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The distribution of four homeobox proteins in the bovine stomach compartments during the fetal period

Distribución de cuatro proteínas homeobox en los compartimentos gástricos bovinos durante el período fetal

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ABSTRACT

Homeobox proteins undertake important roles in the determination of the cell identity during embryonic development, the differentiation of embryonic stem cells, morphogenesis, and the formation and development of the mammalian gastrointestinal tract. Based on these data, this study was designed to determine the immunohistochemical localization and expression levels of HOXA10, HOXA11, HOXC6 and HOXB6, which are subunits of the homeobox proteins, in the rumen, reticulum, omasum and abomasum during fetal development. For this purpose, fetuses obtained from private slaughterhouses and were grouped according to their crown-rump length (CRL) measurements, and the gestational trimester they belonged to, as follows: first trimester (69-89 days old/10 fetuses), second trimester (99-178 days old/10 fetuses) and third trimester (188-269 days old/10 fetuses). Gastric tissue samples taken from each group underwent routine histological processing and immunohistochemical staining. Immunohistochemical staining demonstrated that the HOXA10, HOXA11 and HOXC6 proteins were expressed at varying levels in the rumen, reticulum, omasum and abomasum, and their expression was stronger in the epithelial and smooth muscle cells. On the other hand, while there was almost no expression of HOXB6 in the epithelial cells of the rumen during the second and third trimesters of gestation, the remaining gastric components were immunonegative. Based on these findings, it was concluded that some homeobox proteins could have critical roles in the development, morphogenesis and histogenesis of fetal bovine gastric compartments, and thus, could contribute to the lifetime performance and productivity of cattle in terms of milk and meat yields.

Key words: bovine; fetus; homeobox proteins; stomach

RESUMEN

Las proteínas HOX/Hox, tiene funciones importantes en la determinación de la identidad celular durante el desarrollo embrionario, la diferenciación y la morfogénesis de las células madre embrionarias, así como en la formación y desarrollo del tracto gastrointestinal de los mamíferos. Con base en estos datos, este estudio fue diseñado para determinar la localización inmunohistoquímica y los niveles de expresión de HOXA10, HOXA11, HOXC6 y HOXB6, que son subunidades de las proteínas homeobox, en el rumen, retículo, omaso y abomaso durante el desarrollo fetal. Para ello, los fetos obtenidos de mataderos privados se agruparon según sus medidas de longitud cráneo-anca (LCC) y el trimestre gestacional al que pertenecían, de la siguiente manera: primer trimestre (69-89 días de edad/10 fetos), segundo trimestre (99-178 días/10 fetos) y tercer trimestre (188-269 días/10 fetos). Las muestras de tejido gástrico tomadas de cada grupo se sometieron a procesamiento histológico de rutina y tinción inmunohistoquímica. Como resultado de la tinción, se identificaron las proteínas HOXA10, HOXA11 y HOXC6; Se determinó que se expresaban con intensidades variables en el rumen, retículo, omaso y abomaso, y especialmente esta intensidad de expresión fue más fuerte en las células epiteliales y de la capa de músculo liso. Mientras que, la expresion de HOXB6 en el rumen fue casi inexistente en las células epiteliales a partir del segundo y tercer trimestre del gestacion, la inmunorreacción resultó negativa en todas las secciones restantes del estómago. Como resultado de estos hallazgos; Sugirió que algunas proteínas homeobox pueden tener una importancia crítica para el desarrollo, la morfogénesis y la histogénesis de los segmentos del estómago bovino fetal y, por lo tanto, contribuir al rendimiento y la productividad del ganado durante toda la vida, especialmente en términos de productividad de carne y leche.

Palabras clave: Bovino; fetos; proteínas homeobox; estómago

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INTRODUCTION

The digestive system of vertebrates is a unique structure that takes in and digests food, absorbs nutrients, and removes waste products from the body. Digestive system activity is closely related to the morphology of the digestive system as well as to the feeding habits of the organism [1]. In this context, the ruminant stomach with four compartments, are of interest as they convert low quality feed into highly nutritious products. Thus, there is need for a better understanding of the functionality, including the morphology, of the ruminant digestive system [2].

At parturition, the forestomach of the newborn, namely, the rumen, reticulum and omasum are undeveloped and therefore nonfunctional. On the other hand, during the neonatal period, the abomasum, otherwise known as the true stomach, is well-developed and highly functional. In this period, the abomasum, which is the fourth gastric compartment, constitutes the largest part (almost 70%) of the ruminant digestive system. The immature metabolic digestive system of neonatal calves resembles that of a young monogastric animal in terms of functionality. Before calves enter the rumination period, their rumen is small and loose, and the ruminal papillae are yet in a primitive state. During the phase of transition to rumination, the rumen grows 4- to 8-fold of its birth size yet still does not acquire the rumen wall thickness characteristic of the adult stage. With the advance of age, the spaces between the ruminal papillae and reticular cristae and the omasal laminae enlarge and become evident [3 , 4].

This developmental process of the ruminant stomach enables the rumen microorganisms to convert carbohydrates, proteins and other fermentable substances into volatile fatty acids, ammonium, methane, carbondioxide and microbial proteins. The ruminant forestomachs act as fermentation chambers, and thereby play an important role in the bacterial digestion of cellulose. These compartments also display peristaltic movements and contractions and enable the separation of fluids and solids. Histologically, the wall of the gastric compartments referred to as the ruminant forestomachs are composed of four layers, namely, the tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa. The tunica mucosa is composed of a lamina epithelialis of keratinized stratified squamous epithelium, a lamina propria and a lamina muscularis. The rumen differs from the other gastric compartments in that the ruminal mucosa does not contain a lamina muscularis. In all gastric compartments, the tunica muscularis comprises an inner stratum of circularly arranged muscles and an outer stratum of longitudinally arranged muscles. The stomach with four compartments are encapsulated by a tunica serosa. On the other hand, the abomasum differs from the forestomachs as it resembles the stomach of monogastric species and the tunica mucosa is lined by a simple, glandular and prismatic epithelium [5 , 6].

