The Noradrenergic Innervation and Steroidogenic Activity of Porcine Cystic Ovaries

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Short title: In Cystic Ovary Increases the Noradrenergic Innervation

Summary

The aim of the present study was to investigate the distribution and density of noradrenergic nerve fibres (NNFs), content of catecholamines (CATs) and steroids in the cystic ovaries of gilts receiving DXM from middle luteal phase. *Cystic status* of ovaries was induced by *i.m.* DXM injections on days 7-21 of the estrous cycle. During the same time, gilts in the control group received saline. The ovaries were collected on predicted day 11 of the second studied estrous cycle. The cystic ovaries were supplied by more numerous NNFs than the control gonads. Moreover after DXM injections, the content of CATs and progesterone and androstendione (A_4) in the cystic wall were elevated, while the levels of A_4, testosterone and estradiol-17β in the cystic fluid were lowered. Our results show that in the porcine cystic ovaries, induced by DXM injections from middle phase of estrous cycle, increased the density of NNFs and level of CATs, and that it was accompanied by changes in the content of steroids. Moreover, this study is a

1 further confirmation that the morphological and functional changes of cystic
2 ovaries are partly dependent on phase of the estrous cycle in which the induction
3 of the ovarian cysts was initiated.
4 **Key words:** Ovarian cysts, Noradrenergic innervation, Steroids, Pigs
5

6 **Introduction**

7 Cystic ovarian disease (COD) is one of the most common reproductive disorders in
8 female of farm animals, affecting 6 to 19% of dairy cattle (Kesler and Garverick 1982) and
9 2.4-40% of sows (for a review see Cech and Dolezel 2007; Szulańczyk-Mencel *et al.* 2010)
10 which may result in temporary or permanent infertility. Despite extensive investigations,
11 the etiopathogenesis of COD is not completely understood. It is assumed that cystic
12 condition is mainly caused by disturbances in the function of hypothalamic-pituitary-
13 ovarian (HPO) axis leading to impairment of the synthesis, release and storage of various
14 hormones of this functional unit (Silva *et al.* 2002; Peter 2004). One of the reasons of
15 changes in the HPO axis activity and ovarian cysts formation may be stressors activating of
16 the hypothalamic-pituitary-adrenocortical (HPA) axis. An increase in corticotropin-
17 releasing hormone secretion suppresses the activity of the hypothalamic GnRH pulse
18 generator, leading to a decrease in release of GnRH and LH (Li *et al.* 2005). Moreover,
19 elevated level of adrenocorticotropic hormone (ACTH) reduces pulsatory LH release from
20 pituitary, stimulates produce and release of glucocorticoids from the adrenal cortex that
21 inhibit the release of GnRH and/or LH and ovarian activity (Turner *et al.* 2002; Madej *et*
22 *al.* 2005). The experimental evidence also strengthen the role of sympathetic innervation in
23 the etiopathogenesis and/or persistence of ovarian cystic condition. Numerous studies,
24 performed under physiological conditions, showed that intraovarian sympathetic nerve
25 fibres localized around follicles, corpora lutea (CLs), blood vessels and interstitial gland as

well as within ground plexus, participate in regulation of steroidogenesis, follicular development and ovulation (Majewski 1997; Morán *et al.* 2003; Jana *et al.* 2007). The effect of stressors in the pathogenesis of polycystic ovary is mediated by sympathetic discharge originating at the paraventcicular nucleus (Fiedler *et al.* 2006). It was revealed that in the cystic ovaries of humans (Nakamura 1990; Heider *et al.* 2001), rats (Barria *et al.* 1993; Lara *et al.* 2000), gilts (Jana *et al.* 2005) and cows (Paredes *et al.* 2011) the sympathetic nerve activity is increased. Moreover, the important role of the nerve fibres in the etiopathogenesis of the ovarian cysts provided the fact that the resection of the ovarian medulla fragment contained the part of nerves supplying the ovary (Nakamura 1990) or laparascopic laser cauterization of this place (Balen and Jacobs 1994) in women with polycystic ovary syndrome (PCOS), in which the hormonal therapy was ineffective, induced ovulation. Similar effect was also observed in rats with PCOS after unilateral sectioning of the superior ovarian nerves (Morales-Ledesma *et al.* 2010). Furthermore, the hyperactivation of ovarian sympathetic nerves seen in estradiol valerate (EV)-induced polycystic ovaries in rats is related to an overproduction of nerve growth factor (NGF) and its low affinity receptor in the gonads (Lara *et al.* 2000; Manni *et al.* 2005).

Our earlier study showed that in the ovaries of gilts, in which COD was induced by *i.m.* dexamethasone phosphate disodium salt (DXM) injections from 16 of the first studied estrous cycle to day 9 of the second studied cycle, the density of noradrenergic nerve fibres and the content of catecholamines increased, which was accompanied by distinct changes in the steroidogenic activity of the gonads (Jana *et al.* 2005). It was presented that the morphological and functional changes of cystic ovaries are dependent on both the kind of hormones inducing this pathological state, and also the phase of the estrous cycle, in which administration of this hormone was started (Frautschy and Liptrap 1988; Gee *et al.* 1991). Also, the content of noradrenaline (NA) in the porcine ovary depends on the phase of the

estrous cycle and the period of the pregnancy (Łakomy 1987; Łakomy 1988). Moreover, the exogenous sex steroids influence the NA amount in the ovaries of immature gilts (Łakomy 1987).

