Journal of Animal and Veterinary Advances 11 (24): 4585-4592, 2012
ISSN: 1680-5593

Expression and Distribution Pattern of Ghrelin in the Gastrointestinal Tract of Saanen Dairy Goat

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Abstract: Ghrelin, a gastric acylated peptide has been shown to express in gastrointestinal tract of many animals. However, little is known about its expression pattern in gastrointestinal tract of ruminants such as goat. In this study, researchers investigated the expression and distribution of ghrelin in the gastrointestinal tract of goat (Saanen dairy goat) including esophagus, rumen, reticulum, omasum, abomasus, duodenum, jejunum, ileum, appendix, colon and rectum by PCR, Immunohistochemistry (IHC) and Western blot. The PCR detection showed that ghrelin mRNA existed in all the above tissues and was significantly higher in the abomasus than in other tissues detected ($p<0.05$). Immunohistochemistry assay showed that ghrelin-positive cells were observed in the mucosal layer of all segments examined. The ghrelin-positive cells were most numerous in the abomasus, less abundant in the duodenum, occasional in jejunum, ileum, appendix, colon and rectum and rare in esophagus, rumen, reticulum, omasum and abomasus. Western blot analysis confirmed the ghrelin protein expression pattern in the gastrointestinal tract of the goat. These results demonstrate that ghrelin is expressed in the gastrointestinal tract of Saanen dairy goat, especially in the abomasus.

Key words: Ghrelin, goat, gastrointestinal tract, immunohistochemistry, Western blot

INTRODUCTION

Ghrelin is an acylated 28 amino acid hormone synthesised primarily in the stomach. It is generated by processing of a 117 amino acid peptide, preproghrelin by specific proteases and is stored in secretory vesicles of endocrine cells (Kojima *et al*., 1999). Ghrelin has been shown to be further processed by addition of an octanoyl group to a serine residue and this acylation is important for ghrelin endocrine/biological activity (Kojima *et al*., 1999). Accumulating evidences suggested that ghrelin regulates food intake, acid secretion, glucose metabolism and reproductive function (Cui and Waldum, 2007; Sirotkin *et al*., 2008; Granata *et al*., 2010; Jacob *et al*., 2010a, b). Other evidences also indicated that ghrelin is related to some diseases such as gastrointestinal disease, autoimmune thyroid diseases and neuroendocrine tumors (Corbetta *et al*., 2003; Alexandridis *et al*., 2009; Sawicka *et al*., 2010).

Ghrelin is predominantly produced in the stomach which is the major source of circulating ghrelin (Kojima *et al*., 1999; Date *et al*., 2000). Ghrelin-positive cells have been found to be localized in the mucous membranes of the gastrointestinal tract of rat, human, chicken, duck and African ostrich chicks (Date *et al*., 2000; Neglia *et al*., 2005; Wang *et al*., 2009; Shao *et al*., 2010). Ghrelin-positive cells usually are classified into opened and closed-type cells (Sakata *et al*., 2002). Besides the gastroenteric tract, ghrelin is also expressed in the central nervous system (Caminos *et al*., 2003; Cowley *et al*., 2003) as well as in other peripheral tissues (Harrison *et al*., 2007; Hattori, 2009; Ueberberg *et al*., 2009). There are many studies about the distribution of ghrelin mRNA in humans, rodents and other animals (Hattori, 2009; Shao *et al*., 2010; Wang *et al*., 2011). Ghrelin has been proved to be existing in the sheep hypothalamus, pituitary and ovary (Huang *et al*., 2006; Du *et al*., 2009). However, few studies have been carried out to investigate ghrelin expression and distribution pattern in ruminants.

Saanen dairy goat is a well-known breed of dairy goat which has the characteristic of crude feed tolerance and high milk production. However, the role of ghrelin in Saanen dairy goat still remains largely unknown. Before

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researchers can determine the function of ghrelin in the digestive organs of Saanen dairy goat, it is necessary to analyze its expression patterns and distribution in the digestive organs. Therefore, the present experiment was designed to determine the expression and distribution pattern of ghrelin mRNA and protein in the goat gastrointestinal tract using PCR, IHC and Western blot methods.