Homeobox genes encode the transcription factors referred to as homeodomains, which function as DNA-binding domains of 60 amino acids. These proteins take part in the regulation of several embryonic development processes such as axis formation, limb development and organogenesis [7]. The HOX proteins, known as the subunits of homeobox proteins, have been well investigated in the genome of two mammalian species, the mouse and the human. In total 39 Hox proteins have been identified in both species. These proteins are expressed as Hox for mice and HOX for humans and are clustered at four genomic loci under the names the Hox/HOX-A, B, C and D complexes, and each cluster is composed of 9-13 genes [8]. HOX/Hox proteins are major transcriptional regulators, which are involved in multiple processes ranging from embryogenesis to carcinogenesis [9].

These proteins have also been reported to affect the acquisition of cell identity during embryonic development, embryonic stem cell differentiation, and morphogenesis as well as the skeletal and nervous systems. Furthermore, they have been demonstrated to play a key role in the formation of the mammalian gastrointestinal tract, particularly in the differentiation of the muscle layer and epithelium [10 , 11 , 12]. It has been shown that the HOXA10 and HOXA11 proteins play a crucial effect in the organogenesis, development and differentiation of the reproductive system [13].

While no literature report is available on the potential role and distribution of homeobox proteins in the normal digestive system, it has been reported that HOXA10 and HOXA11 are associated with the presence, development and metastasis of gastric tumors [14 , 15]. Similarly, HOXB6 and HOXC6 are known to have very important roles in organogenesis, embryogenesis and cell differentiation [12 , 16 , 17]. Furthermore, it has been suggested that these proteins could have various physiological roles in the formation and development of tumors in certain organs (stomach, colon, lungs, prostate) and tissues [12 , 18 , 19].

The present study bears significance as it is the first investigation on the distribution of homeobox proteins, known to have significant roles in several processes including embryogenesis, organogenesis, morphogenesis and carcinogenesis as well as in several systems including the skeletal and nervous systems, in the gastric compartments of the bovine fetus. This study was aimed at: a) the immunohistochemical demonstration of the distribution of the HOXA10, HOXA11, HOXC6 and HOXB6 proteins in the rumen, reticulum, omasum and abomasum during fetal development, b) the determination of the potential physiological roles of the selected homeobox proteins during the prenatal period, and their various functional correlations for the different trimesters of gestation, c) the establishment of a better understanding of the mechanisms underlying gastric development in humans and mammals, and the investigation of the potential contribution of homeobox proteins to the structural components of the gastric compartments.

MATERIALS AND METHODS

Supply of the study material and preparation of the tissue samples

The study material comprised of 30 clinically healthy bovine fetuses (*Bos taurus*), which belonged to different gestational trimesters and were obtained from private slaughterhouses. The ages of the fetuses were determined using the formula ''y= 54.6 cm + 2.46 (x) cm'', based on the measurement of the crown-rump length (CRL). In this equation, ''x'' refers to the CRL and ''y'' expresses the fetal age in days (d) [12].

Once their ages were determined, the fetuses were assigned to three groups, as follows, according to the gestational trimester they belonged to: group of fetuses belonging to the first trimester (69-89 d old/10 fetuses), group of fetuses belonging to the second trimester (99-178 d old/10 fetuses), and group of fetuses belonging to the third trimester (188-269 d old/10 fetuses). In view of the specific trimester the fetus belonged to, each fetus underwent tissue sampling from the gastric compartments

(rumen, reticulum, omasum and abomasum). The tissue samples were fixed in 10% formol-alcohol solution for 18 h. Next, they were dehydrated through a graded series of alcohol, starting from 80% alcohol. Subsequently, the dehydrated tissue samples were passed through methyl benzoate, benzole and benzole-paraplast series, and embedded in paraffin. Later the paraffin blocks were cut into 5-micrometer-thick serial sections on a rotary microtome (Leica RM-2125, Germany). The sections were mounted onto slides coated with APES (3 amino propyl triethoxysilan; Sigma–Aldrich Chemicals, St. Louis, MO, USA) for immunohistochemical staining.

Immunohistochemical procedure

The serial sections mounted onto the APES-coated slides underwent immunohistochemical (IHC) staining according to the streptavidin-peroxidase technique and using the Zymed Histostain Plus Bulk Kit (South San Francisco, CA, USA, cat no: 85-9043). Firstly, the sections were allowed to dry, and they were deparaffinized twice in xylol, each time for 5 min, rehydrated in graded alcohols for 3 min in each grade, and washed in distilled water. Then, the endogenous peroxidase activity of the tissue samples was quenched with a 3% H_2O_2 solution in methanol for 20 min. Subsequently, the sections were washed three times, each time for 5 min, in phosphate-buffered saline (PBS, pH: 7.4; 0.01 M).

This was followed by the incubation of the preparations in a citrate buffer (pH: 6) for 30 min at 95ºC and until the temperature fell to room level for the designation of the antibody-binding antigenic sites. Later, to prevent the non-specific binding of the primary antibody, the sections were treated with a blocking solution (Ultra V Blok, catalog: TA-125-UB, Thermo Scientific) for 15 min. Once the solution was discarded, the sections were incubated overnight at +4 °C with antibodies diluted at a concentration of 1:100 (TABLE I). After being washed in PBS for another 3 times, each time for 5 min, at room temperature, the sections were incubated with biotinylated secondary antibody (Biotinylated Goat Anti-Polyvalent, catalog: TP-125-BN, Thermo Scientific) for 20 min at room temperature. This was followed by another 3 washes in PBS, each time for 5 min, and the treatment of the sections with streptavidin peroxidase (Thermo Fisher Scientific, catalog: TA-125-HR) for 20 min at room temperature. Finally, the sections were added 3.3 diaminobenzidine (DAB Substrate, Thermo Scientific, catalog no: TA-125-HD) and were maintained as such for a period from 5 to 15 min, depending on the reaction speed. After being washed in distilled water, the sections were applied Mayer's hematoxylin for 2 min for nuclear staining. Later, the sections were washed under running tap water for 5 min, dehydrated through a graded series of alcohol, cleared in xylol and mounted in Entellan. The stained preparations were examined and imaged under a Nikon Eclipse E400 research microscope (Nikon, Tokyo, Japan) with a DS-RI1 video camera (DS-U3, Nikon, Tokyo, Japan) attachment.

