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Expression of visfatin in the ovarian follicles of prepubertal and mature gilts and *in vitro* effect of gonadotropins, insulin, steroids, and prostaglandins on visfatin levels

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ABSTRACT

Recent studies have demonstrated that visfatin participates in the regulation of female reproduction. Due to the lack of data concerning the level of visfatin in the ovarian follicles of pigs, one of the most economically important livestock species, the aim of this study was to investigate the expression and localisation of visfatin and the follicular fluid concentration in the ovarian follicles of prepubertal and mature gilts. We also aimed to examine the *in vitro* effects of gonadotropins (LH, FSH), insulin, progesterone (P_4), oestradiol (E_2), prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) on visfatin levels. In the present study, we have demonstrated that visfatin expression is dependent on the maturity of the animals and the stage of ovarian follicle development. Visfatin signal was detected in individual follicular compartments from primordial to antral follicles and even in atretic follicles. We have shown that the expression of visfatin in granulosa cells was higher than in theca cells. The level of visfatin is upregulated by LH, FSH, E_2, and P_4 and downregulated by insulin, while prostaglandins have modulatory effects, dependent on the dose and type of ovarian follicular cells. To summarise, our research has shown that visfatin is widely expressed in the ovarian follicles of prepubertal and mature pigs, and its expression is regulated by gonadotropins, insulin, steroids, and prostaglandins, suggesting that visfatin appears to be an important intraovarian factor that could regulate porcine ovarian follicular function.

1. Introduction

Visfatin, also called nicotinamide phosphoribosyltransferase (NAMPT), was discovered in 2005 as a protein with insulin-mimetic properties; however, it was already known as a pre-B cell colony-enhancing factor (PBEF) [1]. It is a 52 kDa protein with 491 amino acids in humans, as well as in mice and pigs. Moreover, the porcine visfatin gene consists of 11 exons and has exactly the same structure as the human orthologue [2]. There are two distinguish isoforms of visfatin: intracellular (iNAMPT), as an enzyme in the nicotinamide adenine dinucleotide synthesis pathway, and extracellular (eNAMPT), which can act by binding to a specific receptor [3]. So far, several studies indicate that visfatin can exert action by the insulin receptor (INSR) [1,4,5], as well as the Toll-like receptor 4, and regulate cytokine secretion in an inflammatory state [6,7]. Visfatin is widely expressed in the whole organism; almost any tissue can be the source of visfatin, but its expression level is tissue-specific. Thus, in humans, the highest number of copies of mRNA encoding visfatin was found in peripheral blood mononuclear cells and in liver and lung homogenates [8]. Additionally, its presence has been demonstrated in the spleen, placenta, muscles, kidneys, and bone marrow [8,9]. Similarly, Palin et al. [10] showed the presence of visfatin mRNA in the liver, heart, skeletal muscles, pituitary, uterus, and ovary of pigs. Visfatin is abundantly secreted by adipose tissue, and its level increases during adipogenesis [11]. The wide expression of visfatin

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in tissues translates into a multidirectional function in the organism. Due to its enzymatic nature and participation in NAD^+ biosynthesis, it is primarily involved in cellular metabolism, cell survival, and mitochondrial biogenesis [12,13]. Many findings indicate its insulin-mimetic properties evidenced by observation of increased glucose uptake after treatment with visfatin in the *in vitro* cultures of osteoblasts [4], renal mesangial cells [5], and adipocytes [1]. Moreover, this adipokine is involved in the stimulation of pro-inflammatory cytokines [14], but it is also a pro-survival factor of neuronal cells [15], promotes angiogenesis [16], and regulates the circadian rhythm [17]. The concentration of visfatin in human circulation varies from 9.8 ng/mL to 282 ng/mL according to various studies [18]; however, it is known that its elevated level is observed in obesity [19], inflammatory state [20], type II diabetes [21], and polycystic ovarian syndrome (PCOS) [22], indicating its participation in the pathophysiology of these diseases.

Recent studies have demonstrated that visfatin participates in the regulation of the female reproductive system. The presence of the transcript or visfatin protein has been noted in mice, cows, and hens, as well in human ovaries [23,24]. More precisely, its expression was demonstrated in human granulosa cells (Gc), which are regulated by human chorionic gonadotropin (hCG) and prostaglandin E_2 (PGE_2) [25,26]. In water buffalo, visfatin is present in the corpus luteum (CL) and stimulates progesterone (P_4) secretion [27]. In addition, visfatin increases basal and insulin-like growth factor 1(IGF1)-induced P_4 secretion in human Gc [26], while it has the opposite effect in chicken [28]. Its positive influences on the maturation of oocytes were confirmed in mice [29] and also in women undergoing *in vitro* fertilization (IVF) [25]. Despite this interest, no one, to the best of our knowledge, has studied visfatin in ovarian follicles of pigs, one of the most economically important livestock species. Reproduction in pigs is highly dependent on the animal's nutritional status. In sows, a restrictive diet has a serious impact on the developmental competences of oocytes. First of all, they had smaller ovarian follicle size, less expansion of cumulus-oocyte complexes, and lower levels of IGF1 and steroids in the follicular fluid (FF) [30]. Moreover, it is observed that animals with a lower weight reach puberty later and have lower reproductive parameters manifested through a smaller number of offspring with smaller birth weights [31]. Our previous study indicates the plasma levels and expression of visfatin in the hypothalamus structures responsible for the production of gonadotropin-releasing hormone (GnRH) [32], as well as fluctuating levels of visfatin in the porcine CL [33], indicating that visfatin content depends on the animal's hormonal environment related to the phase of the oestrous cycle and early pregnancy.

The aim of this study was to investigate the expression of visfatin at the gene and protein levels, concentration in FF, and its localisation in ovarian follicles in prepubertal and mature gilts. Furthermore, we have examined the *in vitro* effect of gonadotropins, insulin, steroids, and prostaglandins on protein levels of visfatin in the ovarian follicular cells.

2. Materials and methods

2.1. Reagents

M199 medium (cat. no M2154), fetal bovine serum (FBS; cat. no. F9665), insulin (cat. no. I5523), luteinizing hormone (LH, cat. no. L6420), follicle-stimulating hormone (FSH, cat. no. F4021), P_4 (cat. no. P8783), oestradiol (E_2, cat. no. E2257), PGE_2 (cat. no. P0409), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, cat. no. P5069), TRI reagent (cat. no. T9424), Laemmli buffer (cat. no. 38733), normal donkey serum (cat. no. S30-M) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Bovine serum albumin (BSA, cat. no. ALB001) was obtained from BioShop Inc., Burlington, Canada. Tissue Protein Extraction Reagent (TPER, cat. no. 78510), antibiotic-antimycotic solution, trypsin, and electrophoresis marker (cat. no. 26616) were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Bradford protein assay kit, was obtained from Bio-Rad Laboratories Inc. Hercules, CA, USA. 1% Triton™ X-100 (cat. no. M143) was purchased in Avantor Performance Materials Inc. Radnor, PA, USA.

2.2. Sample collection and in vitro experiments

The study was carried out on cross-breed gilts, (Large White × Polish Landrace) prepubertal (aged 4–6 months and weighing 100–110 kg) and mature (aged 7–8 months and weighing 140–150 kg), obtained from the local slaughterhouse. The use of animals was in accordance with the Act of the 15th of January 2015 (Journal of Laws Dz. U. 2015 no. item 266) on the Protection of Animals Used for Scientific or Education Purposes and Directive 2010/63/EU of the European Parliament and the Council of the 22nd of September 2010 on the protection of animals used for scientific purposes, so an agreement of ethical commission was not necessary. Within a few minutes of slaughter, the ovaries were removed, placed in PBS (pH = 7.4, 4 °C) with a mixture of antibiotics, and transported on ice to the laboratory within 1–1.5 h.

