Plasma Level and Expression of Visfatin in the Porcine Hypothalamus During the Estrous Cycle and Early Pregnancy

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Plasma level and expression of visfatin in the porcine hypothalamus during the estrous cycle and early pregnancy

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TK, NS and AR contributed to the conception and design. TK contributed to article writing. NS, AR and EZ contributed to revising the article. MK, KD, BW, EZ, EM, ER, KK, MG, KB, GK, KS and BK contributed to data collection. The authors read and approved the final version of the manuscript.

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Abstract

Background: Visfatin exists in two forms: the intracellular form which is a rate limiting enzyme engaged in nicotinamide adenine dinucleotide biosynthesis and the extracellular form considered as an adipokine, produced mainly by the adipose tissue. Visfatin could be an energy sensor involved in regulating female fertility, which creates a hormonal link integrating the control of energy homeostasis and reproduction.

Methods: The study compares the expression levels of visfatin gene (quantitative real time PCR) and protein (Western blotting and fluorescent immunohistochemistry) in the selected areas of the porcine hypothalamus responsible for gonadotropin releasing hormone synthesis: the mediobasal hypothalamus (MBH) and preoptic area (POA), and visfatin concentrations in the blood plasma (enzyme-linked immunosorbent assay). The tissue samples were harvested from gilts on days 2-3, 10-12, 14-16 and 17-19 of the estrous cycle, and on days 10-11, 12-13, 15-16, 27-28 of pregnancy. Differences between groups were analyzed by one-way ANOVA followed by Tukey's *post hoc* test.

Results: During the estrous cycle, visfatin mRNA expression in the MBH was higher on days 2-3 and 17-19, while the visfatin protein concentration on days 17-19. During early pregnancy, the most pronounced gene and protein expression in the MBH was found on days 15-16 and 10-11, respectively. In the POA during the estrous cycle, visfatin gene expression was the highest on days 17-19, and the protein level of visfatin on days 14-16. During early pregnancy, the highest expression of visfatin gene in the POA was observed on days 15-16, whereas the protein concentrations on days 27-28. Blood plasma concentrations of visfatin during the estrous cycle were higher on days 2-3 in relation to other studied phases of the cycle. During early pregnancy, the highest visfatin contents in the blood plasma were observed on days 12-13. Visfatin gene and protein expression in MBH and POA, and visfatin plasma concentrations differed during early pregnancy in relation to days 10-12 of the cycle.

Conclusions: This study demonstrated visfatin expression in the porcine hypothalamus and its dependence on hormonal milieu related to the estrous cycle and early pregnancy.

Keywords: visfatin, hypothalamus, estrous cycle, early pregnancy, pig

1. Background

Visfatin, also termed nicotinamide phosphoribosyltransferase (NAMPT), was identified in 2005 by Fukuhara et al. [1]. It exists in two forms: the intracellular form which is a rate limiting enzyme engaged in nicotinamide adenine dinucleotide (NAD) biosynthesis from nicotinamide and the extracellular form considered as an adipokine. Until now no visfatin receptor has been identified. On the other hand, it is suggested that visfatin can bind and activate insulin receptor (INSR), and downstream signaling pathways [2]. The adipokine and insulin do not compete for binding to INSR which implies that these two hormones recognize different regions of the receptor [3]. Apart from the adipose tissue, visfatin was also expressed in tissues creating the hypothalamic-pituitary-gonadal (HPG) axis: in the mouse hypothalamus [4], in the pituitaries collected from sheep [5] and mice [4], in the ovarian follicular cells of humans, cows and mice [4, 6–8] as well as bovine corpora lutea [8].Visfatin was also found in the porcine tissues, including the pituitary and ovary [9]. Plasma and peri-renal adipose tissue NAMPT levels were not different between the two breeds of pigs fat Meishan and lean Large White, suggesting that this adipokine is not involved in the fattening in swine [10]. The occurrence of visfatin mRNA and protein in various tissues suggests that non-adipose tissue may also contribute to serum visfatin levels. Visfatin expression in the adipocytes can be affected by hormonal factors such as the steroid hormones, tumor necrosis factor α (TNFα), growth hormone (GH) and dexamethasone [9, 11, 12], while in the human granulosa cells by human chorionic gonadotropin (hCG) and prostaglandin E_2 [6]. It is suggested that visfatin gene expression can be controlled by species-specific regulatory mechanism [9] and the adipokine concentrations in human adipose tissue are affected by hormonal status related to pregnancy [13]. The circumstances showing that hormonal milieu typical for pregnancy may affect visfatin production are demonstrated in a study by Mastorakos et al. [14] indicating the increase in visfatin plasma concentrations with advancing gestational age of women. However, the effect of hormonal changes associated with the individual phases of the estrous cycle and periods of pregnancy on the levels of visfatin transcript and protein has not been analyzed in the hypothalamus.

Visfatin is likely to have pleiotropic properties. It plays a role in the control of energy homeostasis, inflammation and cell differentiation. Circulating visfatin concentrations correlate positively with body mass index and obesity [15, 16]. As an insulin-mimetic hormone visfatin stimulates glucose uptake in muscle cells and adipocytes [17] and suppresses glucose release from hepatocytes [18]. Visfatin, like other adipokines [19–22], could be an energy sensor involved in regulating female fertility. Administration of visfatin

during the superovulation of mice increased developmental competence of oocytes and fertility potential [23]. In women undergoing ovarian stimulation, a correlation was found between visfatin concentration in the ovarian follicular fluids and the number of oocytes retrieved [6]. An effect of visfatin on the ovarian steroidogenesis was also demonstrated [7,8]. It also seems that visfatin plays an important role in the implantation and placentation [17, 24]. It is plausible that the action of visfatin during the estrous cycle and early pregnancy (maternal recognition of pregnancy, implantation, placentation) is partly achieved through its influence on the endocrine HPG axis, including the hypothalamus, what has not been investigated so far.

Visfatin may act through the regulation of the hypothalamic structures secreting gonadotropin releasing hormone (GnRH) – the key factor controlling the pituitary and ovaries. We hypothesized that hypothalamic visfatin expression is dependent on animal hormonal status. Therefore, the goal of the present study was to investigate visfatin gene and protein expression in the porcine hypothalamic structures: mediobasal hypothalamus (MBH) and preoptic area (POA) engaged in GnRH generation, and plasma visfatin concentrations during the estrous cycle and early pregnancy.