MATERIALS AND METHODS

Tissue preparation: Six, 15 months old Saanen dairy goats of both sexes were used in the present study. The gastrointestinal tract was sectioned in small samples of the following segments: esophagus, rumen, reticulum, omasum, abomasus, duodenum, jejunum, ileum, appendix, colon and rectum. The tissues were washed in phosphate-buffered saline and frozen in liquid nitrogen for mRNA and western blot analyses. The rest of the tissues were fixed in 4% paraformaldehyde phosphate-buffered solution (PBS, 0.1 mol L^{-1}, pH 7.4) at 4°C for IHC. All experimental procedures involving animals were conducted under the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Real-time PCR analysis: Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, US) and 2 μg each RNA sample was reverse-transcribed using First-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, US). Quantitation of genes coding for ghrelin was performed using SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) by Bio-Rad iQ5 Real Time PCR System (BIO-RAD, USA). The following primers were designed to amplify the goat ghrelin (product size: 171 bp; Accession No. AB060699): sense primer, TGGGAAGT CAGGAGGAAGGT; antisense primer, AGGCGTACAG GGACAGCA and goat β-actin (product size: 203 bp; Accession No. U39357): sense primer, GTCACCAAC TTGGGACGACA; antisense primer, AGGCGTACAG GGACAG CA. Reactions were carried out in 25 μL volume containing 1×SYBR Premix Ex Taq™ II, sense and anti-sense primers (0.4 mM) and target cDNA (4 ng). The cycling conditions were 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec. A negative control was included in each run and the specificity of amplification reaction was checked by melting curve (T_m value) analysis. The relative quantification of gene expression was analyzed by the two ddCt method (Livak and Schmittgen, 2001). The resulting PCR products were analyzed by electrophoresison 2% agarose gels.

Finally, The PCR products were sequenced to ensure that the target gene sequence was amplified. The *β-actin* gene was used as an endogenous control.

Immunohistochemistry: The specimens were rinsed in 30% sucrose/PBS solution overnight and frozen in OCT sections (8 μm thick) were cut and thawed onto poly-L-lysine-coated slides. The sections were processed according to the Streptavidin-Peroxidase Conjugate (SP) Method (Buckland, 1986). Briefly, the sections were incubated in 2% hydrogen peroxide in methanol at 4°C for 15 min to quench endogenous peroxidase followed by washing in PBS. Then, the sections were blocked with normal serum and incubated with a rabbit polyclonal antibody to ghrelin (dilution 1:200; Santa Cruz, USA) at 4°C over night. Sections were treated sequentially with mouse anti-rabbit IgG-biotin conjugate (dilution 1:300; Santa Cruz, USA) and streptavidin/HRP complex (dilution 1:200; Bios, China) 1 h at room temperature, respectively. The sections were reacted in a diaminobenzidine-tetrachloride kit (DAB kit, Maxim, China) for visualization of antigens. Finally, the slides were washed in distilled water and counterstained in Mayer's haematoxylin then dehydrated, cleared and mounted. The specificity of the immunocytochemical reactions was assessed by:

- Replacement of the ghrelin antibody with normal rabbit serum at different concentrations
- Substitution of the anti-ghrelin antibody with normal rabbit IgG
- Omission of the primary antibody
- Omission of the secondary biotinylated antibody
- Incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity

Western blot analysis: Samples of all parts of the gastrointestinal tract were homogenized with ice-cold RIPA lysis buffer with 1 mM Phenyl-Methyl Sulfonylfluoride (PMSF). Protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, US). Equivalent amounts of proteins were loaded and electrophoresed on a 16.5% Tricine gel (Schagger and von Jagow, 1987) and the separated proteins were transferred to Polyvinylidene Difluoride (PVDF) membranes (Millipore Corp, Atlanta, GA, US). The membranes were then blocked with 5% skimmed milk for 1 h at room temperature. They were then sequentially incubated with the rabbit anti-ghrelin (Santa Cruz, USA)

at 1:300 dilutions overnight at 4°C. After washing twice with PBS, the membranes were incubated with HRP-conjugated anti-rabbit IgG (Boehringer Mannheim. Germany) at 1:2000 dilutions for 1.5 h at room temperature. After thorough washing, the immunocomplexes were visualized using ECL reagent (Pierce, Rockford, IL, US) followed by autoradiography on X-ray films. The β-actin was used as an endogenous control.

Statistical analysis: Data are shown as mean±SEM of at least three independent experiments done in triplicate. Results were analyzed by one-way Analysis of Variance (ANOVA). A value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Distribution of ghrelin mRNA in the gastrointestinal tract of the goat: The 15 months old Saanen dairy goats were used to investigate the distribution of ghrelin mRNA in the gastrointestinal tract by RT-PCR. Ghrelin mRNA was found to be existed throughout the gastrointestinal tract (Fig. 1a). Furthermore, real-time PCR (Fig. 1b) showed that the highest level of ghrelin mRNA was detected in the abomasus, moderate level of ghrelin mRNA was detected in the duodenum while lower level of ghrelin mRNA was detected in the other segments of the gastrointestinal tract ($p<0.05$).