To determine the mRNA and protein abundance, as well as the concentration in FF, of visfatin, we collected ovarian follicles (n = 6 per group, from 6 different animals) as follows: small follicles (SF; 3–4 mm in diameter) and medium follicles (MF; 4–5 mm in diameter) from prepubertal gilts and SF (2–4 mm in diameter), MF (4–6 mm in diameter) and large follicles (LF; 8–12 mm in diameter) from mature pigs on Days 4 to 6, 10 to 12, and 17 to 19 of the oestrous cycle, respectively (Supplementary Fig. 1). Additionally, we mechanically isolated theca cells (Tc) and Gc from LF of mature pigs to compare levels of visfatin in two main compartments of follicles. The phase of the oestrous cycle was confirmed based on the morphology of the ovaries [34]. Isolated follicles were immediately frozen in liquid nitrogen and stored at −70 °C for further real-time polymerase chain reaction (qRT-PCR) and western blot analysis. To examine the cellular immunolocalisation of visfatin, whole ovaries (n = 4 per group, from 4 different animals) were collected from prepubertal pigs and mature animals on Days 2 to 3, 4 to 6, 10 to 12, 14 to 16, and 17 to 19 of the oestrous cycle (24 ovaries in total) and immersed in 4% buffered paraformaldehyde for immunofluorescence staining.

To determine the *in vitro* effects of LH, FSH, insulin, E_2, P_4, PGE_2, and $PGF_{2\alpha}$ on visfatin protein expression and concentration in culture media, we used the culture of Tc and Gc collected from LF on Days 17 to 19 of the oestrous cycle of pigs (n = 6 per group, six independent *in vitro* cultures). Ovarian cells were prepared using a technique described by Stoklosowa et al. [35]. Briefly, Gc were isolated mechanically, while Tc were isolated enzymatically using a solution of 0.25% trypsin with EDTA. The viability of cells checked by Trypan blue exclusion test was found to be 90% to 85%. Cells were cultured in M199 medium supplemented with 5% FBS and inoculated at concentrations of 4×10^4 cells/well for Tc and 5×10^4 cells/well for Gc in 96-well tissue culture plates. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After 24 h, the medium was replaced with fresh 1% FBS, and cells were treated with LH at a dose of 100 ng/mL [36] or FSH at a dose of 100 ng/mL [36], insulin at a dose of 10 ng/mL [37], P_4 at doses of 10, 100, or 1000 nM [38], E_2 at doses of 1, 10, or 100 nM [39], or prostaglandins E_2 and $F_{2\alpha}$ at doses of 100, 250, or 500 ng/mL [40] for 24 h. Following incubation, culture media were harvested, centrifuged at 1000×*g* for 10 min at 4 °C, and the supernatants were collected and stored at −20 °C for analysis of visfatin concentration, while the cells were lysed using Laemmli buffer and then stored at −20 °C for analysis of visfatin protein expression.

2.3. RNA isolation and qRT-PCR

The procedure of visfatin gene expression in the porcine ovarian follicles was conducted as described by Kamiński et al. [32]. Total RNA of ovarian follicles was extracted using TRI reagent according to the manufacturer's instructions. The quantity and quality of the isolated RNA were determined spectrophotometrically. Obtained RNA, at the

Table 1
Characteristics of primers used in the experiment.

Gene symbol	Primers sequences	Reaction Conditions	Number of cycles	Primer (nM)	Target sequence accession number
NAMPT	F: 5′-CCAGTTGCTGATCCCAACAAA-3′ R: 5′-AAATTCCCTCCTGGTGTCCTATG-3′	Activation: 50 °C, 2 min Activation: 95 °C, 10 min 1. Denaturation: 95 °C, 15 s 2. Annealing: 60 °C, 1 min	40	300	XM_003132281.5
UBC	F: 5′-GGAGGAATCTACTGGGGCGG-3′ R: 5′ -CAGAAGAAACGCAGGCAAACT-3′	Activation: 95 °C, 10 min 1. Denaturation: 95 °C, 15 s 2. Annealing: 60 °C, 1 min 3. Elongation: 70 °C, 1 min	40	400	XM_003483411.3
18sRNA	F: 5′-TCCAATGGATCCTCGCGGAA-3′ R: 5′-GGCTACCACATCCAAGGAAG-3′	Activation: 95 °C, 10 min 1. Denaturation: 95 °C, 15 s 2. Annealing: 60 °C, 1 min 3. Elongation: 70 °C, 1 min	40	400	AY265350.1

NAMPT: visfatin*; UBC:* Ubiquitin C*;* 18sRNA*:* 18S ribosomal RNA*;* F: forward; R: reverse.

Table 2
Specifications of antibodies used for the study.

Antibody	Host	Dilution	Catalogue number	Supplier
anti-visfatin	rabbit	1:700 (WB) 1:250 (IFC)	ab233294	Abcam, Cambridge, UK
anti-β-actin	mouse	1:1000 (WB)	A5316	Sigma-Aldrich, St. Louis, MO, USA
anti-rabbit horseradish peroxidase-conjugated	goat	1:1000 (WB)	7074	Cell Signaling Technology, Danvers, MA, USA
anti-mouse horseradish peroxidase-conjugated	horse	1:1000 (WB)	7076	Cell Signaling Technology, Danvers, MA, USA
Alexa Fluor® 488 AffiniPure anti-rabbit	goat	1:1000 (IFC)	111-545-003	Jackson ImmunoResearch Labs, West Grove, PA, USA

Abbreviations: WB – western blot, IFC – immunofluorescence staining.

amount of 1 μg for one reaction, was reverse transcribed into cDNA using the Omniscript RT Kit (Qiagen, Düsseldorf, Germany) with 0.5 μg oligo(dT)$_{15}$ primers (Roche, Basel, Switzerland) in a total volume of 20 μL at 37 °C for 1 h, then terminated at 93 °C for 5 min. The analysis of qRT-PCR was performed using an AriaMx Real-Time PCR System (Agilent Technologies, CA, USA). Primers' specifications and concentrations, as well as reaction conditions, are defined in Table 1. The reaction mixtures at the final volume of 20 μL consisted of 20 ng of cDNA, forward and reverse primers at the appropriate concentrations, 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, MA, USA), and RNase-free water. Constitutive expression of reference genes (ubiquitin C gene: *UBC;* 18S ribosomal RNA gene: 18sRNA) in the appropriate research groups was confirmed. RNase free water, instead of cDNA, was used as a negative control. All of the samples were prepared in duplicates. The specificity of amplified products was determined by melting-curve analysis. Relative gene expression levels of *NAMPT* were determined according to Livak and Schmittgen [41], using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method.

2.4. Western blot

Tissue preparation and western blot analysis were performed according to our protocol [32] with some modifications. In brief, porcine ovarian follicles and isolated Gc and Tc from LF were homogenised in 200 μL of TPER. Equal amounts of the lysate (30 μg protein/sample) were separated in 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, 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 diluted at 1:700 or anti-β-actin antibody diluted at 1:1000 (Table 2). Subsequently, the membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated for 1 h at room temperature (RT) with horseradish peroxidase-conjugated antibody diluted at 1:1000. β-actin was used as a loading control. Signals were detected by chemiluminescence method using the WesternBright Sirius HRP substrate (Advansta Inc. San Jose, CA, USA) and visualised in the Chemidoc™ XRS + System (BioRad Laboratories, Hercules, CA, USA). Densitometric analysis of all bands was performed in ImageJ software (US National Institutes of Health, USA).

