGCAGCCAAAUGGdTdT	866	TGTTCCCGCAGCCAAATGG
s	GCUAUCACUUCAAACUGAAdTdT	1019	GCTATCACTTCAAACTGAA
a	UUCAGUUUGAAGUGAUAGCdTdT	1019	TTCAGTTTGAAGTGATAGC
s	CCUGAUAGGAGAAACUAUUdTdT	963	CCTGATAGGAGAAACTATT
a	AAUAGUUUCUCCUAUCAGGdTdT	963	AATAGTTTCTCCTATCAGG

siRNA were designed and used at different concentrations (0, 10, 25, 50, 100, 250, 500, 750 and 1000 nM) mixed with opti-MEM. Endometrial cells were transfected in a six-well plate for 24 h and harvested after 24 h. Furthermore, endometrial cells were centrifuged at 600 x *g* for 10 min to obtain endometrial cell pellets which were used for further assessments of mRNA abundance to determine the extent of COX-2 gene knockdown. In the control group, endometrial cells were incubated with siPort NeoFX without siRNA to monitor cytotoxicity and cell death. This experiment was repeated in triplicate with all three siRNAs.

2.4. Transfection of caprine-cox-2 siRNA into goat endometrial epithelial cells

Epithelial cells were seeded at 9.5×10^5 viable cells at 1640 RPMI, 38.5 °C and 5% CO_2 for 24 h in a CO_2 incubator. About 4 µl of siPORT transfection agent (NeoFX- NeoTnFX agent siPORT, Lipofectamine 2000 Invitrogen, USA) per well was mixed with 96 µl of Opti-MEM (Invitrogen, USA) medium and incubated at room temperature for 10 min. Then 4 µl of siRNA (final concentration 0, 10, 25, 50, 100, 500, 750 and 1000 nM) per well were added to 96 µl of opti-MEM medium. These two solutions were then mixed at a 1:1 ratio and incubated at room temperature for 10 min. About 200 µl per well of transfection complexes were transferred to a six well plate and allowed to incubate for 24 h. With the control treatment, the cells were cultured as previously described without siRNA.

2.5. Quantitative analysis of COX 2 mRNA relative abundance

2.5.1. Total RNA isolation from endometrial cells and cDNA synthesis

Total RNA was isolated from endometrial cells using the RNA Easy Mini kit (Qiagen Netherland) as per the manufacturer's instruction. The isolated RNA was labelled properly and stored at −80 °C until further use. First strand cDNA was synthesized from total RNA using superscript III. Following the manufacturer's instructions, the first strand synthesis system for RT − PCR (Invitrogen, USA) was used (oligodT (50 µm), dNTP (10 mM), RT buffer, mgCl2 (2.5 mM), reverse transcriptase (RT)(200U), and RNAse inhibitor (40U), DTT (0.1 M)] in a 20 µL volume. The synthesized cDNA was stored at −20 °C until it was required for quantifying COX-2 mRNA relative abundances in the endometrial cells in culture. The Step One Plus Real time PCR system (Applied Biosystem, USA) was used to investigate the effect of COX-2siRNA transfection in cultured endometrial cells on the relative abundance of COX-2 mRNA transcript. As the reference gene, β-actin (Ac. No. AY141970) was used in this study. Furthermore, the relative abundances of mRNA transcript for this protein do not vary significantly due to cellular treatment. The qPCR reactions were performed using Fast SYBER Green Master MIX (Applied Bio systems) and gene specific primers for both housekeeping and target genes. Each procedure was performed (in duplicates) in a 25 µL reaction containing 10 µL qPCR master mix, 10 pM gene specific forward and reverse primers, 2 µL cDNA as template and a final volume of 25 µL nuclease - free water. The PCR conditions used to amplify all the genes were initially denatured at 95 °C for 20 s with 40 cycles of denaturation; at 95 °C for 3 s followed by annealing and extension at 60 °C for 1 min. Melting curve analysis was conducted to confirm qPCR specificity. The relative abundance of mRNA transcripts was recorded after normalizing for β- actin abundance using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001) in which the CT value from controls served as the calibrator. The qPCR was conducted three times in three different culture plates.

