REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

Intramuscular injections of male pheromone 5α-androstenol change the secretory ovarian function in gilts during sexual maturation

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SUMMARY

In addition to the standard olfactory pathway typical for signaling pheromones, the existence of a humoral pathway for the priming action of pheromones has been earlier postulated. In this study *in vivo* experiment was performed to establish whether intramuscular injections of boar pheromone, 5α-androstenol (5α-androst-16-en-3-ol), might change the development and secretory function of the ovarian follicles during sexual maturation of gilts. Gilts from groups I (n=15) and II (n=13) received androstenol (10 μg/gilt/injection; i.m.) three times a week from day 192 to 234 of age. Similar, control gilts (group C; n=13) received saline. Additionally, the nasal cavity of animals from group II was irrigated with zinc sulfate solution to depress olfactory function. The reproductive organs and follicular fluid

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were collected on day 240 of age. There were no significant differences among groups concerning the weight of the ovary and uterus, the length of the uterine horns and intensity of cytochrome $P450_{scc}$ and $P450_{arom}$ immunoexpression. However, gilts treated with boar pheromone had a higher ($p<0.01$) total number of follicles > 3 mm in diameter and a lower index of atresia. In addition, androstenol-treated animals were characterized by higher concentrations of progesterone (the 1-3 mm and 3-6 mm follicles; $p< 0.01$) and estradiol (follicles 3-6 mm; $p<0.001$) than those of controls. The results of the present study demonstrate that intramuscular injections of androstenol stimulate the development and secretory function of the ovarian follicles in gilts during sexual maturation. They also support the hypothesis that androstenol, as a priming boar pheromone, may influence reproductive processes in female pigs acting as a chemical signal via humoral pathway.
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Key words: boar pheromone, androstenol, sexual maturation, ovarian follicle

INTRODUCTION

A gilt's snout coming in contact with a boar influences female behavioral and hormonal status during the estrous cycle and accelerates the estrus [21]. Contact with a boar appears to be the most effective stimulus accelerating the attainment of puberty by prepubertal gilts [3, 9, 10, 15]. Two steroid pheromones, 5α-androstenone (5α-androst-16-en-3-one) and 5α-androstenol (5α-androst-16-en-3-ol) have been identified in boar saliva. The concentration of androstenol (50 μg/ml) in saliva was found to be 10-20 times higher than that of androstenone [1]. It was suggested that 5α-androstenol may play a role in stimulating the puberty in gilts [10].

It is generally accepted that pheromones act by stimulating the dendritic receptors of chemosensory neurons in the olfactory neuroepithelium which is located in the dorso-caudal region of the nasal cavity and in the vomeronasal organ [4, 5, 7, 16, 17]. Interaction of pheromones with their specific receptors on the membrane of chemosensory neurons leads to the release of an alpha subunit of G-proteins linked to adenyl cyclase which opens cAMP-gated cation channels and alters the membrane potential [4].

However, it was demonstrated that the boar pheromone, 5α-androstenol, may be resorbed from the nasal cavity to venous blood in cycling gilts. Its local transfer into the hypophysis and certain brain structures have been shown, too. After radiolabeled 5α-androstenol was administered into the nasal cavity of either the isolated heads of gilts [11] or anaesthetized gilts [23], the pheromone was found in arterial blood supplying the hypophysis and brain. In these experiments the selective accumulation of androstenol was found in the hypothalamus, olfactory bulb, amygdala, septum, neurohypophysis and adenohypophysis. It was recently learned that androstenol directly affects the secretory function of the hypothalamic neurones. The incubation of selected brain structures of the prepubertal gilts with androstenol *in vitro* caused a significant increase in GnRH release from neurons of paraventricular nucleus and medial basal hypothalamus (Wąsowska et al., unpublished data). It was also demonstrated that 5α-androstenol injected intramuscularly into 160 day-old gilts for 60 consecutive days accelerated the onset of puberty by 16-20 days [12]. Therefore, it has been suggested that androstenol may influence hormonal regulation of female reproductive processes by a humoral pathway [11, 12]. This steroid has been considered to be the priming pheromone in gilts [11] in addition to the standard olfactory pathway which is typical for signaling pheromones acting by stimulating the dendritic receptors.