TABLE I. Primary antibodies used for immunohistochemistry (IHC)

Primer Antibodies	Clonality / Isotype	Host	Reactivity	Dilution	Catalog number
HOXA10	Polyclonal/IgG	Rabbit	Human, Mouse	1/100	St John's Laboratory, model no: STJ193159
HOXA11	Polyclonal/IgG	Rabbit	Human	1/100	Invitrogen, PA5-57341
HOXC6	Polyclonal/IgG	Rabbit	Rat, Cat, Human	1/100	Invitrogen, PA5-41479
HOXB6	Polyclonal/IgG	Goat	Human, Mouse, Rat, Dog, Bovine, Pig	1/100	St John's Laboratory, model no: STJ73348

Semiquantitative evaluation

The intensity scoring of the immunostaining was performed semiquantitatively. Intensity scores were determined based on the positive staining intensities of the cells as follows: 0 (Negative), 1 (Weak Immunoreactions), 2 (Moderate Immunoreactions), 3 (Strong Immunoreactions). Immunohistochemical assessment was performed by three independent blinded observers (UT, MEA & HS) and the mean scores of the three observers were calculated. Positive immunoreactions for HOXA10, HOXA11, HOXC6 and HOXB6 were identified in the high expression areas by scanning the rumen, reticulum, omasum and abomasum sections at 40x, 100x, 200x and 400x magnification. Five randomly selected areas were evaluated per section and the average of these individual results was taken as one value. Different components of the rumen, reticulum, omasum and abomasum, including epithelial cells, stromal cells and smooth muscle cells, were assessed. Blood vessels were not evaluated in detail, but their general appearance in some sections was described. In this study, 1250 values were averaged for each tissue and 2010 values were averaged per animal.

Statistical analysis

Analyses were evaluated with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). All values are shown as mean ± standard deviation (SD). Data normality was calculated with the Shapiro-Wilk test. The nonparametric Kruskal-Wallis test was used to analyze whether there was any significant difference in the immunohistochemical staining intensity score for HOXA10, HOXA11, HOXC6 and HOXB6 of the luminal epithelium, stromal cells and smooth muscle cells of the bovine fetal stomach compartments during the different trimesters of gestation or between these cell and tissue types. The Mann-Whitney U-test was used to detect differences in the staining score of each antibody among the cell types. Thereby, the final results were expressed as mean ± SD, and statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Histologically, bovine stomach sections consist of three layers called tunica mucosa, muscularis and serosa. The folds formed by the stratified squamous epithelium of the tunica mucosa towards the rumen are named as the ruminal papillae in the rumen, the reticular crista in the reticulum, and the omasal lamina in the omasum. It has been observed that these folds are short and present at varying numbers during the first trimester of gestation and increase in both height and density over the course of gestation as a result of cellular proliferation and differentiation. The abomasum was observed to have a glandular mucosa lined by a simple columnar epithelium, beneath of which gastric glands developed as of the second gestational trimester.

Based on immunohistochemical stainings, it was determined that while positive immunoreactions of varying intensity were present in the fetal rumen, reticulum, omasum and abomasum for HOXA10, HOXA11 and HOXC6 throughout gestation, HOXB6 was excluded from the statistical evaluation as it was only very weakly expressed in the luminal epithelial cells, and not expressed at all in the stromal cells and smooth muscle cells.

Homeobox proteins undertake critical roles in multiple tissue and cell processes, including among others, development, apoptosis, differentiation, motility, receptor signaling and angiogenesis [20]. These proteins are also known to have very important functions in the establishment of cell identity and cell position during embryonic development. It has been reported that changes in these proteins could lead to major developmental defects in the formation of body parts and cell identity [21]. Known to be positioned at the onset of mammalian development, Hox proteins are reported to be expressed in the ectoderm-derived layer (nervous system), mesoderm-derived layer (genitourinary system) and endoderm-derived layer (digestive system) and to take part in the development of these layers [22]. HOX/Hox proteins have also been described to be involved in the development of the skeletal and nervous systems and genital organs, as well as in the modelling of the mammalian gastrointestinal tract. It has been demonstrated that these proteins play a critical act in the differentiation of both the muscle layer and the epithelium of the gastrointestinal tract [11]. However, the presence and immunohistochemical distribution of the HOXA10, HOXA11, HOXC6 and HOXB6 proteins in the fetal bovine gastric compartments have been demonstrated for the first time in the present research.

Reports indicate that, in human fetuses, the HOXA10 and HOXA11 proteins are generally localized along the paramesonephric canal and contribute to embryogenesis as well as to the development and differentiation of the uterus [23]. Previous studies have demonstrated the expression of these proteins in the female genitals, skeleton and kidneys of (*mouse*) [16], the uterus of humans [24], (*apes*) [25], (*rattus*) [26], and (*swine*) [27], the bovine placenta [12], (*felis catus*) testes [28], and bovine fetal liver [16], and have suggested their contribution to the development of the normal physiological functions of these organs.

In a previous study on the healthy human gastric mucosa, it was determined that the weak expression of the HOXA10 protein increased in cases of gastric cancer and the differentiation of gastric cancer. Furthermore, the HOXA10 protein has also been reported to be expressed in the colon mucosa with normal metaplasia [29, 30]. In another study, it was suggested that HOXA10, which enables the normal development and differentiation of hematopoietic stem cells, when expressed at an increased level increases the proliferation of gastric cancer cells, strengthens the tumor and prevents apoptosis in mice [15].