2.5. Immunofluorescence staining

Tissue preparation for fluorescent immunostaining was conducted as described by Rytelewska et al. [42]. One day before cryostat sectioning, tissues were passed to −20 °C, incubated overnight, then cut at −25 °C into 10 μm-thick sections using a Microm HM 525 Cryostat (Thermo Fisher Scientific, Waltham, MA, USA). Slides were stored at −80 °C for further analysis. Immunofluorescence staining was started from the defrosting and air-drying of tissue slides at RT for 30 min. The slides were rinsed three times with 0.1 M PBS and incubated with a blocking buffer (containing 10% normal donkey serum, 1% BSA, and 1% Triton™ X-100) in a humid chamber at RT for 1 h. Following incubation, the slides were rinsed three times with 0.1 M PBS. After that, tissue sections were incubated overnight with anti-visfatin primary antibodies (Table 2) diluted at 1:250 in TBST in a humid chamber at 4 °C. For the negative control, primary antibodies were omitted, and tissues were incubated in 0.1 M PBS. On the next day, the slides were rinsed three times with 0.1 M PBS and incubated with Alexa Fluor® 488 antibodies diluted at 1:1000 in a humid chamber at RT for 1.5 h. Following subsequent rinsing with 0.1 M PBS, the slides were air-dried for 15 min and covered with histology mounting medium Fluoroshield™ with DAPI for nuclear counterstaining. The labelled tissue sections were analysed with an Olympus BX51 research microscope (Olympus, Tokyo, Japan) equipped with an EXFO x-CiteSeries 120Q fluorescence illuminator (Excelitas Technologies Corp., Waltham, MA, USA) using appropriate filters set for DAPI and AlexaFluor®488. Images were acquired with an Olympus DP72 microscope digital camera and CellFsoftware (Olympus, Tokyo, Japan). The analysis was performed in 4 biological replicates, and each of them in 3 technical replicates.

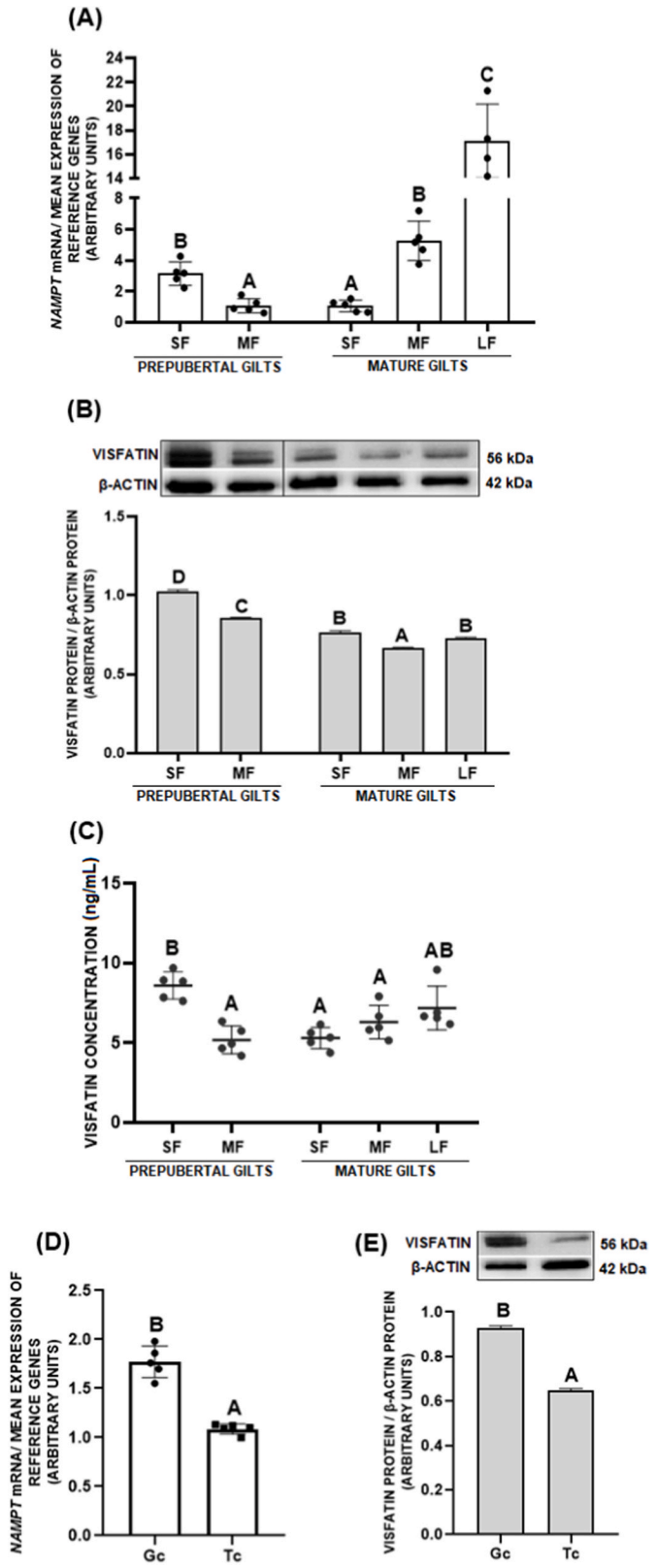

Fig. 1. Relative abundance of visfatin mRNA (A), protein (B), and concentration in follicular fluid (C) in the small (SF), medium (MF) and large (LF) ovarian follicles collected from prepubertal and mature gilts and comparison of visfatin mRNA (D) and protein abundance (E) in granulosa (Gc) and theca cells (Tc) isolated from LF of mature pigs. mRNA expression was determined using quantitative real-time PCR. Protein visfatin abundance was analysed by the western blot method. Results are shown as representative immunoblots and a bar graph with densitometry measurements of relative visfatin protein content normalised with β-actin protein. Visfatin concentration in follicular fluid was evaluated using an ELISA assay. The data are presented as the mean ± SEM (n = 6). Statistical analysis was carried out using Student's t-test or one-way ANOVA, followed by Tukey's test. Different capital letters indicate significant differences at $P < 0.05$.

