Ghrelin, an acylated peptide, was first identified as the endogenous ligand for the GH secretagogue receptor (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.

**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 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
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 MgCl₂ (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. Adjusted Ct value= \((\text{mean } 40-\text{Ct target}) \times (\text{Slope target})/(\text{GAPDH df})\) (slope GAPDH)

where, df is \((40\text{-GAPDH/mean of GAPDH})\)

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 HQ592320 respectively).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Expected size (bp)</th>
<th>Annealing temperature(°C)</th>
<th>PCR cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>Sense 5′-gcgggctccagctttctgag-3′</td>
<td>308</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-tccgggctaaccagctgccct-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin receptor</td>
<td>Sense 5′-acgtcggcacgtgagtctga-3′</td>
<td>369</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-cagcgcggtgatggtgagca-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5′-CCTGGAGAAAACCTGCCAAATG-3′</td>
<td>210</td>
<td>58</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-GCCAAATTCATTGTCGTACCA-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. RT-PCR for ghrelin gene
Fig. 2. RT-PCR for ghrelin receptor gene
Fig. 3. RT-PCR for GAPDH gene

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>New CL</th>
<th>Growing CL</th>
<th>Fully developed CL</th>
<th>Degenerating CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>14.7±0.7\textsuperscript{a}</td>
<td>18.2±1.6\textsuperscript{b}</td>
<td>21.4±0.7\textsuperscript{c}</td>
<td>11.6±1.3\textsuperscript{d}</td>
</tr>
<tr>
<td>Ghrelin receptor</td>
<td>11.1±1.2\textsuperscript{b}</td>
<td>12.1±0.9\textsuperscript{a}</td>
<td>15.1±0.8\textsuperscript{b}</td>
<td>6.5±0.9\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values (mean±SE) in the row bearing different superscripts differ significantly (P<0.05).

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, \textit{in vitro} studies on the effect of ghrelin on early embryonic development may be helpful in the progress of \textit{in vitro} fertilization (IVF) and embryo culture, as its role in embryonic development is still not clear (Dupont \textit{et al.} 2010).

REFERENCES