On the other hand, HOXA11, known to have key roles in cell proliferation and migration, shows an expression level similar to that of HOXA10, which increases in cases of the malignant tumors of the mammary glands, ovaries, endometrium and lungs [31, 32, 33]. It has been shown that, in humans, while the expression of HOXA11 in the normal gastric mucosa is negative, it is positive and strong in cancerous gastric mucosa [34]. In another study on the human gastric mucosa, it was determined that the expression of the HOXA11 gene with DNA methylation occurred at a higher level in cancerous gastric mucosa compared to the normal mucosa [35].

Based on these findings, it was observed that previous research on the distribution of the HOXA10 and HOXA11 proteins in the stomach was rather limited and generally related to the development of gastric cancer in humans and mice. While these proteins have been reported to be present in the normal gastric mucosa of humans [29, 30, 34], literature review showed that there was no study available on their expression and functions during normal gastric development in other species. In the present study, it was determined that the HOXA10 vand HOXA11 proteins induced immunoreactions in some cells and structures of the bovine gastric compartments throughout fetal development.

Rumen

The HOXA10 and HOXA11 proteins were observed to induce strong immunoreactions localized to the cytoplasm and nucleus of the rumen epithelial cells during the first trimester of gestation (FIG. 1 A, B, a, b). The intensity of these immunoreactions decreased to a level below moderate for HOXA10 in the second trimester but increased to a moderate or moderate to strong level in the third trimester (FIG. 1 E, e, I, ı). The intensity of the immunoreactions for the HOXA11 protein over the course of gestation was observed to be moderate or slightly stronger than moderate in the second trimester, and moderate or slightly weaker than moderate in the third trimester (FIG. 1 F, f, K, k). Furthermore, the immunoreactions for HOXC6, which were slightly stronger than moderate in the first trimester, showed slight weakening in the second and third trimesters and remained at the same level thereafter (FIG. 1 C, c, G, g, L, l).

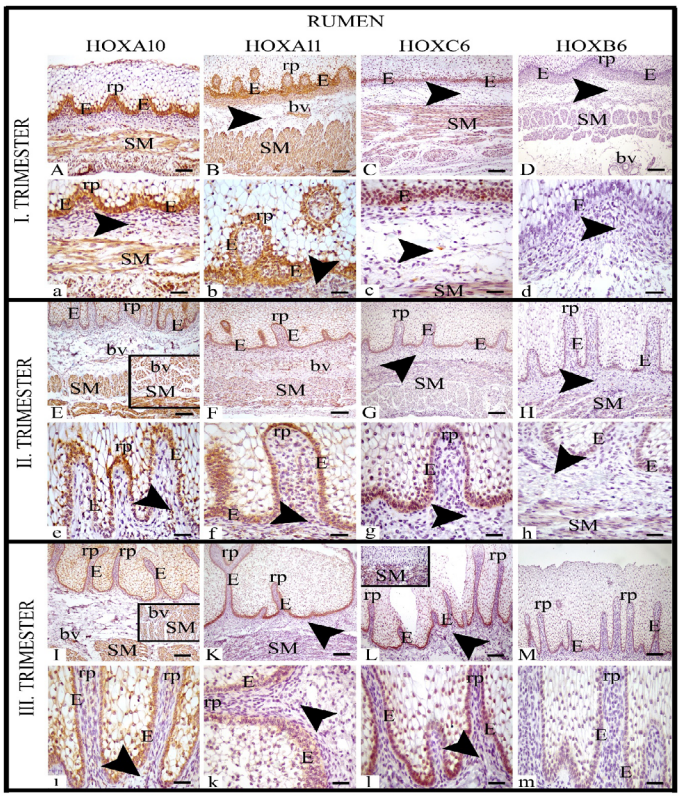

FIGURE 1. Immunolocalisation of HOXA10, HOXA11, HOXC6 and HOXB6 in fetal bovine rumen, in the first trimester of pregnancy (69 days) (A, B, C, D, a, b, c, d), second trimester (99 days) (E, F, G, H, e, f, g, h), third trimester (249 days) (I, K, L, M, ı, k, l, m). E: Epithelium, Black arrowhead: Stroma cell, SM: Smooth Muscle layer, rp: ruminal papillae, bv; blood vessel. Scale Bar: 100 µm (A, B, C, D, E, F, G, H, I, K, L, M), magnification: 10X; 25 µm (a, b, c, d, e, f, g, h, ı, k, l, m), magnification: 40X.

The intensity of immunoreactions induced by HOXA10 and HOXA11 in the stromal cells was moderate or close to moderate in the first trimester but progressively weakened in the second and third trimesters. Moreover, immunoreactions for HOXC6 were very weak and almost absent in the stromal cells throughout gestation. In the smooth muscle cells, the HOXA10 protein was observed to induce moderate immunoreactions throughout gestation.

The strong immunoreactions for HOXA11 in the first trimester weakened to a moderate level of intensity in the second trimester and to an intensity ranging from moderate to weak in the third trimester. On the other hand, the moderately intense immunoreactions for HOXC6 in the first trimester were observed to weaken to a level of almost disappearance in the second and third trimesters. Furthermore, some blood vessels presented with positive immunoreactions for HOXA10 during the second and third trimesters of gestation, and for HOXA11 during the first and second trimesters (FIG. 1).