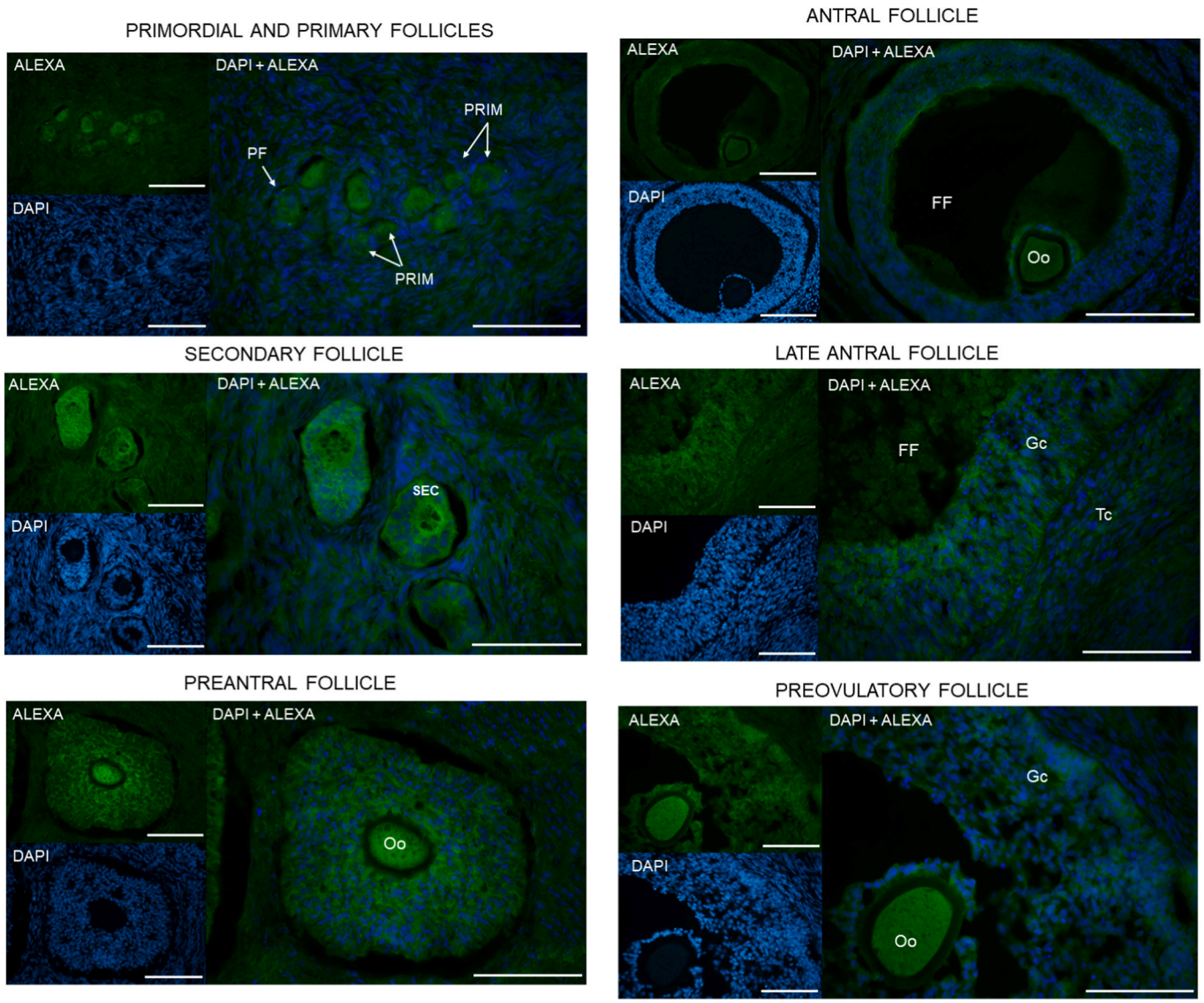

Fig. 2. The localisation of visfatin in the porcine ovarian follicles. Immunoreactivity of visfatin was determined by fluorescent immunohistochemistry. First image: visfatin expression, visualised by Alexa Fluor® 488 as green fluorescence; second: nuclei stained with DAPI as a blue fluorescence, and third: merged images of channels. Images indicate the localisation of visfatin in primordial (PF), primary (PRIM), and secondary (SEC) ovarian follicles, as well as in oocyte (Oo), follicular fluid (FF), granulosa (Gc) and theca cells (Tc) of preantral, antral, late antral, preovulatory, and atretic follicles. Immunofluorescence staining was repeated on four pigs (n = 4). NC: negative control: scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. ELISA

The concentrations of visfatin in FF and culture media were determined using a commercially available ELISA kit (cat. no. ELK8479, ELK Biotechnology, Wuhan, China) according to the manufacturer's protocol. The sensitivity of the assay was 0.51 ng/mL and intra- and inter-assay precision was less than 8% and 10%, respectively. Absorbance values were measured at 450 nm using a Varioskan LUX Multimode Microplate Reader and SkanIt Software 6.1.1 (ThermoFisher Scientific, Waltham, MA, USA).

2.7. Statistical analysis

All experimental data are presented as mean ± SEM of experiments that were performed in six replicates (n = 6). All data were also tested for the assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). To compare the expression of visfatin between ovarian follicles at different stages of development, concentration in FF, and the effect of steroids and prostaglandins on visfatin level in Tc and Gc, one-way ANOVA was used followed by Tukey's test, while for examination of insulin and gonadotropins effects on visfatin level compared to the control group, we used Student's t-test (GraphPad Prism 8.0.1 Software, Inc.). Different letters were considered to indicate statistically significant differences at the level of $P < 0.05$.

3. Results

3.1. Visfatin mRNA and protein expression as well as a concentration in FF of ovarian follicles of prepubertal and mature gilts

In prepubertal gilts, gene and protein expression of visfatin in ovarian follicles and the concentration in FF were higher in SF (8.58 ± 0.84 ng/mL) than in MF (5.18 ± 0.87 ng/mL) ($P < 0.05$; Fig. 1A–C). On the other hand, in mature gilts, the transcript level of visfatin increased

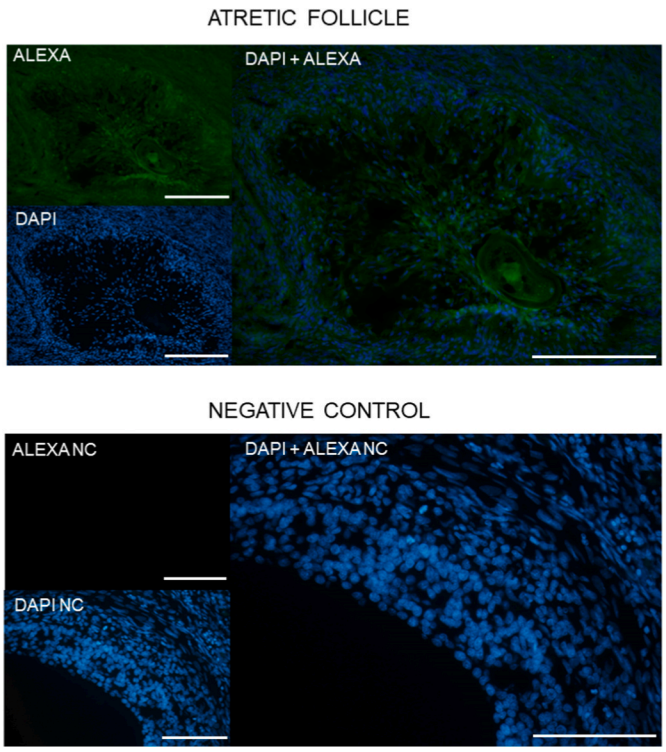

Fig. 2. (*continued*).

along with the size of the ovarian follicle, and the highest level was observed in LF ($P < 0.05$; Fig. 1A). The highest abundance of visfatin protein was noted in an SF of prepubertal animals, while in mature pigs, we observed higher protein content in SF and LF vs MF ($P < 0.05$; Fig. 1B). In mature animals, we did not observe significant changes in the visfatin concentration in FF (SF: 5.31 ± 0.59 ng/mL; MF: 6.30 ± 0.94 ng/mL; LF: 7.18 ± 1.22 ng/mL) (Fig. 1C). Interestingly, we observed that both mRNA and protein expression of visfatin were higher in Gc than Tc collected from an LF on Days 17 to 19 of the oestrous cycle ($P < 0.05$; Fig. 1D and E).

3.2. Visfatin cellular immunolocalisation in porcine ovarian follicles

The signal of visfatin was found in primordial, primary, secondary, preantral, antral, preovulatory, and even atretic follicles (Fig. 2). More specifically, the presence of visfatin was noted in various compartments building ovarian follicles, including Gc, Tc, and the oocyte, where we observed a very strong signal. Additionally, we noticed that visfatin is present in the FF of the antral follicle (Fig. 2).