2. Methods

2.1. Animals and tissues collection

Tissue samples were harvested from animals intended for commercial slaughter and meat processing. The animals used in the study were maintained and transported under conditions defined in the Act of 15th January 2015 (Poland) on the protection of animals used for scientific or educational purposes. Mature cross-breed gilts (Large White x Polish Landrace) aged 7 to 8 months and weighing 140 to 150 kg, obtained from the private breeding farm, were used in the study. Diet was balanced (crude protein, metabolizable energy, exogenous amino acids and minerals) in accordance with the Polish nutritional standards for domestic pigs, access to freshwater ad libitum. To investigate the visfatin expression, a total of forty animals were divided into eight experimental groups (n=5 per group), as follows: days 2-3 (early-luteal phase, the presence of corpora haemorrhagica), 10-12 (mid-luteal phase, the phase when the corpus luteum activity is the highest and similar to that noted during pregnancy), 14-16 (late-luteal phase, the phase of luteolysis) and 17-19 (follicular phase) of the estrous cycle, as well as days 10-11 (migration of the embryos within the uterus), 12-13 (maternal recognition of pregnancy), 15-16 (beginning of implantation) and 27-28 (end of implantation) of pregnancy. Gilts were monitored daily for an estrus behavior in the presence

of a boar. The day of the onset of the second estrus was marked as day 0 of the estrous cycle. The phase of the estrous cycle was also confirmed based on the morphology of ovaries [25]. In the case of pregnant pigs, natural insemination was performed on days 1-2 of the estrous cycle. In the case of days 15-16 and 27-28 of pregnancy, the stage of pregnancy was also confirmed by the presence and morphology of conceptuses/trophoblasts [26]. Within a few minutes after slaughter, blood samples were collected into heparinized tubes and centrifuged at 2500 × g for 15 min at 4°C. The obtained plasma was stored at -80°C for further measurement. Subsequently, the hypothalamus was removed and the MBH and POA were excised as previously described [27]. The mediobasal hypothalamus was defined as a block of tissue bounded rostrally by the optic chiasma, caudally by the mammillary body, laterally by the hypothalamic sulci and dorsally by a 5 mm-deep cut. The preoptic area was limited rostrally approximately 5 mm anterior to the optic chiasma and caudally by the rostral border of the MBH. The mediobasal hypothalamus and POA obtained on days 15-16 of pregnancy and 10-12 of the cycle were divided into two parts, from which one half was intended for immunofluorescent staining (placed in 4% buffered paraformaldehyde; pH=7.4, 4°C), and the other half was frozen in liquid nitrogen and stored at -80°C until processing for RNA and protein analyzis.

2.2. The analyzis of visfatin localization in the porcine hypothalamus using fluorescent immunohistochemistry

The tissue blocks were fixed by immersion for 36 h in 4% buffered paraformaldehyde (pH=7.4; 4°C). Following fixation, the brains were washed in 0.1 M phosphate-buffered saline (PBS) and then cryoprotected for 3-6 days in graded solutions (19% and 30%) of sucrose (Sigma-Aldrich, USA) at 4°C until fully infiltrated. The tissues were frozen and cut into 12 μm thick cryostat coronal sections and stored at -80°C. Localization of the hypothalamic nuclei was calculated based on Felix et al. [28]. Frozen brain sections were processed for routine single-immunofluorescence labelling. All steps of the staining procedures were conducted in humid chambers at room temperature. The sections were air-dried for 30 min, washed 3 times in PBS and incubated for 1 h with blocking buffer (0.1 M PBS, 10% normal horse serum, 0.01% BSA (bovine serum albumin), 1% Tween, 0.05% thimerosal, 0.01% NaN_3). Then, the sections were incubated overnight with rabbit polyclonal antibodies against visfatin (1:600; cat.-no. ab233294, Abcam, UK). Following subsequent rinsing in PBS (3 × 15 min), the sections were incubated (1 h) with the Alexa Fluor 555 donkey anti-rabbit antibodies (1:1000; cat.-no. A-31572, Thermo Fisher Scientific, USA). After that, slides were washed in PBS and coverslipped in buffered carboxyglycerol

(pH=8.6). The sections were analyzed with an Olympus BX51 microscope equipped with an Olympus XM10 digital camera (Tokyo, Japan). Images were acquired with cellSens Dimension 1.7 Image Processing software (Olympus Soft Imaging Solutions, Münster, Germany). Standard controls, i.e. the omission and replacement of primary antisera by non-immunosera were applied to test antibody and method specificity. Lack of any immunoreactions indicated specificity.

2.3. The analyzis of NAMPT *gene expression in the porcine hypothalamus using quantitative real-time PCR (qPCR)*

Total RNA was isolated using the TRI reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. The quantity and quality of the isolated RNA were determined spectrophotometrically (Infinite M200 Pro, Tecan, Switzerland). One microgram of RNA was reverse transcribed into cDNA using the Omniscript RT Kit (Qiagen, Germany) with 0.5 µg oligo(dt)$_{15}$ primer (Roche, Germany) in a total volume of 20 µl at 37°C for 1 h and then terminated at 93°C for 5 min. Quantitative real-time PCR (qPCR) analyzis was performed using an AriaMx Real-Time PCR System (Agilent Technologies, USA) with Power SYBR Green Master Mix (Applied Biosystems Inc., USA), as described previously [29]. Specific primer pairs used to amplify parts of *NAMPT*, *UBC* (ubiquitin C) and *18sRNA* (18S ribosomal RNA) genes are detailed in the Table 1. The *UBC* and *18sRNA* were used as reference genes. Preliminary studies confirmed that their expressions were stable in both studied tissues during all the studied stages of the estrous cycle and early pregnancy (similar Ct values without statistically significant differences). The qPCR reaction mixtures at the final volume of 20 µl contained 20 ng of cDNA, the appropriate forward and reverse primers at the concentrations detailed in the Table 1, 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and RNase free water. The qPCR conditions are detailed in the Table 1. Negative controls contained RNase free water instead of cDNA. All reactions were amplified in duplicates. The analyzis of melting curve was used to confirm the specificity of amplification. The relative gene expression level was calculated using the comparative cycle threshold method (ΔΔCt, [30]) and normalized using the geometrical means of reference genes expression levels of *UBC* and *18sRNA*. The Ct values for all non-template controls were under the detection threshold.

