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Localisation of leptin, ghrelin, their receptors, obestatin and GPR39 in the cow placenta during pregnancy*

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Abstract: Several peptides are expressed in the uterine endometrium and placenta during implantation, suggesting that chemical mediators play an important role in the feto-maternal relationship. Peptide hormones such as leptin, ghrelin, and obestatin are produced in the bovine placenta and play major roles in nutrient distribution and regulation of energy balance, as well as in placental and fetal growth. We evaluated the spatio-temporal expression patterns of leptin, ghrelin, obestatin, GPR39, and their receptors (OB-R, GHS-R) in the bovine placental and interplacentomal tissues during pregnancy in the uteri of 44 pregnant Holstein cows. During pregnancy, leptin, ghrelin, and obestatin, GPR39, as well as their receptors (OB-R, GHS-R), were present in fetal trophoblasts (uninucleate trophoblasts (UTC), binucleate and multinucleate trophoblasts (BNC/TGC)). Epithelial cells of the maternal placenta showed moderate leptin and OB-R expression at similar levels across the three gestational periods. Ghrelin was expressed in fetal uninucleate trophoblasts (UTC) and multinucleate trophoblasts (BNC/TGC), especially during the second period of pregnancy. Strong cytoplasmic expression of obestatin and GPR39 was observed in UTC and BNC/TGC throughout gestation. Moderate to strong expression of leptin, ghrelin, obestatin, GPR39 and their receptors was detected in luminal and glandular epithelial cells in the interplacentomal region during pregnancy. Immunohistochemical analyses demonstrated that these peptide hormones and their receptors were found in the cytoplasm of many cells in the fetal and maternal tissues during pregnancy. These results suggest that leptin, ghrelin, obestatin, and their receptors could be involved in the growth and development of the placenta via placental and interplacentomal expression.

Keywords: *GHS-R, Leptin, Maternal epithelium, Obestatin, Placenta, Trophoblasts.*

Introduction

In viviparous species, there is need for a connection to allow for the physiological exchange of nutrients, gases, hormones and waste products between the fetus and fetal barriers (Ravelich et al., 2004; Seo et al., 2023). The placenta provides this connection, and this organ is particularly well-developed in eutherian (placental) mammals. Thus, normal formation and expansion of the placenta is of critical importance in the enlargement and nutrition of the fetus. All eutherian mammalian placentae are derived from the chorioallantoic membrane, but differ in type and structure (Casarotto et al., 2025; Kuczwara et al., 2023; Ravelich et al., 2004). The ruminant placenta is made of separate placentomes, which are composed of the fetal cotyledons and maternal caruncle (Casarotto et al., 2025; Kuczwara et al., 2023). In cows, the synepitheliochorial placenta serves as a key site for fetomaternal interactions that regulate and maintain embryonic development during pregnancy. In parallel with trophoblast invasion, bovine placentomes contain two distinct cell types: chorionic and maternal epithelial cells (Casarotto et al., 2025; Schlafer et al., 2000). After migrating to the maternal epithelium, TGCs, originating from the acytokinetic mitosis of uninucleate trophoblasts, contribute to the generation of fetomaternal hybrid cells, that transfer various metabolic substances to the endometrium, by merging with epithelial cells and later undergoing degeneration (Casarotto et al., 2025; Kuczwara et al., 2023). The coordinated exchange of substrates between the fetus, placenta and endometrium is critical to both the growth, development and functioning of the feto-placental unit, and the production of the hormones required for the maintenance of pregnancy and fetal growth (Casarotto et al., 2025; Kuczwara et al., 2023; Schlafer et al., 2000). The distribution of nutrients among the maternal and feto-placental compartments is regulated by several endocrine and metabolic hormones (Ravelich et al., 2004; Seo et al., 2023)

Leptin is a 16-kDA peptide hormone, which regulates food intake and weight gain. This hormone is synthesized primarily in white fat tissue, and to a limited extent in brown fat tissue (Ninpetch et al., 2022). Leptin exerts its functions via leptin receptors (OB-Rs). These receptors, which are encoded by a single gene, have two main connection variants. The OB-Rb form, otherwise known as the "long isoform", is biologically most active. The short isoform, OB-Ra, which lacks access to most of the intracellular space that mediates the transmission of signals, is synthesized by the use of alternative exons. The OB-Rb and OB-Ra share the same extracellular ligand-binding areas and show a similar affinity to leptin (Buchbinder et al., 2001). Leptin and its receptors are expressed in the placentae of humans, baboons, sheep, pigs, mice and rats (reviewed in Forhead and Fowden, 2009). Leptin is involved in maintaining or regulating the metabolic adaptation required for optimal fetal growth and development throughout pregnancy (Ravelich et al., 2004).

Ghrelin is a naturally occurring octanoylated peptide hormone, which is composed of 28 amino acids and generated as a result of modification by ser3-O-octanoylation (Schalla and Stengel, 2021). This modification is required not only for ghrelin activity, but also for the activation of its specific receptor (Maranesi et al., 2025). Ghrelin exerts its effect when it is connected the growth hormone-secreting receptor (GHS-R). There are two different isoforms of the ghrelin receptor: GHS-R1a and GHS-R1b (Maranesi et al., 2025; Schalla and Stengel, 2021). Although the significance of this hormone's tissue distribution is not fully understood, ghrelin is thought to play a role in regulating cell differentiation, proliferation, and apoptosis (Harrison et al., 2007). It appears that ghrelin regulates the endocrine and metabolic reactions to nutrition and the energy balance in ruminants (Roche et al., 2007; Sugino et al., 2002). There is limited information available regarding the localization of ghrelin and its receptor in organs and tissues, and their role in cellular development processes in these species. In adult animals, placental tissue undergoes distinctive development over a relatively short period. Recent studies suggest that the localisation of ghrelin and GHS-R affects the regulation of ovine fetal development (Harrison et al., 2007). Kitamura et al. (2003) and that ghrelin, circulating in the blood of the human fetus, may partly originate from the placenta, and can be mobilized for feto-maternal energy transfer and growth.

Obestatin, produced by the oxyntic gastric mucosa, is a 23-amino acid anorexigenic peptide hormone generated by the enzymatic processing of prepro-ghrelin. Obestatin is thought to have an effect opposite to that of ghrelin, inducing satiety and reducing food intake. However, discussions continue on this hypothesis (Szlis et al., 2025). Although the identity of the obestatin receptor is controversial, GPR39 is a member of the G protein-coupled receptor (GPCR) family. Considering its inhibitory effect on feeding and digestive activity, obestatin is thought to inhibit the stimulatory effects of ghrelin by interacting with the orphan GPR39 receptor (Cao et al., 2023; Zhang et al., 2005). However, it has recently been shown that obestatin does not attach to GPR39 and that obestatin has no effect on cells transfected with GPR39. These findings provide evidence that obestatin is not a binding ligand for GPR39 (Zhang et al., 2007). To date, the distribution of obestatin and GPR39, in mammalian maternal and fetal placental tissues, including those of humans, has not been investigated.