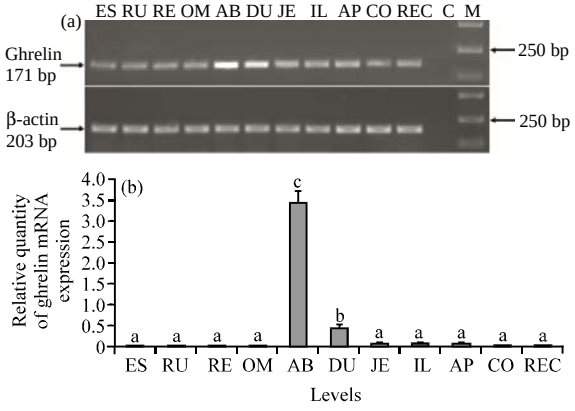

Fig. 1: a) RT-PCR of ghrelin gene in the gastrointestinal tract; b) The expression levels of ghrelin mRNA in different gastrointestinal tract tissues. ES: Esophagus; RU: Rumen; RE: Reticulum; OM: Omasum; AB: Abomasums; DU: Duodenum; JE: Jejunum; IL: Ileum; AP: Appendix; CO: Colon; RE: Rectum; C: negative control; M: DNA marker (DL 2000). [a-c]Different letters indicate significant differences among segments according to Duncan's multiple range test ($p<0.05$)

Distribution of ghrelin-positive cells in the gastrointestinal tract of the goat: Ghrelin-positive cells were observed in mucosal layers of all gastrointestinal tract segments examined including esophagus, rumen, reticulum, omasum, abomasus, duodenum, jejunum, ileum, appendix, colon and rectum (Fig. 2-4). Rare ghrelin-positive cells were detected at the base of the stratified squamous epithelium in the esophagus, rumen, reticulum and omasum and generally appeared to be basal cells (Fig. 2a-d). The most numerous ghrelin-positive cells were detected in the epithelium of the compound glands in the abomasus and some of the ghrelin-positive cells were opened-type cells which showed tapered shape while others were closed-type cells which showed round shape (Fig. 3a). Less abundant ghrelin-positve cells that showed short column shape were detected in the epithelium of the Brunner's gland in the duodenum and some opened-type ghrelin-positive cells were found to be located in the crypt of the duodenum (Fig. 3b). Occasional ghrelin-positive cells were observed in the epithelium of the crypt of the jejunum, ileum, appendix, colon and rectum and most of these cells were opened-type (Fig. 3c, d and 4a-c). No ghrelin-positive cell was found in the negative control of abomasums and duodenum (Fig. 2-4).

Western blot analysis of ghrelin protein in the gastrointestinal tract of the goat: To further confirm ghrelin expression pattern in the gastrointestinal tract of the goat, all segments of the goat gastrointestinal tract was detected by western blot. The results showed that a protein estimated molecular mass of approximately 13 kDa could be detected in all of segments (Fig. 5a) which was similar to the estimated molecular mass of ghrelin (Menzies *et al*., 2009). Further, Western blot analysis revealed that the highest level of ghrelin protein was detected in the abomasus and the moderate level of ghrelin proteinwas observed in the duodenum while lower level of ghrelin protein was found in the other segments of the gastrointestinal tract (Fig. 5b, $p<0.05$) which was in agreement with the immunohistochemical data.

Ghrelin have been found in the stomach, duodenum, jejunum, ileum and colon in human, rats, chicks, ducks and African ostrich (Date *et al*., 2000; Sakata *et al*., 2002; Neglia *et al*., 2005; Wang *et al*., 2009; Shao *et al*., 2010). In the current study, PCR revealed that ghrelin was expressed in goat gastrointestinal tract including esophagus, rumen, reticulum, omasum, abomasus, duodenum, jejunum, ileum, appendix, colon and rectum. Comparison of the present data with those obtained from other species shows there are some similarities as well as