2.4. The analyzis of visfatin protein expression in the porcine hypothalamus using Western blot

About 30-mg-pieces of porcine MBHs and POAs were homogenized in 500 µl of T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, USA). Equal amounts of lysate (50 µg protein/sample) were separated in Mini-Protean TGX System Precast Protein Gels (Bio-Rad Laboratories Inc., USA) and then transferred to Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad Laboratories Inc., USA). The membranes were blocked for 1 h in 0.02 M Tris-buffered saline containing 5% BSA and 0.1% Tween 20, then incubated overnight at 4˚C with primary anti-visfatin antibody (cat.-no. ab233294; Abcam, UK) diluted at 1:700. Subsequently, the membranes were washed with TBST (Tris-buffered saline containing 0.1% Tween ® 20 Detergent) and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated antibody (cat.-no. 7074; Cell Signaling Technology, USA) diluted at 1:1000. The anti-β-actin antibodies (cat.-no. A5316; Sigma-Aldrich, USA) diluted at 1:5000 were used as a loading control. Signals were detected by chemiluminescence using the Western blotting Luminol Reagent (Advansta Inc., USA), and visualized using the Chemidoc™ XRS+ System (BioRad Laboratories Inc., USA). All visible bands were quantified using a densitometer and ImageJ software (US National Institutes of Health, USA).

2.5. The analyzis of visfatin concentration in the porcine blood plasma using enzyme-linked immunosorbent assay (ELISA)

The concentrations of visfatin protein in plasma were determined using a commercial ELISA kit (cat.-no. MBS736963, MyBioSource, USA) according to the manufacturer's protocol. The range of standard curve was 0 to 50 ng/mL. The sensitivity of the assay, defined as the least protein concentration that could be differentiated from zero samples, was <0.1 ng/mL. According to the manufacturer, no significant cross-reactivity or interference between visfatin and homologous proteins assayed has been observed. Absorbance values were measured at 450 nm using an Infinite M200 Pro reader with Tecan i-control software (Tecan, Switzerland). The data were linearised by plotting the log of visfatin concentrations versus the log of the optical density and the best fit line was determined by regression analyzis. Intra-assay coefficient of variation of the ELISA assay for visfatin was 5.29%.

2.6. Data analyzis

All experimental data were presented as means ± standard error of the mean (SEM) from five different observations. Differences between groups were analysed by one-way ANOVA followed by Tukey's honest significant difference *post hoc* test. Statistical analyses were performed using Statistica Software (StatSoft Inc., Tulsa, USA). Values for $p<0.05$ were considered as statistically significant.

3. Results

3.1. The distribution of visfatin in the porcine hypothalamus

The immunofluorescence staining has shown the presence of visfatin in some regions of the pig hypothalamus both during the estrous cycle (days 10-12 – the mid-luteal phase; Fig. 1) as well as during early gestation (days 15-16 of pregnancy – the beginning of implantation; Fig. 2). In the supraoptic nucleus (SON), periventricular nucleus (PPN), sexually dimorphic nucleus of the preoptic area (SDN), paraventricular nucleus (PVN), and additionally the diagonal band of Broca (DBB), a part of basal forebrain, visfatin showed very intensive immunoreactivity and it was usually confined to the the nucleus as well as neuroplasm of the cell bodies. Weaker immunoreactivity was observed in the lateral (LPA) and medial preoptic area (MPA). In the suprachiasmatic nucleus (SCN), ventromedial nucleus (VMH) and arcuate nucleus (ARC) visfatin was located in the nucleus, sporadically was found in cell neuroplasm.

3.2. Gene and protein expression of visfatin in the mediobasal hypothalamus

During the estrous cycle, visfatin gene expression was higher on days 2-3 and 17-19 in comparison to days 14-16. In turn, the protein concentration of visfatin was the highest on days 17-19 compared to days 2 to 12 ($p<0.05$; Fig. 3 A,B).

During early pregnancy, the lowest expression of visfatin gene was observed on days 27-28, however between days 10-11, 12-13 and 15-16 it was gradually increasing. Concerning the protein of visfatin, the highest concentration was observed on days 10-11 in relation to days 15-16 ($p<0.05$; Fig. 3 C,D).

Comparing visfatin gene expression throughout the early pregnancy with days 10-12 of the estrous cycle, visfatin mRNA content during all periods of pregnancy was significantly lower. However, visfatin protein content in pregnant MBH samples was at similar level as on days 10-12 of the cycle ($p<0.05$; Fig. 3 E,F).

3.3. Gene and protein expression of visfatin in the preoptic area

During the estrous cycle, visfatin gene expression was the highest on days 17-19, lower on days 14-16, and the lowest on days 2-3 and 10-12. Concerning the protein level of visfatin, the highest content was noted on days 14-16, lower on days 2-3, and the lowest on days 10-12 and 17-19 ($p<0.05$; Fig. 4 A,B).

During early pregnancy, the highest expression of visfatin gene was observed on days 15-16, and the lowest on days 12-13. In turn, the protein concentrations were the highest on days 27-28, and the lowest on days 10-11 and 15-16 of gestation ($p<0.05$; Fig. 4 C,D)

Comparing the studied periods of early pregnancy with days 10-12 of the estrous cycle, we noted that visfatin gene expression on days 15-16, 27-28 and 10-11 of pregnancy was significantly enhanced, while the expression on days 12-13 of pregnancy was considerably lower compared to this phase of the estrous cycle. In turn, visfatin protein content on days 27-28 of pregnancy was essentially higher compared to the cycle, while during the other periods of pregnancy it was at a similar level as on days 10-12 of the cycle ($p<0.05$; Fig. 4 E,F).

3.4. Visfatin concentrations in the blood plasma

During the estrous cycle, significantly higher concentration of visfatin was noted on days 2-3 in relation to other studied phases of the cycle ($p<0.05$; Fig. 5A). During early pregnancy, the highest visfatin contents in the blood plasma were observed on days 12-13 as compared to days 15 to 28 ($p<0.05$; Fig. 5B), whereas the lowest on days 27-28 compared to days 10 to 13. The comparison of the studied stages of early pregnancy and days 10-12 of the estrous cycle has shown that the concentrations of visfatin in the blood plasma on days 15-16 and 27-28 were statistically lower than on days 10-12 of the cycle ($p<0.05$; Fig. 5C).

