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Long-term estradiol-17β exposure decreases the cholinergic innervation pattern of the pig ovary☆

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ABSTRACT

Elevated levels of endogenous estrogens in the course of pathological states of ovaries, as well as xenoestrogens, may lead to hyperestrogenism. It has previously been demonstrated that long-term estradiol-17β (E_2) administration in adult gilts affected the population of sympathetic intraovarian nerve fibers. The aim of this study has been to determine the effect of long-term E_2 exposure on the cholinergic innervation pattern of porcine ovaries. Intraovarian distribution and the density of nerve fibers immunoreactive (IR) to vesicular acetylocholine transporter (VAChT) and/or neuronal isoform of nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), somatostatin (SOM) were determined. From day 4 of the first estrous cycle to day 20 of the second studied cycle, experimental gilts were intramuscularly injected with E_2, while control gilts received corn oil. The ovaries were then collected and processed for double-labelling immunofluorescence. After E_2 administration, the total number of fibers IR to VAChT, nNOS and VIP decreased significantly. The numbers of VAChT-, nNOS- and VIP-IR fibers within the ground plexus were significantly lower, while they were significantly higher around small or medium tertiary follicles. In the E_2-affected ovaries, the numbers of nNOS- and VIP-IR fibers were significantly higher near secondary follicles and VAChT-IR in the vicinity of medullar blood vessels. In turn, around the latter structures there were significantly lowered populations of nNOS- and VIP-IR nerve fibers. These results suggest that the elevated E_2 levels that occur during pathological states may affect the cholinergic innervation pattern of ovaries and their function(s).

1. Introduction

The porcine ovary receives its nerve supply from sympathetic, parasympathetic and sensory components of the peripheral nervous system. The parasympathetic ovarian innervation derives from the cranial part of paracervical ganglion (PCG). Intraovarian parasympathetic nerve fibers are localized around preantral and antral follicles, corpora lutea (CL), blood vessels and interstitial glands as well as within ground plexus. These fibers express acetylcholine (ACh), major parasympathetic neurotransmitter and its co-transmitters: nitric oxide (NO), vasoactive intestinal peptide (VIP), somatostatin (SOM), substance P and galanin (Majewski, 1997). These substances are involved in the regulation of ovarian functions under physiological conditions. For example, ACh participates in follicular development and steroidogenesis in rats (Orozco et al., 2010; Daneri et al., 2013). In pigs, NO regulates steroidogenesis (Masuda et al., 2001) and the contractility of the ovarian arteries (Barszczewska and Jaroszewski, 2004). VIP regulates rat steroidogenesis (Parra et al., 2007) and follicular development (Chen et al., 2013). Similarly, SOM is involved in follicle growth and development in rats (Nestorović et al., 2014) and mice (Gougeon et al., 2010).

Many pathological states appearing in the ovaries and other organs such as the pituitary, adrenal gland and uterus in women and animal females are accompanied by disturbances in the ovarian steroidogenic activity, resulting in changes in the circulating levels of steroid hormones. The development of ovarian follicular cysts (Kucharski et al., 2002; Kengaku et al., 2007) as well as tumors (Tanaka et al., 2007; Matias-Guiu, 2010) result in a significant increase in the peripheral blood concentrations of estradiol-17β

Abbreviations: ACh, acetylcholine; CL, corpora lutea; E_1, estrone; E_2, estradiol-17β; IR, immunoreactive; NaN_3, sodium azide; NGF, nerve growth factor; nNOS, nitric oxide synthase; P_4, progesterone; PCG, paracervical ganglion; SOM, somatostatin; T, testosterone; VAChT, vesicular acetylocholine transporter; VIP, vasoactive intestinal polypeptide.

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(E_2) and/or estrone (E_1) inducing hyperestrogenenism. In addition, xenoestrogens, contained in many foods, may mimic or antagonize the activity of endogenous estrogens and disturb the normal neuroendocrine function and decrease fertility in females (Dickerson and Gore, 2007).

It is known that estrogens exert their action on the ovarian innervation. However, to date, mainly morphological and functional features of the sympathetic nerve fibers under the influence of estrogens have been studied in rats (Rosa-E-Silva et al., 2003; Casais et al., 2012) and gilts (Koszykowska et al., 2011a, 2011b, 2013). There is little information concerning the role of estrogens in the regulation of the ovarian parasympathetic nervous system. In fact, the pig, due to its embryological, anatomical and physiological similarity to humans, constitutes an especially valuable species for biomedical research (Swindle et al., 2012). A rise in the activity of acetylcholinesterase (ACh degrading enzyme) in the ovaries of E_2-treated pre-pubertal gilts as well as a decrease in its activity after the application of E_2 together with progesterone (P_4) has been reported (Lakomy et al., 1986a, 1986b). Our previous reports revealed that long-term E_2 treatment (induction of hyperestrogenism) of adult gilts down-regulates the populations of cholinergic PCG perikarya supplying the ovaries and the population of these perikarya-expressing estrogen receptors (ERs; Jana et al., 2013a, 2013b). Considering the above-mentioned findings, it is hypothesized that elevated levels of endogenous estrogens during pathological processes (also xenoestrogens) may also affect the parasympathetic innervation pattern in the ovaries, and finally the gonadal functions. In this study, the effect of long-term E_2 exposure on the distribution and density of nerve fibers containing vesicular ACh transporter (VAChT) and/or nNOS, VIP, SOM in the ovaries of gilts was examined.