Reproductive success is important for livestock operations (LeBlanc, 2008). Failure to achieve the desired reproductive success on farms causes significant economic losses. A short calving-to-calving interval is critical in terms of profitability (De Vries, 2006). In cows, failure of the zygote to implant in the uterus and failure of placenta formation and pregnancy are the main causes of reproductive losses (reviewed in D'Occhio et al., 2020). In gravid animals, the favorable growth, development and functioning of the placenta are directly related to cell division processes and form the basis of optimal fetal growth (Sugino et al., 2002). The distribution of nutrients among the maternal and feto-placental compartments is regulated by several endocrine

hormones, including placental leptin, ghrelin, and obestatin (Harrison et al., 2007; Roche et al., 2007), which are also effective in fetal and placental growth (Harrison et al., 2007). Nevertheless, data are limited on whether the localisation and expression of leptin, ghrelin, their receptors (OB-R, GHS-R), obestatin and GPR39 in the bovine placenta development during pregnancy. Considering that leptin, ghrelin, their receptors, obestatin and GPR39 are structural components of the genital organs and plays a role in various stages of female reproductive histophysiology, we examined the localization, expression and possible roles of these peptide hormones and their receptors in the bovine placenta.

Materials and Methods

Ethical approval: This study used slaughterhouse material and therefore does not require ethical committee approval in alignment with the principles of the Guideline on the Use of Tissue Samples issued by the National Ethics Committee (dated February 15, 2014, No. 28,914).

Tissue collection: Uteruses were obtained from slaughterhouses from pregnant bovine (n = 44) at different stages. Immediately after slaughter, the abdomen was opened, the uterus was removed, and the fetuses were extracted. Fetal age was calculated to identify which gestational period the uteri belonged to. For this purpose, following the measurement of the crown-rump length (CRL, in cm), calculations were made according to Harris et al. (1983), using the equation y:54.6+2.46(x), which is based on the relationship between CRL and fetal age. In this equation, y represents fetal age, and x represents crown-rump length. According to fetal ages determined by this method, the fetuses were assigned to three groups, including those, which were in the 1st period of gestation, 2nd period of gestation, and 3rd period of gestation (Fig. 1).

When collecting the study material, following the extraction of the gravid uterus, the gravid uterine horn was laterally incised to remove the fetus. After the fetal membranes were cut and the fetal fluid was discharged. A sample was taken for the CRL measurement of each fetus.

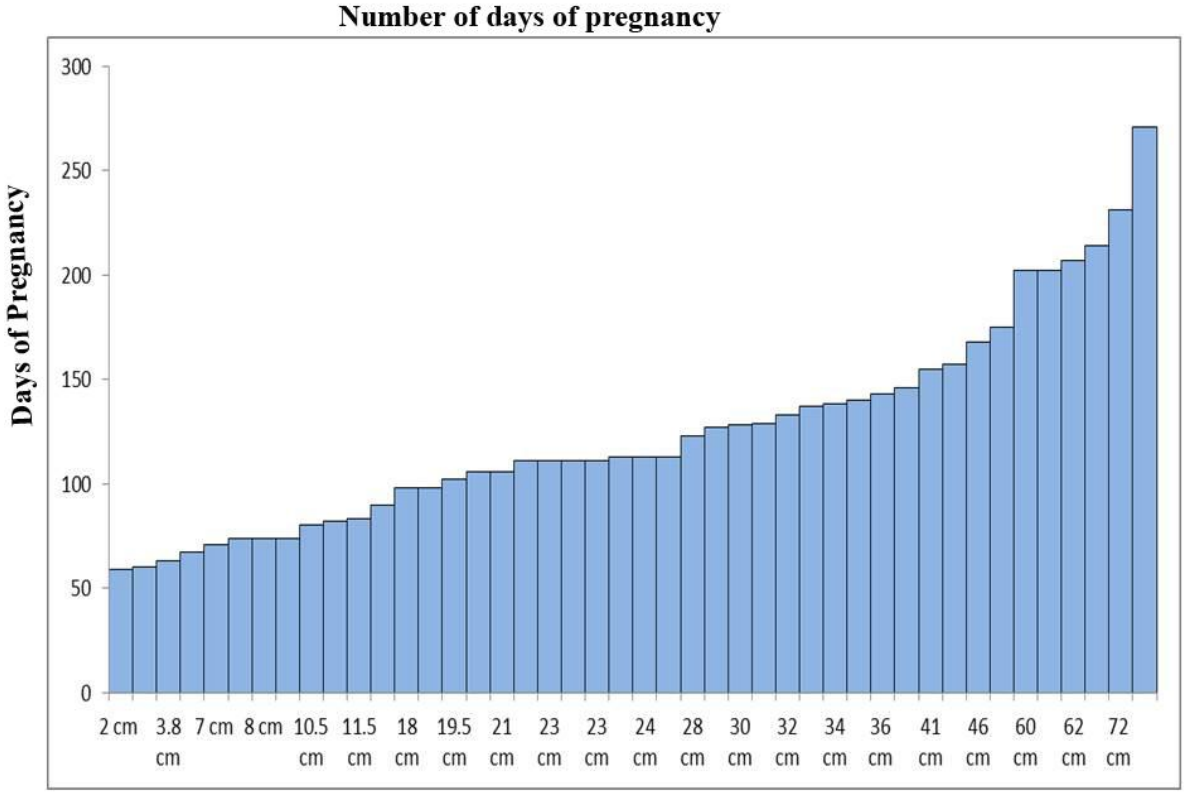


Figure 1. Pregnancy periods according to forehead-rump lengths.

Histological processing and examination: Immediately after the cows were slaughtered, the placenta was completely removed, and then small samples were cut from the placenta and the interplacental region, and the tissues were fixed in 10% neutral formalin for 24 hours. After routine histological procedures, it was embedded in paraffin. 5 µm thick sections were taken from the prepared blocks. Six slides were prepared for each sample. Sections were mounted on glass slides containing 3-aminopropylethoxysilane and left to dry overnight at room temperature. Next, the slides were stained immunohistochemically, using antibodies specific to the indicated factors.

Immunohistochemical staining: The Streptavidin-peroxidase method was used to determine the presence of leptin, ghrelin, their receptors, obestatin, and GPR39 in placental sections. These stains were performed as

previously described in our studies on these peptide hormones (Sağsöz et al., 2020). Following routine procedures, primary antibodies was followed by incubation 1/200 diluted at 4^0C overnight with leptin (Ab117751) rabbit polyclonal, ghrelin (Ab129383) rabbit polyclonal, obestatin (Ab41704) rabbit polyclonal, leptin receptor (OB-R) (Ab104403) rabbit polyclonal, ghrelin receptor (GHS-R) (Ab188986) rabbit polyclonal, and GPR39 (PA3-027) rabbit polyclonal primary antibodies (Abs).

To demonstrate the accuracy of immunohistochemical staining, negative and positive controls were used. Negative controls were treated normal rabbit serum IgG instead of primary Ab and bovine gastric sections were used as positive controls. The stained slides were evaluated and photographed using a Nikon digital camera attached to a Nikon-Eclipse 400 DSRI research microscope.