4. Discussion

The presented research was the first experiment to report the expression of visfatin gene and protein in the structures of porcine hypothalamus responsible for GnRH production during the estrous cycle and early pregnancy. Based on our immunohistochemical analyses, visfatin was localized in the nucleus and cytoplasm of cells creating both studied hypothalamic structures. These structures, including medial preoptic area, diagonal band of Broca, lateral hypothalamic area, paraventricular nucleus, periventricular zone, suprachiasmatic nucleus and mediobasal hypothalamus, are also known as the place of GnRH neurons location in the pig brain [31, 32]. The expression pattern of visfatin gene in MBH and POA during the cycle and pregnancy was not parallel with the pattern of visfatin protein expression. Taking into account that in mammals the correlation between gene and protein expression frequently does not exceed 40% [33], this observation is not surprising. The mentioned phenomenon can be attributed to transcriptional, post-transcriptional and translational regulations and, as a consequence, discrepancies in mRNAs and proteins stability [34, 35].

In previous studies, visfatin gene expression was noticed in the mouse and chicken hypothalamus [4, 36], analyzed however as a whole, without division into particular hypothalamic structures. Visfatin expression was also found in the pituitary of male sheep [5] and female mice [4]. In the mouse pituitary, visfatin was present mainly in gonadotroph cells, responsible for the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production [4]. To our best knowledge, this is the first study investigating visfatin gene and protein expression in the hypothalamus coupled with consideration of physiological status/hormonal milieu of animals characteristic for the estrous cycle and early pregnancy. The crucial hormonal factors related to the estrous cycle are progesterone (P_4) and estradiol (E_2). Most of them are ovarian origin, but minority is produced in the brain, including hypothalamus, as neurosteroids [37]. Thus the local concentration of steroids in the hypothalamus is a mixture of peripherally derived steroid hormones, converted peripheral steroids and neurosteroids [38, 39]. Both neurons, oligodendrocytes and astrocytes are steroidogenically active cells and their primary steroidogenic product is neuroprogesterone [37]. It was indicated that astrocytes are the main source of the essential neuroprogesterone generated within the hypothalamic structures [40]. Expression pattern of visfatin gene and protein expression in MBH and POA is different throughout the estrous cycle and early pregnancy, which may suggest that the regulation of visfatin generation in both hypothalamic structures is also different. It seems possible that the observed in the MBH an increase of visfatin protein content on days 14-16 and 17-19 of the cycle is caused by stimulatory action of E_2, which plasma level is enhanced. In turn in the POA, the noticed suppressed visfatin expression on days 10-12 and 17-19 of the cycle can be coupled with P_4 concentrations in the blood and hypothalamus. On days 10-12 of the porcine estrous cycle, plasma level of P_4 is the highest which may suggest that hypothalamic concentration of this steroid is also enhanced, whereas on days 17-19 the same inhibitory effect is achieved by P_4 produced mainly in the hypothalamus. Studies by Micevych et al. [41] indicated that during proestrus the increased level of estrogens significantly induces neuroprogesterone synthesis in the hypothalamus. Circulating E_2 stimulated also hypothalamic mRNA level and activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), the key enzyme for P_4 production. It was also suggested that locally synthesized hypothalamic neuroprogesterone, apart from its action *via* progesterone receptors (PR), may be 5α-reduced, converted to allopregnanolone, and bind to $GABA_A$ receptors ($GABA_AR$) [41]. It is postulated that this kind of $GABA_AR$ stimulation is involved in the induction of GnRH release [42].

It is strongly suggested that regulatory mechanism controlling visfatin expression is tissue- and species-specific [9, 36]. The relationship between visfatin expression in particular

tissues and hormonal milieu of the organism is to a high degree unclear. On one hand, visfatin concentration in cerebrospinal fluid did not differ between man and women [43]. Similarly, there was no significant differences in visfatin mRNA expression in the mouse pituitary between the estrous and diestrus phases [4]. Generally, these reports indicate lack of any dependence of visfatin generation on hormonal status of the body, especially on the influence of sex steroid hormones. On the other hand, considerable evidence has accumulated to implicate the involvement of hormonal environment in the control of visfatin expression. It has been indicated that human maternal plasma concentration of visfatin was increased during pregnancy, especially between the two last and the first trimester [14]. One of reasons of the observed increase may be many fold higher visfatin gene expression in omental fat of pregnant women at term compared to nonpregnant subjects [13]. Similarly in rats, visfatin mRNA content was elevated in the white fat on day 21 of pregnancy [44]. The results of our study also indicate the relationship of pregnancy with plasma visfatin level. Our observations are slightly different compared to the mentioned above studies – we noticed a decrease in plasma visfatin concentrations on days of implantation in relation to earlier days of pregnancy and days 10-12 of the estrous cycle. The reason of these differences may be, of course, varied and species-dependent regulation of visfatin production throughout gestation, but the simplest explanation of the observed inconsistencies is distinct (very early in the case of our studies) period of pregnancy taken into consideration in our and cited above works. Additional examples were obtained based on experiments with 3T3-L1 pre-adipocytes and adipocytes. Studies with pre-adipocytes have shown that visfatin gene expression decreased under influence of insulin, P_4 and testosterone and increased in response to dexamethasone [12]. In turn, treatment of 3T3-L1 adipocytes with GH, TNFα, cyclic adenosine monophosphate (cAMP) and beta-adrenergic agonists caused downregulation of the adipokine expression [11], whereas the effect of sex hormones was insignificant [12]. More detailed studies concerning possible influence of sex steroid hormones on visfatin expression in adipocytes indicated that estriol added to cell cultures increased visfatin mRNA concentration and the effect of P_4 and E_2 was negligible, but these three steroid used in combination essentially enhanced the gene expression [45]. Moreover, unlike the mouse pituitary, visfatin expression in the mouse uterus fluctuated during the estrous cycle. It has been shown that visfatin protein concentration was increased in proestrus and metestrus, and decreased in estrus and diestrus. According to the authors, the reason of the observed changes can be the increased plasma estrogens levels in proestrus and their decrease in diestrus. Simultaneously, treatment with E_2 of ovariectomized mice caused visfatin up-regulation, whereas P_4 down-regulated visfatin expression. The co-treatment with both steroids enhanced visfatin protein content in the

ovariectomized mouse uterus [46]. Taken together, the sum of our results and the results reported above suggest that expression of the visfatin gene may be hormonally regulated.