The findings mentioned above and literature data allow to hypothesize that changes in the noradrenergic innervation pattern as well as steroidogenic activity of cystic ovaries, induced by DXM treatment from middle the luteal phase of the estrous cycle, will be most probably different from those, found after induction of cyst formation from the follicular phase (Jana *et al.* 2005). Therefore, the aim of the present study was to determine the changes in the noradrenergic innervation pattern and in the content of catecholamines and steroid hormones in the porcine cystic ovaries induced by DXM treatment from middle luteal phase of the estrous cycle.

Methods

Animals and experimental procedure

We followed the principles of animal care (NIH publication No. 86-23, revised in 1985) as well as the specific national law on animal protection. The experiment was carried out on 12 crossbred gilts (Large White x Landrace), aged 7-8 months and weighing 90-100 kg, with two controlled subsequent estrous cycles. Behavioural estrus was detected by using the boar-tester. The animals were then individually housed in stalls, under conditions of natural light and room temperature. They were fed with a commercial grain mixture and tap water *ad libitum*. The gilts were randomly assigned to one of two groups: control, receiving saline (CON, n=6) and DXM-treated (DXM, n=6).

In the DXM group, the cystic status of ovaries was induced according to the protocol described earlier by Gee et al. (1991) with following modifications: the gilts in the present study received DXM (3.3 μg/kg of body weight, in total volume of 6 ml; Dexasone®, Norbrook Laboratory, Newry, UK) every 12 hours (h), starting on day 7 (day

1 of the study) to day 21 of the first studied estrous cycle (*i.e.* during 15 consecutive days). During the same period of time, animals of the CON group were injected with 6 ml of saline. The gilts were then slaughtered by electrical shock (ENZ 300 Metalowiec, Bydgoszcz, Poland) and exsanguinated on predicted day 11 of the second studied cycle (*i.e*. on day 26 of the experiment). The ovaries were immediately dissected out and their weight, volume, measurements as well as the numbers of follicular structures and CLs were estimated. The follicles were divided into two size classes: small (1-3 mm in diameter) and medium (4-6 mm in diameter). Follicular structures exceeding 1.0 cm in diameter were classified as cysts (Nalbandov 1952). The morphological examination of ovaries was described earlier (Kozłowska *et al.* 2009), and it showed that DXM injections on days 7-21 of the estrous cycle caused, comparing to the control group, formation of partly luteinized follicular cysts, a decrease in the number of small follicles and the lack of medium follicles and CLs. Afterwards, blocks of ovarian tissue were processed for further immunochemical studies as follows: they were fixed by immersion in Zamboni's fixative for 30 min, washed in 0.1 M phosphate buffer, stored in 18% sucrose for several days and then frozen (-80°C) and stored until sectioning. Follicular fluid was collected and stored at -21°C to determine concentrations of progesterone (P_4), androstendione (A_4), testosterone (T), estrone (E_1) and estradiol-17β (E_2). Next, the cystic and follicular wall as well as CL samples were shock-frozen in liquid nitrogen and then stored at -80°C in order to analyse the contents of the steroid hormones. To estimate the content of catecholamines in follicular and cystic fluid, the samples were collected immediately after excision of the ovary and estimation of above-mentioned parameters (*ca.* 1 min) was carried in tubes containing 1N HCl to prevent oxidation and they were later kept on ice. Next, pieces of follicular or cystic wall were dissected out, weighed and, in order to estimate the content of catecholamines, were placed in tubes (4°C) containing aqueous solution of 0.4% perchloric

acid, 0.1% EDTA, 0.1% sodium metabisulfite and 0.01% ethanol, prepared *ex tempore* to prevent oxidation of amines and to precipitate proteins. Fluid and tissue samples were stored at -80˚C until assayed by high-performance liquid chromatography (HPLC).

Double-labelling immunofluorescence

Ten-µm-thick cryostat (Reichert-Jung, Nuβloch, Germany) sections of the ovaries were subjected to routine double-immunofluorescence staining technique to visualise the distribution of noradrenergic population of nerve fibres containing dopamine-β-hydroxylase (DβH) and/or neuropeptide tyrosine Y (NPY). The sections were air-dried at room temperature (RT) for 45 min and rinsed (3 x 15 min) with PBS (phosphate buffered saline, pH 7.4). Next, sections were blocked with a blocking mixture containing 1% Triton X100, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN_3 and 10% normal goat serum in 0.01M phosphate-buffered saline for 1h at RT to reduce non-specific background staining. After a wash, sections were incubated overnight in the humid chamber at RT with a mixture of primary antisera raised in different species and recognizing DβH (mouse monoclonal, working dilution 1:400; MAB308, Chemicon, USA) and NPY (rabbit polyclonal, working dilution 1:10 000; NA1233-0100, Affinity, UK). Primary antisera were then visualized by a mixture of FITC-conjugated donkey anti-mouse IgG-specific (working dilution 1:400; 715-095-150, Jackson Immunoresearch, USA) and CY3-conjugated donkey anti-rabbit IgG-specific antisera (016-160-084, Jackson Immunoresearch, USA); after the incubation, sections were washed again and then covers were lipped with carbonate-buffered glycerol (pH 8.6). The specificity of primary antisera was tested as follows: sections were incubated with antibody that had been preabsorbed with synthetic antigen (10 µg of antigen per ml diluted antiserum); the primary antibody was omitted from the incubation; either normal rabbit or rat serum was substituted for the primary antibody.