Semi-quantitative assessment: The expression of leptin, ghrelin, their receptors, obestatin and GPR39 was assessed semi-quantitatively in cells displaying positive reactions. Scoring of staining intensity was performed similarly to our previous studies (Sağsöz et al., 2020). Immune-reactive responses were examined by two different experts (B.B. and H.S.), and the average values were obtained. All sections, leptin, ghrelin, their receptors, obestatin and GPR39 localizations were performed using an attachment-equipped research light microscope with a DS-RI1 video camera at X100, X200, and X400 objective magnifications. Three regions were randomly selected from each uterus and placenta section and recorded on a computer using the NIS Elements D Imaging Program, as previously described. Three areas were randomly selected and analyzed for all tissues. In this context, luminal and glandular epithelium, stromal and smooth muscle cells were examined in uterine samples. In contrast, trophoblasts and mesenchymal cells were evaluated in the fetal placenta section and maternal epithelium and stromal cells were evaluated in the maternal placenta section.

Statistical analyses: The study data were analyzed using SPSS 15.0 (SPSS for Windows, SPSS Inc., Chicago, IL, USA). Data are given as mean ± SD. Statistical significance is indicated as $P<0.05$. The Kruskal-Wallis test was performed to recognize differences between semi-quantitative data obtained from uterine and placental tissue samples during pregnancy. If a difference was found, the Mann-Whitney U test was used to determine whether it originated in the uterine or placental layers or in the gestational period.

Results

While the negative controls did not show any immunohistochemical reactions indicative of expression (Fig. 2), the bovine abomasal tissue used as a positive control displayed immunoreactions confirming expression (Fig. 2).

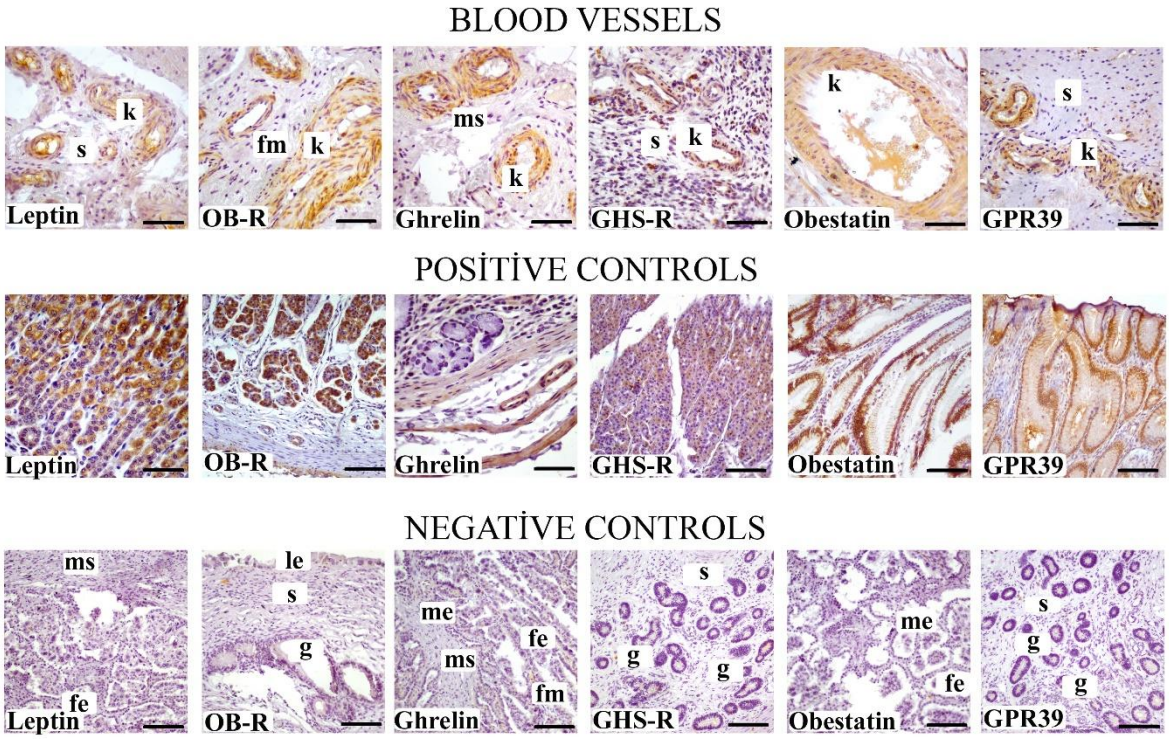


Figure 2. Localization of leptin, ghrelin, obestatin, GPR39 and their receptors (OB-R, GHS-R) to blood vessels in the bovine placental and interplacentomal regions during pregnancy. Positive reactions for leptin, ghrelin, obestatin and their receptors in glandular epithelial and smooth muscle cells of bovine abomasum used as positive controls. Negative controls were produced with rabbit IgG and no immunostainings for leptin, ghrelin, obestatin, GPR39 and their receptors (OB-R, GHS-R) in the bovine placental and interplacentomal regions. k, blood vessels; fe, fetal epithelium (trophoblasts); me, maternal epithelium; fm, fetal mesenchyme; ms, maternal stroma; le, luminal epithelium; g, uterine glands; s, stroma. Scale Bars: 25 µm (For blood vessels and negative controls) and Scale Bars: 50 µm (For positive controls).

Cellular immunostaining for the peptide hormones and their receptors

Leptin and OB-R: Leptin was expressed in the fetal placenta throughout gestation in the cytoplasm of trophoblasts within the chorionic villi, and this expression was of moderate intensity during the different gestational periods. BNC had stronger leptin expression than UTC. Fetal mesenchymal cells showed weak cytoplasmic immunoreactivity for leptin across all three gestational periods. In the maternal placenta, epithelial cells showed moderate leptin expression, with levels relatively similar across the three gestational periods. On the other hand, maternal stromal cells had a weak leptin expression in all three gestational periods (Fig. 3a-f). Throughout pregnancy, OB-R localization was stronger in the maternal placenta than in the fetal placenta. Compared to the maternal epithelium, OB-R expression was weak in the cytoplasm of the uninucleate and binucleate trophoblasts, fetal mesenchyma, stromal mesenchyma, and stromal cells. OB-R was strongly localized in the cytoplasm of maternal epithelial cells throughout the three gestational periods. (Fig. 3m-s).