It has been suggested that besides local synthesis, additional source of visfatin in the central nervous system may be transport across the blood-brain barrier. However, the concentrations of visfatin in human cerebrospinal fluid are at the level of approximately 10% of those in plasma. Moreover, visfatin levels in plasma and cerebrospinal fluid were negatively correlated as visfatin concentrations in cerebrospinal fluid decreased with increasing plasma visfatin contents in obese subjects [43]. Similarly in our study, the concentration pattern of visfatin in both examined hypothalamic structures and in the plasma of pigs is completely different. It may suggest that the transport across the blood-brain barrier transport does not play a major role, and the local hypothalamic regulation of visfatin production is autonomic and distinct in relation to other tissues, especially the adipose tissue which seems to be the main source of the adipokine.

The visfatin role in the hypothalamus is to a high degree unknown. As previously mentioned, visfatin is both an intracellular enzyme and a secreted hormone. Especially its localization in cell nuclei seems to be intriguing and may suggest, based on data provided by Svoboda et al. [47], that NAMPT, through stimulation of NAD biosynthesis and sirtuins activation, takes part in the regulation of DNA repair, chromatin structure, transcription, replication, telomerase length and circadian rhythm. It has been indicated day-night differences of visfatin expression in the ovine pars tuberalis and an increased visfatin expression, under melatonin influence, in this structure connecting the hypothalamus and pituitary [5]. It seems that links between energy sensing and NAD cycle, and the regulation of circadian clock function exist also in other parts of the central nervous system [48]. Visfatin, acting at the hypothalamic level as an energy metabolism sensor, may be involved in the control of food intake, principally fasting- and ghrelin-induced food intake [49]. Other studies, using visfatin knockin mice, indicated an enhanced NAD^+ levels in multiple tissues, including the hypothalamus, and an improvement in physical activity, sleep quality and cognitive functions [50] Nevertheless, visfatin effect on pituitary secretory functions remains unknown. Its expression in the structures responsible for GnRH production may suggest that visfatin is also engaged in the control of GnRH generation. This hypothesis, however, should be confirmed in further detailed studies

5. Conclusion

Summarizing, visfatin expression in the porcine hypothalamic structures responsible for GnRH production implies its autocrine/paracrine influence on GnRH synthesis and confirms

the potential role of visfatin as neuromodulator of reproductive functions. The variations in the expression levels of visfatin noticed during the estrous cycle and early pregnancy may suggest the effect of steroid hormones, both peripheral and hypothalamic origin. It cannot be ruled out either, that the influence of steroids is achieved through their action on hypothalamic neural systems, like GABAergic, dopaminergic, serotoninergic systems engaged in the control of GnRH production. Further research focused on steroid hormones and their receptors concentrations in particular hypothalamic nuclei involved in GnRH synthesis are also required.