Double-immunolabelled nerve fibres were analyzed under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. The distribution and density of intraovarian DβH- and NPY-immunoreactive (IR) nerve fibres were estimated in the surrounding zone of follicles, cysts, CLs, blood vessels, interstitial glands and within the autonomic ground plexus. In order to evaluate differences in the pattern of perifollicular nerve fibres, follicles were, depending on their stage of development, microscopically classified according to Wulff *et al.* (2002) and Barboni *et al.* (2004) into following classes: *primordial* – without granulosa cells, *primary* – surrounded by a single layer of cuboidal granulosa cells, *secondary* – with two or more granulosa cell layers without antral cavity, and *tertiary* – with antrum. Additionally, the tertiary follicles were divided into two size classes: up to 3 (small) and 4-6 (medium) mm in diameter. As mentioned earlier, follicular structures exceeding 1.0 cm in diameter were classified as cysts (Nalbandov 1952). The DβH- and NPY-IR nerve fibres supplying the particular ovarian structures were counted on nine randomly chosen ovarian sections from each gilt, and then the mean (±SEM) numbers of fibres for each group (from fifty-four sections) were calculated.

Catecholamine analyses

The contents of NA, dopamine (DA) and adrenaline (AA) in the follicular and cystic fluid and wall as well as in the CLs were estimated according to Skipor et al. (2004). Briefly, 25 µl of the prepared tissue supernatant and fluids were injected into a C18 reverse-phase column (Hypersil BDS, Thermo Electron Corporation, USA) of HPLC equipped with electrochemical detector TOA ICA-3000 SYSTEM (Tokyo, Japan). The mobile phase consisted of citrate-sodium phosphate buffer (pH 4.2) containing 0.3 mM octane sulfonate, 0.01 M EDTA, 1.5% methanol (Merck, Poland) and 1.0% acetonitrile (Merck, Poland) The flow rate was set at 0.8 ml/min. Separated catecholamines were then

detected by a glassy carbon electrode, at the potential of 850 mV vs. Ag/AgCl reference electrode. Recovery of the DL-isoproterenol hydrochloride (Sigma, Poland), as an internal standard, was 75%. Final data was corrected for procedural losses.

Hormone analyses

Extraction of P_4, A_4, T, E_1 and E_2 from the follicular and cystic wall and the CLs was performed by means of the method described by Tsang *et al.* (1990). The contents of the studied hormones in the follicular and cystic fluid as well as extracts prepared from the follicular and cystic wall, and also CLs were estimated by the radioimmunoassay procedures and published for P_4 and E_2 by Hotchkiss *et al.* (1971), for A_4 and E_1 by Dziadkowiec *et al.* (1982a) and for T by Kotwica and Williams (1982). Characteristics of antibodies (obtained from the Institute of Animal Physiology, University of Warmia and Mazury in Olsztyn, Poland) have previously been provided for P_4 by Dziadkowiec *et al.* (1982b) and for the remaining steroids by Szafranska *et al.* (2002). The sensitivity of the assay for P_4, A_4, T, E_1 and E_2 was 15, 5, 2.5, 2.5 and 5 pg/ml, respectively. Intra- and interassay coefficients of variation for P_4, A_4, T, E_1 and E_2 were 4.5 and 10.6%, 6.0 and 13.4%, 7.6 and 11.9%, 6.5 and 11.8%, 7.0 and 13.8%, respectively. Standards of P_4, A_4, T, E_1 and E_2 derived from Sigma Chemicon Co, while the [^{3}H]-labelled P_4, A_4, T, E_1 and E_2 originated from Amersham plc UK.

Statistical analysis

To calculate the statistical significance in the mean (±SEM) contents of catecholamines and steroid hormones in follicular and cystic fluid and wall as well as the numbers of DβH- and NPY-IR nerve fibres supplying the particular ovarian structures between the CON and DXM groups, one-way analysis of variance (ANOVA) followed by the Bonferroni test was chosen (InStat GraphPad, San Diego, CA). Differences with probability of $P<0.05$ were considered significant.

Results

Distribution and density of the noradrenergic nerve fibres in the control and cystic ovaries

In the ovaries of DXM-treated gilts the number of DβH and NPY- IR nerve fibres, often forming fascicle, around cysts (Fig. 1B) was higher ($P<0.001$) than in the vicinity of medium follicles of the CON group (Fig. 1A). Furthermore, in the DXM group an increase in the number of nerve terminals mentioned above was found enclosing the interstitial gland ($P<0.001$, Fig. 1H) and near cortical ($P<0.001$, Fig. 1D – arteries; $P<0.001$, Fig. 1F – veins) and medullar ($P<0.05$, Fig. 2D – arteries; $P<0.001$, Fig. 2F – veins) blood vessels. After DXM administration populations of the DβH- and NPY- IR nerve fibres, often forming big fascicles, were greater ($P<0.001$) within medullar part of ground plexus (Fig. 2B) than in the CON group (Fig. 2A). In turn, application of DXM did not significantly change the populations of DβH and NPY-IR nerve fibres within cortical part of ground plexus and around secondary and small tertiary follicles. Data concerning the distribution and density of noradrenergic nerve fibres are presented in Table 1.