It was also found that intramuscular injections of androstenol during periestrous period affected the ovarian morphology in hypoosmatic cycling gilts [24]. However, the mechanism of the action of 5α-androstenol on the secretory function of the ovary has never been investigated. This study was designed to establish whether intramuscular injections of boar pheromone, androstenol, might change the secretory ovarian function of the gilts during sexual maturation, and whether the destruction of the olfactory epithelium (hypoosmia) may influence this effect.

MATERIALS AND METHODS

Animals

The study was conducted in accordance with national guidelines for the care and use of research animals. The experiments were carried out from August

2001 to February 2002. Female piglets from 10 litters, born within one week of one another, were weaned at 42 days of age and then individually marked. All gilts were housed on a commercial farm, in an enclosed barn with no boar present. The females from each litter were randomly assigned to one of the following treatment, commenced at a mean age of 192 days and continued for 42 days:

- group C (control, n=13): irrigation of the nasal cavity with saline and intramuscular injections of saline;
- group I (n=15): irrigation of the nasal cavity with saline and intramuscular injections of boar pheromone, androstenol;
- group II (n=13): irrigation of the nasal cavity with zinc sulphate solution and intramuscular injections of boar pheromone, androstenol. Zinc sulphate is routinely used to evoke anosmia in several species [6, 8, 14, 20].

The gilts of group C were penned in a separate part of the barn, more than 100 m away from groups I and II. Gilts were fed a standard ratio for growing pigs. The housing was identical for all three groups.

Irrigation of the nasal cavity

The gilts were anaesthetized with atropine (0.05 mg/kg, i.m., Biowet, Gorzów Wielkopolski, Poland), azaperone (Stresnil, 2 mg/kg, i.m., Jansen Pharmaceutica, Beerse, Belgium) and ketamine hydrochloride (Narkamon, 4 mg/kg, i.m., SPOFA, Praha, Czech Republic) and then placed on their backs. Ten ml of 0.17 M zinc sulphate solution (group II) was administered into each nasal cavity via external nares, according to the method used earlier in rhesus monkeys [20], rabbits [14] and mice [6, 8]. The nasal cavities of gilts from groups C and I were irrigated with saline. After the irrigation the gilts were left in the dorsal position for over five minutes.

Intramuscular injections of androstenol or saline

Androstenol (5-androst-16-en-3-ol; Sigma, St. Louis, USA) dissolved in ethanol was diluted in saline (to a concentration of 20 μg/ml) on the day of injection. To avoid manipulation with the volatile substances on the farm,

the syringes were filled with 0.5 ml of androstenol solution (corresponding to 10 µg of androstenol) in the laboratory and closed with the injection needles. The gilts from groups I and II were injected intramuscularly with androstenol solution and the gilts from group C with saline. These injections were performed three times a week during the 42 day-period. The dose of androstenol used in this experiment is equivalent to its content in 200 µl of boar saliva [1]. In our earlier study [12] intramuscular injections of such dose of androstenol to gilts from 190 to 220 day of age significantly accelerated their puberty.

Collection of samples

On day 240 of age (six days after the last injection of androstenol or saline) the gilts were weighed and then killed by electrical shock (ENZ-Metalowiec, Bydgoszcz, Poland) and exsanguination. The mean body mass of gilts was as follows: group C - 113.9 ± 3.5 kg, group I - 113.1 ± 2.8 kg and group II - 116.0 ± 3.9 kg. The ovaries and uteri were removed and weighed. The length of uterine horns was measured. The number and diameter of ovarian structures (follicles and corpora lutea) were recorded and classified according to criteria presented by Leiser et al. [13]. One ovary from each gilt was used for harvesting the follicular fluid to determine the concentration of steroid hormones. The follicular fluid was collected individually from the follicles of different sizes (1-3 mm, 3-6 mm and >6 mm in diameter) and pooled separately for each ovary according to follicle size. Samples of follicular fluid were centrifuged (1 000×g) and stored at -70°C until steroid assays were completed. After the follicular fluid was collected, the ovary was frozen in liquid nitrogen and stored at -70°C for analysis of cytochromes $P450_{scc}$ ($P450_{scc}$) and $P450_{arom}$ ($P450_{arom}$) expression. The second ovary was submitted to histological analysis.