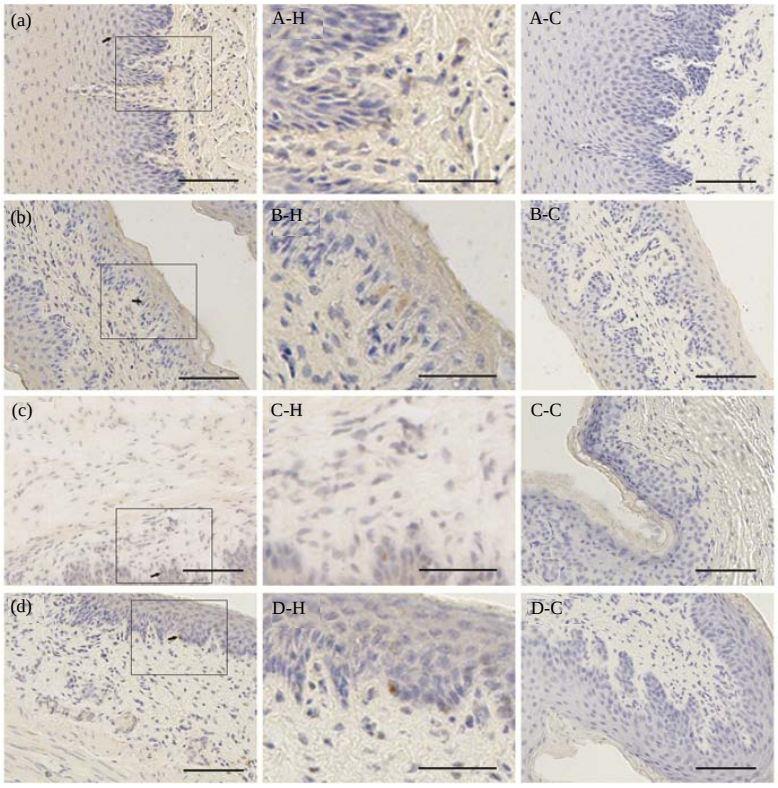

Fig. 2: Immunohistochemical staining of ghrelin in the gastrointestinal tract. A-D) Rare ghrelin-positive cells (black arrows) were detected at the base of the stratified squamous epithelium in the esophagus, rumen, reticulum and omasum. (A-H)-(D-H) is the higher magnification of A-D) section, (A-C)-(D-C) is the negative control section of (A)-(D), no ghrelin-positive cell was detected in negative control sections. Scale bars: A-D, (A-C)-(D-C), 100 μm; (A-H)-(D-H), 50 μm

some differences. For example, Huang *et al.* (2006) reported that ghrelin mRNA express in the rumen, reticulum, omasum, abomasum and duodenum of sheep. Gnanapavan *et al.* (2002) observed ghrelin mRNA expression in esophagus, ileum and colon of the human using RT-PCR. Lee *et al.* (2002) also reported that ghrelin mRNA existed in stomach-fundus and colon of rat. Whereas in ducks, ghrelin mRNA expression was not detected in ileum and large intestine. Ghrelin mRNA in the abomasum was significantly higher than those in the other segments of the gastrointestinal tract. The predominant expression of ghrelin mRNA is in abomasum of Saanen dairy goat which is consistent with the results found in humans, rats, chickens and other vertebrates (Kojima *et al.*, 1999; Date *et al.*, 2000; Wada *et al.*, 2003; Huang *et al.*, 2006). These results suggest that distribution patterns ghrelin mRNA shows a high degree of conservation among various vertebrates.

Additionally, researchers also demonstrated the distribution of ghrelin protein in the gastrointestinal tract of the Saanen dairy goat using immunohistochemistry and Western blot analysis. The antiserum researchers used in this study was rabbit anti-human ghrelin (Santa Cruz, USA). This polyclonal antibody raised against amino acids 21-60 mapping at the N-terminus of ghrelin of human origin. A search of the NCBI goat protein database identified goat ghrelin (Accession No. BAD34669). Amino acids 21-34 mapping at the N-terminus of goat ghrelin, AMAGSSFLSPEHQ exhibits 100% identity to rat and human ghrelin (Ida *et al.*, 2010). They reported that the major form of goat ghrelin is a 27 amino acid peptide that is octanoylated (C8:0) at Ser(3) and lacks Gln(14) which is present in rat and human ghrelin. Thus, it is reasonable to assume that this antiserum cross-reacts with goat ghrelin. Using this antiserum, researchers detected the largest number of the ghrelin-positive cells localized in the abomasus and the next largest number localized in the duodenum. The ghrelin-positive cells could be detected through the gastrointestinal tract of the Saanen dairy goat which are not congruent with studies results that