Content of catecholamines in the cystic and follicular fluid

The NA concentration in the cystic fluid was lower ($P<0.001$) as compared to medium follicles of the CON group. The contents of DA and AA in the cystic fluid were below the threshold of detection. After DXM injections the level of NA in the fluid from small follicles was greater ($P<0.001$) than in the CON group. The concentration of DA in fluid from small follicles was similar in the CON and DXM groups. Moreover, the content of AA in the fluid from small follicles in both the CON and DXM groups as well as the levels of DA and AA in the fluid from medium follicles of the CON group were below the threshold of detection (Tab. 2).

Content of catecholamines in the cystic and follicular wall and CLs

The concentrations of DA, NA and AA in the cystic wall were higher ($P<0.001$) than in the wall of medium follicles of the CON group. The greater ($P<0.001$) content of NA was revealed also in the wall of small follicles of the DXM group. The amounts of DA and AA in the wall of small follicles were similar in both the CON and DXM groups. In turn, the content of NA in CLs in the CON group was below the threshold of detection (Tab. 2).

Content of steroid hormones in the cystic and follicular fluid

After DXM administration, the content of P_4 in the cystic fluid was higher ($P<0.001$) than in medium follicles of the CON group. The levels of A_4 ($P<0.001$), T ($P<0.05$) and E_2 ($P<0.01$) in the cystic fluid as well as P_4 ($P<0.001$) and A_4 ($P<0.01$) in the fluid from small follicles of the gilts receiving DXM, were lower than in the CON group. In turn, the contents of E_1 in the cystic fluid and T, E_1 and E_2 in the fluid from small follicles varied insignificantly between the examined groups (Tab. 3).

Content of steroid hormones in the cystic and follicular wall

DXM injections caused an increase ($P<0.001$) in the concentrations of P_4 and A_4 in the wall of cysts, and P_4 in the wall of small follicles. Comparing to the CON group, the concentration of T declined ($P<0.01$) in the cystic wall. No significant differences were noted in the E_1 and E_2 contents in the cystic wall and in the A_4, T, E_1 and E_2 concentrations in the wall of small follicles between both the CON and DXM groups (Tab. 3).

Discussion

The present study shows that in the porcine cystic ovaries, induced by DXM administration on days 7-21 of the estrous cycle, the number of noradrenergic nerve fibres was increased and accompanied by alterations in the content of catecholamines and steroid hormones in ovarian structures.

We have found that the number of DβH- and/or NPY-IR nerve fibres increased in the cystic-changed ovaries. This is in agreement with previous studies performed on the cystic ovaries of women (Nakamura 1990; Heider *et al.* 2001) and gilts receiving DXM from follicular phase of the estrous cycle (Jana *et al.* 2005). It is difficult to indicate the mechanism(s) underlying the increase of the number of noradrenergic nerve fibres in the cystic ovaries found in our study. We suggest that this situation may be a consequence of a local ovarian mechanism that results in neuronal plasticity. Thus, the higher density of nerve fibres may be referred to the elevated production of NGF, which plays a crucial role in development, survival and differentiation of sympathetic and sensory neurons and in the regulation of axon and dendrite growth (Huang and Reichardt 2001). This assumption is based on the findings that the contents of NGF and its receptor (p75) were augmented in rat EV-induced polycystic ovaries (Lara *et al.* 2000) and porcine cystic ovaries evoked by DXM (Jana et al., unpub. data). In addition, in rats this was accompanied by an increase in both the level of tyrosine hydroxylase (TH) mRNA expression in the celiac ganglion and the number of ovarian noradrenergic nerve endings (Lara et al. 2000). Thus, changes in the chemical coding of neurons may resulted from direct effects of steroid hormones on these cells, as it has been shown that estrogen receptors (ERs) are expressed by ovarian neurons of the caudal mesenteric ganglion (CaMG; Koszykowska *et al.* 2011a) and sympathetic chain ganglia (SChG; Koszykowska *et al.* 2011b) in adult gilts. In turn, androgen or progesterone receptors were localized in the neurons in the dorsal root ganglia (DRG) of male (Keast and Gleeson 1998) and female (Chan *et al.* 2000) rats, respectively. Furthermore, the long-term E_2 treatment lead to the decrease in the number of both noradrenergic and ERs expressing ovarian neurons in the porcine CaMG and SChG (Koszykowska *et al.* 2011 a, b). It is worth adding that in the gilts, from which the cystic ovaries were collected for the present study, the peripheral blood levels of sex steroids

increased (Jana et al., unpub. data). It is possible that the increase in the numbers of DβH- and/or NPY-IR nerve fibres studied the cystic ovaries, can be also a consequence of the DXM administration. This assumption is supported by studies showing that in rats DXM lead to the increase in the density of TH-IR nerve fibres in the uterus (Bianchimano *et al.* 2007) and the expression of NPY mRNA and protein in the isolated islets of Langerhans (Jamal *et al.* 1991) as well as that the part of neurons in the lumbar DRG possesses nuclear glucocorticoid receptors (DeLeón *et al.* 1994). Changes in the pattern of noradrenergic innervation of cystic ovaries, revealed in the present study, referred only to the density of DβH- and/or NPY-IR nerve fibres surrounding particular ovarian structures, but not their distribution. Thus, the increase in the number of noradrenergic nerve fibres was found especially around the cysts, interstitial glands, blood vessels and in the area of ground plexus in the medulla. These observations correspond to our earlier study (Jana *et al.* 2005), in which cystic changes in porcine ovaries were induced by DXM injections starting at follicular phase of the cycle. However, in the present study the number of noradrenergic nerve fibres around cysts was significantly lower, whereas it was significantly higher in the vicinity of the interstitial gland and cortical veins when compared with the mentioned study (Jana *et al.* 2005).