3.3. In vitro effect of gonadotropins on visfatin protein expression and concentration in culture media of Gc and Tc

We observed that both FSH and LH at a dose of 100 ng/mL increased the protein expression of visfatin in Gc and Tc, as well as the concentration of visfatin in the culture media (FSH in Gc: 5.6 ± 1.01 ng/mL *vs* 3.45 ± 1.03 ng/mL in control, and in Tc after LH: 3.85 ± 1.05 ng/mL *vs* 2.21 ± 0.38 ng/mL in the control group) ($P < 0.05$; Fig. 3C, D).

3.4. In vitro effect of insulin on visfatin protein expression and concentration in culture media of Gc and Tc

Insulin at a dose of 10 ng/mL reduced the protein abundance of visfatin in both Gc and Tc ($P < 0.05$; Fig. 4A and B). In the case of visfatin concentration in culture media, we observed that insulin significantly decreased its level only in Tc (1.34 ± 0.56 ng/mL *vs* 2.21 ± 0.38 ng/mL in the control group) and had no effect in Gc (4.09 ± 1.13 ng/mL *vs* 3.96 ± 0.86 ng/mL in the control) ($P < 0.05$; Fig. 4C and D).

3.5. In vitro effect of P_4 and E_2 on visfatin protein expression in Gc and Tc and concentration in media after culture of the follicular cells

Progesterone increased the protein abundance of visfatin in Gc at a dose of 10 nM and in all doses in Tc ($P < 0.05$; Fig. 5A and B). Interestingly, we noted that P_4 at a dose of 100 nM significantly decreased protein expression of visfatin in Gc ($P < 0.05$), while a dose of 1000 nM had no effect (Fig. 5A). Moreover, P_4 at doses of 10 and 100 nM upregulated the visfatin level in culture media of Gc (7.45 ± 2.49 ng/mL and 6.34 ± 2.10 ng/mL *vs* 3.45 ± 1.20 ng/mL in control, respectively) and at all used doses in Tc (4.53 ± 1.06 ng/mL, 3.83 ± 1.21 ng/mL, 3.85 ± 0.30 ng/mL *vs* 2.21 ± 0.38 ng/mL for control, respectively) ($P < 0.05$; Fig. 5C and D).

We observed that E_2 at all doses of 1–100 nM increased the abundance of visfatin protein compared to the control group in both Gc and Tc ($P < 0.05$; Fig. 5A and B). On the other hand, the concentration of visfatin in the culture media was increased by E_2 only at a dose of 100 nM in Gc (5.89 ± 1.77 ng/mL *vs* 3.45 ± 1.20 ng/mL in control) and 1 nM in Tc (4.43 ± 1.46 ng/mL *vs* 2.21 ± 0.38 ng/mL in control) ($P < 0.05$; Fig. 5C and D).

3.6. In vitro effect of prostaglandins E_2 and $F_{2\alpha}$ on visfatin protein expression in Gc and Tc and concentration in media after culture of the follicular cells

Both prostaglandins at all doses significantly reduced the visfatin protein expression in Gc, while in Tc, they have opposite effect: they increased visfatin protein abundance ($P < 0.05$; Fig. 6A and B). Concerning the concentration of visfatin in the culture media, we observed that PGE_2 at 100 and 500 ng/mL decreased the level of visfatin in Gc (1.64 ± 0.64 ng/mL and 1.26 ng/mL ± 0.89 ng/mL *vs* 3.45 ± 1.20 ng/

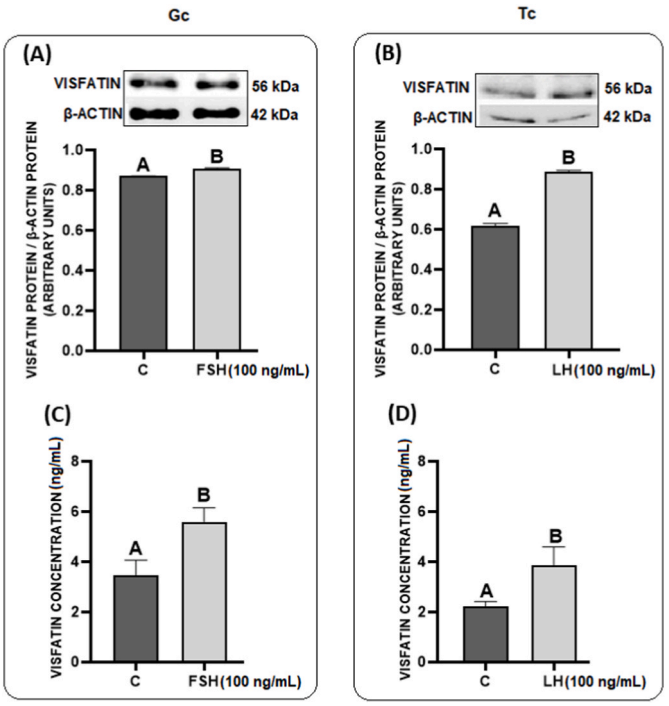

Fig. 3. *In vitro* effect of follicle-stimulating hormone (FSH) or luteinizing hormone (LH) at a dose of 100 ng/mL on visfatin protein abundance in the granulosa (Gc) (A) and theca cells (Tc) (B) and visfatin concentration in the culture media of Gc (C) and Tc (D) isolated from large ovarian follicles of mature gilts. The visfatin protein abundance was analysed by the western blot method. Results are shown as representative immunoblots and a bar graph with densitometry measurements of relative visfatin protein content normalised with β-actin protein. Visfatin concentration in culture media was evaluated using an ELISA assay. The data are presented as the mean ± SEM. Figures are representative of the six independent experiments based on six *in vitro* cultures of Gc and Tc (n = 6 per each structure). Statistical analysis was performed using Student's t-test. Different capital letters indicate significant differences at $P < 0.05$.

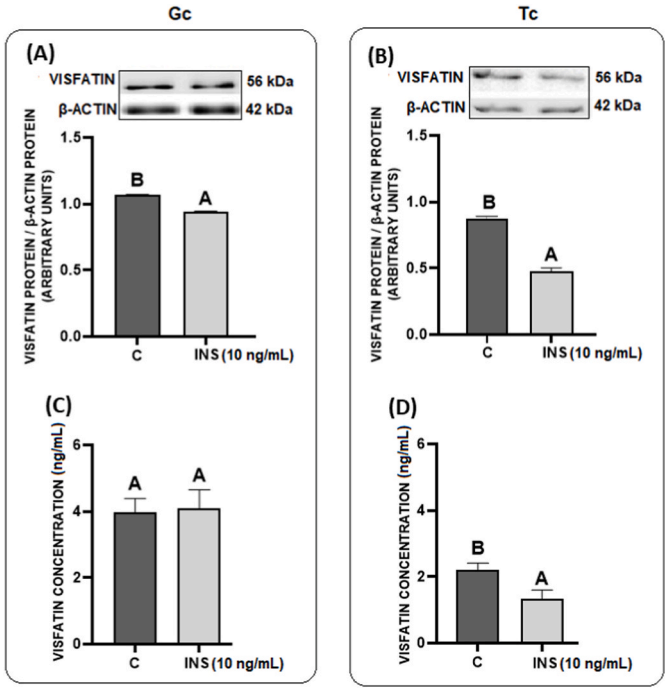

Fig. 4. *In vitro* effect of insulin (INS) at a dose of 100 ng/mL on visfatin protein abundance in the granulosa (Gc) (A) and theca cells (Tc) (B) and visfatin concentration in the culture media of Gc (C) and Tc (D) isolated from large ovarian follicles of mature gilts. The visfatin protein abundance was analysed by the western blot method. Results are shown as representative immunoblots and a bar graph with densitometry measurements of relative visfatin protein content normalised with β-actin protein. Visfatin concentration in culture media was evaluated using an ELISA assay. The data are presented as the mean ± SEM. Figures are representative of the six independent experiments based on six *in vitro* cultures of Gc and Tc (n = 6 per each structure). Statistical analysis was performed using Student's t-test. Different capital letters indicate significant differences at $P < 0.05$.