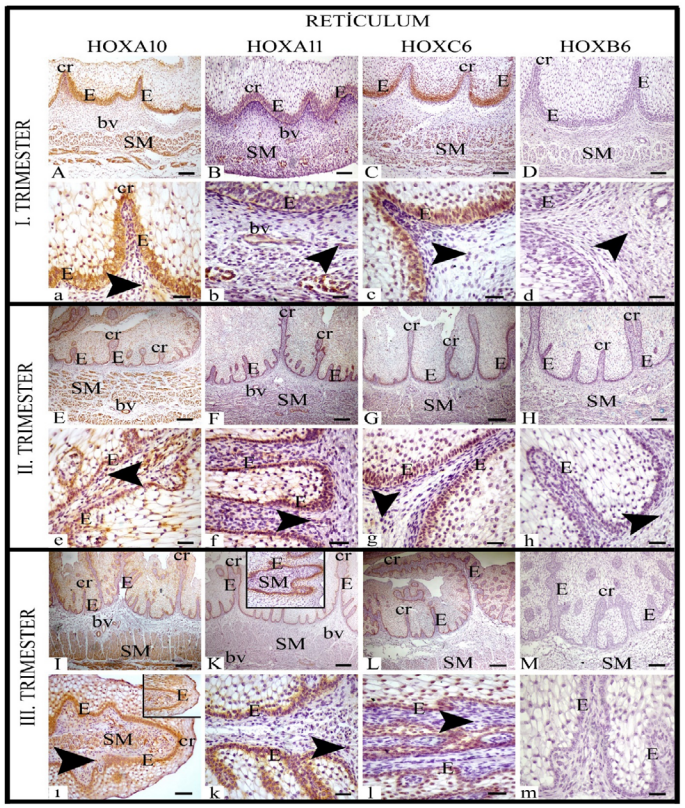

FIGURE 2. Immunolocalisation of HOXA10, HOXA11, HOXC6 and HOXB6 in fetal bovine reticulum, in the first trimester of pregnancy (86 days) (A, B, C, D, a, b, c, d), second trimester (123 days) (E, F, G, H, e, f, g, h), third trimester (227 days) (I, K, L, M, ı, k, l, m). E: Epithelium, Black arrowhead: Stroma cell, SM: Smooth Muscle layer, cr: crista reticuli, bv; blood vessel. Scale Bar: 100 µm (A, B, C, D, E, F, G, H, I, K, L, M), magnification: 10X; 50 µm (ı), magnification: 20X; 25 µm (a, b, c, d, e, f, g, h, k, l, m), magnification: 40X.

Reticulum

During the first trimester, the HOXA10 and HOXC6 proteins were determined to produce strong immunoreactions localized to the nucleus and cytoplasm of the epithelial cells (FIG. 2A, a, C, c). While the intensity of these immunoreactions decreased to a moderate level for HOXA10 as gestation advanced, it significantly and progressively decreased to a moderate and then to a weak level for HOXC6. On the other hand, immunoreactions for HOXA11 during the first and second trimesters increased from weak to moderate in intensity and maintained a moderate level throughout the third trimester of gestation (FIG. 2 B, b, F, f, K, k). The immunoreactions produced by the HOXA11 and HOXC6 proteins in the stromal cells were observed to be very weak throughout gestation. The immunoreactions, which were moderate for HOXA10 in the first trimester weakened as gestation advanced. In smooth muscle cells, the HOXA10 protein induced strong immunoreactions during the first and third trimesters, and moderately intense immunoreactions in the second trimester. Immunoreactivity for HOXA11 was weak in the smooth muscle cells throughout gestation. Moreover, HOXC6 immunoreactivity, which was close to moderate in the smooth muscle cells during the first trimester, were observed to become weak in the second and third trimesters. In some blood vessels, HOXA10 immunoreactivity was observed in the first trimester, while HOXA11 immunoreactivity was observed in the first and third trimesters. (FIG. 2).

These immunoreactions, which were observed to be strong in the epithelial cells of the rumen, reticulum, omasum and abomasum during the first trimester, showed statistically insignificant decrease and increase as gestation advanced ($P>0.05$). Furthermore, in the fetal rumen and reticulum the luminal epithelial cells displayed stronger HOXA10 and HOXA11 expressions compared to the stromal cells ($P<0.05$). Similarly, the expressions of HOXA10 and HOXA11 were stronger in the luminal epithelial cells, when compared to the smooth muscle cells, yet this difference was statistically insignificant.

These findings obtained in the present study suggest that HOXA10 and HOXA11 may positively contribute to the proliferation of the luminal epithelial and smooth muscle cells, and some of the stromal cells of the bovine gastric compartments throughout fetal development, as well as to the developmental regulation and normal functioning of these organs.

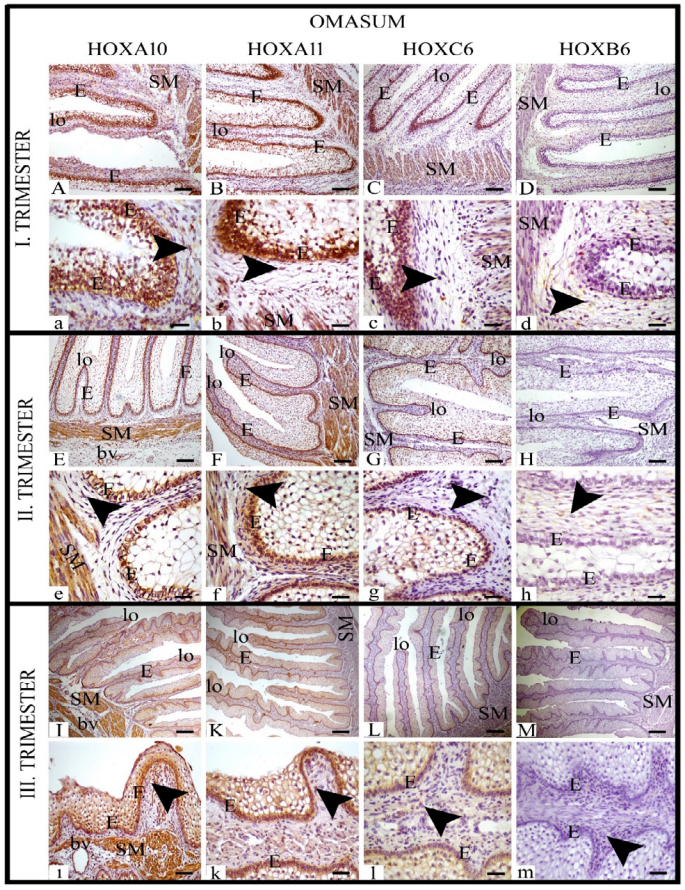

FIGURE 3. Immunolocalisation of HOXA10, HOXA11, HOXC6 and HOXB6 in fetal bovine omasum, in the first trimester of pregnancy (82 days) (A, B, C, D, a, b, c, d), second trimester (178 days) (E, F, G, H, e, f, g, h), third trimester (257 days) (I, K, L, M, ı, k, l, m). E: Epithelium, Black arrowhead: Stroma cell, SM: Smooth Muscle layer, lo: lamina omasi, bv; blood vessel. Scale Bar: 100 µm (A, B, C, D, E, F, G, H, I, K, L, M), magnification: 10X; 25 µm (a, b, c, d, e, f, g, h, ı, k, l, m), magnification: 40X.

