Molecular characterization and expression profile of ghrelin gene during different reproductive phases in buffalo (*Bubalus bubalis*)

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**ABSTRACT**

Ghrelin, a novel motilin-related endogenous ligand for growth hormone secretagogue receptor, is implicated in various biological functions, including regulation of female reproduction. But the presence of ghrelin and its role in reproductive functions in buffalo, a species with poor reproductive efficiency, is not known. In the present study full-length ghrelin cDNA was isolated from bubaline abomasum, which encodes the entire propeptide of 116 amino acids. The deduced amino acid sequence of ghrelin of buffalo showed >95% and 31% identity with that of ruminants (cattle, sheep, and goat) and humans, respectively. Analysis of synonymous and nonsynonymous nucleotide substitutions in the coding region of ghrelin indicated that these sequences of different species have been under purifying selection. The 3995-bp amplicon of ghrelin gene consisting of 4 exons and 3 introns was cloned with genomic DNA from buffalo. Further, ghrelin expression was determined by quantitative real-time PCR, in situ hybridization, and immunohistochemistry in bubaline endometrial tissues at different stages of the estrous cycle and early pregnancy. Our results indicated the persistent expression of ghrelin mRNA and protein in the endometrium during stage I (day 3–5), stage II (day 6–15), and stage III (day 16–21) of the estrous cycle and also during early pregnancy. Immunohistochemistry and quantitative real-time PCR experiments indicated the relatively higher expression of ghrelin in the endometrium during stage II (day 6–15) of the estrous cycle and early pregnancy than during stage I (day 3–5) and stage III (day 16–21) of the estrous cycle, but no statistically significant difference in ghrelin expression was observed among stages. To conclude, the results of the present study indicate the persistent expression of ghrelin in the uterine endometrium throughout the estrous cycle and in early pregnancy which might be helpful in determining its role in buffalo reproduction.

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1. Introduction

Ghrelin, an endogenous ligand for growth hormone secretagogue (GHS) receptor [1], is predominantly secreted by the stomach, duodenum, and jejunum [1–3]. It has pleiotropic functions, including various reproductive processes [4]. Recent studies indicated its involvement in female reproduction [5,6]. Among the various reproductive functions, ghrelin regulates implantation of the embryo [7] and modulates the secretion of many reproductive hormones [8–10].

Ghrelin is expressed in different reproductive tissues, including the uterus [11] and ovary [12]. Expression of ghrelin receptor (GHS-R) is also detected in the endometrial tissue [7] and ovary [13]. A recent study reported the presence of ghrelin mRNA, ghrelin protein, and GHS-R1A in different parts of reproductive tracts, including the endometrium and oviduct in Holstein heifers [14]. In
addition to the basal expression of ghrelin in the reproductive tissues, expression of ghrelin mRNA in the human endometrial tissue is pronounced during early pregnancy compared with the proliferative or secretory phase [7]. Conversely, a lower ghrelin and GHS-R expression in human endometrial tissues is associated with decreased fertility [15]. Further, ghrelin is reported to accelerate the decidualization, suggesting its involvement in remodeling of the endometrium [11]. However, recent studies reported the inhibitory effects of ghrelin on progesterone secretion by corpus luteal cells [16,17]. In addition, it is proposed that persistently elevated levels of ghrelin associated with negative energy balance might also affect reproductive function [5]. From the above-mentioned studies, the potential involvement of ghrelin in various reproductive functions, particularly embryonic development, is apparent.

Water buffalo (*Bubalus bubalis*), one of the important dairy animals in most of the Asian countries, contributes to more than one-third of total milk production in Asia [18,19]. The productive efficiency of this species is mainly affected by reproductive problems such as reduced conception rate and high early embryonic mortality [20,21]. However, the primary causes responsible for early embryonic mortality are still unknown. Uterine endometrial tissue is the maternal interface of fetal–maternal interaction, and various locally produced factors regulate the embryo receptivity of endometrium [22]. Taking into account the importance of ghrelin, its involvement in various reproductive processes in general and embryonic development in particular, and lack of any report on the ghrelin expression pattern in the reproductive organs of buffalo, we have characterized the cDNA as well as genomic sequence of the bubaline ghrelin gene and further investigated the effects of stages of estrous cycle and early pregnancy on its expression in buffalo.