List of abbreviations

18sRNA - 18S ribosomal RNA

3β-HSD - 3β-hydroxysteroid dehydrogenase

ARC - arcuate nucleus

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

DBB - diagonal band of Broca

E_2 - estradiol

ELISA - enzyme-linked immunosorbent assay

FSH - follicle-stimulating hormone

$GABA_AR$ - $GABA_A$ receptors

GH - growth hormone

GnRH - gonadotropin releasing hormone

hCG - human chorionic gonadotropin

HPG - hypothalamic-pituitary-gonadal axis

INSR - insulin receptor

LH - luteinizing hormone

LPA - lateral preoptic area

MBH - mediobasal hypothalamus

MPA - medial preoptic area

NAD - nicotinamide adenine dinucleotide

NAMPT - nicotinamide phosphoribosyltransferase

NaN_3 - sodium azide

P_4 - progesterone

PBS - phosphate-buffered saline

POA - preoptic area of the hypothalamus

PPN - periventricular nucleus

PR - progesterone receptors

PVN - paraventricular nucleus

qPCR - quantitative real-time PCR

SCN - suprachiasmatic nucleus

SDN - sexually dimorphic nucleus of the preoptic area

SEM - standard error of the mean

SON - supraoptic nucleus

TBST - Tris-buffered saline with 0.1% Tween ® 20 Detergent

TNFα - tumor necrosis factor α

UBC - ubiquitin C

VMH - ventromedial nucleus

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Figures

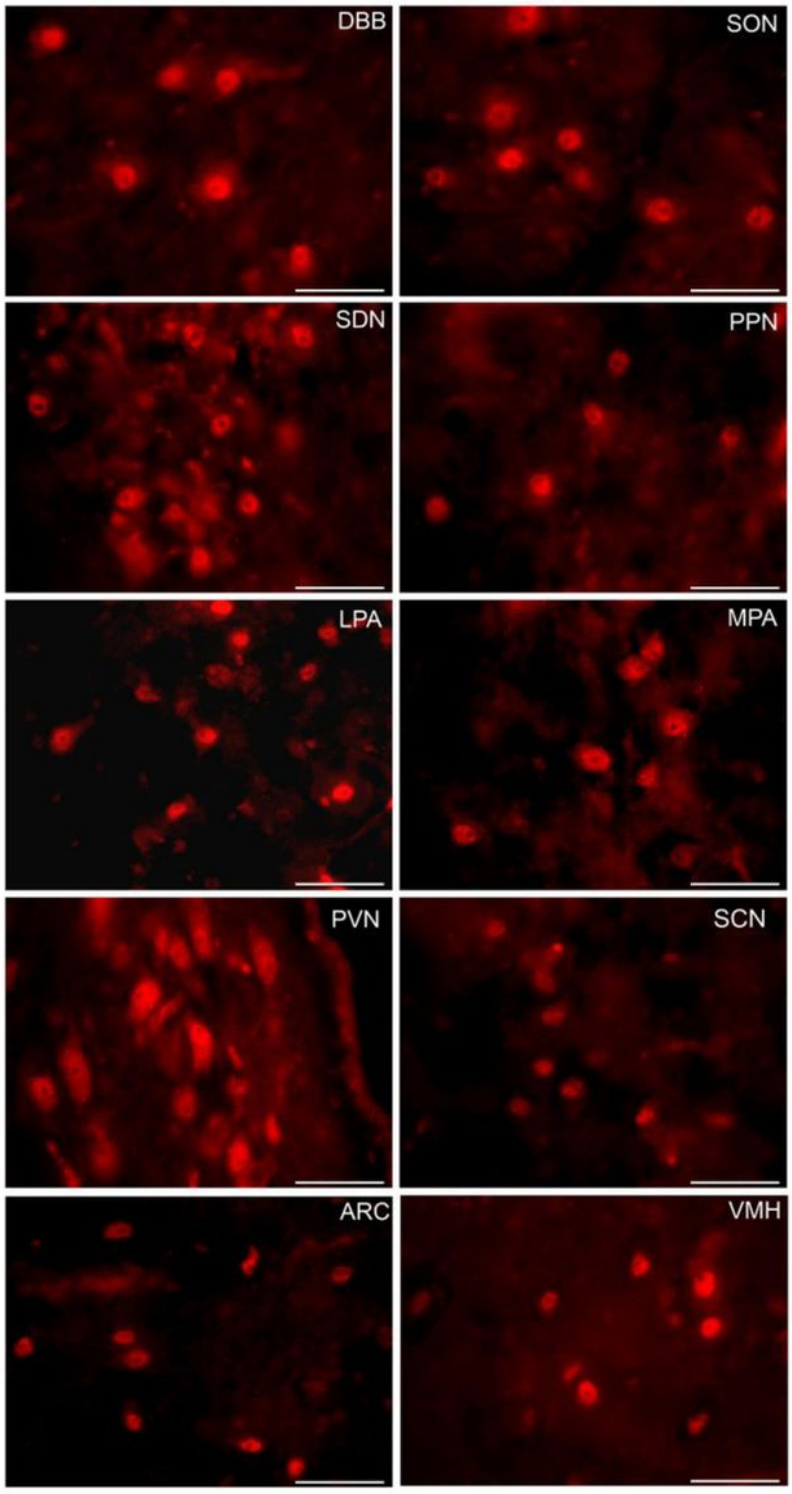

Figure 1

The visfatin localization in the porcine hypothalamus on days 10-12 of the estrous cycle Immunoreactivity of visfatin was determined by fluorescent immunohistochemistry. Particular images indicate the immunolocalization of visfatin in the diagonal band of Broca (DBB), supraoptic nucleus

(SON), preoptic area (SDN), periventricular nucleus (PPN), lateral (LPA) and medial preoptic area (MPA), paraventricular nucleus (PVN), suprachiasmatic nucleus (SCN), arcuate nucleus (ARC), as well as ventromedial nucleus (VMH). Scale bar: 50 μm.

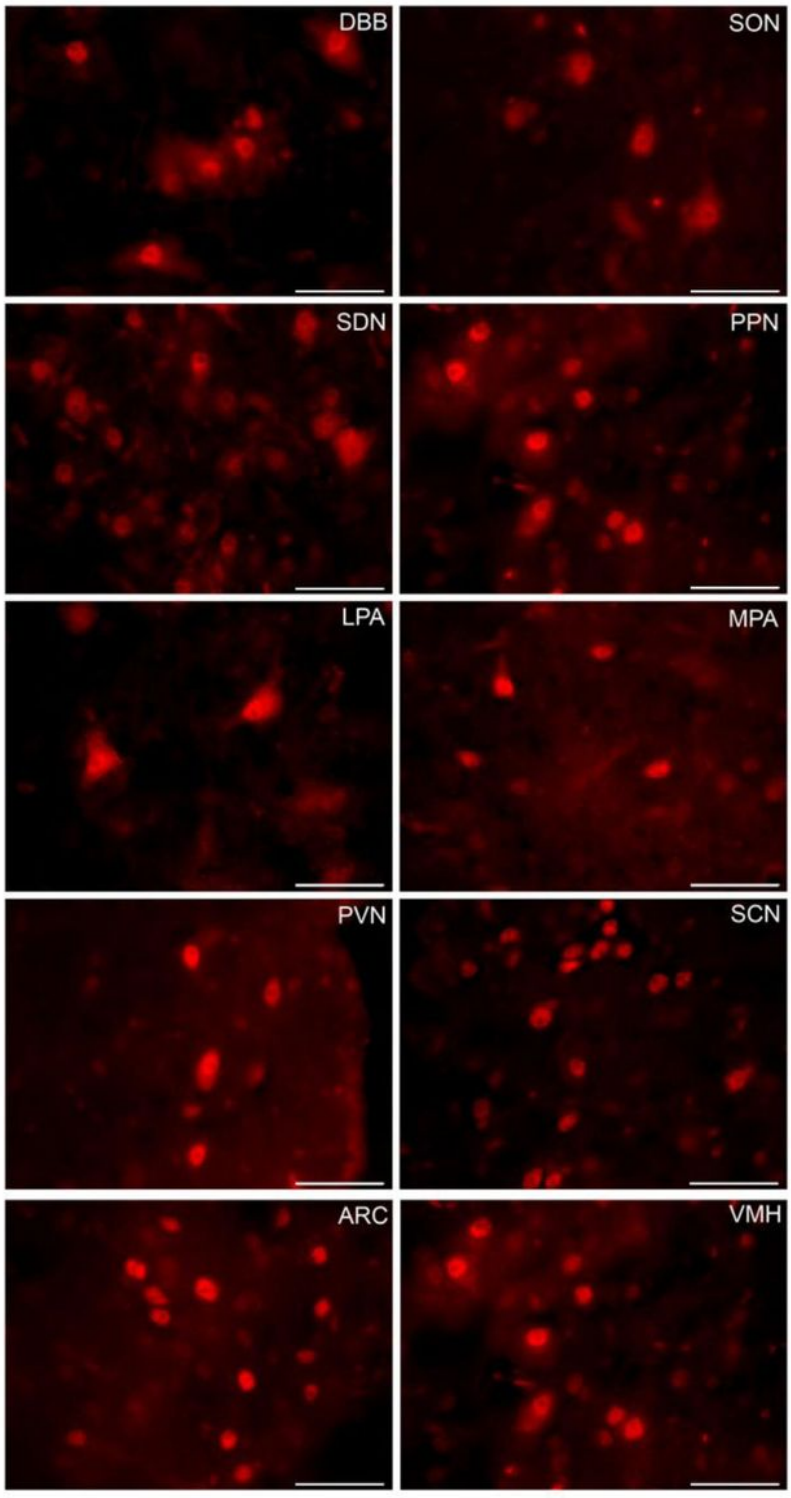

Figure 2

The visfatin localization in the porcine hypothalamus on days 15-16 of early pregnancy Immunoreactivity of visfatin was determined by fluorescent immunohistochemistry. Particular images indicate the