Omasum

The HOXA10 and HOXA11 proteins induced strong immunoreactions in the omasal epithelium during the first trimester, which decreased to either a moderate or close to moderate level during the second and third trimesters (FIG. 3 A, a, E, e, I, ı, B, b, F, f, K, k). However, in the first trimester, HOXC6 produced close to moderate immunoreactions, which were observed to be weaker during the second and third trimesters (FIG. 3 C, c, G, g, L, l). In the stromal cells, there was scarcely any immunoreactivity for HOXC6 and HOXA11 throughout all three trimesters. On the other hand, the weak immunoreactions observed for HOXA10 during the first and second trimesters were observed to be stronger and of close to moderate intensity in the third trimester. In the smooth muscle cells, during the first and second trimesters, the HOXA10 and HOXA11 proteins induced moderate and stronger than moderate immunoreactions, which increased in intensity for HOXA10 but decreased in intensity for HOXA11 in the third trimester. However, the immunoreactions, which ranged from weak to moderate for HOXC6 in the smooth muscle cells during the first trimester, decreased in intensity during the second and third trimesters to a constant weak level (FIG. 3).

Abomasum

In the abomasal epithelial cells, while the immunoreactions induced by the HOXA10 and HOXC6 proteins were either moderate or moderate to strong in the first trimester, those induced by HOXA11 were rather strong (FIG. 4 A, a, B, b, C, c). While HOXA10 produced strong immunoreactivity in the second and third trimesters, HOXA11 induced moderate immunoreactions in the second trimester and very strong immunoreactions in the third trimester (FIG. 4 E, e, F, f, I, ı, K, k). The weaker than moderate immunoreactivity for HOXC6 during the second gestational trimester increased to a strong intensity in the last trimester (FIG. 4 G, g, L, l).

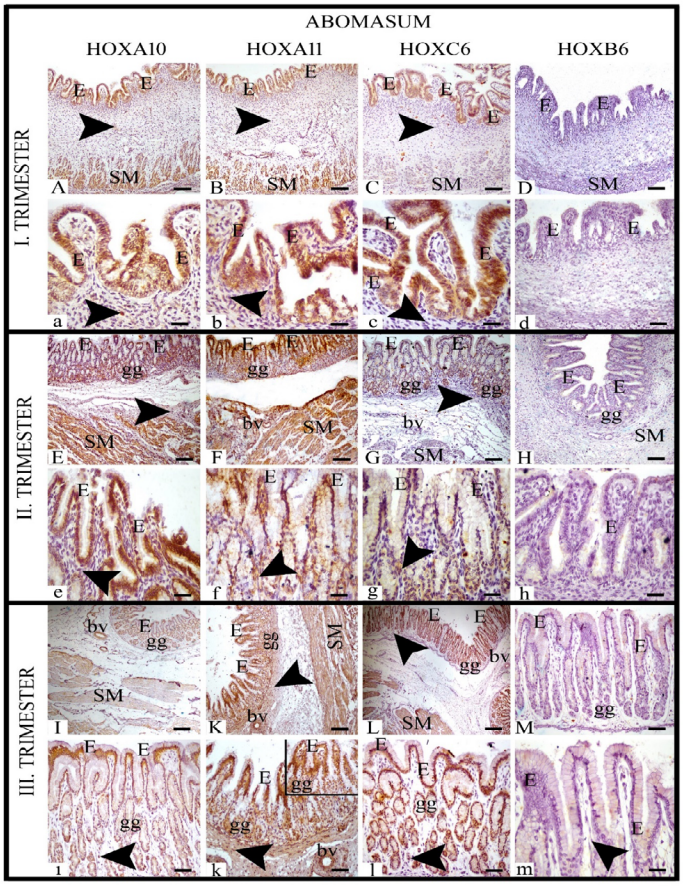

FIGURE 4. Immunolocalisation of HOXA10, HOXA11, HOXC6 and HOXB6 in fetal bovine abomasum, in the first trimester of pregnancy (73 days) (A, B, C, D, a, b, c, d), second trimester (106 days) (E, F, G, H, e, f, g, h), third trimester (269 days) (I, K, L, M, ı, k, l, m). E: Epithelium, Black arrowhead: Stroma cell, SM: Smooth Muscle layer, gg: gastric gland, bv; blood vessel. Scale Bar: 100 µm (A, B, C, D, E, F, G, H, I, K, L, M), magnification: 10X; 50 µm (d, ı, k, l,), magnification: 20X; 25 µm (a, b, c, e, f, g, h, m), magnification: 40X.

Moreover, the immunoreactions for HOXA10, HOXA11 and HOXC6 proteins in the epithelial cells were localized to the basal cytoplasm and nucleus during the third trimester. In the stromal cells, there was almost no immunoreactivity for the HOXA10 and HOXC6 proteins throughout gestation. While HOXA11 immunoreactions displayed similarity to those for the HOXA10 and HOXC6 proteins during the first and second trimesters, they were observed to increase in intensity during the third trimester to a level stronger than moderate. In the smooth muscle cells, the HOXA10 protein was observed to induce close to moderate

