Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
28 (5): 543-552, 2022
DOI: 10.9775/kvfd.2022.27526

Research Article

The Expression Profile of Some Homeobox Proteins in the Bovine Liver During Prenatal Development

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Article ID: KVFD-2022-27526 Received: 05.04.2022 Accepted: 26.08.2022 Published Online: 10.09.2022

Abstract: Homeobox proteins play critical roles in controlling processes such as morphogenesis and organogenesis in many organisms. Some of these proteins are known to affect the formation, development and regeneration of the liver. In this context, the present study was aimed at demonstrating the localization and expression intensity of some homeobox proteins in the bovine fetal liver during the different stages of gestation, determining whether or not these proteins are found in the structural components of the liver, and identifying their potential physiological roles. The study material comprised of 27 clinically healthy bovine fetuses, which were obtained from slaughterhouses and belonged to different stages of gestation. The fetuses were grouped according to their crown-rump length (CRL) measurements. Liver samples were taken from each study group and subjected to routine histological processing, followed by immunohistochemical staining. The staining results showed that, throughout gestation, the expression intensities of the homeobox proteins HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX were stronger in the hepatocytes, compared to the hepatic artery, vena interlobularis and bile ducts. However, the expression intensity of HLX was determined to have significantly decreased during the second and third trimesters of gestation, compared to the first trimester. In conclusion, the expression of the investigated homeobox proteins at differing and similar levels in the hepatocytes, hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during gestation could be interpreted as an important indicator of these proteins being involved in the development and physiological activity of the fetal liver.

Keywords: *Bovine, Fetus, Hepatocytes, Homeobox proteins, Liver*

Prenetal Gelişim Süresince Sığır Karaciğerindeki Bazı Homeobox Proteinlerinin Ekspresyonu

Öz: Homeobox proteinleri, birçok organizmada morfogenezis ve organogenezis gibi süreçlerin kontrol edilmesinde kritik roller üstlenmektedir. Bu proteinlerin bir kısmının karaciğerin oluşumu, gelişimi ve rejenerasyonuna da etki ettiği bilinmektedir. Bu nedenle çalışmamız; Homeobox proteinlerinin sığır fötal karaciğerinde gebeliğin farklı dönemlerinde bazı homeobox proteinlerinin lokalizasyonu ve ekspresyon yoğunluğunu göstermek, karaciğerin yapısal bileşenlerine katılıp katılmadığını ve olası fizyolojik rollerini belirlemek amacı ile yapılmıştır. Çalışmada kesimhanelerden temin edilen gebeliğin farklı dönemlerine ait ve klinik olarak sağlıklı 27 adet fetüs kullanıldı. Kullanılan fetüslerin gruplandırılması da alın-sağrı uzunluğu (Crown-Rump Lenght; CRL) ölçümüne göre yapıldı. Belirlenen her gruptan alınan karaciğer örnekleri rutin histolojik prosedürlerinden geçirilerek immunohistokimya boyamasına tabi tutuldu. Boyama sonucunda gebelik dönemlerine göre karaciğer hepatositleri, arteria hepatika, vena interlobularis ve ductus biliferilerdeki HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 ve HLX ekspresyon yoğunlukları karşılaştırıldığında gebelik süresince hepatositlerdeki reaksiyonun diğerlerine oranla daha güçlü olduğu görüldü. Ancak, HLX ekspresyon yoğunluğunun gebeliğin 2. ve 3. trimesterlarında gebeliğin 1. trimesterına göre anlamlı bir şekilde azaldığı belirlendi. Sonuç olarak bazı homeobox proteinlerinin fötal sığır karaciğerindeki hepatositlerde, arteria hepatika, vena interlobularis ve ductus beliferuslarda gebeliğin her döneminde benzer ve farklı düzeylerde ekspresse olması bu proteinlerin fötal karaciğerin gelişiminde ve fizyolojik aktivitesinde rol oynadıklarının önemli bir kanıtı olabilir.

Anahtar sözcükler: *Sığır, Fetüs, Hepatositler, Homeobox proteinler, Karaciğer*

Introduction

The liver, which is the largest and most functional visceral organ of the body, develops from the intestinal endoderm in the mid-third week of embryonic development [1,2], and owing to its hematopoietic role, displays a rapid development in the prenatal period, such that it constitutes nearly 10% of the fetal weight by the 10th week of gestation.

How to cite this article?

Topaloğlu U, Sağsöz H, Akbalik ME, Ketani MA, Güney Saruhan B, Aydin N: The expression profile of some homeobox proteins in the bovine liver during prenatal development. *Kafkas Univ Vet Fak Derg,* 28 (5): 543-552, 2022.
DOI: 10.9775/kvfd.2022.27526

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The hematopoietic activity of the liver continues until the last two months of the prenatal period, and progressively decreases until parturition, such that only very small hematopoietic islets are observed at the time of birth [2,3].