2.5.2. Confirmation of qPCR amplicons

The qPCR amplicons of targeted genes were confirmed by using 2% agarose gel electrophoresis.

2.6. PGF2α and PGE2 hormone analysis in cultured media

The culture media for each concentration was collected from cell culture plates after 24 h of incubation with COX-2 siRNA. There was quantification of amounts of PGE2 and PGF2α using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol. The $PGF_{2\alpha}$ concentrations were determined in 50 ml aliquots of culture medium after a 10 fold dilution with extraction buffer using the ELISA kits (Neogen, USA). The sensitivity of the assay was 0.002 ng/ml. The cross reactivity of the antisera against 6-keto prostaglandin F_{1a}, 13, 14 dihydro-15 keto-prostaglandin F_{2a}, prostaglandin D_2 and prostaglandin E_2 were 3.05%, 0.05%, 0.05% and < 0.01%, respectively. The intra- and inter-assay coefficients of variation were less than 15%. The concentrations of PGE_2 were determined in 50 ml aliquots of culture medium after there was a five-fold dilution with the extraction buffer. The sensitivity of the assay was 0.002 ng/ml. The cross reactivity of the antisera against 6-keto prostaglandin F_1, 13,14 dihydro-15 keto-prostaglandin F_{2a}, prostaglandin D_2 and prostaglandin $F_{2\alpha}$ was > 0.01%. Intra- and inter-assay coefficients of variation were less than 13%.

2.7. Statistical analysis

Each experiment was conducted in triplicate. The data were expressed as means ± SE. An ANOVA and the Tukey's multiple comparison test (Graph Pad Prism 5 statistical package, USA) were used to analyze the differences in $PGF_{2\alpha,}$ and PGE2 concentrations and transcript abundance following COX-2 gene knock down. The $P < 0.05$ values was considered to be statistically significant (Fig. 1).

3. Results

In the present study, there was assessment of the pattern of relative abundance of COX-2 mRNA transcript in cultured goat

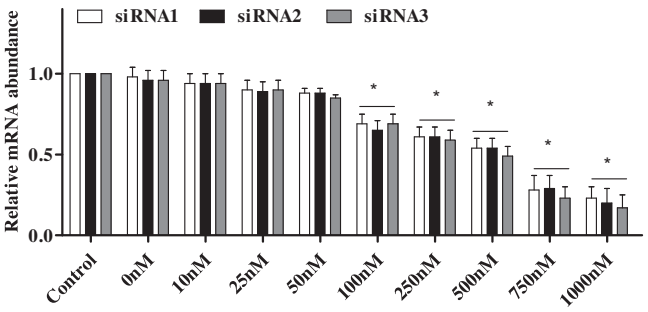


Fig. 1. Relative abundance of COX-2 mRNA transcript in endometrial cells of goats with different caprine COX-2 siRNA concentrations; COX-2 mRNA relative abundances in endometrial cells of goats was relative to β actin mRNA abundance; Experiments were repeated three times; Values represent the mean ± SEM; Concentrations with asterisk * indicate a difference ($P < 0.05$).

endometrial epithelial cells following transfection with different concentrations of caprine COX-2 siRNAs at 0, 10, 25, 50, 100, 250, 500, 750 and 1000 nM. The relative abundances of COX-2 mRNA transcript were not suppressed by transfection of the endometrial cells with 0–50 nM siRNA for 24 h. The relative abundances of COX-2 mRNA transcript, however, was less when there was transfection of the endometrial cells with 100–1000 nM COX-2 siRNA. There were lesser relative abundances of COX-2 mRNA transcript with the siRNA-3 transfection resulting in a greater (77%) suppression of relative abundance than with the siRNA2 (72%) and siRNA1 (71%) transfections at the 750 nM concentration (Fig.2). There was a further decrease (81%) in relative abundance of COX-2 mRNA transcript with the 1000 nM transfections in comparison to the control group. The determination of COX-2 mRNA transcript abundance relative to the abundance of β-actin mRNA transcript using qPCR analysis indicated that relative abundance of COX-2 mRNA decreased by an average of 73% when there was with 750 nM and 81% with 1000 nM transfections with COX-2 siRNAs in the endometrial epithelial cells compared with the mRNA abundance in the control samples.

