ABSTRACT

Fertility disorders are a problem that is still current among both women and farm animals. One of the observed causes of infertility is improper function of the corpus luteum (CL). The CL is a transitional gland that develops during each estrous cycle and is present during pregnancy. It is the main source of progesterone (P_4), a hormone necessary to prepare the uterine wall for embryo implantation and subsequent maintenance of its intrauterine development. Disorders that arise during angiogenesis and endocrine function of the CL are associated with luteal phase failure, problems with conception, and miscarriages. As is known, reproduction is an extremely energy-consuming process, especially in females. Therefore, the identification of biological molecules that regulate specific processes occurring in the hypothalamic-pituitary-gonadal axis and energy homeostasis are particularly needed. The group of metabolic hormones includes, among others, visfatin and phoenixin-14 (PNX-14). Both hormones regulate food intake, glucose, and insulin levels, participate in inflammatory reactions, and affect adipogenesis and fat metabolism. As indicated by the latest reports, visfatin and PNX-14 regulate reproductive functions in females, starting from the regulation of gonadotropin-releasing hormone, and gonadotropins secretion as well as influencing locally in the ovary on folliculogenesis, steroidogenesis, and oocyte maturation. It has also been shown that visfatin and PNX-14 are expressed in luteal cells, but their function in the CL has not been identified. Taking into account the data indicating the positive influence of visfatin and PNX-14 on the physiology of ovarian follicles, as well as their observed high expression in the CL of the species studied so far, the research hypothesis underlying this doctoral dissertation was formulated as follows: visfatin and PNX-14 are expressed in porcine CL during the entire luteal phase and also regulate processes necessary for the proper functioning of the CL, such as hormone secretion, angiogenesis, apoptosis and activation of signalling pathways. Therefore, the objectives of this doctoral dissertation were as follows: *AIM 1*: Determination of mRNA and protein expression and immunolocalisation of visfatin, insulin receptor (INSR), PNX-14, and GPR173 in porcine CL as well as a concentration of visfatin and PNX in plasma during the estrous cycle. *AIM 2*: Investigation of luteinizing hormone (LH), insulin, P_4, prostaglandin E_2, and $F_{2\alpha}$ (PGE_2, $PGF_{2\alpha}$) effect on the level of visfatin and PNX-14 in luteal cells. *AIM 3*: Determination of the role of visfatin and PNX-14 in CL physiology by examining the following processes: activation of signalling pathways, steroidogenesis, prostaglandin secretion and signalling, angiogenesis, and apoptosis of luteal cells.

The research model was sexually mature crossbred gilts of the breed Large White × Polish Landrace, aged 7–8 months. CLs were collected on days 2–3 (early luteal phase, presence of hemorrhagic corpora), days 10–12 (middle luteal phase, the phase when CL activity is highest), and days 14–16 of the estrous cycle (late luteal phase, CL luteolysis). The expression of visfatin/INSR, PNX-14/GPR173 mRNA, and protein and their localisation in luteal cells were investigated by RT-qPCR, Western blot, and immunohistochemistry, respectively. The levels of visfatin and PNX in porcine plasma were measured using ELISA kits. *In vitro* cultures of luteal cells were performed to investigate factors that regulate visfatin and PNX levels and the role of visfatin and PNX-14 on luteal function. Commercially available ELISA tests were also used to measure the levels of steroids, prostaglandins, and angiogenic factors in the culture medium. The RIA test was used to measure the level of P_4. Caspase 3 and 7 activity was determined by the Caspase-Glo 3/7 assay, and DNA fragmentation by the Cell Death Detection ELISA. To study the molecular mechanism of visfatin and PNX action in luteal cells, pharmacological blockers of INSR and signalling pathways: extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase A (PKA), protein kinase B (AKT) and 5'AMP-activated kinase (AMPK) and siRNA to silence GPR173 were used.

Visfatin was shown to decrease INSR phosphorylation and increase ERK1/2, AKT, and AMPK. PNX-14 stimulated the expression of its receptor GPR173, ERK1/2, and AKT, reduced PKA, and modulated AMPK and PKC phosphorylation. Both visfatin and PNX-14 had a positive effect on the endocrine function of luteal cells, stimulating the levels of STAR protein and the steroidogenic enzymes CYP11A1, HSD3B, and CYP19A1, and consequently also increasing the secretion of P_4 and E_2. It was also observed that visfatin increased LH-stimulated steroidogenesis, whereas PNX-14 decreased it. Interestingly, both hormones tested upregulated the secretion of luteotropic PGE_2, reduced the secretion of $PGF_{2\alpha}$, and modulated the levels of its receptors PTGER2 and PTGFR. Visfatin also reduced the secretion of angiogenic factors such as vascular endothelial growth factor A (VEGFA), basic fibroblast growth factor (bFGF2), angiopoietin-1 (ANG-1) and the protein expression of their receptor. PNX-14, on the other hand, had a positive effect on the process of blood vessel formation by stimulating the secretion of bFGF2 and ANG-1 and modulating the expression of VEGFR, FGFR, and TIE2 receptors. Visfatin and PNX-14 were identified as anti-apoptotic factors in porcine CL; they decreased the transcript level of caspases 8, 9 and 3, BAX factor and the activity of executive caspases 3 and 7, while increasing the level of pro-apoptotic factor BCL2. PNX-14 also reduced the level of DNA fragmentation in luteal cells. The observed effects of visfatin in CL resulted from the

action of the enzymatic form of visfatin - iNAMPT, as well as the extracellular form - eNAMPT, through interaction with INSR. In addition, ERK1/2 was involved in the effect of visfatin on the secretion of steroids, prostaglandins and VEGFA factor, as well as caspase 3 and 7 activity; AKT was involved in the secretion of P_4 and caspase 3 and 7 activity, while APMK mediated the stimulatory effect of visfatin on secretion of P_4 and E_2. PNX-14 acts in CL by activating the GPR173 receptor and protein kinase signalling pathways. Its effect primarily occurs through the ERK1/2 pathway, which is involved in processes such as steroid secretion and $PGF_{2\alpha}$ production. Additionally, the AKT pathway mediates the stimulation of bFGF2 secretion and inhibits caspase 3 and 7 activity and the AMPK pathway is involved in executor caspase activity.

In summary, although each of the studied hormones has its unique structure and mode of action, many similarities were observed in the action of visfatin and PNX-14 on luteal cells, like luteotropic action, supporting the development and functioning of CL in pigs. The conducted *in vitro* studies are an excellent beginning to a deeper understanding of the role of both hormones in the regulation of farm animal reproduction by conducting *in vivo* studies. Given the increasing problems with fertility, the knowledge gained as a result of the implementation of the doctoral thesis may contribute to the development of better methods and therapies for the treatment of infertility caused by CL failure.