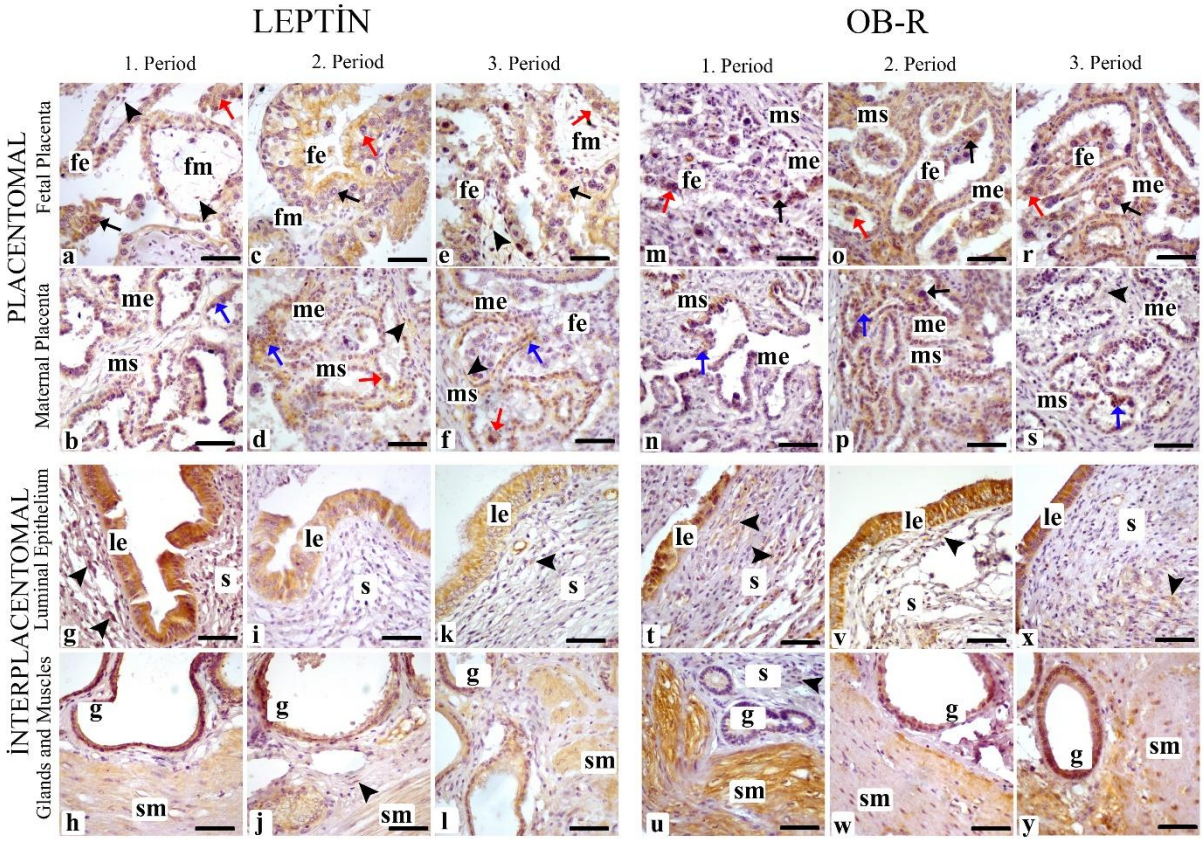


Figure 3. Expression of leptin and its receptor (OB-R) in the bovine placental and interplacentomal tissues during pregnancy. In placental tissues (a-f), leptin is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (a,c,e), and by maternal epithelial and stromal cells in the maternal placenta (b,d,f). In interplacentomal tissues (g-l), leptin is localized to the luminal and glandular epithelial cells, stromal and smooth muscle cells. In placental tissues (m-s), OB-R is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (m,o,r), and by maternal epithelial and stromal cells in the maternal placenta (n,p,s). In interplacentomal tissues (t-y), OB-R is localized to luminal and glandular epithelial cells, stromal and smooth muscle cells. Leptin and OB-R are found in the entire cytoplasm of trophoblasts, fetal mesenchymal cells, maternal epithelial and stromal cells, and uterine epithelial, stromal and smooth muscle cells. Black arrow, leptin- and OB-R-positive uninucleate trophoblasts (UTC); red arrow, leptin- and OB-R-positive binucleate trophoblasts (BNC); blue arrow, leptin- and OB-R-positive maternal epithelial cells; arrowhead, leptin- and OB-R-positive fetal mesenchymal and stromal cells. fe, fetal epithelium (trophoblasts); me, maternal epithelium; fm, fetal mesenchyme; ms, maternal stroma; le, luminal epithelium; g, uterine glands; s, stroma; sm, smooth muscles cells. For leptin; (a,b,g,h) 74 days of pregnancy, (c,d,i,j) 123 days of pregnancy, (e,f,k,l) 207 days of pregnancy. For OB-R; (m,n,t,u) 82 days of pregnancy, (o,p,v,w) 98 days of pregnancy, (r,s,x,y) 271 days of pregnancy. Scale Bars: 25 µm.

The interplacental region of the uterine mucosa showed strong expression of leptin and its receptor in the cytoplasm of luminal and glandular epithelial cells throughout the three trimesters of pregnancy. No differences were observed among expression. Compared to epithelial cells, uterine stromal and smooth muscle cells had weak cytoplasmic leptin expression of varying levels during the different gestational periods (Fig. 3g-l). However, compared with leptin expression, OB-R expression decreased with leptin expression, and OB-R expression decreased in luminal and glandular epithelial cells as pregnancy progressed. OB-R showed weak expression in some stromal cells during each of the three stages of pregnancy. It was determined that the cytoplasms of smooth muscle cells in the myometrium strongly expressed OB-R throughout pregnancy (Fig. 3t-y).During pregnancy, the endothelium and smooth muscle