3.1. Effect of endometrial cell transfection with COX-2 siRNA on PGF2α secretion

The concentration of $PGF_{2\alpha}$, and PGE_2, in culture medium 24 h after the transfection with three different siRNAs was determined by using an enzyme immunoassay. The data for effects of different concentrations of COX-2 siRNA-1, 2 and 3 on $PGF_{2\alpha}$ secretion are depicted in Fig. 2. The transfection of endometrial cells of goats with COX-2 siRNA resulted in a decrease in $PGF_{2\alpha}$ secretion from these cells. Transfection with all three siRNAs resulted in a decrease in $PGF_{2\alpha}$ concentrations when the transfection was at the 500 to 1000 nM concentration in comparison to the control group (Fig. 3). There was a lesser $PGF_{2\alpha}$ concentration after COX-2 siRNA transfection at 500 nM siRNA for 24 h as compared to that of the control sample. There was a further reduction in $PGF_{2\alpha}$ concentrations with the 1000 nM transfection.

3.2. Effect of endometrial cell transfections with cox-2 siRNA on PGE2 secretion

The PGE2 concentrations in the control and siRNA transfected endometrial cells were not affected during the 24 h of culture (Fig. 4).

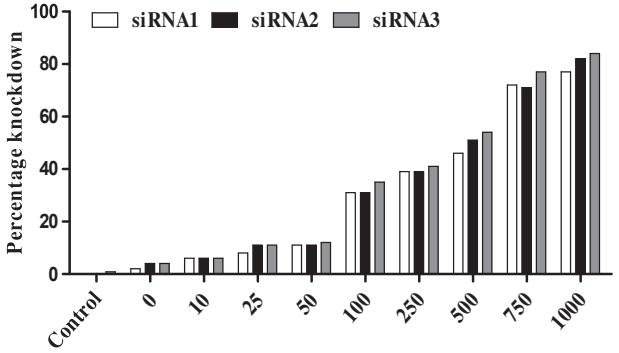


Fig. 2. Effect of different concentrations of COX-2 siRNAs on the relative abundance of COX-2 mRNA transcript' There were lesser relative abundances of COX-2 mRNA transcript with 100 nM of siRNA transfections with the greatest suppression in COX-2 mRNA relative abundances being with the 1000 nM COX-2 siRNA transfection.

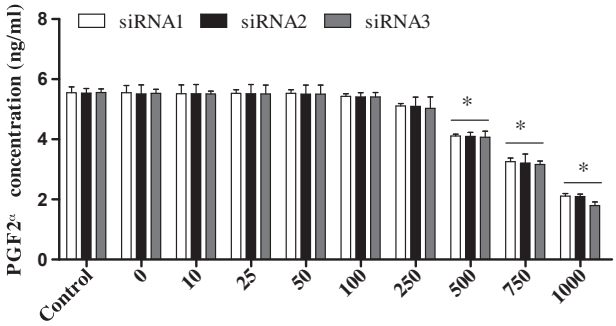


Fig. 3. Effect of different concentrations of COX-2 siRNA transfections on the PGF2α secretion in endometrial cells of goats; Data expressed as mean ± SEM; Experiments were repeated three times.

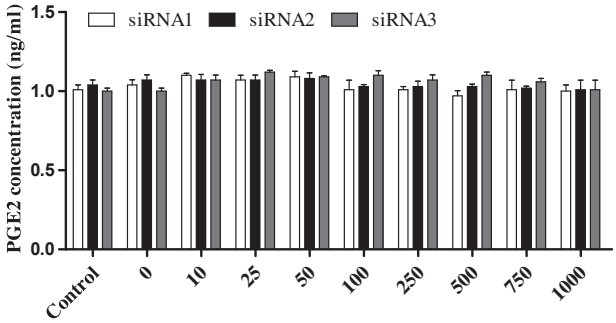


Fig. 4. Effect of different concentrations of COX-2 siRNA transfections on the PGE_2 secretion from endometrial cells of goats; Values represent the mean ± SEM; Differences between the control and treated groups are non-significant ($P > 0.05$).