The liver plays a critical role in both viability and some digestive processes. This organ is involved in metabolic processes such as hematopoiesis and blood volume regulation in the embryonic period, as well as in protein synthesis, immunity, the endocrine control of growth signal pathways, and physiological processes such as metabolite deposition, bile secretion and detoxification [4,5].

The liver is composed of different types of embryonic cells (hepatocytes, biliary epithelial cells-cholangiocytes, stellate cells, Kupffer cells and hepatic sinusoidal endothelial cells). Each of these different cell types have unique tasks, which complement each other in the functioning of the liver. Hepatocytes, which comprise the primary epithelial cell population of the liver, make up the majority (60%) of the hepatic volume and undertake multiple tasks. Hepatocytes have been demonstrated to be regulatory cells that are critical to nutrient transport as well as fetal growth and development. On the other hand, Kupffer cells are described as resident hepatic macrophages. These cells are capable of responding to pathogenic stimuli carried by the hepatic portal circulation, and depending on a series of contributing factors, may play pro- or anti-inflammatory roles in hepatic wound healing [5,6].

Homeobox genes encode the homeodomain proteins, which regulate development, differentiation and morpho-genesis in various organisms, including animals and plants [7]. By means of hematopoietic differentiation, tissue-specific homeobox proteins are reported to show effect on cell division, cell development and hepatic regeneration [8]. HOX proteins, which are a subunit of the homeobox proteins, are classified under subtypes, which are referred to as HOX/Hox A/a, B/b, C/c and D/d and are localized to different chromosomes, in humans and mice [9].

HOXA10, which is a sub-member of the HOX proteins and belongs to cluster A on chromosome 7, plays critical roles in gene expression, morphogenesis, differentiation, fertility, embryonic viability and hematopoietic lineage [10]. This particular protein has also been indicated to regulate the proliferation, migration and invasion of cells in various organ and tissue tumors [11].

HOXA11 is a transcription factor, which provides certain positional identities to cells and takes part in the regulation of the developmental system. Moreover, HOXA11 has also been reported to regulate uterine development in females, and to be expressed in the thymus, placenta, lungs, prostate and liver [12]. HOXB6, similar to other mammalian HOX proteins, serves as a DNA-binding transcription factor [13]. Thereby, HOXB6 has been reported to have influence on neurogenesis, renal development and hematopoiesis, as well as on the proliferation and differentiation of multiple cells and tissues [13,14]. The physiological functions determined for other members of the homeobox protein family include splenogenesis and the development of certain sensory neurons for TLX1, the development of the forebrain and craniofacial structures, osteogenesis, chondrogenesis, neurogenesis and hematopoiesis for Dlx-5, and the development of visceral organs such as the gallbladder, liver and intestines as well as the differentiation of hematopoietic cells for HLX [15-19]. The primary regulators of hepatic development were identified by the use of rodent, fish and frog models in preliminary research. These regulators include extracellular signal molecules, intracellular signal transduction pathways and transcription factors. While members of the family of transcription factors have been described as being proteins required for hepatic specification, homeobox proteins have been listed among the major regulatory factors of hepatic development [20]. In this context, the present study was aimed at i) determining the localization and expression intensity of some homeobox proteins during the development of the liver in the bovine fetus ii) identifying the gestational stage during which the intensity of expression, demonstrated by immunohistochemistry, differs iii) and determining the potential physiological roles of the selected homeobox proteins.

Material and Methods

The study material comprised of 27 clinically healthy Holstein bovine fetuses without sex differentiation, which belonged to different gestational stages and were obtained from private slaughterhouses. Fetal age was estimated by measuring the crown-rump length (CRL) and using the formula described by Harris et al.[21]. Following age estimation *(Table 1),* the fetuses were assigned to one of the three groups established for the different gestational trimesters as follows: the first trimester (days 69-89 of gestation/9 fetuses), the second trimester (days 99-178 of gestation/9 fetuses), and the third trimester (days 190-269 of gestation/9 fetuses). Hepatic tissues samples were taken from the fetuses included in each group. These tissue samples were first fixed in 10% formalin-alcohol solution for 18 h, then dehydrated through a graded series of alcohol, cleared in methyl benzoate and benzene, and embedded in paraffin. Five-micrometer-thick cross-sections were cut from the paraffin blocks. For immunohistochemical staining, these sections were mounted on glass slides coated with 3-aminopropyltriethoxysilane (APS) (Sigma-Aldrich Chemicals, St. Louis, MO, USA).