immunolocalization of visfatin in the diagonal band of Broca (DBB), supraoptic nucleus (SON), preoptic area (SDN), periventricular nucleus (PPN), lateral (LPA) and medial preoptic area (MPA), paraventricular nucleus (PVN), suprachiasmatic nucleus (SCN), arcuate nucleus (ARC), as well as ventromedial nucleus (VMH). Scale bar: 50 μm.

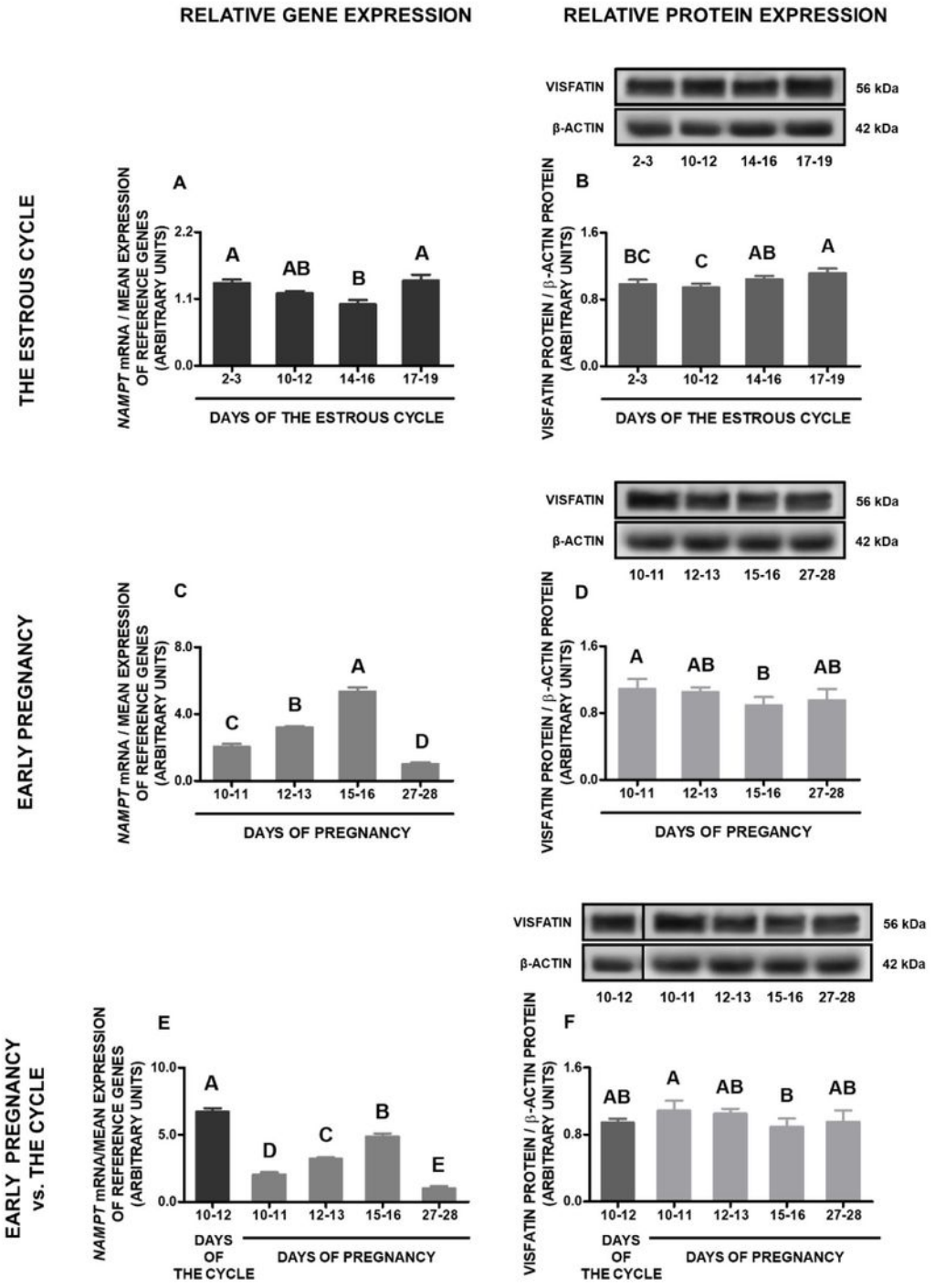

Figure 3

Visfatin gene and protein expression in the porcine mediobasal hypothalamus (MBH) during the estrous cycle and early pregnancy Gene and protein expression of visfatin in the porcine MBH was determined during the estrous cycle on days: 2-3, 10-12, 14-16 and 17-19 (A, B), during early pregnancy on days: 10-11, 12-13, 15-16 and 27-28 (C, D) and compared between early pregnancy and days 10-12 of the estrous cycle (E, F). Gene expression was analyzed by qPCR. Protein expression was analyzed by Western blotting; upper panels: representative immunoblots; lower panels: densitometric analyzis of visfatin protein relative to actin protein. Results are presented as means ± SEM (n=5). Bars with different superscripts are significantly different (one-way ANOVA at $p<0.05$ followed by Tuckey post hoc test at $p<0.05$).