An augmentation in the density of the DβH- and/or NPY-IR nerve fibres found in the present study in the vicinity of the cysts was accompanied by an increase in the level of NA and AA in the wall of these structures, which was earlier revealed after DXM administration at follicular phase of the cycle; however, they significantly higher than those observed in our previous study (Jana *et al.* 2005).

Interestingly, the whole concentration of NA in the cystic fluid was significantly lower, when as compared to earlier findings, while the increase in the NA content in the wall of small tertiary follicles was in line with our previous data (Jana *et al.* 2005). Moreover, an

increase in the level of DA (in the cyst wall) as well as of NA (in the fluid of small tertiary follicles) was found in the present study when compared to results obtained previously (Jana *et al.* 2005). A rise in the NA concentration was also reported in rat cystic ovaries (Barria *et al.* 1993; Lara *et al.* 1993; Rosa-e-Silva *et al.* 2003) and in the cystic fluid in cows (Paredes *et al.* 2011). We suggest that the increase in the content of catecholamines found in the present study could be a consequence of intensified synthesis and release of these substances. A high NA content in the cysts may also result from an enhanced release of catecholamine from numerous noradrenergic fibres supplying the cysts, which was suggested earlier by Lara *et al.* (1993). Moreover, such high concentration of NA in the wall and/or fluid of cysts and follicles may resulted from the ability of steroidogenic cells to take up NA, store it, and upon depolarization, release it, which was presented in ovarian granulosa cells (Greiner *et al.* 2008; Saller *et al.* 2012). An additional source of NA in the cysts and follicles may be adrenal medulla (for a review see Stener-Victorin *et al.* 2005). In turn, the higher DA content in the cystic wall may be caused by the increased inflow of this catecholamine into the ovary with blood stream, which may be judged from the study of Rothschild et al. (1984), who observed an increase in the plasma level of free DA in humans receiving DXM.

The present study also revealed changes in the concentration of steroid hormones in the ovarian structures of DXM-treated gilts. We noticed a higher content of P_4 in the cysts (wall, fluid), which is in line with our earlier study, in which cystic changes in gilt ovaries were induced by DXM administrated at follicular phase of the cycle (Jana *et al.* 2005). The content of P_4 increased also in the theca interna layer of the follicles in polycystic ovaries of women (Gilling-Smith *et al.* 1994). The elevated content of P_4 in the wall of cysts and small follicles, found in our study, was accompanied by an increase in the contents of catecholamines and levels of $P450_{scc}$ and/or 3β-HSD protein expression, which was earlier

reported by Kozłowska *et al.* (2009). The interrelationship can be explained by stimulatory effect of NA on expression and activity of $P450_{scc}$ and 3β-HSD (Miszkiel and Kotwica 2001; Kotwica *et al.* 2002). It has also been reported that DA and NA augmented P_4 production and secretion from bovine (Kotwica *et al.* 1996) and porcine (Wiesak *et al.* 1991) luteal cells. It is also possible that the increase in the P_4 content in the cysts and follicles, observed in the present study, could be the result of NPY effect, as reported previously by Baranowska *et al.* (1999). We have also found that the contents of A_4 in cystic fluid, T in cystic wall and fluid, and A_4 in the fluid from small tertiary follicles decreased after DXM injections from the middle luteal phase of the cycle. Similar phenomenon was reported earlier in the porcine cystic ovaries formed by DXM injections starting at follicular phase of the cycle (Jana *et al.* 2005). Moreover, a similar decrease in the concentration of T was previously observed in the porcine cysts (Scharfe *et al.* 1994). In turn, Paredes *et al.* (2011) revealed an increase in basal and isoproteneol (β-receptor agonist)- and hCG-stimulated release of T from bovine cystic wall. The present study showed also that the content of A_4 in the cystic wall was significantly higher, while it was markedly lower after induction of cystic changes at follicular phase of the cycle (Jana *et al.* 2005) as compared to the control groups. It is possible that this increase in the A_4 content in cystic wall (present study), could be caused by higher conversion rate of P_4 to A_4 (Gilling-Smith *et al.* 1994). Moreover, Jakimiuk *et al.* (2001) showed a rise in the expression of 17α-hydroxylase/C (17-20) lyase mRNA in the theca interna layer of follicles (3-7 mm in diameter) from polycystic ovaries of women. We suppose that the increase in the content of A_4 found by us in the ovarian structures of DXM-treated gilts, may also be caused by NA, as it was presented in rat cystic ovaries (Barria *et al.* 1993). In the present study a reduction in the E_2 content in the cystic fluid was demonstrated, similarly as after formation of cystic condition by DXM administrated at follicular phase

of the cycle (Jana *et al.* 2005). However, we were not able to find significant changes in the contents of E_2 in the cystic wall and E_1 in the cystic wall and fluid, while the amounts of these steroid were significantly lower in the treated with DXM gilts from follicular phase of the cycle (Jana *et al.* 2005).