immunoreactions during all three trimesters. Likewise, while HOXA11 produced moderate or slightly stronger than moderate immunoreactions during the first and second trimesters of gestation, immunoreactivity was determined to reach a rather strong intensity level in the last trimester. However, the weak immunoreactivity observed for HOXC6 during the first and second trimesters increased to a moderate level of intensity in the third trimester. Furthermore, the gastric glands, which were observed to start to form and develop in the second trimester, displayed moderately intense immunoreactions for HOXA10, HOXA11 and HOXC6 proteins. These positive immunoreactions grew stronger for all three proteins in the third trimester. Moreover, the endothelial cells lining some blood vessels displayed positive immunoreactivity for the HOXA10 and HOXC6 proteins in the last trimester and for HOXA11 during both the second and third trimesters (FIG. 4). Furthermore, in the fetal omasum and abomasum, the luminal epithelial cells displayed stronger HOXA10 and HOXA11 expressions compared to the stromal cells ($P<0.05$). Furthermore, the expressions of the HOXA10 and HOXA11 proteins in some blood vessels of the rumen, reticulum, omasum and abomasum during the different trimesters of gestation suggest that these proteins could also be involved in the proliferation of the vascular endothelial cells, as well as in angiogenesis. Moreover, it was concluded that these proteins may have critical roles in the development and physiological functioning of the gastric glands, which start to form as of the second trimester.

While almost no immunoreactivity was detected for the HOXB6 protein in the ruminal epithelial cells during the second and third trimesters, immunoreactivity was negative for the remaining gastric compartments (FIG. 1-4 D, d, H, h, M, m).

Previous studies have reported the involvement of the HOXC6 protein in mammary gland development and milk production. Known to be expressed in normal tissues and cells, HOXC6 has also been determined to display excessively increased expression in cases of mammary, pulmonary and prostatic cancers. Thus, this protein has been observed to be directly associated with the presence and development of cancer [36]. It has also been reported that, although not being expressed in the mucosal cells of the normal human stomach, the HOXC6 protein is expressed in the mucosal cells of cancerous gastric mucosal cells, suggesting the involvement of this protein in the pathogenesis of gastric cancer [20].

On the other hand, in another study, it was determined that HOXC6 was expressed in the nucleus and cytoplasm of normal human gastric mucosal cells, and that its expression increased in cases of gastric cancer [37]. Literature reviews showed that reports on the presence of HOXC6 in the normal stomach was limited to humans as species, and that these reports were mostly related to the presence and pathogenesis of cancer. In parallel with the report of [20], the present study demonstrated that the HOXC6 protein produced positive immunoreactions in the fetal gastric compartments. While these immunoreactions ranged from moderate to strong in the epithelial cells of the rumen, reticulum, omasum and abomasum during the first trimester, over the course of gestation their intensity increased in the abomasum but decreased in the other gastric compartments ($P>0.05$).

The moderate HOXC6 immunoreactions detected in the smooth muscle cells of the rumen, reticulum and omasum during the first trimester weakened in intensity over the course of gestation. However, the weak HOXC6 immunoreactions detected in the abomasum during the first trimester were observed to increase to a moderate level of intensity as gestation advanced ($P>0.05$). In the stromal cells, there was almost no immunoreactivity throughout gestation, such that when compared to the epithelial and smooth muscle cells, the intensity of the immunoreactions was significantly lower ($P<0.05$).

These findings explained in detail suggest that HOXC6 could have major roles in the regulation of the development of the fetal bovine gastric compartments and the proliferation and differentiation of the luminal epithelial and smooth muscle cells. Moreover, the periodic differences observed in the intensity of cellular immunoreactions suggested that the cellular effects of HOXC6 changed and that this protein could be included in the cellular structure, and thereby, affect physiological processes. Furthermore, HOXC6 was ascertained to induce immunoreactions in the abomasal gastric glands, the intensity of which increased over the course of gestation. Based on this finding, it was concluded that HOXC6 could positively contribute to the development, growth and physiological functioning of these glands.

Statistical findings for the different cell types and gestational periods

The intensity scores for the expression of the HOXA10, HOXA11, HOXC6 and HOXB6 proteins in the fetal rumen, reticulum, omasum and abomasum throughout gestation are summarized in TABLE II. It was determined that, during all three trimesters, the expressions of HOXA10, HOXA11 and HOXC6 were stronger in the luminal epithelial cells of the fetal rumen, reticulum, omasum and abomasum, compared to the stromal cells ($P<0.05$). Similarly, while the expressions of HOXA10, HOXA11 and HOXC6 were stronger in the luminal epithelial cells, compared to the smooth muscle cells, this difference was statistically insignificant ($P>0.05$) (TABLE II). The comparison of the gestational trimesters demonstrated that there was no difference between the first, second and third trimesters for the expressions of HOXA10, HOXA11 and HOXC6 ($P>0.05$) (TABLE II).

TABLE II. Intensity scores for HOXA10, HOXA11, HOXC6 and HOXB6 expression in rumen, reticulum, omasum and abomasum of bovine fetuses during pregnancy. Means ± SD.