2. Materials and methods

2.1. Experimental animals and sample collection

All the experimental procedures were approved by Institute Animal Ethics Committee, Indian Veterinary Research Institute, Izatnagar, Bareilly, India. To amplify the bubaline ghrelin gene, genomic DNA was extracted from venous blood of buffalo as described previously [23]. Abomasal tissues, for cDNA cloning, and uteri, for mRNA expression analysis, were collected during the month of September (at the end of the monsoon season) from the local municipal abattoir and immediately (within 2 h of slaughter) transported to the laboratory on ice. The specimens with any abnormality were discarded. Uteri were washed with diethylpyrocarbonate-treated sterile PBS. On the basis of color, vasculature, size, and consistency of corpus luteum [24–26], the uteri were classified into one of the following three stages of the estrous cycle: stage I, day 3 to 5; stage II, day 6 to 15; and stage III, day 16 to 21. The uteri were opened longitudinally for collection of endometrial tissue. In case of cyclic uteri, the endometrial tissues were scraped with RNase-free glass slides. In case of gravid uteri, the embryo or fetus along with whole fetal membrane was carefully removed, and the intercaruncular endometrial tissues from the uterus were collected. Day of pregnancy or approximate age of the fetus was determined on the basis of both crown-to-rump length and weight of the fetus [27]. Total RNA was isolated from the endometrial tissues using TRI reagent (Sigma, St Louis, MO, USA) according to the manufacturer’s instructions. The isolated RNA samples were treated with DNase using DNA-free DNase Treatment and Removal Reagents (Ambion, Austin, TX, USA). The concentration and purity of RNA preparation were determined spectrophotometrically at OD260 and OD280, and the integrity of the RNA was examined by electrophoresis. Tissues from uteri were also used for immunohistochemistry (IHC) and in situ hybridization (ISH) experiments.

2.2. Cloning and characterization of bubaline ghrelin gene

Degenerate primers for ghrelin (forward, 5'-TCCATCT-GCCTCCAGCCAGGAAGCC-AT-3' ; reverse, 5'-TCAGAGCTG-CCTTGCTGGTCGGAATGTG-3') were designed on the basis of ghrelin mRNA sequence of goat (Accession no. AB089200), cattle (Accession no. NM_174067), and sheep (Accession no. NM_001009721) available at GenBank (www.ncbi.nlm.nih.gov). Amplification of buffalo ghrelin gene was performed with Long Range PCR Mix (catalog K0181; Fermentas, Glen Burnie, MD, USA), whereby the reaction mixture contained 100 ng of genomic DNA, 1 × Long Range PCR buffer (2.5 μL), 2.0 mM concentrations of MgCl2, 200 μM dNTPs, 10 pM of each primer, 1 U of Long Range PCR enzyme mix, 0.5% dimethylsulfoxide, and quantum-sufficient nuclease-free water. A negative control with no template DNA was also included. For amplification of the ghrelin gene, 2-step PCR was performed with an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing and extension at 68°C for 4 min 30 s, and a final extension at 68°C for 10 min. The amplified product was checked by agarose gel (1%) electrophoresis in 1 × Tris-acetate-EDTA buffer after staining with ethidium bromide. Ready-to-load 100-bp DNA ladder (GeneRuler; Fermentas) was used as a molecular weight marker for electrophoresis. After electrophoresis, the stained gels were recorded with a digital fluorescent image recorder (Syngene, Frederick, MD, USA).

The amplicon of complete ghrelin gene was purified by using Gel clean up Kit (Eppendorf, Hamburg, Germany), and the purified PCR product was cloned into the pTZ57R/T vector (MBI; Fermentas) according to the manufacturer’s specification. Positive recombinant clones were identified with blue and white screening. Further, the presence of the insert was confirmed by plasmid PCR, followed by restriction digestion with EcoRI and BamHI restriction enzymes (Fermentas). The positive clone was sequenced with an ABI PRISM automated sequencer (version 2.0) under standard cycle conditions of Sanger’s dideoxy chain termination method with standard M13 forward and reverse primers. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotides as well as deduced amino acid sequences were aligned with those of other species available in the GenBank database with the
Phylogenetic and molecular evolutionary analyses of deduced amino acids of ghrelin of various species were performed with MEGA software version 4 [28]. Reliability of the derived phylogenetic tree was tested by the bootstrapping test of phylogeny. Time of divergence between cattle and buffalo ghrelin was estimated on the basis of Poisson-correlated distance as described previously [29]. Cattle and buffalo are assumed to have diverged 4.03 million years ago, based on paleontologic data [30]. The putative signal sequence of the bubaline preproghrelin was determined with SignalP 3.0 from Center for Biological Sequence Analysis, Technical University of Denmark [31]. To check whether purifying selection was operating, z-tests were conducted as described in the literature [28,32]. The number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN), and their variances, Var(dS) and Var(dN), were calculated, and then the null hypothesis that H0: dN = dS was tested with a z-test: Z = (dN - dS)/SQRT[Var(dS) + Var(dN)]

2.3. Reverse transcription PCR

Total RNA was reverse-transcribed with Reverse Transcription System (Promega) according to the manufacturer’s instructions. Briefly, the cDNA was synthesized from approximately 2 μg of total RNA with the use of oligo-dT primers and avian myeloblastosis virus reverse transcriptase in a final volume of 20 μL. The resultant first strand of cDNA was stored at −20°C until further use.