Histological analysis

The ovaries were fixed in Bouin's fluid, cut into three parts and than routinely embedded in paraffin. From each part of the ovary, 6 series sections

(6 μm) were cut and stained with hematoxylin-eosin. The diameter of the ovarian structures (expressed in mm) was measured employing the ocular micrometer. The sections were analyzed to establish the number of healthy and atretic follicles of each size (<1 mm, 1-3 mm, 3-6 mm, >6 mm) to calculate the index of atresia. The index of atresia for each follicle size in particular groups was calculated as a percentage of atretic follicles to total number of the follicles (healthy + atretic) in the ovary.

Hormone assays

The concentration of hormones in follicular fluid samples was determined by radioimmunoassays without extraction. Steroid antibodies were obtained from the Department of Animal Physiology, University of Warmia and Mazury, Olsztyn. Antibodies against estradiol (BS/88/754) and testosterone (BS/88/312) were characterized by Szafrańska et al. [26]. The specificity of antibodies for progesterone was reported by Stefańczyk-Krzymowska et al. [22]. The sensitivity of the assays was: estradiol - 2.5 pg/ml, testosterone - 2.3 pg/ml and progesterone - 0.02 ng/ml. The inter- and intra-assay coefficients of variations were: estradiol - 3.1% and 3.6%, testosterone - 4.0% and 6.6% and progesterone - 3.8% and 5.6%, respectively.

Western blotting analysis

Membrane fraction for immunoblotting was obtained using the procedure described by Stępień et al. [25], with some modifications. Briefly, examined follicles, preovulatory follicles (positive control) and liver (negative control) were placed in freshly made, ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100, 10 μg/ml aprotinin, 52 μM leupeptin, 1 mM pepstatin A, 1mM EDTA, 1 mM PMSF; Sigma-Aldrich Co., St Louis MO, USA) and homogenized on ice. Homogenates were then centrifuged for 10 minutes at 1 000×g at 4°C. The supernatant was centrifuged for one hour at 30 000×g at 4°C and the pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4). The resulting suspension

was stored at -70°C for further analysis. The protein level was determined by Bradford's method [2].

Equal portions of protein (30 μg) were dissolved in SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol and 2% β-mercaptoethanol), heated at 95°C for two minutes and separated on 10% SDS-PAGE. Separated proteins were electroblotted onto 0.45 μm nitrocellulose membrane in a transfer buffer (20 mM Tris-HCl buffer, pH 8.2; 150 mM glycine, 10% methanol). The non-specific binding sites were blocked with 5% non-fat dry milk in TBS buffer (100 mM Tris-HCl, pH 7.4; 150 mM NaCl) containing 0.1% Tween-20 (TBS-T buffer) for 1.5 hour at room temperature. After incubation, the nitrocellulose membrane was washed once with TBS-T. Next the membrane was incubated overnight at 4°C with primary antibodies: rabbit anti-rat $P450_{scc}$ polyclonal antibody (1:1 000; AB 1244, Chemicon International, Temecula CA, USA) or rabbit anti-human placental $P450_{arom}$ antibodies (1:2 000; R-2-4; provided by Hauptman-Woodward Medical Research Institute, Inc., Buffalo, USA). Then, the membrane was washed again with TBS-T. The antibodies against both $P450_{scc}$ [18] and $P450_{arom}$ [27] were characterized earlier. The membranes were incubated with anti-rabbit alkaline phosphate conjugated IgG (1:20 000; Sigma-Aldrich Co., St Louis MO, USA) for 1.5 hour at room temperature and washed three times with TBS. The immune complex was visualized using a standard alkaline-phosphate visualization procedure [19]. All immunoblots were quantitated by scanning on KODAK 1D Image Analysis Software (USA). The intensity of bands detected by one-dimensional image analysis is reported in arbitrary units.

Statistical analysis

The data, except for the index of atresia, are presented as means (± SEM). All morphometrical parameters (mean weights of the uterus and ovary, length of the uterine horns, mean number of the follicles >3 mm) were analysed by one-way analysis of variance followed by Bonferroni t test (Prism Software GraphPad, San Diego, USA). The same method was used to compare histological parameters (mean number of healthy and atretic follicles in the ovary) and hormones concentration in follicular fluid.