Immunohistochemistry

The serial sections, after being mounted onto adhesive glass slides, underwent immunohistochemical (IHC)

Table 1. Estimation of fetal age

Parameter	Number of Samples																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Fetal crown-rump length (CRL, in cm)	6	7.5	8	10	11	12.5	13	13.5	14.5	18	19.5	21	22.5	24	28	34.5	47.5	50	55	58	60	65	66.5	70	79	82	87
Fetal age (Day)	69	73	75	79	82	86	87	88	90	99	102	106	110	114	123	140	172	178	190	198	202	214	219	227	249	257	269

Table 2. Primary antibodies used for immunohistochemistry (IHC)

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
HOXB6	Polyclonal/IgG	Goat	Human, Mouse, Rat, Dog, Cattle, Pig	1/100	St John's Laboratory, model no: STJ73348
TLX1	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	Invitrogen, cat no: PA5-34553
Dlx-5	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	St John's Laboratory, model no: STJ92725
HLX	Polyclonal/IgG	Rabbit	Human	1/100	Invitrogen, PA5-44857

staining using the streptavidin-peroxidase procedure. Once dried, the sections were first deparaffinized (2x5 min in xylol), then rehydrated through a graded series of ethanol, and transferred into distilled water. Subsequently, tissue endogenous peroxidase activity was blocked by maintaining the sections in 3% H_2O_2 solution in methanol for 20 min followed by 3x5 min washes in phosphate-buffered saline (PBS) (pH: 7.4, 0.01 M). Next, the preparations were incubated in citrate buffer solution (pH: 6) at 95ºC for 30 min to expose the antigenic regions for antibody binding, and at the end of the incubation period, were left in the same solution until being cooled to room temperature. Subsequently, the sections were incubated in a blocking solution (Ultra V Blok, catalogue number: TA-125-UB, Thermo Scientific) for 15 min to block the non-specific binding of the primary antibody, and after the discard of the solution, were incubated with the primary antibodies listed in *(Table 2)* overnight at 4ºC. The next day, after being washed 3x5 min in PBS, the sections were incubated with biotinylated secondary antibody (Biotinylated Goat Anti-Polyvalent, catalogue number: TP-125-BN, Thermo Scientific) at room temperature for 20 min. Following another round of 3x5 min washes in PBS, the sections were treated with streptavidin peroxidase (Thermo Fisher Scientific, catalogue number: TA-125-HR) at room temperature for 20 min. Subsequently, 3.3 diaminobenzidine (DAB Substrate, Thermo Scientific, catalogue number: TA-125-HD) was dropped onto the slides and treatment was allowed for 5-15 min. After being washed in distilled water, nuclear staining was performed with Mayer's hematoxylin for 2 min. Next, the sections were washed under running tap water for 5 min, dehydrated through a graded series of alcohol, cleared in xylol, and finally embedded in Entellan and covered with a coverslip. The accuracy of the immunohistochemical method applied was demonstrated with the use of positive controls, which comprised of bovine uterine and feline testicular tissue samples. On the other hand, the negative controls comprised of hepatic tissue samples, which were incubated with PBS instead of primary antibody.

Semi-quantitative Assessment

The immunoreactions demonstrated for some homeobox proteins in the hepatic tissue samples were observed at different magnifications (10X, 20X and 40X) under a Nikon Eclipse E400 (Nikon, Tokyo, Japan) research microscope equipped with a digital camera (Nikon Coolpix 4500), and were assessed semi-quantitatively for the intensity score. The intensity scores were determined on the basis of the intensity of the positive staining of the cells. All regions of the liver were screened by two independent senior researchers (UT and HS) for the scoring of the immunohistochemical staining. Scoring was performed on a 3-point scale as follows: 0 - negative (no staining observed in the cells at high microscopic magnification), 1 - weak (stained cells observed only at high microscopic magnification), 2 - moderate (stained cells easily observed at low microscopic magnification), 3 - strong (stained cells observed at very low microscopic magnification) [22]. Semi-quantitative assessment was made for each hepatic portal area components and adjacent hepatocytes (hepatocytes, branch of the hepatic artery, branch of the vena interlobularis and bile ducts).

Statistical Analysis

Statistical analyses were made with the SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) software package. All values are given in mean ± standard deviation. Data normality was assessed with the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was used to analyze any statistically

significant difference in the immunohistochemical staining intensity score for HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX of the hepatocytes, hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during the different trimesters of gestation or between these cell and tissue types. Differences between the cell types for the staining intensity score of each antibody were determined with the Mann-Whitney U test. The results are given in mean ± standard deviation (SD) and statistical significance was set at $P<0.05$ *(Fig. 1, Fig. 2-k)*.

Results

Immunohistochemical staining demonstrated varying intensities of positive reactions for the proteins HOXA10, HOXA11, HOXB6, Dlx-5, TLX1 and HLX in the bovine fetal liver during the different stages of gestation.

