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Expression profile of ghrelin and ghrelin receptor in cyclic goat ovary

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ABSTRACT

Ghrelin, a 28-amino acid acylated peptide, was identified as the endogenous ligand for the GH secretagogue receptor and recently the role of ghrelin in reproduction was established. Total RNA was extracted from corpus luteum (CL) of the slaughterhouse derived goat ovaries of different stages of cyclicity. First strand cDNA synthesis was done using constant quantity of total RNA (8µg / 20 µl reaction mixture) and M-MuLV-RT at 36°C for 1h. Semi quantitative PCR analysis as well as real time PCR (Q-PCR) analysis revealed, persistent expression of ghrelin and its receptor throughout the estrous cycle. Ghrelin mRNA corresponded the activity of CL i.e. minimum expression in the degenerating CL and it was at peak in the fully grown CL and similar trend was also observed for ghrelin receptor. Further, ghrelin and its receptor was absent in prepubertal ovary while it was present in cyclic goat ovaries. It can be concluded that ghrelin may have role in the regulation of ovarian follicular development and early embryonic development.

Key words: Ghrelin, Goat, mRNA expression, Ovary, Receptor

Ghrelin, an acylated peptide, primarily secreted by the stomach, was first identified as endogenous ligand of GH secretagogue receptor (GHS-R) (Kojima *et al*. 1999). Ghrelin was also identified in kidney (Mori *et al*. 2000), hypothalamus (Guan *et al*. 1997), pituitary (Howard *et al*. 1996), testes (Gaytan *et al*. 2004), ovary (Xilingaowa *et al*. 2009), foetal tissues (Volante *et al*. 2002) and placenta (Gualillo *et al*. 2001). Based on its action as modulator of feeding behavior, energy metabolism and reproductive physiology, its role in control of gonadal axis was hypothesized. Ghrelin regulates Leydig cell proliferation (Barreiro *et al*. 2004), directly affects basic ovarian functions in chicken (Sirotkin and Grossmann 2008), decreases hCG/LH and testosterone secretion (Tena-Sempree *et al*. 2002) and inhibits early embryonic development (Kawamura *et al*. 2003, Dupont *et al*. 2010). Ghrelin mRNA levels significantly varied depending on the phase of the cycle — lowest expression levels in proestrous and maximum values in the diestrous phase in rat (Caminos *et al*. 2003) and similarly in sheep (Duggal *et al*. 2002). Ovarian ghrelin gene expression is markedly influenced by pregnancy (Caminos *et al*. 2003). Intense and specific ghrelin immunoreactivity was observed in the cytoplasm of steroidogenic luteal cells and non-apoptotic cells of corpus luteum (CL) of previous cycle (Xilingaowa *et al*. 2009). Expression of ghrelin paralleled pattern of progesterone secretion (Duggal *et al*. 2002) suggesting its possible role in reproduction. Such information regarding goat is not available, so the present study was designed to observe the expression profile of ghrelin and its receptor at different stages of developing CL.

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MATERIALS AND METHODS

Tissue collection and total RNA extraction: Goat ovaries were obtained from local slaughter-house and transported in ice, and categorized into different stages on the basis of presence of CL and its stage of activity. The ovaries were washed in sterilized normal saline solution and in 50% alcoholic solution. Samples for RNA isolation were obtained from respective CL. Total RNA were isolated from these samples by using Trizol reagent (invitrogen) as per the standard protocol. Total RNA was quantified using Nanodrop 1000 (Thermoscientific) at 260nm.

Reverse transcription: RNA samples giving OD values at 260/280 nm between 1.8 to 2.0 were used for further cDNA synthesis. cDNA synthesis was performed using constant quantity (8000ng) of total RNA, 2 µl Moloney-Murine luekemia virus reverse transcriptase (MMLV-RT), 0.2 µg random hexamer, 4µl 5× RT buffer, 20U RNasin, 2 µl dNTP mix (10 mM) and total volume of reaction mixture made to 21 µl. The reaction was carried out at 25°C for 10 min and at 42°C for 1 h followed by a denaturation step at 70°C for 10 min with flash cooling. The reaction mixture was stored at –20°C.