In our previous (Kozłowska *et al.* 2009) and in the present studies we have revealed that the morphology (macroscopic changes, the noradrenergic innervation pattern) and steroidogenic activity of the porcine cystic ovaries induced by DXM treatment at middle luteal phase are in part different from those found after DXM administration started at follicular phase of the cycle (Jana *et al.* 2005). Most probably, these differences may result from other phase of the estrous cycle (different stage of follicular development and hormonal status), in which the injections of DXM started. This supposition is supported by previous studies indicating that application of ACTH or glucocorticoids in pigs before or after the time of ovulatory follicle selection resulted in differential macroscopic and steroid hormone changes of cystic ovaries (Gee *et al.* 1991). Thus is additionally supported by findings showing that the contents of catecholamines changed distinctly in porcine ovaries in the course of the estrous cycle (Lakomy 1987) as well as that the density of noradrenergic innervation of gilt follicles dependent on their developmental stage (Majewski 1997). Considering the partly different morphological and functional changes found between the cystic ovaries, induced by DXM from the middle luteal (present study, Kozłowska et al. 2009) and follicular (Jana *et al.* 2005) phase of the estrous cycle, we speculate that differential cystic changes occurring in natural conditions may result, at least partly, from the phase of the cycle, in which the activation of the HPA axis and sympathetic innervation took place. However, this assumption must be further elucidated in detail.

In conclusion, data of the present study clearly show that in the porcine cystic ovaries, formed after DXM injections at middle luteal phase of the estrous cycle, both density of noradrenergic nerve fibres as well as the concentration of catecholamines have increased. It was simultaneously accompanied by changes in the content of steroid hormones. Our study is a further confirmation that morphological and functional changes of cystic ovaries are partly dependent on phase of the estrous cycle in which the induction of the ovarian cysts was initiated.

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Author Contributions

All authors contributed equally to the intellectual content of this paper.

Conflict of interest

None.

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Table 1. Mean (±SEM) number of DβH- and/or NPY-IR nerve fibres in the ovaries of gilts from the control (CON, n=6) and DXM-treated (DXM, n=6) groups

Ovarian tissue	DβH		NPY	
	Group			
	CON	DXM	CON	DXM
	Cortex			
Ground plexus	13.3±0.9	12.8±1.1	11.9±1.31	11.6±1.2
Follicles:				
primordial	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
primary	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
secondary	1.1±0.1	1.05±0.01	0.0±0.0	0.5±0.01
tertiary with a diameter of:				
- up to 3 mm	2.5±0.5	3.1±0.4	2.9±0.2	3.0±0.1
- 4-6 mm	2.8±0.4	l.s.	2.7±0.1	l.s.
Cysts	l.s.	15.1±1.4B***	l.s.	13.6±1.1B***
Corpora lutea	0.0±0.0	l.s.	0.0±0.0	l.s.
Arteries	2.8±0.3	12.5±0.3***	2.5±0.3	11.6±0.15***
Veins	1.8±0.1	11.1±0.1***	1.9±0.2	10.2±0.21***
Interstitial gland	15.5±0.4	22.6±0.6***	13.3±0.1	21.2±0.12***
	Medulla			
Ground plexus	4.1±0.3	96.8±13.1B***	3.6±0.4	91.1±5.1B***
Arteries	3.9±1.1	7.9±1.2*	3.6±0.1	7.7±0.5*
Veins	1.01±0.01	4.8±0.1***	1.02±0.05	4.3±0.2***

*P<0.05; ***P<0.001 - indicate significant differences between the examined groups for the same structures and between follicles measuring 4-6 mm in diameter in the CON and cysts in the DXM group; l.s. – lack of structures, B – bunches of fibres.

Table 2. Mean (±SEM) concentrations of catecholamines in the follicular fluid [ng/ml] and in follicular wall and corpora lutea [ng/g of tissue] of gilts from the control group (CON, n=6) as well as in the follicular and cystic fluid and wall of gilts from the DXM-treated group (DXM, n=6)

Group	Structure:	**Dopamine**		**Noradrenaline**		**Adrenaline**	
		fluid	*wall*	*fluid*	*wall*	*fluid*	*wall*
CON	*Follicle size - 1-3 mm*	0.04±0.01	3.3±2.3	0.2±0.02	3.2±0.1	b.t.d	0.4±0.4
DXM		0.02±0.004	2.4±1.02	1.5±0.1***	21.1±5.6***	b.t.d	1.3±0.2
CON	*Follicle size - 4-6 mm*	b.t.d	4.3±1.5	3.9±0.1	1.2±0.2	b.t.d	0.4±0.1
DXM		l.s.	l.s.	l.s.	l.s.	l.s.	l.s.
CON	*Corpus luteum*	-	2.4±1.9	-	b.t.d	-	0.5±0.1
DXM		l.s.	l.s.	l.s.	l.s.	l.s.	l.s.
CON	*Cysts*	l.s.	l.s.	l.s.	l.s.	l.s	l.s.
DXM		b.t.d	16.7±2.5***	0.004±0.001***	129.2±3.2***	b.t.d	6.2±0.6***

***P<0.001 - indicates significant differences between the examined groups for the same structures and between follicles measuring 4-6 mm in diameter in the CON and cysts in the DXM group; l.s. – lack of structure; b.t.d. – value below the threshold of detection.