Strong immunoreactions were observed for HOXA10, HOXA11 and HOXB6 in the hepatocytes during all three trimesters of gestation *(Fig. 1-a,b,c)*. Immunoreactions for HOXA10 in some hepatic arteries were weak any during

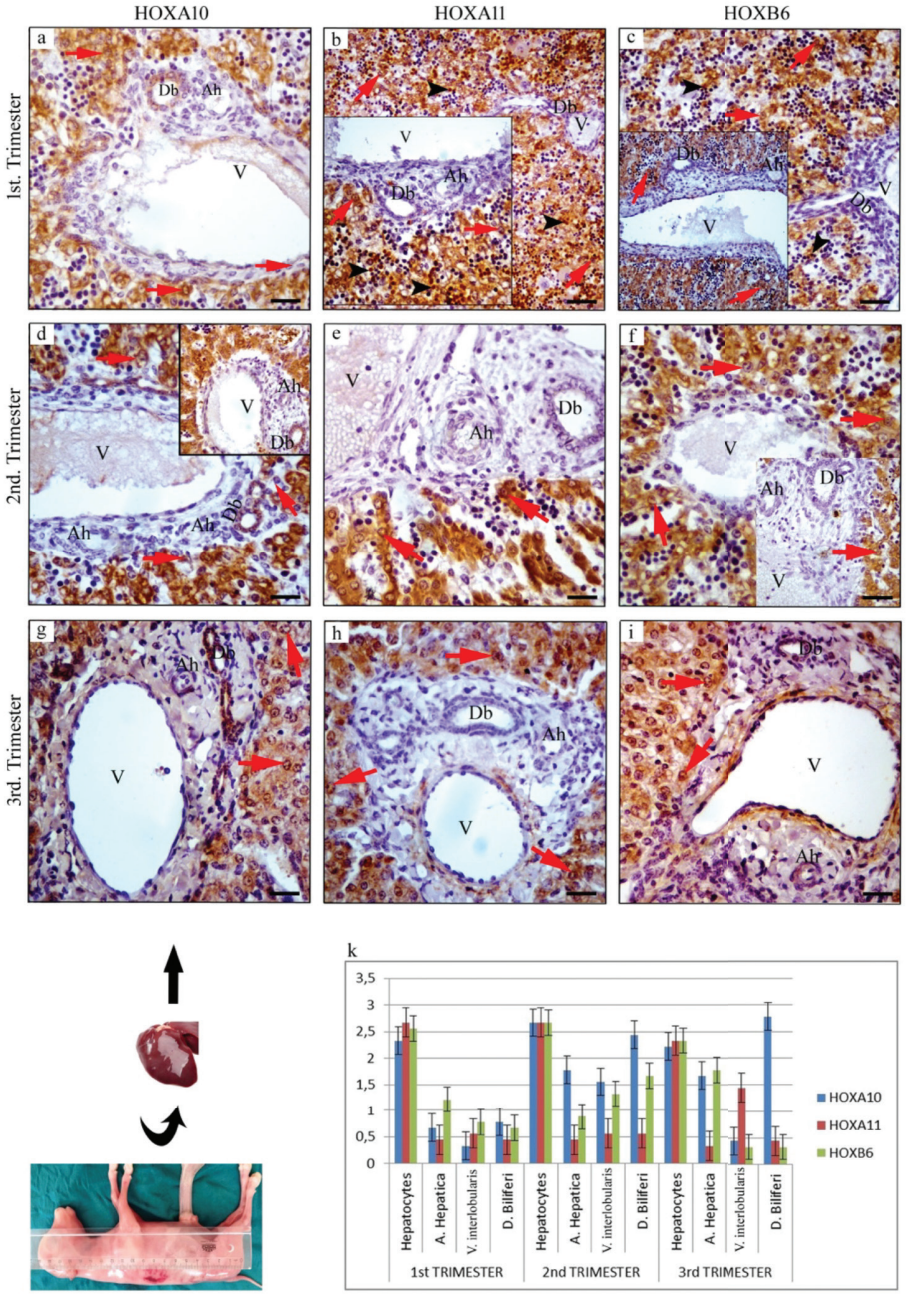

Fig 1. Expression of HOXA10, HOXA11 and HOXB6 in fetal bovine liver, in the 1st trimester (73 days) (a, b, c), 2nd trimester (102 days) (d, e, f), 3rd trimester (214 days) (g, h, i), Statistical graph of HOXA10, HOXA11 and HOXB6 staining intensity (k). *Red arrow:* Hepatocytes, Black arrowhead: Lymphocyte, Ah: Arteria hepatica, V: Vena interlobularis, Db: Ductus biliferi. Scale Bar: 25 µm (a, d, e, f, g, h, i), 50 µm (b, c)