2.4. Cloning of ghrelin cDNA

Ghrelin cDNA was amplified from first-strand cDNA derived from total RNA of the abomasal source. The entire coding region of bubaline ghrelin cDNA was amplified with primers (forward, 5’-TCCATCTGGCTTCAAGGCAGGCAGCAT-3’; reverse, 5’-TCAGAGCTGGCTTGGCTGCGGAAGTGT-3’) designed as described in the previous section. PCR amplification was performed in a total volume of 25 μL that contained 5 pM of each primer, 1 μL of cDNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM MgCl2, 2.5 mM dNTPs, and 1 U of Taq DNA polymerase (Fermentas). Amplification was performed in a Thermal Cycler (Eppendorf, Hamburg, Germany) for 35 cycles with the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 68°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. Cloning and sequencing of the ghrelin cDNA was done as described in the previous section.

2.5. RT-qPCR to determine the expression of ghrelin mRNA

Expression of the ghrelin mRNA in the uterine endometrium during different reproductive stages was quantified with quantitative real-time PCR (RT-qPCR). Endometrial tissues were collected from uteri at each of the 3 stages of estrous cycle and at early pregnancy (d 30–40). For each reproductive phase, tissue samples were collected from 3 animals. Subsequently, total RNA was isolated from the tissues as described earlier, and the first strand of cDNA was generated with the use of the total RNA as template. The gene-specific primer pairs (forward, 5’-CGAGCTGGAATCCCCTTTTA-3’; reverse, 5’-GACCCCTGACAGCTTGAT-3’) were designed with Primer Express version 3.0 on the basis of the sequence of the bubaline ghrelin gene that was used to amplify the 471-bp amplicon. A pair of primers (forward, 5’-AGCTCCCATGGATGTA-3’; reverse, 5’-TCCCAGACCTTGTGC-3’) was used to amplify β-actin as an endogenous control [33]. The RT-qPCR experiment was performed with Applied Biosystems 7900HT Fast Real-Time PCR System. All PCR reactions were performed in duplicates with total volume of 5 μL. The reaction mixture contained 1 × Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA), 5 pM of each gene-specific primer, and 2.5 μL of cDNA template. PCR cycling conditions were as follows: initial denaturation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 1 s; annealing/extension at 60°C for 20 s. To examine the DNA contamination, for each RNA sample, a control reaction was set up in which reverse transcriptase enzyme was omitted during cDNA synthesis. To determine the specificity of the PCR reaction, a dissociation curve was generated after completion of amplification. The PCR efficiency for ghrelin and β-actin were 93 and 104, respectively.

2.6. Localization of ghrelin mRNA with ISH

Probe synthesis was performed according to the manufacturer’s instructions with the use of PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany) and ghrelin cDNA as template. Briefly, a 230-bp fragment of ghrelin cDNA was amplified by PCR. All PCR reactions were performed in a total volume of 25 μL which contained 5 μL of cDNA, 2.5 μL of 10× PCR buffer, 2.5 μL of 10× PCR DIG Labeling Mix, 10 pM of each primer (forward: GHLN203F, 5’-CGAGGACAGCTGTAATCCCCTTTTA-3’; reverse: GHLN400R, 5’-AAATTGTCCTCCAAAGGAGCAGCTTACGTTGTGC-3’), and 0.4 μL of enzyme mix (3.5 U/μL), and the rest of the reaction mixture was made up with nuclease-free water. The cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 60 s, and a final extension at 72°C for 10 min. With the use of agarose gel electrophoresis, incorporation of dUTP was confirmed by increased molecular weight of the amplicon. In situ hybridization was done in paraffin-embedded transverse uterine tissue sections as described previously [34]. In negative control sections, the hybridization probe was omitted.