Table 3. Mean (±SEM) concentrations of steroid hormones in the follicular fluid [ng/ml] of gilts from the control group (CON, n=6) and in follicular wall and corpora lutea [ng/g of tissue] as well as in the follicular and cystic fluid and wall of gilts from the DXM-treated group (DXM, n=6)

Group	Structure:	**P_4**		**A_4**		**T**		**E_1**		**E_2**	
		fluid	*wall*	*fluid*	*wall*	*fluid*	*wall*	*fluid*	*wall*	*fluid*	*wall*
CON	*Follicle size - 1-3 mm*	292±44.1	1050±158.2	21.2±1.7	6.3±0.4	8.0±2.8	18.9±8.1	17.4±5.1	2.2±0.1	2.9±0.3	6.1±0.8
DXM		57.8±89.3***	45821±16214***	6.7±0.5**	26.6±10.5	3.3±0.1	1.5±0.2	18.4±14.7	3.1±0.2	7.6±2.8	0.9±0.02
CON	*Follicle size - 4-6 mm*	258.1±17.5	1553±144.3	24.3±4.2	3.3±0.4	9.4±1.6	61.6±21.1	5.3±1.7	1.3±0.3	10.5±1.5	9.8±0.4
DXM		l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.
CON	*Corpus luteum*	-	6521±1664	-	1.2±0.2	-	15.8±6.0	-	1.1±0.1	-	5.3±0.6
DXM		l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.
CON	*Cysts*	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.	l.s.
DXM		347.2±16.8***	2472±58.2***	5.8±1.3***	278.5±1.5***	2.3±0.1*	1.43±0.1**	2.3±0.01	7.3±0.1	2.8±1.7**	7.5±1.5

*P<0.05; **P<0.01; ***P<0.001 - indicate significant differences between the examined groups for the same structures and between follicles measuring 4-6 mm in diameter in the CON and cysts in the DXM group; l.s. – lack of structure.