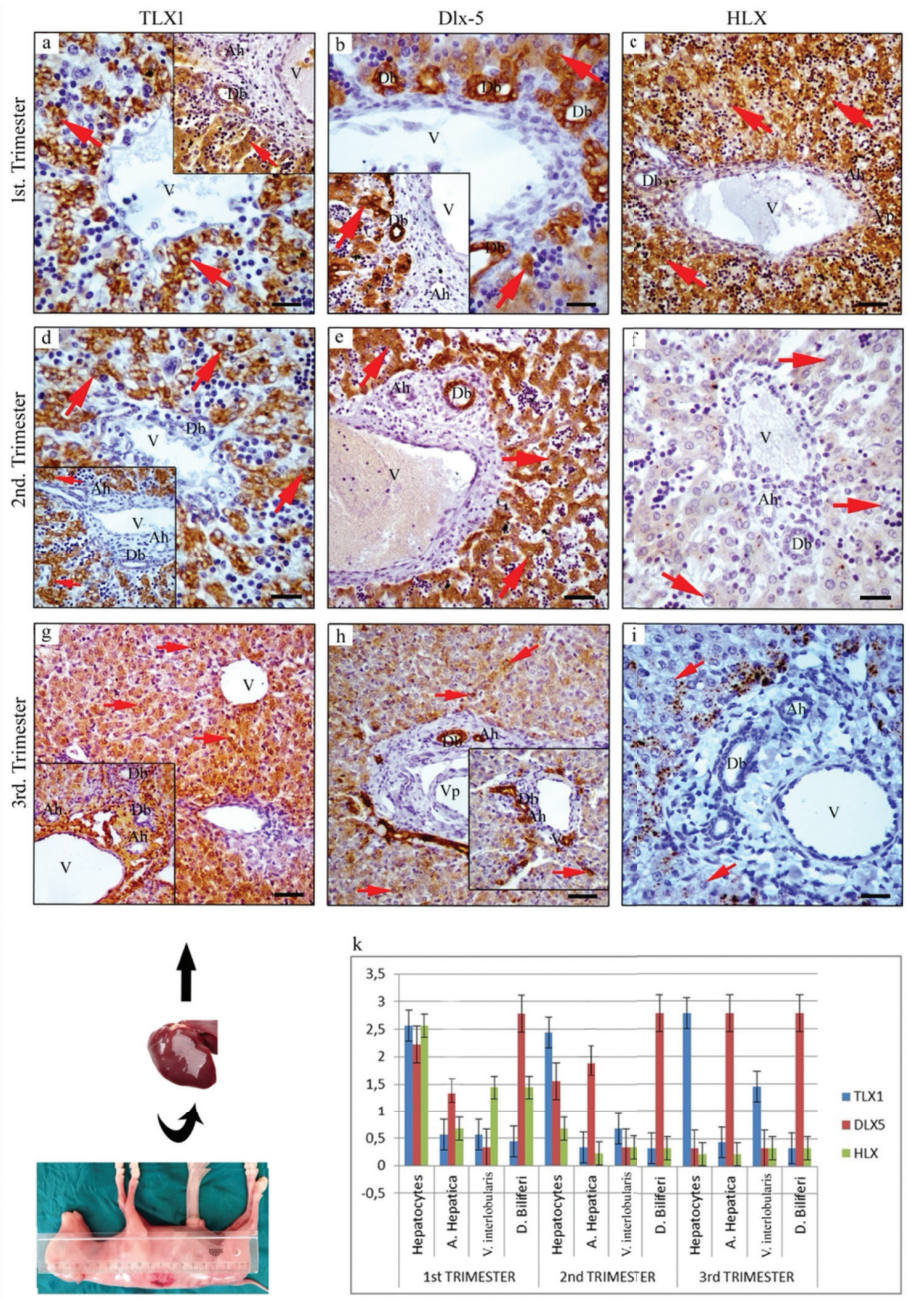

Fig 2. Expression of TLX1, Dlx-5 and HLX in fetal bovine liver, in the 1st trimester (69 days) (a, b, c), 2nd trimester (114 days) (d, e, f), 3rd trimester (227days) (g, h, i), Statistical graph of TLX1, Dlx-5 and HLX staining intensity (k). *Red arrow:* Hepatocytes, Ah: Arteria hepatica, V: Vena interlobularis, Db: Ductus biliferi. Scale Bar: 25 µm (a, b, d, f, h, i), 50 µm (c, e, g, h)

the first trimester and moderate during the second and third trimesters. Immunoreactions in some vena interlobularis were weak any during the first and third trimesters and moderate during the second trimester. On the other hand, immunoreactivity in the intrahepatic bile ducts was weak during the first trimester and strong during the second and third trimesters *(Fig. 1-a,d,g)*. Immunoreactions were weak observed for HOXA11 in the hepatic artery and intrahepatic bile ducts throughout the three trimesters of gestation. Furthermore, immunoreactions in the vena interlobularis were weak during the first and second trimesters and moderate during the third trimester *(Fig. 1-b,e,h)*. HOXB6 immunoreactivity in the hepatic artery was weak throughout gestation, but relatively stronger during the last trimester compared to the first and second trimesters. On the other hand, in some vena interlobularis and intrahepatic bile ducts, immunoreactions were weak any during the first and third trimesters, and stronger during the second trimester *(Fig. 1-c,f,i)*. Furthermore, immunoreactions for HOXA11 and HOXB6 in the lymphocytes were strong during the first trimester of gestation *(Fig. 1-b,c)*.