3.3. Effect of endometrial cell transfections with cox-2 siRNA on abundance of B-actin mRNA transcript

The effect of endometrial cell transfections with COX-2 siRNA, on abundance of β actin mRNA was similar with the three siRNAs. The abundance of β-actin mRNA transcript was unaffected by COX-2 siRNA transfection throughout the experiment for all samples (Fig. 5).

3.4. Effect of cox-2siRNA transfection on morphology of endometrial cells

The morphology of *in vitro* cultured endometrial epithelial cells in control and cox-2 siRNA-treated groups was observed after 24 h of siRNA transfection to study morphological changes in the cultured cells. There, however, were no morphological changes in the cultured endometrial epithelial cells with Cox-2siRNA transfections with the 1000 nM concentration (Fig.6).

4. Discussion

The present study was the first to be conducted regarding the effect of COX-2 siRNA on relative abundances of COX-2 mRNA transcript and prostaglandin secretion in goat endometrial cells. Three different COX-2 siRNAs were designed to study the effects on PGF2α and PGE2 synthesis and relative abundance of COX-2 mRNA transcript. Results of the present study indicate that with transfection of all three siRNAs there was a lesser abundance of COX-2 mRNA transcript when transfections were with or at greater than the 100 nM concentration of siRNA as compared to lesser concentrations of siRNA and the control. The abundance of COX-2 mRNA, however, was not affected at 24 h after transfection of endometrial epithelial cells with 0 or 50 nM of siRNA. With transfection

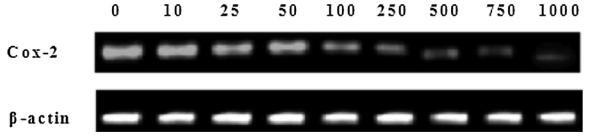
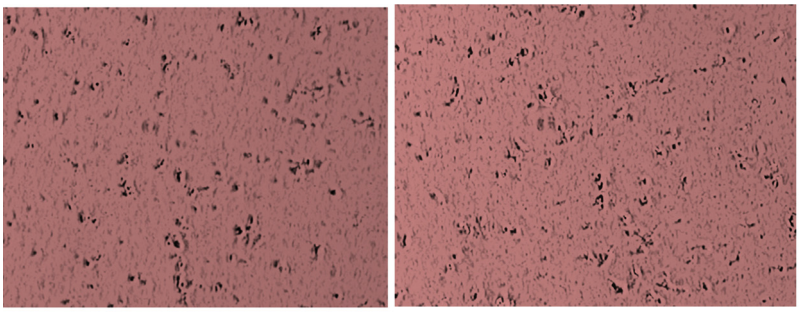


Fig. 5. Effect of different concentrations of caprine COX-2 siRNA-3(nM) transfections on COX -2 mRNA relative abundances in endometrial cells of goats; Specific RT-PCR products for COX -2 mRNA (upper) and β-actin (lower) separated using agarose gel electrophoresis; Concentration at 0 nM indicates the control while concentrations at 10, 25, 50, 100, 250, 500, 750 and 1000 nM indicate the siRNA transfected cells; COX -2 mRNA relative abundances were clearly suppressed at the 100–1000 nM concentration transfections.



a. Cultured endometrial cells without caprine COX-2 siRNA. Magnification:100X

b. Endometrial cells transfected with COX-2-siRNA at 1000nM. Magnification:100X

Fig. 6. Morphological changes in the cultured endometrial epithelial cells when cox-2siRNAs was transfected at the 1000 nM concentration transfection; No morphological changes were observed 24 h after the cox2 siRNA transfections.