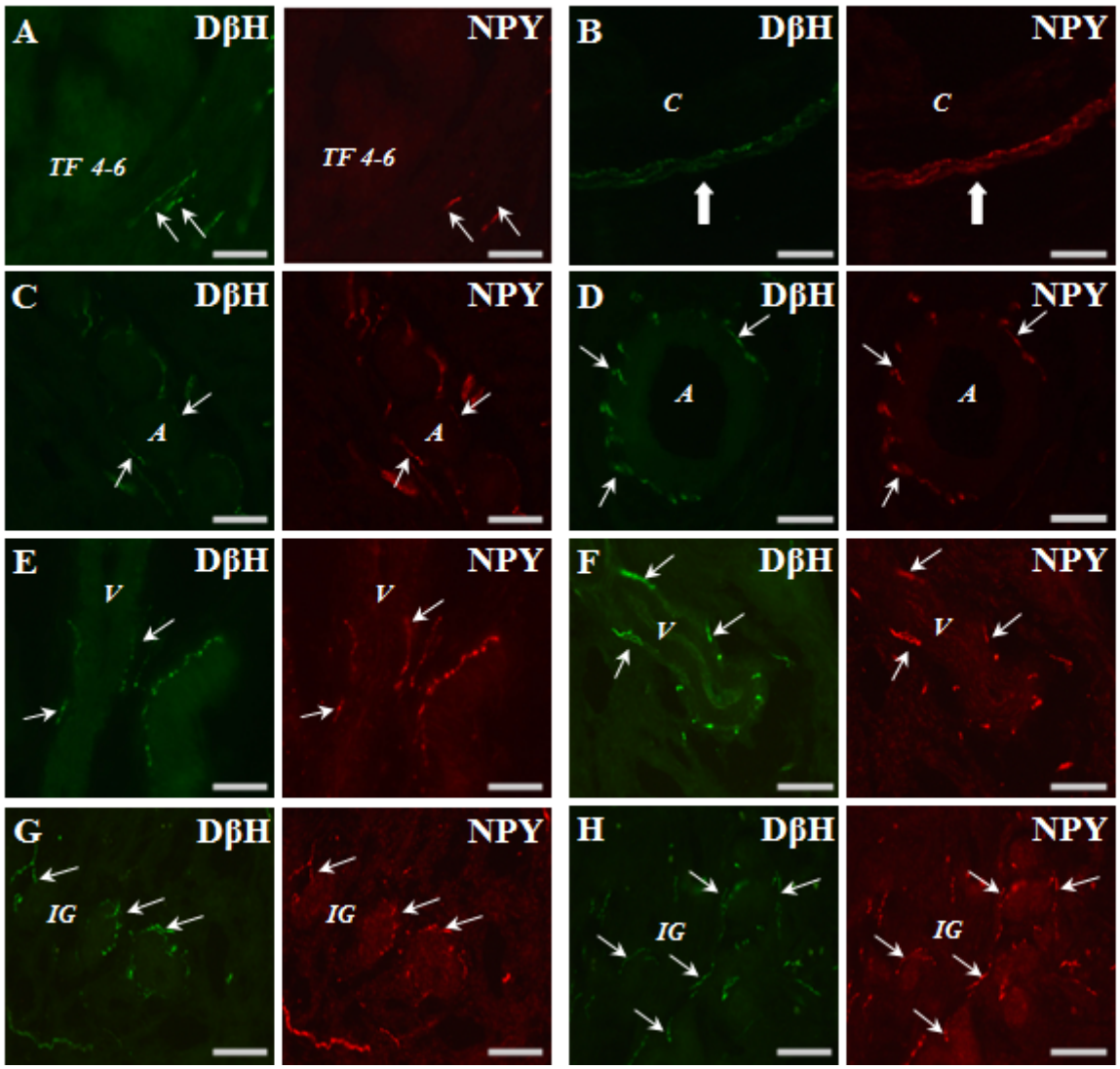

Fig. 1. Immunohistochemical localization of DβH- and/or NPY-immunoreactive (IR) nerve fibres in the ovarian cortex of the control (CON) and DXM-treated (DXM) gilts. After DXM administration numerous DβH- and/or NPY-IR nerve fibres are present in vicinity of the cyst (Fig. B) when compared to tertiary follicle measuring 4-6 mm in diameter of the control animal (Fig. A). Also in the DXM group, greater populations of DβH- and/or NPY-IR nerve fibres are visible near artery (Fig. D), vein (Fig. F) and interstitial gland (Fig. H) compared to the CON group (Figs. C, E, G; respectively). thin arrow – nerve fibre, thick arrow bunch of fibres, *TF 4-6* – tertiary follicle measuring 4-6 mm in diameter, *C* – cyst, *A* – artery, *V* – vein, *IG* – interstitial gland; scale bars in each picture = 25 μm.

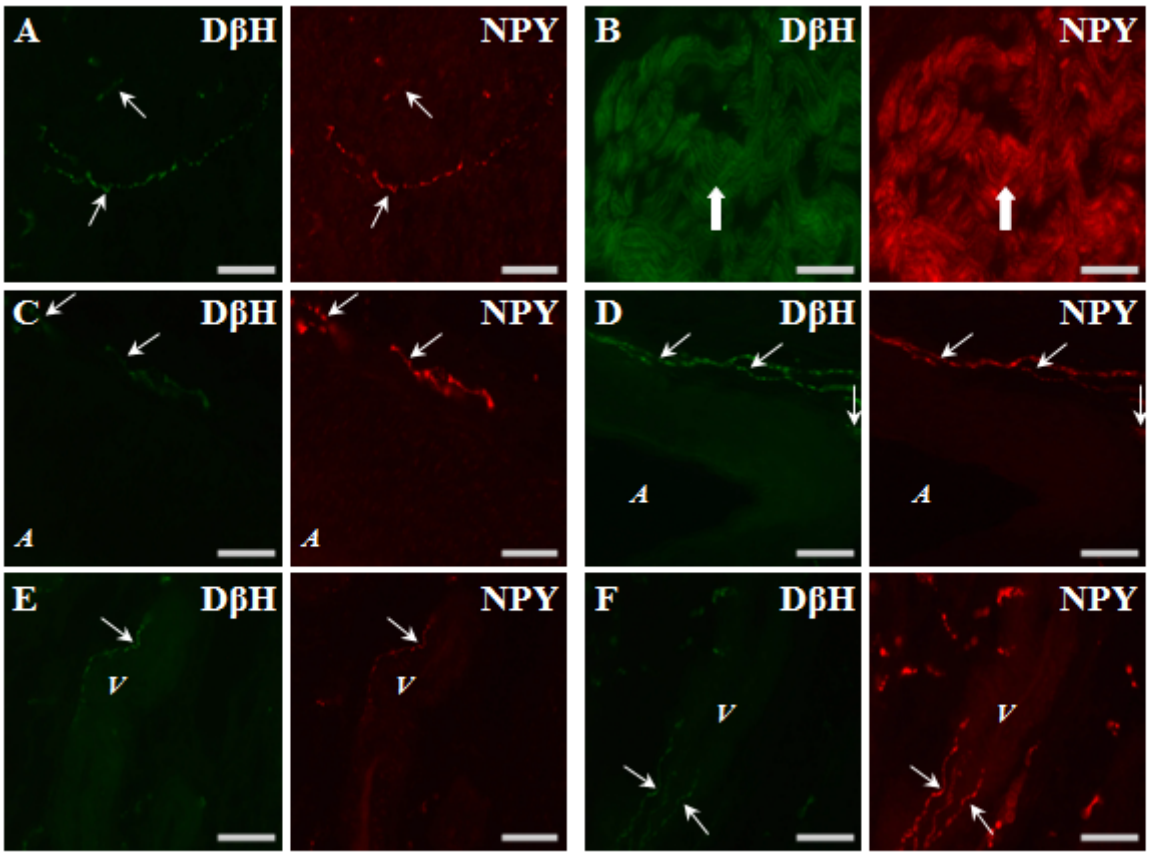

Fig. 2. Immunohistochemical localization of DβH- and NPY-immunoreactive (IR) nerve fibres in the ovarian medulla of the control (CON) and DXM-treated (DXM) gilts. In the ovaries of DXM-treated gilts, greater populations of DβH- and NPY-IR nerve fibres are visible in the area of ground plexus (Fig. B), close to the artery (Fig. D) and vein (Fig. F) compared to the CON group (Figs. A, C, E; respectively). thin arrow – nerve terminal, thick arrow – bunch of fibres, *GP* – ground plexus, *A* – artery, *V* – vein, scale bars in each picture = 25 μm.