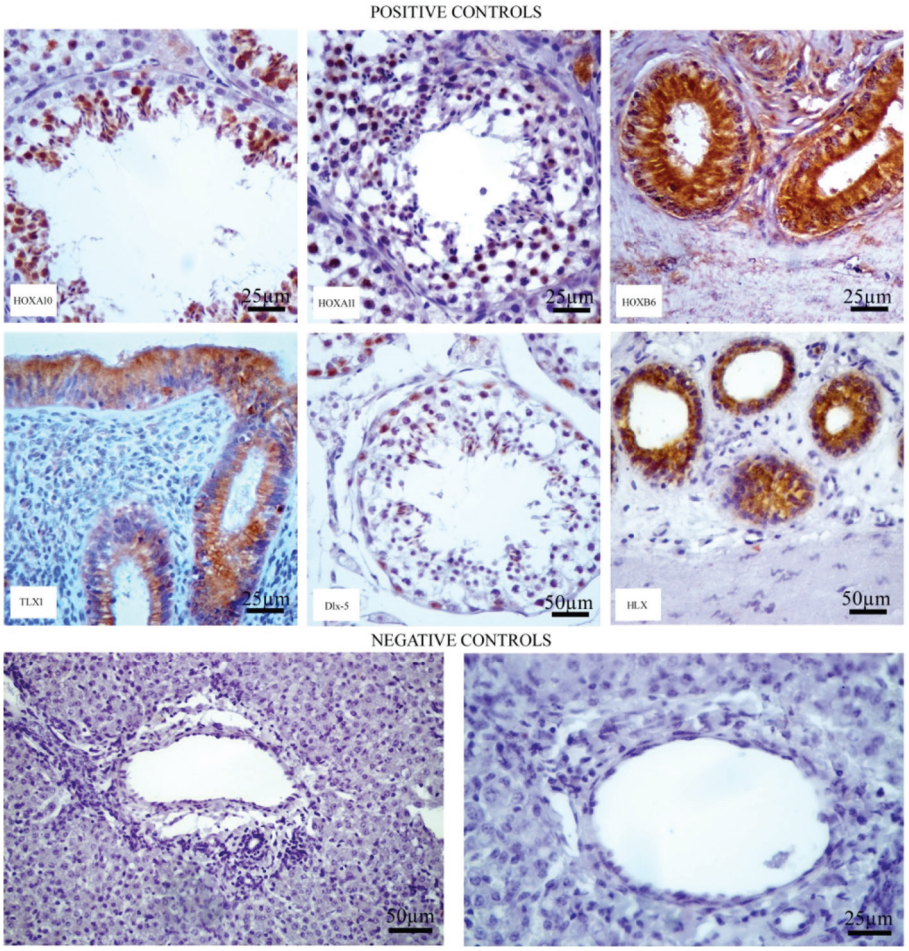

Fig 3. Positive expression of HOXA10, HOXA11, Dlx-5 proteins in testis, HOXB6, TLX1 and HLX proteins in uterine tissue. Negative expression of HOXA10, HOXA11, Dlx-5, HOXB6, TLX1 and HLX proteins in fetal bovine liver

During all three trimesters, the intensity of immunoreactions for TLX1 in the hepatocytes ranged from moderate to strong. On the other hand, immunoreactivity in some hepatic artery and intrahepatic bile ducts was weak any throughout gestation. In the vena interlobularis, immunoreactions ranged from weak to moderate during the third trimester, and were relatively stronger in intensity compared to the first and second trimesters *(Fig. 2-a,d,g)*. In the hepatocytes, immunoreactions for Dlx-5 were of moderate intensity during the second and third trimesters, and were stronger during the first trimester. On the other hand, Dlx-5 immunoreactions were moderate to strong in some hepatic artery and intrahepatic bile ducts during all three trimesters ($P<0.05$), but were weak any in the vena interlobularis *(Fig. 2-b,e,h)*. While the HLX protein induced strong immunoreactions in the hepatocytes during the first trimester ($P<0.05$), immunoreactivity was weak any and even negative in some hepatocytes during the second and third trimesters. Immunoreactions for HLX were weak any in the hepatic artery throughout gestation. Furthermore, immunoreactions in the vena interlobularis and intrahepatic bile ducts were weak during the second and third trimesters, but were relatively stronger during the first trimester *(Fig. 2-c,f,i)*.

The accuracy of the staining was confirmed by the use of positive controls (bovine uterine and feline testicular tissues) and negative controls *(Fig. 3)*.