mL in the control group, respectively), while $PGF_{2\alpha}$, only at the dose of 250 ng/mL, caused a statistically significant effect by increasing the level of visfatin (5.23 ± 1.78 ng/mL *vs* 3.45 ± 1.20 ng/mL in the control group, respectively) ($P < 0.05$; Fig. 6C). In Tc, PGE_2 did not affect the concentration of visfatin, while $PGF_{2\alpha}$ increased its level at all doses used (4.16 ± 0.97 ng/mL, 6.00 ± 1.87 ng/mL, and 4.06 ± 0.36 ng/mL *vs* 2.21 ± 0.38 ng/mL in the control group, respectively) ($P < 0.05$; Fig. 6D).

4. Discussion

To the best of our knowledge, this is the first study to investigate the gene and protein expression and the immunolocalisation of visfatin in ovarian follicles of prepubertal and mature gilts. The obtained results showed that visfatin levels depended on ovarian follicle size and animal maturity. The expression of visfatin and its concentration in FF of prepubertal pigs were higher in SF than MF; however, in ovarian follicles of mature animals, we observed a difference between the transcript (LF > MF > SF) and protein (SF > MF) abundance of visfatin and no differences in concentration in FF. The presented results are partly in agreement with the study of Reverchon et al. [43], who observed that mRNA and protein expression of visfatin were higher in bovine Gc of LF than in SF. Also, Tharke et al. [27] noted increasing expression of visfatin along with follicle growth in Gc and Tc. Contrary to these reports, a decrease in visfatin expression in Tc of large hierarchic follicles (F3–F4) was observed in hens and turkeys [28,44]. The observed differences in the pattern of visfatin expression may be due to species specificity or the hormonal status of females; however, the differences observed between the level of mRNA and protein of visfatin, that we noted in our experiment, may result from post-transcriptional or post-translational modifications and the action of factors regulating the level of the produced visfatin protein depending on the needs [45]. Interestingly, we did not observe any changes in the visfatin concentration in FF between the follicles collected from mature pigs. A previous study evaluating visfatin concentration in FF of women undergoing IVF showed that its level was 9.3 ± 3.9 ng/mL, similar to the concentration recorded in plasma, suggesting that visfatin is not significantly secreted by individual follicle compartments [25]. Our previous study demonstrated that plasma concentrations of visfatin are similar to those obtained by us in FF [32]. Moreover, we noted that the concentration of visfatin in the FF and protein expression in ovarian follicles was the highest in SF of prepubertal gilts compared to mature animals, suggesting an important role of visfatin in puberty. Annie et al. [46] suggested the involvement of visfatin in the regulation of ovarian physiology before reaching sexual maturity; immunohistochemical analysis showed intense staining in Tc and oocytes, while mild it was in Gc in the ovaries of prepubertal mice. Moreover, further experiments showed that visfatin upregulated proliferation and inhibited apoptosis and steroidogenesis in the ovaries of prepubertal mice [46]. We also indicated a high expression of visfatin in the ovaries of immature pigs and localisation with a strong signal in primordial, primary, and secondary follicles, which also indicates its potential contribution to ovarian physiology from the early stages in pigs. Interestingly, it is also worth mentioning that measurements of the plasma concentration of visfatin in birds have shown that its concentration decreases with age [28,44].

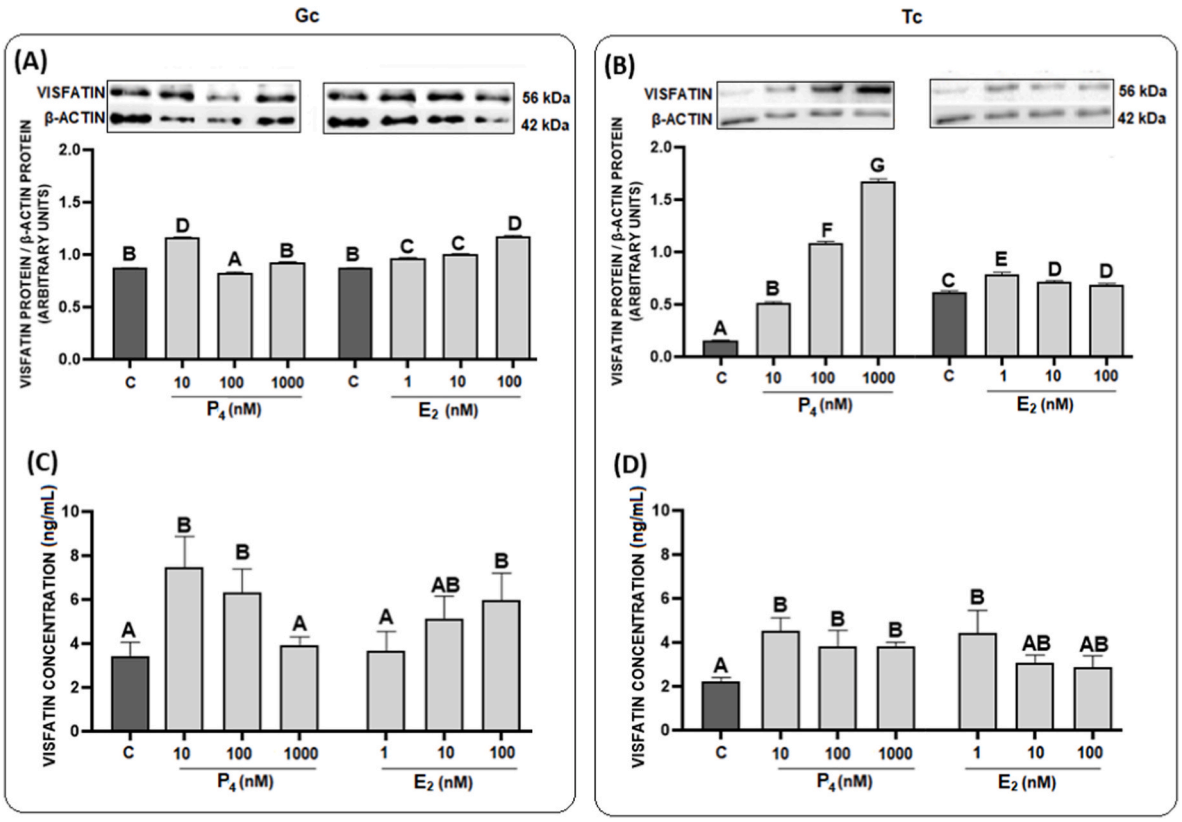

Fig. 5. *In vitro* effect of progesterone (P_4) at doses of 10, 100, and 1000 nM and oestradiol (E_2) at doses of 1, 10, and 100 nM on visfatin protein abundance in the granulosa (Gc) (A) and theca cells (Tc) (B) and visfatin concentration in the culture media of Gc (C) and Tc (D) isolated from large ovarian follicles of mature gilts. The protein abundance of visfatin was analysed by the western blot method. Results are shown as representative immunoblots and a bar graph with densitometry measurements of relative visfatin protein content normalised with β-actin protein. Visfatin concentration in culture media was evaluated using a commercially available ELISA assay. The data are presented as the mean ± SEM. Figures are representative of the six independent experiments based on six *in vitro* cultures of Gc and Tc (n = 6 per each structure). Statistical analysis was performed using one-way ANOVA, followed by Tukey's test. Different capital letters indicate significant differences at $P < 0.05$.