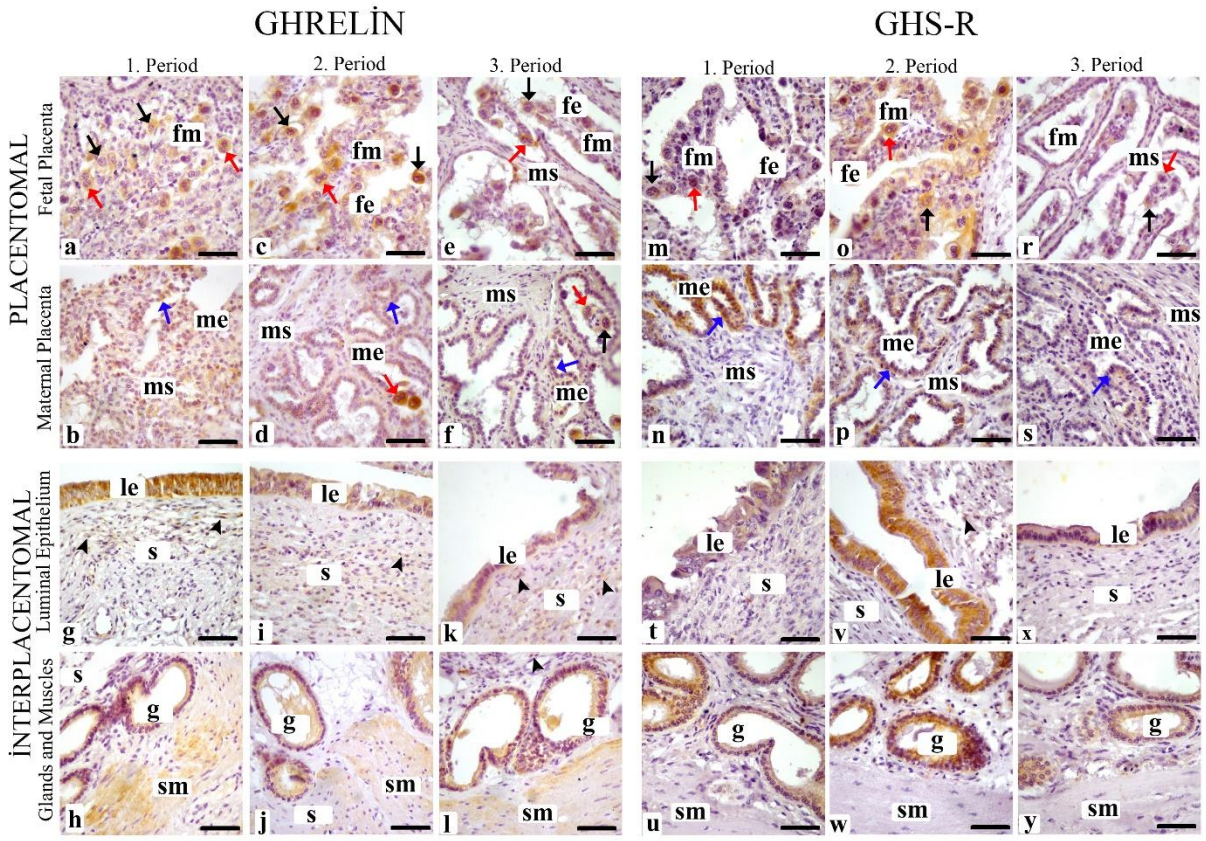


Figure 4. Expression of ghrelin and the growth hormone secretagogue-receptor (GHS-R) in the bovine placental and interplacentomal tissues during pregnancy. In placental tissues (a-f), ghrelin is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (a,c,e), and by maternal epithelial and stromal cells in the maternal placenta (b,d,f). In interplacentomal tissues (g-l), ghrelin is localized to luminal and glandular epithelial cells, stromal and smooth muscle cells. In placental tissues (m-s), GHS-R is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (m,o,r), and by maternal epithelial and stromal cells in the maternal placenta (n,p,s). In interplacentomal tissues (t-y), GHS-R is localized to luminal and glandular epithelial cells, stromal and smooth muscle cells. Ghrelin and GHS-R are found in the entire cytoplasm of trophoblasts, fetal mesenchymal cells, maternal epithelial and stromal cells, and uterine epithelial, stromal and smooth muscle cells. Black arrow, ghrelin- and GHS-R-positive uninucleate trophoblasts (UTC); red arrow, ghrelin- and GHS-R-positive binucleate trophoblasts (BNC); blue arrow, ghrelin- and GHS-R-positive maternal epithelial cells; arrowhead, ghrelin- and GHS-R-positive stromal cells. fe, fetal epithelium (trophoblasts); me, maternal epithelium; fm, fetal mesenchyme; ms, maternal stroma; le, luminal epithelium; g, uterine glands; s, stroma; sm, smooth muscles cells. For ghrelin; (a,b,g,h) 83 days of pregnancy, (c,d,i,j) 168 days of pregnancy, (e,f,k,l) 271 days of pregnancy. For GHS-R; (m,n,t,u) 63 days of pregnancy, (o,p,v,w) 157 days of pregnancy, (r,s,x,y) 214 days of pregnancy. Scale Bars: 25 µm.

cells of the blood vessels in the fetal-maternal and uterine stroma showed a positive immunoreaction for leptin and its receptor (Fig. 2).

Ghrelin and GHS-R: Unlike the first and third trimesters, strong cytoplasmic ghrelin expression was observed in the second trimester in the UTC and BNC, while weak expression was observed in the fetal placenta's mesenchymal cells. Throughout all three trimesters of pregnancy, cytoplasmic expression of ghrelin was weak in the epithelial and stromal cells of the maternal placenta (Fig. 3a-f). In the fetal placenta, throughout gestation, GHS-R localisation in the UTC and BNC was similar to that of ghrelin. GHS-R localisation was moderate in the first gestational period in the maternal epithelial and stromal cells, and its intensity decreased as gestation advanced (Fig. 4m-s).

Throughout pregnancy, uterine luminal and glandular epithelial cells and smooth muscle cells in the interplacental region showed strong cytoplasmic ghrelin expression at similar levels. In contrast, weak cytoplasmic localizations were determined in stromal cells throughout pregnancy (Fig. 4g-l). Throughout all three trimesters of pregnancy, GHS-R expression was strongly observed in luminal and glandular epithelial cells, moderately in stromal cells, and weakly in uterine smooth muscle cells. (Fig. 4t-y).

During pregnancy, strong ghrelin expression and weak GHS-R expression were observed in the cytoplasms of endothelial and smooth muscle cells of blood vessels in the uterus and placenta (Fig. 2).

Obestatin and GPR39: During pregnancy, obestatin expression was observed to be strong in the cytoplasm of the fetal placenta's UTC and some BNCs, and moderate in the cytoplasm of mesenchymal cells. No difference was detected in the intensity of expression among the different gestational periods. Compared to the mesenchymal cells, the UTC and BNC had significantly stronger cytoplasmic expression of obestatin. However, it was found that obestatin was weakly