Discussion

Homeobox proteins are critical to the identity of the various structures/tissues localized to the anterior-posterior axis of the developing embryo, as well as to organogenesis and cell differentiation [23,24]. Known to be expressed during the very early period of mammalian development, Hox proteins have also been observed in all three embryonic germ layers (and are of ectodermal origin in the nervous system, mesodermal origin in the genitourinary system and endodermal origin in the digestive system), and have been reported to undertake critical roles in these layers [25]. Research has shown that some homeobox proteins (Hex) undertake basic roles in endodermal organs, such as the thyroid gland and liver [26]. The present study demonstrated both the expression and the localization of the investigated

homeobox proteins in the bovine fetal liver during gestation and revealed that expression showed relative differences with gestational stage. Thus, in agreement with previous research on homeobox proteins [20], the present study demonstrated that HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX could play major roles in the morphogenesis and cell differentiation of the bovine fetal liver during gestation.

Depending on the chromosomal position of their encoding genes, the proteins HOXA10 and HOXA11 have been indicated to be expressed along the paramesonephric canal in the human fetus. Based on this information, these proteins have been reported to be involved in embryonic development and to affect uterine development and differentiation [27]. Several studies have shown that these particular proteins may have normal functional roles in the female genital system of mice as well as in skeletal and renal tissue development [28], the uterus of rats [29], humans [30], monkeys [31] and pigs [32], and the bovine placenta and feline testis [33,34]. On the other hand, these proteins have also been reported to be involved in the formation and progression of tumors in humans, such that in particular HOXA10 has been indicated to be present at levels higher than that of hepatocytes in hepatic cell cancer [11,35,36]. Furthermore, it has been determined that, apart from being expressed in normal hepatic tissue [12], HOXA11 also affects hepatocyte carcinoma and aids in the proliferation and invasion of these cancer cells [35]. In line with these studies, it has been determined that HOXA10 and HOXA11 are found in adult liver. However, Cauwelier and Speleman [12] and Yu et al.[35] have revealed that these proteins are expressed in the adult liver. In parallel with this report, the present study demonstrated that the homeobox proteins HOXA10 and HOXA11 are expressed in some cells and structures of the bovine fetal liver throughout gestation. While expression was determined to be strong and at similar levels in the hepatocytes during all three trimesters of gestation, it was ascertained that the expression of HOXA10 was stronger in the bile ducts during the second and third trimesters of gestation ($P<0.05$). In the hepatic artery, the expression of HOXA10 was relatively stronger than that of HOXA11, and occurred at stronger intensities during the second and third trimesters ($P>0.005$). The findings of the present study suggest that these proteins may have a modulating effect in the bovine fetal liver and contribute to the development of the liver and the physiological functions of the hepatocytes (nutrient transport). Our findings also suggest the particularly major involvement of HOXA10 in the division and proliferation of vascular endothelial cells and bile duct epithelial cells. Furthermore, HOXA11 having been determined to be strongly expressed in some lymphocyte-like cells during the first trimester of gestation suggests that this protein may contribute to the erythropoietic activity of the liver, the blood-forming organ of the fetal development period, as well as to the defense system and hematopoiesis.

In previous research, HOXB6 has been generally reported to be expressed in human hematopoietic progenitor/stem cells [37,38]. To our knowledge, there is no previous study on the role of HOXB6 in healthy fetal liver tissue. However, in available literature [39], this protein has been described as a SOX9 biomarker involved in the proliferation, differentiation and regeneration of liver progenitor cells, hepatocytes and bile duct epithelial cells in mice. Moreover, it has been reported that HOXB6 transcriptionally regulates the expression of the SOX9 biomarker, and thereby, affects the proliferation and differentiation of liver cells. In another study, it was determined that HOXB6 was expressed during the oncogenic processes of some tissues and organs (esophagus, hepatocytes) and affected the regulation of the proliferation, migration and invasion of cancer cells [40]. Similar to the case in the human and bovine placentae and feline testes, the present study demonstrated that HOXB6 was expressed in the bovine fetal liver, such that the expression intensity was strong in the hepatocytes, but ranged from weak to moderate in the hepatic artery, vena interlobularis and bile ducts during all three trimesters of gestation. Thus, in agreement with the findings of previous cancer research on HOXB6 [40], the present study revealed that this protein could also affect cell division, proliferation and migration in the bovine fetal liver. Similar to HOXA11, the determination of HOXB6 immunoreactivity in lymphocyte-like cells during the first trimester of gestation suggests that this protein could have a synergistic effect with HOXA11.