Polymerase chain reaction (*PCR*): PCR was performed with cDNA obtained through reverse transcription for the genes of interest, viz. ghrelin and ghrelin receptor with

Table 1.Target genes and primers showing annealing temperature and fragment size

Target gene	Primers	Expected size	Annealing temperature(°C)	PCR cycles
Ghrelin	Sense 5'-gcgggctccagctttctgag-3' Antisense 5'-tccgggtcaaactggccttc-3'	308bp	58	39
Ghrelin receptor	Sense 5'-acgctgccggacctggactg-3' Antisense 5'-cagcgcggtgatggtgagca-3'	369bp	58	39
GAPDH	Sense 5'-CCTGGAGAAACCTGCCAAGT-3' Antisense5'-GCCAAATTCATTGTCGTACCA-3'	210bp	58	39

GAPDH as internal control (constitutive gene). The primers for these genes were designed using published sequences and gene tool software (Table 1). For PCR following reactants were added in the given order in a 200 µl prechilled PCR tube. The reaction mixture consisted 2 µl of cDNA, 2.5 µl 10× Taq buffer, 1.5µl $MgCl_2$ (1.25 mM), 0.5µl dNTPs (10 mM), specific primers (0.4 pmol each), 0.25 µl Taq DNA polymerase (0.05 U/µl) and nuclease free water to make reaction mixture 25 µl. Along with samples negative control consisting of all above constituents except cDNA was also prepared. The contents were gently spun to mix and PCR was performed using Thermal Cycler (eppendorf) with cycling program; initial denaturation at 95°C for 5 min and 39 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s. The last cycle was followed by extension at 72°C for 7 min.

Verification of RT-PCR amplicons: For the confirmation of specific amplicons, products of RT-PCR were subjected to electrophoresis on a 1.5% agarose containing 0.2 pg ethidium bromide (EtBr) in 1× TE buffer. After gel electrophoresis at 80 V for 45 min, the fragments were visualized on a UV transilluminator (312 nm). For comparison, a 100 bp DNA ladder was electrophoresed parallely to the amplicons. The image of each gel was recorded using a gel documentation system with CCD camera. Amplicons for the genes were further confirmed by cloning and sequencing of the product (HQ592319 and HQ592320 respectively).

Real time PCR: Real time PCR was performed for the above samples to quantify the target genes expression using real time PCR machine and kit. The same set of primers was used and real time PCR conditions were kept same as for the normal PCR. Negative control consisting of no template was also run along with the samples. Each experiment was repeated thrice and mean of which was considered as final value. The standard curve was obtained for GAPDH, ghrelin and ghrelin receptor genes using 10-fold dilutions of the sample and resultant slope of the curve was utilized for the calculation of the quantitative expression of both the target genes. The cycle at which the sample amplicon reporter dye concentration crossed a prefixed threshold was recorded as the cycle threshold (Ct) value. As there is inverse relationship between initial template concentration and Ct value, the adjusted Ct value was calculated as an indicator of template concentration using method of Kaiser *et al*. (2002) viz.

$$\text{Adjusted Ct value} = (\text{mean 40-Ct}_{\text{target}})\ (\text{Slope}_{\text{target}})/(\text{GAPDH df})\ (\text{slope GAPDH})$$

where, df is (40-GAPDH/mean of GAPDH)

Data were analyzed using one way ANNOVA test and P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

RT-PCR and real time PCR analysis revealed that the mRNA expression of ghrelin and ghrelin receptor in goat CL was consistent throughout the estrous cycle. RT-PCR assay of ghrelin, ghrelin receptor and GAPDH using gene specific primers (Table 1) resulted in the generation of amplicons of expected sizes of 308bp, 369bp and 210bp respectively (Figs 1-3) (gene bank accession: HQ592319 and