2.7. Localization of ghrelin protein with IHC

Tissue fixations, embedding, sectioning, de-waxing, and re-hydrating of tissues were performed as described [35]. Antigen retrieval was done by heating the tissue sections for 10 min in 0.01 M citrate buffer (pH 6.0) [36]. Endogenous peroxidase activity was quenched by incubating sections in 3% H2O2 in methanol for 10 min. Sections were rinsed in water, equilibrated in PBS (pH 7.4) for 5 min, and
then incubated overnight in a humidified chamber at 4°C with anti-human ghrelin primary antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1:100) in blocking serum. Secondary antibody treatment and other protocols were followed as described in the goat ABC staining system (Santa Cruz Biotechnology). Sections were counterstained with hematoxylin and mounted with dibutyl phthalate xylene (DPX). In negative controls, either primary antibody or secondary antibody was omitted. Because the primary anti-human ghrelin antibody (C-18) has also been reported to cross-react with rat ghrelin, sections of rat stomach were used as positive control and processed in parallel.

2.8. Statistical analysis

Levels of expression of ghrelin mRNA during different reproductive phases were expressed as fold change relative to stage I (day 3–5 of estrous cycle) [37]. All numerical data were expressed as a mean ± SEM of three biological replicates from each reproductive phase. Effect of different reproductive stages on the expression of ghrelin is analyzed by one-way ANOVA.

3. Results

3.1. Sequence analysis of ghrelin cDNA and ghrelin gene

We cloned and sequenced the full-length cDNA (Fig. 1A) and the gene (Fig. 3A) encoding the buffalo preproghrelin. The cDNA contains the entire coding sequence of 351 bp in length, encoding 116 amino acids prepropeptide. The predicted prepropeptide has signal sequence of 23 amino acids, and the mature peptide has 27 amino acids (Fig. 1B).

The multiple sequence alignment of buffalo preproghrelin showed 97, 96, 96, 82, 80, 79, 80, 75, 74, 74, 70, 53, 52, 25, 12, and 13% identity at the nucleotide and 96, 95, 95, 76, 70, 68, 68, 68, 68, 68, 55, 49, 35, 36, 31, 20, and 22% identity at the amino acid level with that of cattle, goat, sheep, cat, dog, horse, Japanese macaque, mouse, rat, Asian house shrew, Tammar wallaby, chicken, mallard, human, Nile tilapia, and zebrafish, respectively (Fig. 2). Compared with cattle ghrelin, deduced buffalo ghrelin peptide has P40A, A51T,
and S86A amino acid substitutions. Similar to other mammalian ghrelin genes, deduced amino acid sequence of bubaline ghrelin has the conserved Ser3, an acylation site at the N-terminal region (Fig. 2). The nucleotide sequence of bubaline ghrelin cDNA was deposited in GenBank with Accession number JQ859818. Further, the 3995-bp clone

Fig. 3. (A) PCR-amplified product of ghrelin gene from total DNA isolated from buffalo. (B) Schematic diagram shows exon organization in buffalo ghrelin gene. E, PCR efficiency.

Fig. 4. Phylogram based on ghrelin amino acid sequences of different species by using the Neighbor-Joining Tree method/p-distance model with the use of MEGA4 software.
(Fig. 3A) of ghrelin gene consisting of 4 exons and 3 introns was also submitted to GenBank with Accession number EF583468. Figure 3B summarizes a comparison of different structural characteristics, including exon and intron sizes.

3.2. Phylogenetic and evolutionary analysis

On the basis of the deduced amino acid sequences of ghrelin gene of different species, a phylogram was constructed, and it revealed 4 distinct clades (A, B, C, and D) (Fig. 4). Clade A includes all mammals, clade B includes marsupial species, clade C includes birds, and clade D was formed by fish. Within the mammal clade, ghrelin sequences of ruminants, including buffalo, fall into a single cluster. Evolutionary analysis revealed that the buffalo ghrelin diverged from that of cattle nearly 4.4 million years ago, later than those species divergence. Synonymous and nonsynonymous nucleotide substitution analysis (dS/dN) of ghrelin sequences revealed that the dS value is greater than the dN and P values of the z-test (Table 1). This indicates that the ghrelin coding regions in those species have been under purifying selection.

3.3. Quantitative real-time PCR

Constitutive expression of ghrelin mRNA was observed in the uterine endometrium during all stages of estrous cycle as well as during early pregnancy (Fig. 5). A trend of higher ghrelin mRNA expression was observed during diestrus (10-fold increase) and early pregnancy (21-fold increase) relative to estrus stage (day 3–5). However, one-way ANOVA analysis showed no significant (P = 0.24) difference in the ghrelin mRNA expression across the stages.