The results of the current study indicate a visfatin signal in individual ovarian follicle compartments, ranging from primordial to antral follicles, even in follicles undergoing the process of atresia. We observed visfatin localisation in Gc, Tc, and oocytes of antral and preovulatory follicles. Previous studies have demonstrated the visfatin signal in Gc and Tc of ovarian follicles and in small and large luteal cells of water buffalo [27]. In humans, cattle, and chickens, the visfatin signal was localised in Gc, Tc, cumulus cells, and oocytes [26,28,43]. In mice ovaries, apart from Gc, Tc, oocytes, and CL, visfatin immunoreactive cells have been found in stromal and endothelial cells throughout the oestrous cycle. Interestingly, we noted strong visfatin immunoreactivity in oocytes, which is in agreement with results obtained in humans [26], mice [46], and cattle follicles [43], suggesting an important role of visfatin in oogenesis and oocyte maturation in pigs. In farm animals, such as pigs, the process of oocyte maturation is extremely energy-consuming and requires the activation of pathways related to glucose and fat metabolism [30]. In obese mice, a deficiency of *Nampt* led to severe disruptions in the progression of oocyte maturation and meiosis but also induced metabolic dysfunction in oocytes as well as contributes to the impairment of the development potential of oocytes and early embryos [47]. Furthermore, our previous studies have shown that adipokines, such as vaspin, are directly involved in porcine oocyte maturation [48]. In light of this observation, we suggest that visfatin could be another factor that also plays a role in folliculogenesis and oocyte maturation in pigs.

Previously literature data indicate that the expression of visfatin is higher in the Gc than in Tc in human [26], cow [43], water buffalo [27], and hen follicles [28]. In prepubertal mice [46] and in turkeys [44], higher visfatin expression was observed in Tc. Our results are consistent with most of the available literature because we noted that both the gene and protein level of visfatin was higher in Gc. The increased expression of visfatin in Gc could potentially be related to its function in porcine follicles. In pigs, both Tc and Gc have active aromatase, which means that they can produce E_2 [49]. Nevertheless, Gc, sensitive to FSH, are its primary source [50]. Particularly, high expression of visfatin in Gc may indicate its participation in folliculogenesis through its influence on Gc proliferation and production of E_2. The observed differences in visfatin expression in Gc and Tc could be partly explained by the action of gonadotropins, insulin, E_2, P_4, and prostaglandins (PGE_2 and $PGF_{2\alpha}$).

Follicle-stimulating hormone, as a primary selector and activator of follicular maturation, activates the proliferation of Gc, stimulating them to secrete large amounts of E_2 and inhibin and the expression of LH receptors. In turn, Tc also respond to the action of LH through receptors on their surface and have the ability to synthesise steroid hormones, mainly P_4 and androgens [51]. The action and interaction of these two types of cells ensure the proper development of follicles. According to our findings, both gonadotropins upregulate the level of visfatin protein expression and concentration in culture media of Gc and Tc. *In vitro* studies performed on human Gc indicated that FSH does not affect the mRNA level of visfatin, although, as the authors admit, the Gc used for the study were luteinised, which could cause an incomplete FSH effect, due to the lower number of receptors for this gonadotropin on the cells' surface [25]. In our previous studies, we observed the modulatory role of LH on regulating visfatin protein levels in porcine CL [33]. Literature data indicates that both gonadotropins are involved in the regulation of ovarian expression of other adipokines, like leptin [52], adiponectin

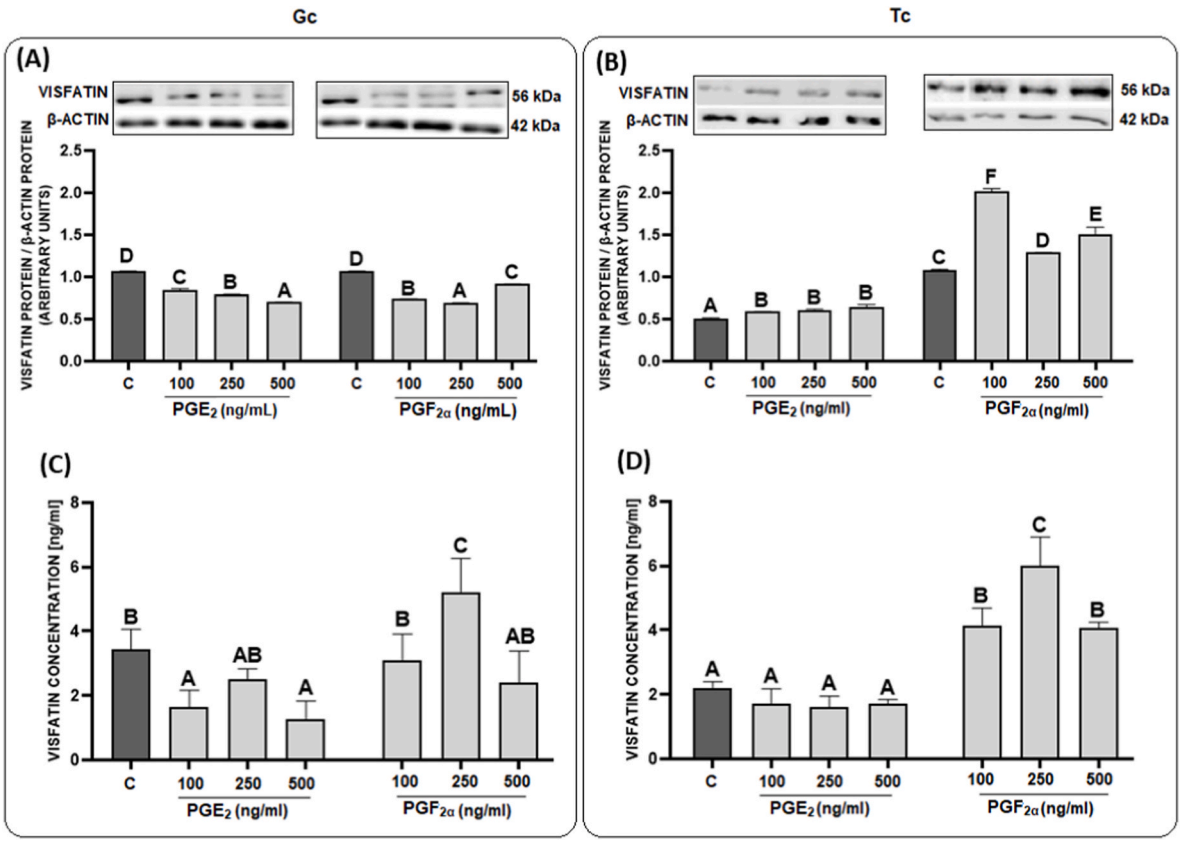

Fig. 6. *In vitro* effect of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at doses of 100, 250, and 500 ng/mL on visfatin protein abundance in the granulosa (Gc) (A) and theca cells (Tc) (B) and visfatin concentration in the culture media of Gc (C) and Tc (D) isolated from large ovarian follicles of mature gilts. The protein abundance of visfatin was analysed by the western blot method. Results are shown as representative immunoblots and a bar graph with densitometry measurements of relative visfatin protein content normalised with β-actin protein. Visfatin concentration in culture media was evaluated using a commercially available ELISA assay. The data are presented as the mean ± SEM. Figures are representative of the six independent experiments based on six *in vitro* cultures of Gc and Tc (n = 6 per each structure). Statistical analysis was performed using one-way ANOVA, followed by Tukey's tests. Different capital letters indicate significant differences at $P < 0.05$.