Although TLX1 is normally not expressed in hematopoietic cells, previous studies on TLX1 and Dlx-5 have shown that this protein is expressed in the fetal spleen and plays an important role in the development of this organ [41]. TLX1 has been described as an oncogene, the disrupted expression levels of which are associated with T-cell acute lymphoblastic leukemia (T-ALL) in humans [42]. In a previous study aimed at demonstrating the effects of TLX1 on cell differentiation and proliferation in mice, this protein was determined to be structurally expressed in fetal liver cells [43]. Similarly, the Dlx-5 gene encodes the transcription factors essential to embryonic and postnatal development. This protein is involved in the morphogenesis of the craniofacial structures, branchial arches, forebrain and sensory organs, postpartum homeostasis and particularly hematopoiesis, and if expressed irregularly, also in oncogenesis (cancer of the ovaries and lungs) [44]. According to the current information about the expression and presence of TLX1 and Dlx5 in the liver, it is known that these proteins have critical roles in the development of some tissues during

the embryonic period. In the present study, TLX1 induced strong immunoreactions in the hepatocytes during all three trimesters of gestation, whilst Dlx-5 induced weaker immunoreactions during the second and third trimesters. This suggests that these proteins could potentially affect the division, proliferation and physiological functions of hepatocytes. On the other hand, the expression of Dlx-5 having been observed to progressively decrease with the advance of gestation was considered to be related to the decrease in metabolic activity, division and proliferation rate of cells with gestational advance. Moreover, immunoreactions for TLX1 being scarcely any in the hepatic artery, vena interlobularis and bile ducts throughout gestation suggests that this protein has no effect on the mitotic activity of vascular endothelial cells and bile duct epithelial cells in the liver. Contrarily, immunoreactions having been observed for Dlx-5 in the bile ducts and moderate to strong in some hepatic artery throughout gestation could be interpreted as this protein contributing to angiogenesis, the division and differentiation of vascular endothelial cells and bile duct epithelial cells, as well as the production and secretion of bile.

HLX/Hlx has been reported to be expressed in mesodermal tissues, particularly the visceral mesenchyme, skeletal myoblasts, sclerotome and mesenchyme of the extremities during embryogenesis [45,46]. It has been reported that, in mice, Hlx is significantly expressed in mesodermal tissues, in particular the mesenchyme of the developing liver, gallbladder and intestines [46]. In previous research aimed at determining the functions of the Hlx gene by means of its targeted mutation in mice, it was observed that not only did the liver and intestines display anemia and hypoplasia, but also hepatocyte differentiation and liver growth were restricted [18]. It has also been demonstrated that while Hlx has critical roles in the development of the liver and intestines in mice, the protein sequence of mouse Hlx shows 86.5% homology to that of human HLX, which suggests that these proteins could have similar roles in both species [47]. Human research has shown that the mutation of HLX may cause various anomalies during embryonic development, including diaphragmatic hernia, short bowel and asplenia. In the present study, it was determined that HLX expression had significantly decreased during the second and third trimesters of gestation, when compared to the first trimester ($P<0.05$). Based on this finding, it was considered that, in parallel with the rapid division, growth and differentiation of cells during early gestation, the expression of HLX in bovine fetal liver cells was strong during this period, and this particular protein had a basic role in physiological processes. Furthermore, the decrease observed in the expression of HLX with the advance of gestation was considered to be associated with the reduced metabolic activity and decreased growth rate of the liver with gestational advance. On the other hand, HLX immunoreactions being scarcely any in the hepatic artery, vena interlobularis and bile ducts suggested that the homeobox protein HLX had no effect on the physiological functions of these structures. Based on these results, it is suggested that, similar to the case in mice and humans, HLX is also involved in the formation and development of the bovine liver, and its deficiency may cause liver anomalies.

In conclusion, the presence of the homeobox proteins HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX in the hepatocytes, some hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during all three trimesters of gestation, and the expression of these proteins at varying levels, both suggest that these proteins may have significant roles in the development and physiological activity of the bovine fetal liver. Different from previous research on homeobox proteins, which have generally focused on cancer and developmental anomalies, the present study has shown that these proteins may positively contribute to the development of organs, such as the liver, as well as to the development of cells, such as hepatocytes, vascular endothelial cells and bile duct epithelial cells, and may be found in structural components. Thereby, this study provides valuable data for future research.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request (U. TOPALOĞLU).

Funding Support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing Interests

The authors declare that there is no conflict of interest.

Authors' Contributions

UT, HS and MEA planned the study, designed the experiments and helped manuscript writing; MAK and NA helped with data analyses and bioinformatics and wrote the manuscript; UT, BGS and NA collected samples and conducted laboratory process; HS and MEA analysed the statistics data. All authors read and approved the final manuscript.

Ethical Approval

The materials used in our study were collected from the slaughterhouses of the province of Diyarbakir, and in accordance with the regulation on the working procedures and principles of animal experimentation ethics committees in the official gazette published on

February 15, 'Procedures with dead animals or tissues, slaughterhouse materials, waste fetuses' are not subject to HADYEK permission.

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