3.4. Localization of ghrelin mRNA and protein

Messenger RNA as well as immunoreactive signals of ghrelin was localized in the uterus (Figs. 6 and 7). Comparatively stronger mRNA and immunoreactive ghrelin signals were observed during stage II, that is, the luteal phase of the estrous cycle (Figs. 6D and 7D) and early pregnancy (Figs. 6F and 7F). Very weak signals were observed during stage III (day 16–21) of the estrous cycle (Figs. 6E and 7E). Signals for both the ghrelin mRNA and immunoreactive protein were found in the glandular epithelium (GE) and stromal cells. No signal specific to ghrelin was observed in negative control sections (Figs. 6B, 7B, and 7G). Specific signals were observed in positive controls of the ISH (Fig. 6G) and IHC (Fig. 7H) experiments.

4. Discussion

Ghrelin is involved in normal embryonic development [38] and has been associated with fertility [15,38]. Recently,
a study showed that ghrelin-deficient mice had reduced fertility [38]. Previous studies have also shown that dysregulated expression of the gene affects the uterine receptivity of the embryo [39], and supraphysiological level of ghrelin negatively affects the embryo growth [40]. Role of endometrium in secretion of ghrelin is largely unknown to date in most of the domestic animal species, including buffalo that has poor reproductive efficiency as a result of early embryonic losses [20,41]. In the present study, as part of our work in exploring the possible physiological role of the ghrelin on reproductive functions in buffalo, we have cloned and characterized the expression of the ghrelin in bubaline endometrial tissue.

The bubaline ghrelin gene shares highest identity with that of cattle and goats at both the nucleotide and amino acid levels. It shows many similar characteristics with its phylogenetically close relative bovine. Like cattle [42], the buffalo ghrelin gene lacks an alternative splicing site as reported in the human ghrelin gene [43]. In addition, the predicted bubaline mature peptide sequence has 27 amino acids as observed in ghrelin mature peptides of other ruminants [42]. As expected, the deduced amino acid sequence of buffalo ghrelin falls into a cluster formed by other ruminants. Evolutionary analysis indicates that the bubaline ghrelin gene is diverged from its close relative cattle ghrelin after species divergence, and purifying selection is operating in this gene. Purifying selection acts against mutations that result in deleterious effects on protein function and eventually reduce the frequency of deleterious alleles.

The present study indicates that the bubaline ghrelin gene expression was persistent in the endometrial tissue at all stages of the estrous cycle. Similarly, persistent endometrial expression of ghrelin mRNA throughout the menstrual cycle has been reported previously in humans, characteristically with the pronounced expression during the secretory phase [11] and early pregnancy [7]. The results obtained with IHC and ISH techniques in this study showed a predominant expression of the gene in GE and stromal cells of bubaline endometrium. This higher level of expression restricted to the GE of the endometrial tissue perhaps indicates the localized nature of ghrelin secretion that possibly acts in an autocrine manner on the endometrial cells in the bubaline species. Expression of both ghrelin gene and its receptor (ie, GHS-R) has been investigated in endometrial epithelial cells of cattle [14] and humans [11]. However, we did not investigate the expression profile of GHS-R in this study, which remains to be determined in the future.

Immunohistochemistry studies showed relatively higher expression of ghrelin protein in the bubaline endometrial tissues during the luteal phase (day 6–15 of estrous cycle) and early pregnancy. Further, the RT-qPCR experiment strengthens our findings that show the similar trend in the expression of ghrelin mRNA among the reproductive stages. However, the physiological implications of the increased trend in the expression of ghrelin during diestrus (10-fold) and early pregnancy (21-fold) is yet to be ascertained. The changes in the sex steroids [7], possibly progesterone, may be responsible for the variation in ghrelin expression during the different phases of the estrous cycle in the bubaline species. On the basis of the

<table>
<thead>
<tr>
<th>Pairwise comparison</th>
<th>dN/dS ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo with cattle</td>
<td>1.8209316</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Buffalo with goat</td>
<td>1.7546880</td>
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<td>Buffalo with rat</td>
<td>6.6221016</td>
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dN/dS, ratio of nonsynonymous to synonymous substitutions.

**Table 1**
Analysis of dN/dS between ghrelin orthologs.
above-mentioned findings, it seems that ghrelin expression is localized in nature and degree of expression in the bubaline endometrial tissues and is stage specific. However, the precise molecular mechanism(s) responsible for the aforementioned differential ghrelin expression remains to be determined.

5. Conclusion

In conclusion, the ghrelin gene is highly conserved in ruminants, including bubaline species. Ghrelin is expressed throughout the estrous cycle and during early pregnancy in the bubaline endometrium. A trend of stage-specific variation in ghrelin expression indicates its variable roles in regulating endometrial functions during estrous cycle and early pregnancy in the bubaline species. Further studies are required to elucidate the temporal relationship among ghrelin expression, steroids, particularly progesterone secretion, and embryonic losses in buffalo.

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References


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