of siRNA (0–200 pM) into cumulus granulosa cells of cattle and mammalian hepatic cells, there has been a decrease in targeted gene expression (Hirano et al., 2004; Lambeth et al., 2005; Tonges et al., 2006; Schulze-Bergkamen et al., 2006; Kobayashi et al., 2007). There was no suppression in the abundance of COX-2 mRNA, however, when there was transfection with less than 100 nM siRNA in the present study. This difference in suppressive efficacy might be due to the effective concentration of the siRNA because of the cell type used in the experiment (Kobayashi et al., 2007). With regard to the inhibition of target gene expression by transfection with siRNA, the inhibitory effect on the expression of a target gene by RNAi is known to differ depending on the sequence of the introduced siRNA (Holen et al., 2002; Reddy et al., 2014). Using siRNA design program for the present study, there was, therefore, three different regions of the caprine COX-2 mRNA considered for the design of three different siRNAs that were used to conduct the present study that focused on COX-2 gene expression in primary endometrial cell culture. There was the most effective COX-2 transfection with the siRNA3 sequence, which had greater efficacy than the siRNA2 and siRNA1 sequences in suppressing COX-2 mRNA transcript abundances. Furthermore, the expression and regulation of the COX-2 gene are tissue, sequence and species specific (Arosh et al., 2002, 2004; Kobayashi et al., 2007).

Cyclooxygenase exists in both a constitutive (COX -1) and an inducible form (COX-2) (McCracken et al., 1999; Arosh et al., 2002). The COX-2 gene expression is associated with production of PGF2α while COX -1 gene expression is associated with PGE2α production (Parent et al., 2003). To determine whether the reduction of COX-2 mRNA abundance reflects COX-2 protein abundance or activity, PGF2α and PGE2 concentrations were quantified in the culture medium in the present study. The PGF2α concentrations were not affected at less than the 250 nM transfection with all three siRNAs after 24 h of incubation; however, PGF2α concentration was lesser when there was transfection with 500 nM siRNA as compared with the transfection with 250 nM and the control. The COX-2 siRNA mediated COX-2 mRNA relative abundance and decreased $PGF_{2\alpha}$ concentrations in the medium in the present study and these results are consistent with those in other reports for different species (Lambeth et al., 2005; Tonges et al., 2006; Mondal et al., 2011). A distinct dose dependent change in PGF2α secretion was observed in siRNA transfected cells, with there being lesser PGF2α concentrations with increased concentrations of the siRNA transfections in endometrial cells of goats. The PGE2 concentrations, however, were not affected when there was COX-2 siRNA transfections at any of the concentration used in the present study. In the present study the siRNAs suppressed COX-2 gene expression without affecting PGE2 production, which is mainly regulated by COX-1. These results indicate the siRNA designed in the present study suppresses PGF2α specifically, and that the concentration of PGE2 was not associated with that of PGF2α.

Large concentrations of siRNA can be toxic to cells (Rao et al., 2009), however, in the present study there were no morphological changes when endometrial cells of goats were transfected with largest concentration (1000 nM) of COX-2 siRNA. In the cell culture conditions used in the present study, the suppression of COX-2 mRNA transcript abundances when there were transfections with the greater siRNA concentrations did not affect the endometrial cell morphology compared to non-transfected cells. In ruminants, endometrial epithelial cells are the primary source of PGF2α, whereas stromal cells release more PGE2 (Pepin et al., 2011). There have been reported to be large concentrations of PGF2α in the sheep endometrium (Vázquez et al., 2010). The application of the RNAi technique made it possible to inhibit COX-2 specifically in endometrial cells to maintain basal concentrations of PGF2α during the early stages of pregnancy. The alteration of specific COX -2/ COX-1 gene expression can provide an ideal method to study the causes of altered PGF2α production during early pregnancy in goats (Campeau and Gobeil, 2011). In summary, gene silencing can be potentially utilized for studying functional analysis of target genes in goat endometrial cells. There, therefore, is the potential to use RNAi in goats to control PGF2α secretion to improve understanding of the biology of early embryonic mortality (EEM) in goats.

5. Conclusion

The results of the present study indicate COX-2 siRNA effectively and specifically suppressed COX-2 mRNA and PGF2α secretions in goat endometrial cells without affecting PGE2 secretion and this may provide the basis for a greater understanding of the functions of COX-2 in the reproductive physiology of goats.

Declaration of Competing Interest

The authors do not have any conflict of interest in the content of the article.

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