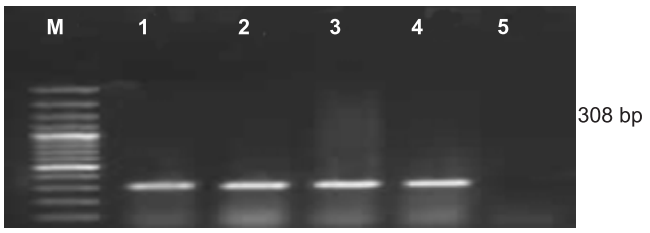

Fig . 1. RT-PCR for ghrelin gene

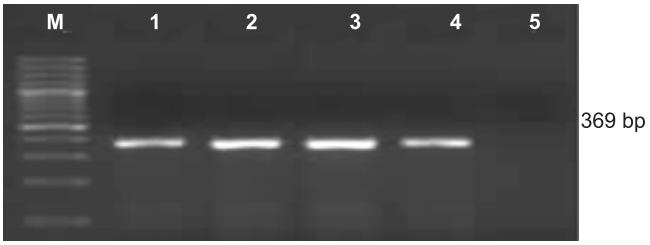

Fig. 2. RT-PCR for ghrelin receptor gene

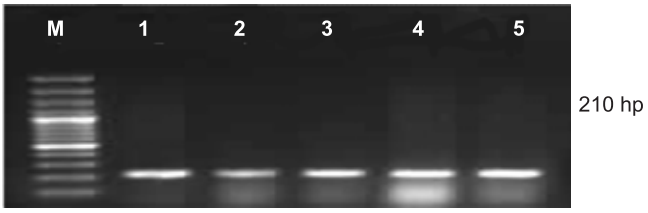

Fig. 3. RT-PCR for GAPDH gene

M: 100bp+ marker; 1: newly formed CL; 2: Growing CL; 3: Fully developed CL; 4: Degenerating CL; 5: prepubertal ovary.

Table 2. Relative expression of ghrelin and ghrelin receptor in different stages of CL

Gene	New CL	Growing CL	Fully developed CL	Degenerating CL
Ghrelin	14.7±0.7[a]	18.2±1.6[b]	21.4±0.7[c]	11.6±1.3[d]
Ghrelin receptor	11.1±1.2[a]	12.1±0.9[a]	15.1±0.8[b]	6.5±0.9[c]

*Values (mean±SE) in the row bearing different superscripts differ significantly (P<0.05).

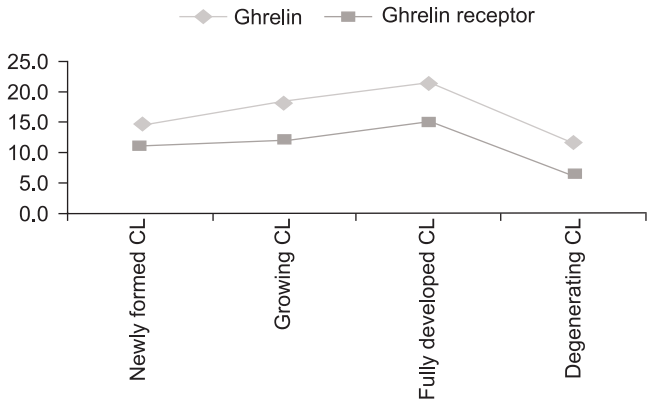

Fig.4. Relative expression of ghrelin and ghrelin receptor in goat CL.

HQ592320 respectively) and negative control gave no amplification (not shown). Real time PCR assay revealed that relative expression of ghrelin and ghrelin receptor varied according to the stage of estrous cycle and it increased gradually according to the growth of CL and it was highest in the fully developed CL and lowest at degenerating CL (Table 2; Fig. 4). Further, ghrelin and ghrelin receptor was absent in pre-pubertal goat ovaries while it was present in the cyclic goat ovarian cortex (Figs 1, 2).

Our results showed first demonstration of expression (mRNA level) of ghrelin and ghrelin receptor in the goat ovary. In this study expression of ghrelin and its receptor was accomplished by molecular methods (real time PCR) and the effect of estrous cycle on the expression of ghrelin and its receptor was evaluated. Similar studies were performed in rat (Caminos *et al*. 2003) and sheep (Xilingaowa *et al*. 2009) and similar trends were reported. Consistent expression of ghrelin and its receptor during all stages of estrous cycle and maximum expression in the fully developed CL and minimum expression at degenerating CL, highly suggests that expression of ghrelin and its receptor are governed by the activity of CL of the current cycle. Coincidental relative expression of ghrelin with plasma progesterone (Duggal *et al*. 2002) and predominant expression of ghrelin in CL within the ovarian tissue (Caminos *et al*. 2003) suggests potential role of ghrelin in the corpus luteum development/function. Ghrelin decreases hCG/LH and testosterone secretion (Tena-Sempere *et al*. 2002) and ghrelin was reported to inhibit early embryo development (Kawamura *et al*. 2003). Thus ghrelin acts as regulator of energy metabolism, endocrine and reproductive control.

It can be concluded from the present study that ghrelin and its receptor are persistent throughout the estrous cycle in goat while absent in prepubertal goat ovary. Thus it may be involved in the functioning of CL and early embryonic development. Further, *in vitro* studies on the effect of ghrelin on early embryonic development may be helpful in the progress of *in vitro* fertilization (IVF) and embryo culture, as its role in embryonic development is still not clear (Dupont *et al*. 2010).

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