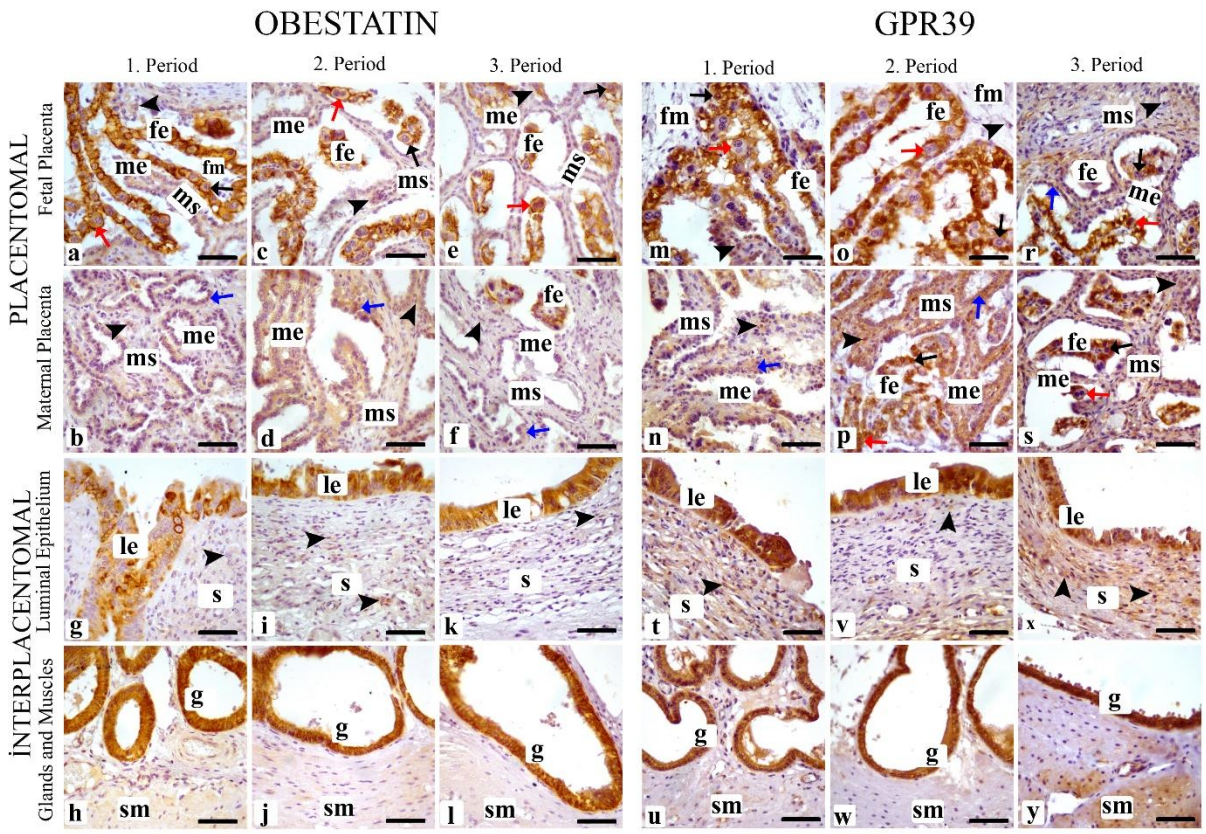


Figure 5. Expression of obestatin and GPR39 in the bovine placental and interplacentomal tissues during pregnancy. In placental tissues (a-f), obestatin is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (a,c,e), and by maternal epithelial and stromal cells in the maternal placenta (b,d,f). In interplacentomal tissues (g-l), obestatin is localized to luminal and glandular epithelial cells, stromal and smooth muscle cells. In placental tissues (m-s), GPR39 is expressed by trophoblasts and fetal mesenchymal cells in the fetal placenta (m,o,r), and by maternal epithelial and stromal cells in the maternal placenta (n,p,s). In interplacentomal tissues (t-y), GPR39 is localized to luminal and glandular epithelial cells, stromal and smooth muscle cells. Obestatin and GPR39 are found in the entire cytoplasm and membrane of trophoblasts, fetal mesenchymal cells, maternal epithelial and stromal cells, and uterine epithelial, stromal and smooth muscle cells. Black arrow, obestatin- and GPR39-positive uninucleate trophoblasts (UTC); red arrow, obestatin- and GPR39-positive binucleate trophoblasts (BNC); blue arrow, obestatin- and GPR39-positive maternal epithelial cells; arrowhead, obestatin- and GPR39-positive fetal mesenchymal and stromal cells. fe, fetal epithelium (trophoblasts); me, maternal epithelium; fm, fetal mesenchyme; ms, maternal stroma; le, luminal epithelium; g, uterine glands; s, stroma; sm, smooth muscles cells. For obestatin; (a,b,g,h) 59 days of pregnancy, (c,d,i,j) 175 days of pregnancy, (e,f,k,l) 231 days of pregnancy. For GPR39; (m,n,t,u) 67 days of pregnancy, (o,p,v,w) 133 days of pregnancy, (r,s,x,y) 207 days of pregnancy. Scale Bars = 25 µm.

expressed in maternal epithelial and stromal cells during all three trimesters of pregnancy (Fig. 4a-f). GPR39 expression in the fetal placenta was observed to be strong in UTC and some BNC cytoplasms, but weak in fetal mesenchymal cell cytoplasms during pregnancy. During pregnancy, no significant differences in GPR39 expression levels were observed between UTC and BNC groups. The epithelial cells of the maternal placenta had stronger GPR39 expression during the first period of gestation, compared to the second and third periods of gestation. The intensity of GPR39 expression progressively decreased in the maternal epithelial cells during gestation. GPR39 was weakly expressed in the cytoplasm of the maternal stromal cells throughout gestation (Fig. 5m-s).

In the interplacental region, obestatin expression was observed at relatively similar levels in the cytoplasm of luminal and glandular epithelial cells and in stromal and smooth muscle cells during all three trimesters of pregnancy (Fig. 4g-l). Additionally, cytoplasmic expression of GPR39 was detected at high levels in luminal and glandular epithelial cells and at moderate levels in stromal and smooth muscle cells during all three trimesters of pregnancy (Fig. 5t-y).

Endothelial and smooth muscle cells in the fetomaternal and uterine stroma blood vessels were observed to exhibit strong GPR39 and varying levels of obestatin expression (Fig. 2).

Variations based on cell types and gestational periods: The calculated intensity scores for leptin, ghrelin, their receptors (OB-R, GHS-R), obestatin, and GPR39 expression during pregnancy are shown in Table 1. In general, the expression of leptin, ghrelin, receptors, obestatin, and GPR39 throughout pregnancy was found to be stronger in

fetal trophoblasts and maternal epithelial cells in the placenta and in luminal and glandular epithelial cells of the uterus in the interplacental region (p<0.05) (Table 1**)**.

Throughout pregnancy, immunolabeling for leptin, ghrelin, their receptors (OB-R, GHS-R), obestatin, and GPR39 was detected more intensely in fetal mesenchymal cells in the fetal placenta compared to UTC and BNC, and in maternal epithelial cells in the maternal placenta compared to maternal stromal cells. Furthermore, in the interplacentomal region, immunostaining for leptin, ghrelin, their receptors, obestatin and GPR39 was more intense in the uterine luminal and glandular epithelial cells, compared to the stromal and smooth muscle cells, throughout gestation (Table 1).

Table 1. Intensity scores of leptin, ghrelin, obestatin, GPR39 and their receptors (OB-R, GHS-R) expression from the feto-maternal tissues and the uterine mucosa in the interplacentomal region during pregnancy. Data are given as the mean±s.d.