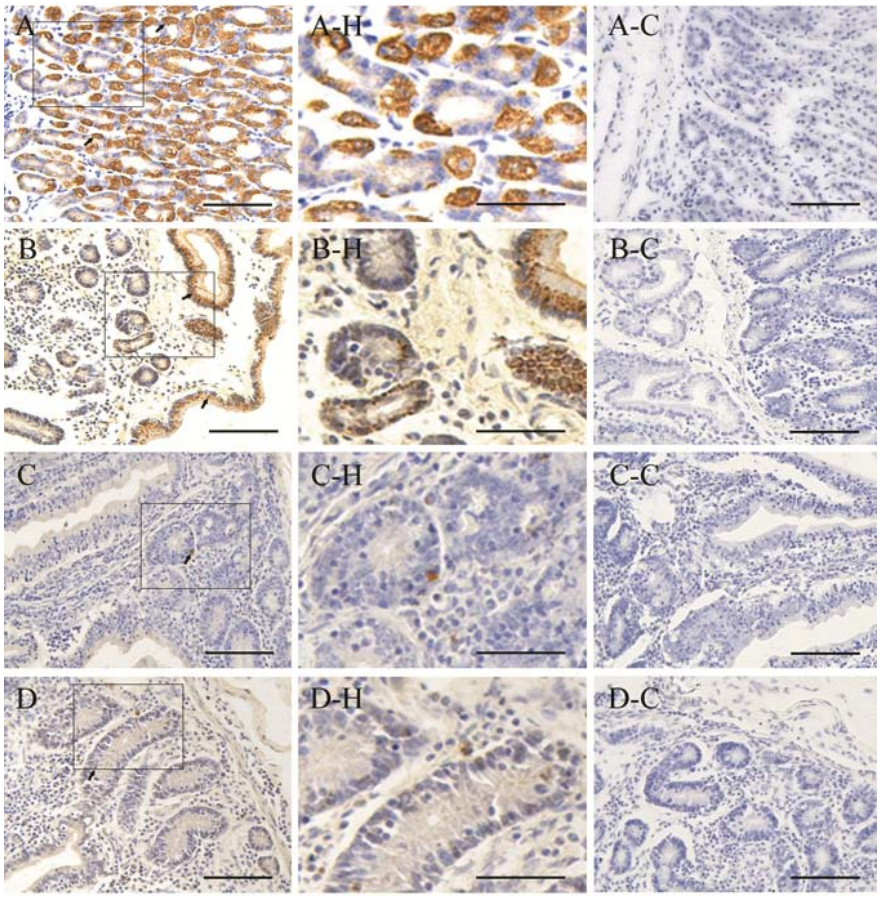

Fig. 3: Immunohistochemical staining of ghrelin in the gastrointestinal tract; A) The most numerous ghrelin-positive cells (arrows) were found in the abomasums mucosa. These cells contained closed-type cells (white arrows) and opened-type cells (black arrows); B) Less abundant ghrelin-positive cells (black arrows) were detected in the epithelium of the Brunner's gland in the duodenum, some ghrelin cells (white arrow) were observed in the crypts of the duodenum; C and D) Occasional ghrelin-positive cells (black arrows) were observed in the epithelium of the intestinal gland in the jejunum and ileum. (A-H)-(D-H) is the higher magnification of A-D section, (A-C)-(D-C) is the negative control section of A-D, no ghrelin-positive cell was detected in negative control sections. Scale bars: A-D, (A-C)-(D-C), 100 μm; (A-H)-(D-H), 50 μm

ghrelin-positive cell are not present in the human colonic and rat rectum (Date *et al*., 2000; Gronberg *et al*., 2008). This discrepancy may be due to differences in sensitivity of detection and/or species in the two studies.

Ghrelin-producing cells in the digestive tract of human and rat have been identified to be endocrine cells which are typical of the oxyntic mucosa and correspond to X/A-like cells (Kojima *et al*., 1999; Date *et al*., 2000). The findings on distribution patterns and morphologic characteristics of ghrelin-positive cells in goat abomasus and intestinal tract suggest that these ghrelin-positive cells correspond to endocrine cells. However, ghrelin-positive cells detected at the base of the stratified squamous epithelium in the esophagus, rumen, reticulum and omasum were not endocrine cells in the present study. Thus, further investigations are required to identify the specific cell types among various species on the basis of the current classification (Solcia *et al*., 2000).