Immunohistochemical Parameter	Pregnancy Trimesters	Stomach Departments	Histological Layers		
			Luminal Epithelium	**Stromal**	**Smooth Muscle**
HOXA10	**1st**	**Rumen**	2.80±0.42^{a}	2.0±0.0^{b}	1.90±0.32^{b}
		Reticulum	2.70±0.48	2.0±0.47	2.60±0.52
		Omasum	2.50±0.53^{a}	1.40±0.52^{b}	2.20±0.42^{ab}
		Abomasum	2.30±0.48^{a}	1.10±0.32^{b}	1.80±0.42^{ab}
	2nd	**Rumen**	1.70±0.48	1.50±0.53	2.20±0.42
		Reticulum	2.30±0.48^{a}	1.30±0.48^{b}	2.30±0.48^{a}
		Omasum	1.80±0.42	1.50±0.53	2.20±0.42
		Abomasum	2.60±0.52^{a}	1.50±0.53^{b}	1.90±0.32^{ab}
	3rd	**Rumen**	2.40±0.52^{a}	1.30±0.48^{b}	2.20±0.42^{ab}
		Reticulum	2.40±0.52	1.60±0.52	2.50±0.53
		Omasum	1.90±0.32	1.80±0.42	2.60±0.52
		Abomasum	2.50±0.53^{a}	0.90±0.32^{b}	2.20±0.42^{a}
HOXA11	**1st**	**Rumen**	2.60±0.52^{a}	1.80±0.42^{b}	2.50±0.53^{ab}
		Reticulum	1.50±0.53	0.80±0.42	1.20±0.42
		Omasum	2.60±0.52^{a}	1.50±0.53^{b}	2.30±0.48^{ab}
		Abomasum	2.60±0.52^{a}	0.90±0.32^{b}	2.30±0.48^{a}
	2nd	**Rumen**	2.20±0.42	1.70±0.48	2.10±0.32
		Reticulum	1.70±0.48	0.80±0.42	1.30±0.48
		Omasum	2.40±0.52^{a}	1.20±0.42^{b}	2.20±0.42^{a}
		Abomasum	2.30±0.48^{a}	1.20±0.42^{b}	2.30±0.48^{a}
	3rd	**Rumen**	1.80±.0.42	1.20±0.42	1.60±0.52
		Reticulum	2.10±0.32^{a}	0.70±0.48^{b}	1.20±0.42^{ab}
		Omasum	1.90±0.32^{a}	0.90±0.32^{b}	1.70±0.48^{ab}
		Abomasum	2.80±0.42	2.40±0.52	2.80±0.42
HOXC6	**1st**	**Rumen**	2.30±0.48^{a}	0.80±0.42^{b}	1.80±0.42^{ab}
		Reticulum	2.50±0.53^{a}	1.20±0.42^{b}	1.80±0.42^{ab}
		Omasum	1.80±0.42	0.70±0.48	1.60±0.52
		Abomasum	2.30±0.48^{a}	0.90±0.32^{b}	1.30±0.48^{ab}
	2nd	**Rumen**	1.80±0.42	0.60±0.52	0.80±0.42
		Reticulum	1.50±0.53	0.70±0.48	1.30±0.48
		Omasum	1.50±0.53	0.60±0.52	1.30±0.48
		Abomasum	1.70±0.48^{a}	0.60±0.48^{b}	0.90±0.32^{ab}
	3rd	**Rumen**	1.80±0.42	0.60±0.52	0.80±0.42
		Reticulum	1.80±0.42^{a}	0.70±0.48^{b}	1.40±0.52^{ab}
		Omasum	1.60±0.52^{a}	0.60±0.52^{b}	1.20±0.42^{ab}
		Abomasum	2.40±0.52^{a}	0.70±0.48^{b}	1.70±0.48^{b}

HOXB6	1st	**Rumen**	N/W	N	N
		Reticulum	N/W	N	N
		Omasum	N/W	N	N
		Abomasum	N/W	N	N
	2nd	**Rumen**	N/W	N	N
		Reticulum	N/W	N	N
		Omasum	N/W	N	N
		Abomasum	N/W	N	N
	3rd	**Rumen**	W/N	N	N
		Reticulum	N/W	N	N
		Omasum	N/W	N	N
		Abomasum	N/W	N	N

Different superscripts (a, b) in the same line indicate significant differences among epithelial, stroma and smooth muscle cells (P<0.05). Intensity scores (IS) of HOXA10, HOXA11 and HOXC6 immunostaining (0: no staining at high magnification, 1: immunostaining only visible at high magnification, 2: readily visible at low magnification, 3: strikingly positive at low power magnification. HOXB6 was excluded from statistical evaluation because its expression was only in the epithelium and was weak. W; weak and N; negative

Previous studies on HOXB6 have demonstrated that this protein induces immunoreactions in human hematopoietic progenitor-stem cells and affects the proliferation and differentiation of murine hepatic cells [9 ,38 ,39]. Furthermore, it has also been shown that HOXB6 is expressed during the oncogenic processes of some organs and tissues (esophagus and hepatocytes) and undertakes critical roles in the proliferation, migration and invasion of cells in these organs and tissues [34]. Previous studies on the digestive system have revealed that the intensity of the expression of the HOXB proteins progressively increases from the esophagus to the intestines [40]. It has also been reported that HOXB6 plays a key role in the gastrointestinal system of humans and mice and is expressed in the normal human esophagus [41].

Moreover, it has been indicated that HOXB6 is expressed in the normal human gastric mucosa and that the expression of this protein increases in tumoral tissues. Thereby, a direct association has been established between HOXB6 and gastric cancer stages and tumor grades [42]. In a study on the development of the chicken stomach, it was determined that HOXB6 was expressed in the mucosa of the stomach and affected its development process [43]. In parallel with the findings of studies on the normal stomach of humans [42] and chickens [43], it has been reported that HOXB6 is also expressed in the bovine placenta [12] and, fetal bovine liver [16] and may contribute to physiological processes. However, in the present study, we determined that, excluding the rumen, HOXB6 was not expressed in any of the fetal bovine gastric compartments throughout gestation. The very weak expression of HOXB6 in the rumen after the second trimester of gestation suggests that this protein joins the structure of ruminal cells and affects the development of the rumen only after this period. Moreover, HOXB6 not being expressed in the other fetal bovine gastric compartments was attributed to the different structure and physiological functions of the bovine rumen, reticulum, omasum and abomasum, compared to the human and avian stomachs.

CONCLUSION

In conclusion, that the HOXA10, HOXA11 and HOXC6 proteins were present at varying levels in the epithelial, stromal and smooth muscle cells of the bovine gastric compartments throughout prenatal development. It was considered that HOXA10, HOXA11 and HOXC6 could have important physiological roles in the regulation of the development of the bovine gastric compartments and the proliferation and differentiation of the cells of these compartments. In view of the milk and meat yields of food-producing animals being directly related to stomach activity, it was concluded that these proteins could have significant effects on the normal gastric development process, and thus, could directly contribute to the sustainability of animal production. Furthermore, it can be suggested that homeobox proteins may play a role in the tumorigenesis of the bovine gastrointestinal system, and they could potentially be used as prognostic biomarkers in gastrointestinal tumors in future studies.

Declaration of Competing Interest

The authors declare no conflict of interest.

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