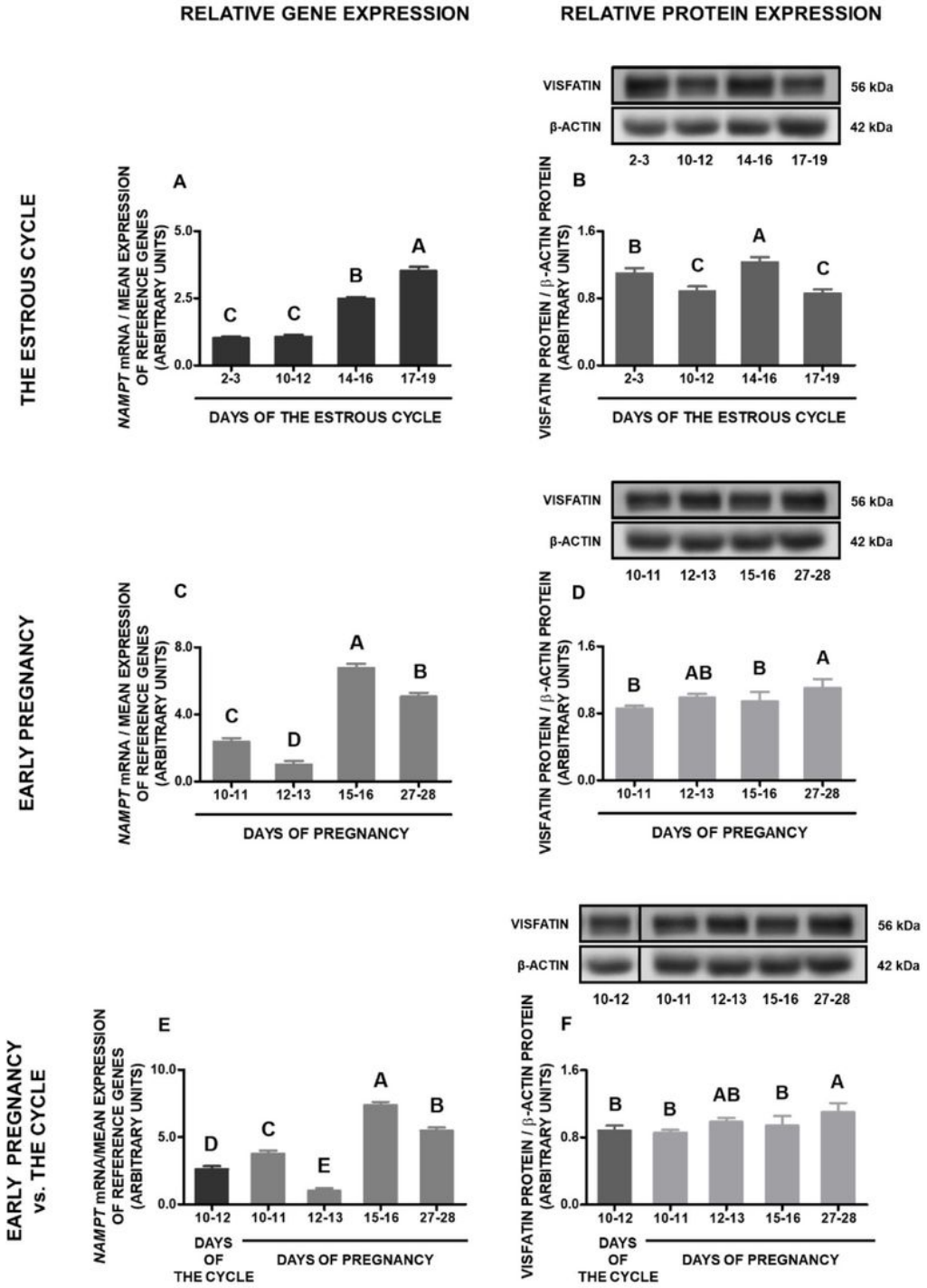

Figure 4

Visfatin gene and protein expression in the porcine preoptic area (POA) during the estrous cycle and early pregnancy Gene and protein expression of visfatin in the porcine POA was determined during the estrous cycle on days: 2-3, 10-12, 14-16 and 17-19 (A, B), during early pregnancy on days: 10-11, 12-13, 15-16 and 27-28 (C, D) and compared between early pregnancy and days 10-12 of the estrous cycle (E, F). Gene expression was analyzed by qPCR. Protein expression was analyzed by Western blotting; upper panels:

representative immunoblots; lower panels: densitometric analyzis of visfatin protein relative to actin protein. Results are presented as means ± SEM (n=5). Bars with different superscripts are significantly different (one-way ANOVA at p<0.05 followed by Tuckey post hoc test at p<0.05).

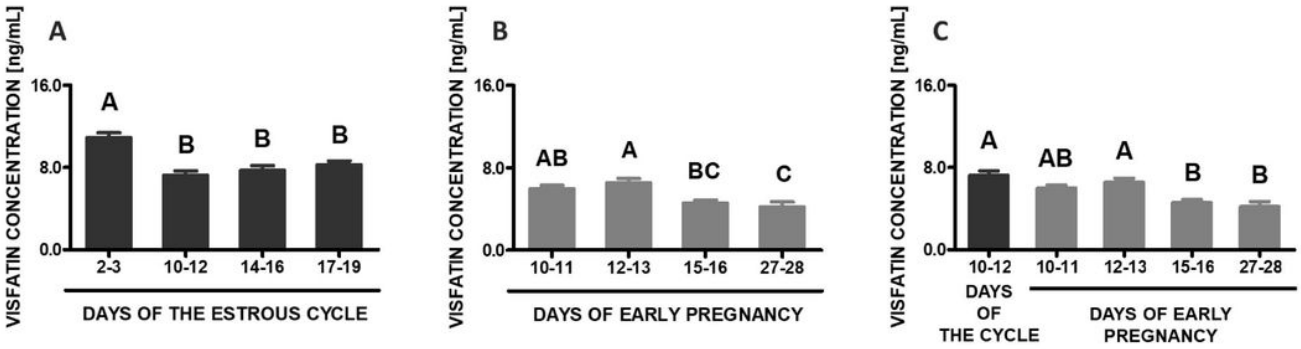

Figure 5

Visfatin concentration in the porcine blood plasma Concentrations of visfatin in the porcine blood plasma was determined during the estrous cycle on days: 2-3, 10-12, 14-16 and 17-19 (A), during early pregnancy on days: 10-11, 12-13, 15-16 and 27-28 (B) and compared between early pregnancy and days 10-12 of the estrous cycle (C). The hormone content in blood plasma was evaluated using ELISA. Results are presented as means ± SEM (n=5). Bars with different superscripts are significantly different (one-way ANOVA at p<0.05 followed by Tuckey post hoc test at p<0.05).

Supplementary Files

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- Table1.pdf