Parametreler	Pregnancy Periods	Uterine mucosa				Placenta fetalis		Placenta maternalis	
		LE	GE	S	SM	TC	FM	ME	MS
	1st	3.0±0.0^{a}	3.0±0.0^{a}	1.65±0.45^{b}	2.20±0.18^{b}	1.85±0.45^{b}	0.65±0.45^{c}	1.65±0.45^{bc}	0.96±0.18^{c}
Leptin	2nd	2.85±0.25^{a}	3.0±0.0^{a}	1.0±0.0^{b}	1.56±0.24^{b}	1.50±0.25^{b}	0.45±0.10^{c}	1.45±0.10^{bc}	1.20±0.18^{bc}
	3rd	3.0±0.0^{a}	2.70±0.56^{a}	1.25±0.65^{b}	1.90±0.45^{b}	1.35±0.12^{b}	0.65±0.45^{c}	1.30±0.25^{bc}	1.10±0.24^{bc}
	1st	2.40±0.10^{a}	2.80±0.20^{a}	1.45±0.45^{b}	2.60±0.35^{a}	2.40±0.15^{a}	0.80±0.20^{c}	1.45±0.45^{b}	1.01±0.23^{bc}
Ghrelin	2nd	2.30±0.15^{a}	2.75±0.25^{a}	1.0±0.0^{b}	2.56±0.24^{b}	2.90±0.25^{a}	0.75±0.12bc	1.20±0.20^{bc}	1.05±0.14^{bc}
	3rd	2.05±0.10^{a}	2.70±0.56^{a}	1.25±0.55^{b}	2.80±0.25^{a}	2.30±0.25^{a}	0.70±0.36^{c}	1.35±0.55^{ac}	1.10±0.25^{ac}
	1st	3.0±0.0^{a}	3.0±0.0^{a}	2.75±0.45^{a}	2.80±0.60^{a}	3.0±0.0^{a}	1.65±0.45^{b}	1.25±0.35^{b}	0.56±0.15^{c}
Obestatin	2nd	2.95±0.25^{a}	3.0±0.0^{a}	2.80±0.45^{a}	2.90±0.65^{a}	2.90±0.25^{a}	1.40±0.10^{b}	1.0±0.0^{b}	0.30±0.20^{bc}
	3rd	2.80±0.25^{a}	2.90±0.50^{a}	2.85±0.65^{a}	2.90±0.45^{a}	2.95±0.60^{a}	1.35±0.45^{b}	1.0±0.0^{b}	0.20±0.10^{bc}
	1st	3.0±0.0^{a}	3.0±0.0^{a}	1.25±0.45^{b}	2.90±0.15^{a}	1.75±0.35^{b}	0.75±0.45^{bc}	3.0±0.0^{a}	1.0±0.0^{bc}
OB-R	2nd	2.85±0.25^{a}	2.90±0.15^{a}	1.0±0.0^{b}	3.0±0.0^{a}	1.40±0.15^{b}	0.55±0.10^{bc}	2.75±0.15^{a}	0.80±0.18^{bc}
	3rd	2.55±0.20^{a}	2.40±0.36^{a}	1.15±0.45^{b}	2.75±0.25^{a}	1.35±0.12^{b}	0.65±0.45^{bc}	2.50±0.25^{a}	0.75±0.24^{bc}
	1st	2.30±0.18^{a}	2.50±0.25^{a}	1.25±0.45^{b}	0.60±0.25^{c}	2.60±0.55^{a}	0.65±0.20^{c}	1.25±0.25^{bc}	0.91±0.23^{bc}
GHS-R	2nd	2.20±0.10^{a}	2.55±0.15^{a}	1.20±0.18^{b}	0.56±0.14^{bc}	2.50±0.85^{a}	0.46±0.23^{bc}	1.10±0.5^{bc}	1.0±0.0^{bc}
	3rd	2.15±0.20^{a}	2.40±0.46^{a}	1.15±0.35^{b}	0.50±0.25^{bc}	2.45±0.67^{a}	0.40±0.26^{bc}	1.05±0.15^{b}	0.85±0.25^{b}
	1st	3.0±0.0^{a}	3.0±0.0^{a}	2.45±0.25^{a}	2.40±0.60^{a}	2.80±0.25^{a}	1.25±0.45^{b}	2.25±0.35^{a}	0.80±0.15^{b}
GPR39	2nd	2.75±0.15^{a}	3.0±0.0^{a}	2.30±0.45^{a}	2.25±0.50^{a}	3.0±0.0^{a}	1.10±0.10^{b}	1.75±0.50^{b}	0.60±0.20^{b}
	3rd	2.85±0.35^{a}	2.85±0.35^{a}	2.35±0.25^{a}	2.85±0.15^{a}	3.0±0.0^{a}	1.15±0.05^{b}	1.70±0.40^{b}	0.80±0.10^{b}

Within rows, different lowercase superscript letters indicate significant differences among luminal and glandular epithelium, stromal cells, smooth muscle cells, fetal trophoblast cells, fetal mesenchymal cells, maternal epithelial cells, maternal stromal cells (P<0.05). Values in the same line that do not share the same number of stars are significantly different (P <0.05; N = 44 for leptin, ghrelin, their receptors [OB-R, GHS-R], obestatin and GPR39). Intensity scores among luminal and glandular epithelium, stromal cells, smooth muscle cells, fetal trophoblast cells, fetal mesenchymal cells, maternal epithelial cells, maternal stromal cells in the placenta and interplacentomal regions were statistically compared in the 1st, 2nd and 3rd periods of pregnancy, (0, no staining of any nuclei even at high magnifi cation; 1, immunostaining only visible at high magnifi cation; 2, readily visible at low magnifi cation; 3, strikingly positive at low power magnifi cation.) **LE;** luminal epithelium, **GE;** glandular epithelium, **S;** stromal cells, **SM;** smooth muscle cells, **TC;** fetal trophoblast cells, **FM;** fetal mesenchymal cells, **ME;** maternal epithelial cells, **MS;** maternal stromal cells.

Discussion

The placenta is a special organ, which not only regulates respiratory gas and nutrient exchange between the mother and fetus, but also serves various endocrine functions. (Harrison et al., 2007). Although peptide hormones involved in the regulation of metabolic functions, such as leptin, ghrelin and obestatin, are mostly known as hormones specific to the digestive system, their expression in the placenta and their presence in the amniotic fluid and cord blood during gestation in some species, including humans, suggest that they are also locally produced by the placenta (Harrison et al., 2007; Roche et al., 2007). In this study, we found that leptin, ghrelin, its receptors (OB-R, GHS-R), obestatin and GPR39 were expressed in both the bovine placenta and bovine uterus throughout gestation, which supports the hypothesis that the placenta may serve as a local source for these peptide hormones in bovine animals.

Contrary to the findings of previous studies in humans (Farley et al., 2010; Toth et al., 2008) and cats (Dall'Aglio et al., 2012), and similar to the findings previously reported in sheep (Buchbinder et al., 2001; Ducsay et al., 2006), dogs (Balogh et al., 2015), baboons (Henson et al. ,1999), rats (Smith and Waddell, 2002) and mice (Hoggard et al., 1997; Hoggard et al., 2000), this research has revealed that leptin and OB-R were expressed in the cells in the placental and interplacentomal regions in cows throughout gestation. In agreement with findings previously reported for humans (Farley et al., 2010; Toth et al., 2008), baboons (Henson et al., 1999), mice (Hoggard et al., 1997; Hoggard et al., 2000), rats (Smith and Waddell, 2002), cats (Dall'Aglio et al., 2012) and dogs (Balogh et al., 2015), In this study showed that, throughout gestation, leptin and OB-R were moderately expressed not only in bovine fetal trophoblasts, but also in maternal epithelial cells. In cows, BNC had a stronger leptin expression than UTC. Consistent with some other reports in humans (Farley et al., 2010), cats (Dall'Aglio et al., 2012) and dogs (Balogh et al., 2015), in this study revealed weak expression in the fetal mesenchymal cells and maternal stromal cells. In contrast, our study showed that in cows, while OB-R localisation was stronger in the maternal placenta, than the fetal placenta, throughout gestation, expression in uninucleate and binucleate trophoblasts and fetal mesenchymal and maternal stromal cells was weaker than that in the maternal epithelial cells (Table 1). In contrast to the report of Ravelich et al. (2004) in cows, the present study found that leptin and OB-R are expressed in

trophoblasts, fetal mesenchymal cells, maternal epithelial and stromal cells in the bovine placenta throughout gestation. The intensity of expression varied with the different periods of gestation. Similar to dogs (Balogh et al., 2015) and cats (Dall'Aglio et al., 2012), in this study found that in the cows uterine mucosa leptin and OB-R are expressed in the luminal and glandular epithelial cells, and stromal and myometrial smooth muscle cells during gestation, such that expression was stronger in the luminal and glandular epithelial cells, compared to the stromal and smooth muscle cells (Table 1).