[53], resistin [54], or vaspin [39].

Although the role of insulin in the ovary is species-specific and differences exist between *in vivo* and *in vitro* observations, insulin signalling is very important for follicle function as it is involved in glucose transport and metabolism and the regulation of steroid synthesis [55]. In porcine Tc, insulin, acting together with LH, stimulates the mRNA expression of the steroidogenic acute regulatory (StAR) protein and 17alpha-hydroxylase/17,20-lyase (CYP17) and provides substrates for the synthesis of E_2 in Gc [56]. Moreover, expression of the insulin receptor (INSR) was detected in ovarian cells of humans, mice, rats, and cattle [55]. In our study, the level of visfatin was reduced after treatment with insulin, both in Gc and Tc. The relationship between insulin and visfatin has already been widely documented. Visfatin can exert a direct effect on insulin secretion as was demonstrated in a mouse pancreatic β-cell line, where it increased insulin secretion and induced INSR phosphorylation and the extracellular signal-regulated kinases (ERK1/2) pathway [57]. Nevertheless, available literature data described insulin mostly as a negative regulator of visfatin levels; for example, hyperinsulinemia resulted in a significant decrease in serum visfatin concentration in healthy men [58]. In another study, visfatin mRNA expression was lowered by insulin in 3T3-L1 pre-adipocytes [59]. Visfatin is often described as an insulin-mimetic hormone; however, mechanisms of downregulation of visfatin expression may be activated to avoid its synergistic effect with insulin. Thus, the negative regulation of visfatin in ovarian follicles observed in our study may be due to the relationship between these two hormones documented so far. In a previous study, we also observed that insulin tended to decrease the secretion and expression of visfatin in porcine luteal cells [33].

Oestradiol is the main hormone produced by the developing follicle, P_4 is also secreted but in smaller amounts, and together they create an environment for ovulation to occur [60]. So far, there is evidence of steroid regulation of visfatin levels in various tissues. In mice, E_2 significantly increased uterine visfatin expression, while P_4 decreased compared to control mice. On the other hand, the use of E_2 and P_4 in combination showed the highest expression of the visfatin protein both compared to the control and E_2-and P_4-treated alone [61]. Similarly, in cultures of 3T3-L1 adipocytes, the combined action of oestriol, E_2, and P_4 increases *Nampt* mRNA expression, again indicating the synergistic effect of these hormones [62]. In porcine luteal cells, we also noted that P_4 increased the level of the visfatin protein and the concentration in the culture media [33]. Similarly, visfatin has been shown to affect the secretion of steroids. It increases E_2 and P_4 secretion *in vitro* in luteal cells of water buffalo [27], but also in bovine and human Gc [26,43], while it lowers them in hen Gc [28]. This suggests that there is a local autocrine/paracrine regulation in ovarian cells among these, but it is strongly species-specific.

In Gc, PGE_2 generally reduced the level of visfatin, while in Tc, it increased its expression, but it has no effect on the secretion into the culture media. Similarly, $PGF_{2\alpha}$ reduced the level of visfatin in Gc, while it increased in Tc. Moreover, we observed differences between the effect of prostaglandins on the protein abundance of visfatin measured in cell lysates and concentration in the culture media. This observation can be partially explained by the existence of two isoforms of visfatin: intracellular and extracellular, which can respond in a different way to

treatment with factors. In addition, it has been documented that the expression of these two forms is cell- and tissue-specific [3]. Comparing the results obtained in the present study to literature data, both prostaglandins reduced protein abundance and concentration of visfatin during the oestrous cycle in porcine luteal cells [33]. In human Gc, PGE_2 caused an increase in the mRNA expression of NAMPT [25]. The role of both prostaglandins in the ovary is multidirectional, but often the opposite. In undifferentiated rat Gc, PGE_2 increased the expression of aromatase gene, but $PGF_{2\alpha}$ had no effect [63]. Moreover, in pigs, PGE_2 is considered to mediate the steroidogenic effects of LH on the development of Gc, leading to increased P_4 production in the pre-ovulatory phase of the oestrous cycle, while $PGF_{2\alpha}$ acts on the Tc, leading to an enhancement of the oestrus symptoms [64]. The different functions during folliculogenesis may lead to a different action of these prostaglandins on visfatin levels in Gc and Tc. Another potential explanation could be the differences in the expression of prostaglandin receptors on the surface of these cells. So far, there are no studies showing the expression profile of prostaglandin receptors in individual follicular compartments in pigs. Nevertheless, comparing ovine small and large luteal cells, whose precursors are Tc and Gc, respectively, the number of receptors for both prostaglandins is many times higher in large luteal cells [65]. This difference in receptor expression may also determine the various prostaglandin effects in Gc and Tc and, above all, the sensitivity of follicular cells on prostaglandin action, which could result in different visfatin abundance and secretion.

Our results clearly indicated that visfatin is locally produced by the porcine ovarian follicular cells and its level is widely regulated by action of factors creating hormonal environment in the ovary. Based on these findings we speculate that visfatin can influence on ovarian physiology including human, mouse, bovine and hen steroidogenesis as well as mouse *in vitro* oocyte maturation, suggested important regulatory role of visfatin in female reproduction [24]. Interesting, molecular mechanism of visfatin action has been described in human Gc; visfatin activates phosphorylation of ERK1/2, P38 and protein kinase B (AKT) [26]. Higer levels of visfatin were noted in plasma [65] and endometrial tissues of PCOS patients, which was positively correlated with the expression of phosphorylation of AKT and ERK1/2 signaling pathways [66]. However, to understanding the molecular mechanism of visfatin action on the porcine ovarian function, more data should be provided.

5. Conclusion

Visfatin is widely expressed in the ovarian follicles of prepubertal and mature pigs and may influence ovarian physiology from the early stages of ovarian follicle formation. Moreover, visfatin levels respond to hormonal changes during the oestrous cycle; gonadotrophins and steroids increase its amount and insulin lowers it, while the action of prostaglandins is dose- and cell type-dependent. Therefore, further research focusing on the role of visfatin in regulating follicular steroidogenesis, folliculogenesis, proliferation, and apoptosis in the porcine ovary is required.

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CRediT authorship contribution statement

Ewa Mlyczyńska: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Patrycja Kurowska:** Methodology, Formal analysis, Investigation, Data curation. **Edyta Rytelewska:** Methodology, Formal analysis, Investigation, Data curation. **Ewa Zaobina:** Methodology, Formal analysis, Investigation, Data curation. **Karolina Pich:** Formal analysis, Investigation, Data curation. **Marta Kieżun:** Formal analysis, Investigation, Data curation. **Kamil Dobrzyń:** Formal analysis, Investigation, Data curation. **Katarzyna Kisielewska:** Formal analysis, Investigation, Data curation. **Grzegorz Kopij:** Formal analysis, Investigation, Data curation. **Nina Smolińska:** Conceptualization, Writing – review & editing. **Tadeusz Kamiński:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Agnieszka Rak:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.07.040.

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