In addition, researchers demonstrated that these ghrelin-producing cells are either opened or closed-type cells according to their relationship with the lumen. Earlier studies have indicated that the opened-type cells is functionally affected by the food content and pH in the lumen whereas the closed-type cells is affected by hormones, local factors, neuronal stimulation and/or mechanical stress (Solcia *et al*., 2000). The occurrence of the opened and closed type cell in the goat abomasus and intestinal tract especially in Brunner's glands suggests

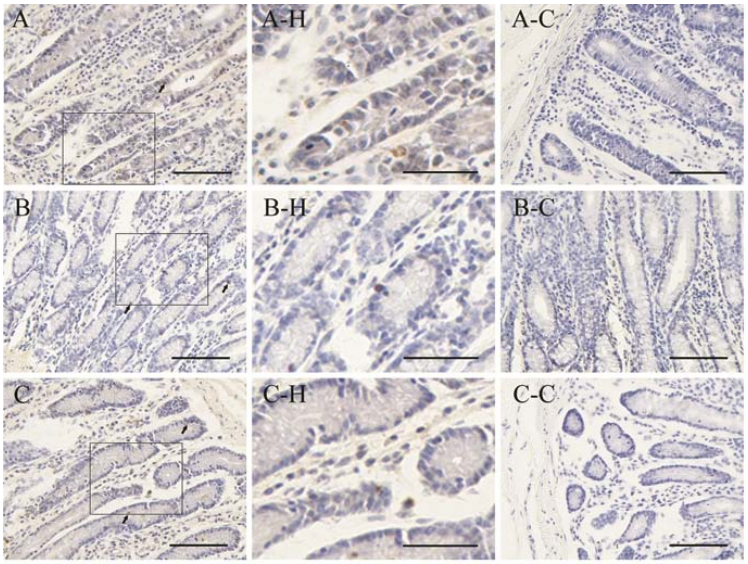

Fig. 4: Immunohistochemical staining of ghrelin in the gastrointestinal tract. A-C) Occasional ghrelin-positive cells (black arrows) were observed in the epithelium of the intestinal gland in the appendix, colon and rectum. (A-H)-(C-H) is the higher magnification of A-C section, (A-C)-(C-C) is the negative control section of A-C, no ghrelin-positive cell was detected in negative control sections. Scale bars: A-C, (A-C)-(C-C), 100 μm; (A-H)-(C-H), 50 μm

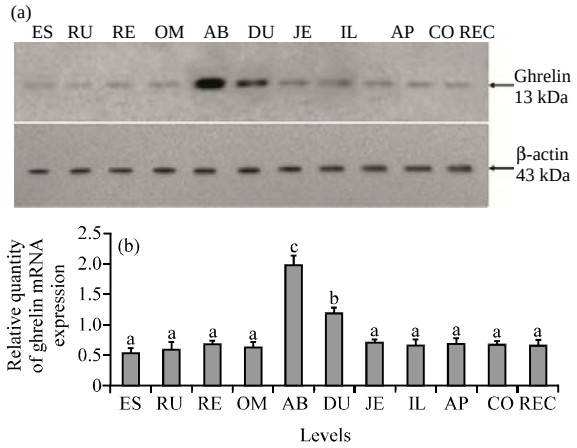

Fig. 5: a) Western blot analysis of ghrelin protein in different gastrointestinal tract tissues. β-actin was used as an internal loading control; b) Relative quantity of ghrelin protein expression. ES: Esophagus; RU: Rumen; RE: Reticulum; OM: Omasum; AB: Abomasum; DU: Duodenum; JE: Jejunum; IL: Ileum; AP: Appendix; CO: Colon; REC: Rectum. [a-c]Different letters indicate significant differences among segments according to Duncan's multiple range test ($p<0.05$)

that ghrelin-producing cells have specific physiological effect in each region and various factors regulate ghrelin synthesis in these cells.

CONCLUSION

Researchers have demonstrated that ghrelin mRNA and protein is expressed throughout the entire gastrointestinal tract of Saanen dairy goat. Ghrelin mRNA was significantly higher in the abomasus than in other tissues. The ghrelin-positive cells were most mumerous in the abomasus, less abundant in the duodenum, occasional in jejunum, ileum, appendix, colon and rectum and rare in esophagus, rumen, reticulum, omasum and abomasus. These results indicate that Saanen dairy goat as a ruminant has itself characteristics in its ghrelin distribution compared with other animals.

ACKNOWLEDGEMENTS

This research was supported by the China Postdoctoral Science Foundation (20090451402) and Sci-Tech Innovation Project of Shaanxi province (2010k01-8-1).

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