Leptin is a polypeptide hormone that regulates body weight and energy levels and participates in reproductive processes in both animals and humans (reviewed in Henson and Castracane, 2000). The presence of leptin and OB-R in multiple tissues supports the hypothesis that this hormone has a significant role in growth and development. Leptin and OB-R being specifically localized to multiple cell types in the placenta is indicative of autocrine and/or paracrine mechanisms in this active tissue. Trophoblasts, which are the predominant cell type in the placenta, are the most probable source of placental leptin and OB-R (in reviewed Henson and Castracane, 2000). Throughout pregnancy, leptin and OB-R within the maternal-feto-placental unit have been reported to serve various physiological functions (in reviewed Pérez-Pérez et al., 2015). Similarly, this study revealed that leptin and OB-R are expressed in both the placental and interplacentomal regions throughout gestation in the bovine placenta. This suggests that these hormones may have functions in cows similar to those described above.

Similar to findings reported in humans (Caminos et al., 2003; Tanaka et al., 2003), mice (Kawamura et al., 2003), rats (Caminos et al., 2003; Gualillo et al., 2001), sheep (Harrison et al., 2007) and water buffaloes (Kandasamy et al., 2013), in this study revealed that ghrelin and GHS-R were mainly localized by trophoblasts in the bovine placenta during all three gestational periods. In cows, ghrelin and GHS-R expressions were strong in UTC and BNC, but weak in mesenchymal cells. Furthermore, ghrelin was weakly expressed in bovine maternal placental cells throughout gestation, and differences in expression intensity were observed across gestational periods. In contrast, GHS-R expression in the maternal epithelial and stromal cells decreased as gestation advanced (Table 1). In humans, ghrelin was strongly localized in the myometrium during weeks 38-39 of pregnancy, and it was considered that this strong expression, even if to a limited extent, could have a relaxant effect on the myometrium (Hehir et al., 2008). Similar to humans (Hehir et al., 2008), ghrelin was also strongly expressed in the bovine myometrium throughout gestation. Again, it was found that ghrelin was strongly expressed in uterine epithelial cells during pregnancy, but weakly expressed in stromal cells, while GHS-R expression was strong in the uterine epithelial cells, moderate in the stromal cells, and weak in the smooth muscle cells throughout gestation.

The presence of ghrelin and GHS-R in the reproductive organs and placenta clearly demonstrates their involvement in reproductive physiology. In humans, while ghrelin and peptide hormone levels are high in cytotrophoblasts during the first trimester of pregnancy, these hormones are not detected in the third trimester (Tanaka et al., 2003). This suggests that ghrelin may act as a major autocrine/paracrine factor for the growth of the human placenta throughout pregnancy, and may support placental formation and development through regulatory effects on proliferation and apoptosis (Caminos et al., 2003; Tanaka et al., 2003). It has been suggested that, throughout gestation, the placental ghrelin-ghrelin receptor system accelerates fetal growth by inducing cell proliferation (Kaygusuz et al., 2013; Nonoshita et al., 2010). Ghrelin has also been reported to inhibit myometrial contractility in humans (Kaygusuz et al., 2013). In this study, the detection of ghrelin and GHS-R in the placental and interplacentomal structures supports the hypothesis that they have similar functional roles in cows throughout gestation.

A literature review has shown that, although very limited information is available on the expression of obestatin and GPR39, there are no published data on this hormone and GPR39 during pregnancy in mammals. In this study, we showed that throughout gestation, obestatin and GPR39 were strongly expressed in UTC and BNC and moderately expressed in mesenchymal cells, and the expression intensity did not differ across gestational periods (Table 1). While obestatin was weakly localized in the maternal epithelial and stromal cells during all three gestational periods, the expression of GPR39 was strong in the maternal epithelial cells and weak in the stromal cells during the first gestational period (Table 1). Furthermore, obestatin was strongly expressed in the uterinal epithelial cells and stromal and smooth muscle cells of the bovine uterus during all three gestational periods. On the other hand, GPR39 expression was strong in the uterinal epithelial cells, and moderate in the stromal and smooth muscle cells of the bovine uterus throughout gestation. Obestatin interacts with ghrelin and motilin, and thereby, contributes to the regulation of gastrointestinal and metabolic functions (Zhang et al., 2005). Obestatin and ghrelin are functional antagonists: while ghrelin stimulates food intake, obestatin suppresses it (Gualillo et al., 2003). Obestatin has been reported to significantly increase progesterone secretion in cultured pig granulosa cells, and induce both in vivo and in vitro testosterone secretion in adult male rats (Jahan et al., 2013). The localization of obestatin and GPR39, in particular to trophoblasts, and maternal epithelial, stromal and smooth muscle cells during the developmental processes of the bovine fetus and placenta suggests that this hormone could be involved in energy homeostasis by regulating local or general metabolic functions. However, it should be noted that current information on the reproductive effects of ghrelin and obestatin, which are antagonists, is largely incomplete. Thus, further research is needed to elucidate the functions of these two peptide hormones and assess their in vivo and in vitro use.

Studies in humans, dogs, cats, sheep, mice, and rats have shown that leptin, ghrelin, and their receptors are expressed in the endothelium and smooth muscle cells of blood vessels. However, the vascular effects of these peptide

hormones remain unclear (Balogh et al., 2015; Buchbinder et al., 2001; Caminos et al., 2003; Dall'Aglio et al., 2012; Ducsay et al., 2006; Farley et al., 2010; Gualillo et al., 2001; Harrison et al., 2007; Henson et al., 1999; Hoggard et al., 1997; Hoggard et al., 2000; Kawamura et al., 2003; Smith and Waddell, 2002; Tanaka et al., 2003; Toth et al., 2008). It has been suggested that ghrelin and leptin may regulate vasodilation, vasoconstriction, and vascular permeability. (Balogh et al., 2015; Dall'Aglio et al., 2012; Gualillo et al., 2001). To date, there have been no reports of obestatin or GPR39 expression in blood vessels. We found that leptin, ghrelin, their receptors (OB-R, GHS-R), obestatin and GPR39 are expressed in blood vessels in the bovine placenta and uterus throughout gestation, suggesting that they may potentially serve the abovementioned functions in blood vessels.

In conclusion, the expression of leptin, ghrelin, their receptors (OB-R, GHS-R), obestatin and GPR39 at varying intensity levels in the bovine placental and interplacentomal tissues throughout gestation, and the localization of these peptide hormones and their receptors to primarily trophoblasts, as well as maternal epithelial, stromal and smooth muscle cells support multiple paracrine/autocrine functions of these hormones in the bovine placenta.

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Conflicts of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Ethical approval

This study used slaughterhouse material, and tissue samples were not subject to ethical committee approval in accordance with the principles of the Regulation on the Working Procedures and Principles of Animal Experimentation Ethics Committees of the Ministry of Forestry and Water Affairs of the Republic of Turkey (dated February 15, 2014, No. 28,914).

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Author Contributions

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Design: HS, BB; Control/Supervision: HS
Data Collection and/or Processing: BB, UT
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