2. Materials and methods

2.1. Animals

The experimental procedures were approved by the Local Ethics Committee of the University of Warmia and Mazury in Olsztyn (Agreement no 21/N). The study was carried out on six crossbred gilts (Large White × Landrace), aged 7–8 months and weighing 90–110 kg, having two controlled subsequent estrous cycles. Behavioral estrus was detected using a boar. Three days before surgical operations, the gilts were transported from a farm to a local animal house and kept in individual stalls under natural light and temperature (April, May). They were fed a commercial grain mixture and tap water *ad libitum*.

2.2. Experimental procedures

On day 3 of the first studied estrous cycle (day 0 of the study) after induction of general anesthesia by azaperone (2 mg/1 kg of body mass, Stresnil, Janssen Pharmaceutica N.V., Belgium) and sodium pentobarbital (30 mg/1 kg of body mass, Vetbutal, Biovet, Poland), a polyvinyl cannula (outer diameter 2.2 mm, inner diameter 1.8 mm, Tomel, Tomaszów Maz., Poland) was inserted into the jugular vein of each gilt to collect blood samples.

The gilts were then randomly assigned to one of two following groups: the control group (group I, n = 3) and the experimental group (group II, n = 3). In the gilts of group I, from day 4 of the first studied estrous cycle (day 1 of the study) to the expected day 20 of the second studied cycle; *i.e.* within 38 consecutive days, 2 ml of corn oil was injected *i.m.* every 12 h (h; at 07:00 and 19:00 h). In the gilts of group II, 1000 μg of E_2 (≥98%, E8875, Sigma-Aldrich, USA) in 2 ml of corn oil was injected at the same time and in the same manner as in group I. The applied dose of E_2 was defined based on our preliminary experiment showing that its application enhances the peripheral blood E_2 concentration by about 4–5-fold. According to the available reports, this E_2 blood level accompanies pathological states such as ovarian cysts (Kucharski et al., 2002; Kengaku et al., 2007) and tumors (Hiroi et al., 2002). For determination of the E_1, E_2 and P_4 concentrations, blood samples were collected from gilts of both groups throughout the whole period of E_2/oil administration (twice a day — 09:00 and 21:00 h). Immediately afterwards, the samples were placed in an ice bath, where they were kept until centrifugation (1500 × *g*, at 4 °C, 10 min). The plasma was decanted and stored at −20 °C until further processing. After the last blood sample collection, the gilts were slaughtered by electric shock (ENZ 300 Metalowiec, Bydgoszcz, Poland) and both ovaries from each gilt were immediately dissected and weighed. Afterwards, the volume, length, width and height of the gonads as well as the numbers of follicles, CL and cysts were estimated. The follicles were divided into three size classes: 1–3 (small), 4–6 (medium) and 7–10 (large) mm in diameter. Follicular structures exceeding 1.0 cm in diameter were classified as cysts (Nalbandov, 1952). The results of macroscopic assessment of ovaries and analysis of hormone concentrations in the peripheral blood of gilts were reported earlier (Koszykowska et al., 2013). Following inspection of the ovarian surfaces, for immunocytochemical studies, ovaries were cut into three parts (two lateral and third middle — containing hilar region) and fixed by immersion in Zamboni's fixative for 30 min, washed with 0.1 M phosphate buffer (PB; pH 7.4) over two days and finally transferred to and stored at 4 °C in 18% buffered sucrose solution (pH 7.4) containing 0.01% sodium azide (NaN_3) until further processing.

2.3. Immunofluorescent procedures

To study the distribution and density of intraovarian nerve terminals expressing VAChT, nNOS, VIP, SOM from every third part of ovary 9 (12-μm-thick), serial sections were cut in a cryostat (Frigocut, Reichert-Jung, Nussloch, Germany). The sections were mounted on chrome alum-coated slides and then subjected to routine double-immunofluorescence technique (Majewski and Heym, 1991) with antibodies listed in Table S1. Standard tests (pre-absorption for the used antisera with the respective antigen at a concentration of 20–50 μg antigen/ml diluted antiserum, omission of primary (Fig. 1Q–T) or secondary antisera and replacement by non-immune sera of all the primary antisera used) were employed to control the specificity of immunofluorescence. In addition, VAChT, nNOS, VIP, SOM stainings in the porcine PCG ovary supplying neurons were applied as positive controls (data not shown). The immunocytochemical staining procedure for one combination of substances examined (VAChT/nNOS, VAChT/VIP, VAChT/SOM) was conducted on three randomly chosen ovarian sections from one-third of the each ovary derived from each animal studied.

Double-immunolabelled nerve fibers were analyzed and photographed under an Olympus BX51 microscope equipped with epifluorescence and the appropriate filter sets for FITC (B1 module, excitation filter 450–480 nm, barrier filter 515 nm) and CY3 (G1 module excitation filter 510–550 nm, barrier filter 590 nm). Pictures were captured by a digital camera connected to a PC and analyzed with the AnalySIS software (version 3.02, Olympus Soft Imaging Solutions, Muenster, Germany). In our study, the distribution and density of VAChT-, nNOS-, VIP-, SOM- IR intraovarian nerve fibers were determined within ground plexus and around follicles, blood vessels, interstitial glands, CL and cysts. For each antigenic combination (VAChT/nNOS, VAChT/VIP, VAChT/SOM), the mean numbers of nerve fibers supplying the