Table 1. Development of the reproductive organs of control and androstenol treated gilts on 240 day of age (six days after the last treatment)

	Group		
	C	I	II
Number of gilts	13	15	13
Number of gilts sexually mature before 240 day of age	2	0	4
Number of gilts with the follicles >3 mm	9	15	10
Mean (± SEM) number of follicles >3 mm per ovary	6.7 ± 1.1^{a}	11.0 ± 0.9^{b}	10.0 ± 0.9^{b}
Mean (± SEM) weight of the uterus [g]	98.2 ± 8.1	111.9 ± 12.3	115.1 ± 17.2
Mean (± SEM) length of the uterine horns [cm]	55.5 ± 1.9	59.6 ± 1.7	58.7 ± 2.3
Mean (± SEM) weight of the ovaries [g]	3.0 ± 0.1	2.6 ± 0.2	3.2 ± 0.1

Means with different superscript letters differ at $p < 0.01$

RESULTS

Macroscopic observation

The macroscopic evaluation of the excised ovaries revealed that only 14% of control and 14% of androstenol-treated gilts (groups I and II) were sexually mature before the 240 day of age (presence of the corpora lutea or corpora albicantia in the ovaries). The ovaries of the remaining control and androstenol-treated gilts contained follicles of various sizes. The number of follicles >3 mm in diameter was significantly higher ($p<0.01$) in gilts treated with androstenol than that in control gilts (tab. 1).

Table 2. Histological evaluation of number of healthy and atretic ovarian follicles[1] in control and androstenol-treated gilts

A

Group	Healthy follicles		
	<1 mm	1-3 mm	3-6 mm
C	8.4 ± 0.4^{a}	3.8 ± 0.5^{a}	0.4 ± 0.09^{a}
I	11.5 ± 0.7^{b}	4.7 ± 0.3^{ab}	0.4 ± 0.07^{a}
II	10.1 ± 1.2^{ab}	5.2 ± 0.3^{b}	0.6 ± 0.04^{a}

B

Group	Atretic follicles		
	<1 mm	1-3 mm	3-6 mm
C	6.9 ± 0.9^{A}	10.7 ± 0.6^{A}	1.7 ± 0.1^{A}
I	5.2 ± 0.3^{A}	7.9 ± 0.4^{B}	1.3 ± 0.1^{A}
II	11.7 ± 0.8^{B}	6.2 ± 0.2^{B}	0.2 ± 0.03^{B}

C

Group	Index of atresia (%)		
	<1 mm	1-3 mm	3-6 mm
C	45.3	73.7	81.7
I	31.2	62.7	74.3
II	53.6	54.1	27.8

[1]mean (± SEM) number of follicles per section is depicted in the table; different superscripts denote significant differences within columns; a, b: $p < 0.05$; A, B: $p < 0.001$

Histological analysis

In histological preparations the follicles over 6 mm in diameter were not found in spite that they were observed during macroscopic observations of the ovaries. Such difference, probably resulting from fixation and staining processes, is often observed.

The number of healthy follicles <1 mm in group I and the 1-3 mm follicles in group II was higher ($p<0.05$) than in the control animals (tab. 2A). Additionally, the number of atretic follicles <1 mm was higher ($p<0.001$) in group II than in group C. There were significantly ($p<0.001$) less 1-3 mm follicles in androstenol-treated groups in comparison to controls. The number of 3-6 mm follicles in group II was lower ($p<0.001$) than in the group C (tab. 2B). The atresia index in the androstenol-treated groups in the 1-3 mm and 3-6 mm follicles was lower as compared to the control group (tab. 2C).

Concentration of steroid hormones in the follicular fluid

The mean concentration of progesterone was higher in the 1-3 mm and 3-6 mm follicles of both groups injected with androstenol ($p<0.01$) than that in the controls (fig. 1A). A significantly higher concentration of estradiol was found in the 3-6 mm follicles in gilts injected with androstenol as compared to the control group ($p<0.001$; fig. 1B). The mean concentration of testosterone differs among neither the groups of gilts nor classes of the follicle development (fig. 1C).

Western blot analysis

All examined classes of follicles from groups C, I and II expressed $P450_{scc}$ and $P450_{arom}$ proteins at significant levels. Western blot analysis demonstrated that the expression of $P450_{scc}$ in the 1-3 mm and 3-6 mm follicles of animals treated with androstenol was lower than in controls (fig. 2A, B). In the follicles with diameter >6 mm in group I, the staining intensity of the $P450_{scc}$ band was higher compared to the control group (fig. 2A, B).

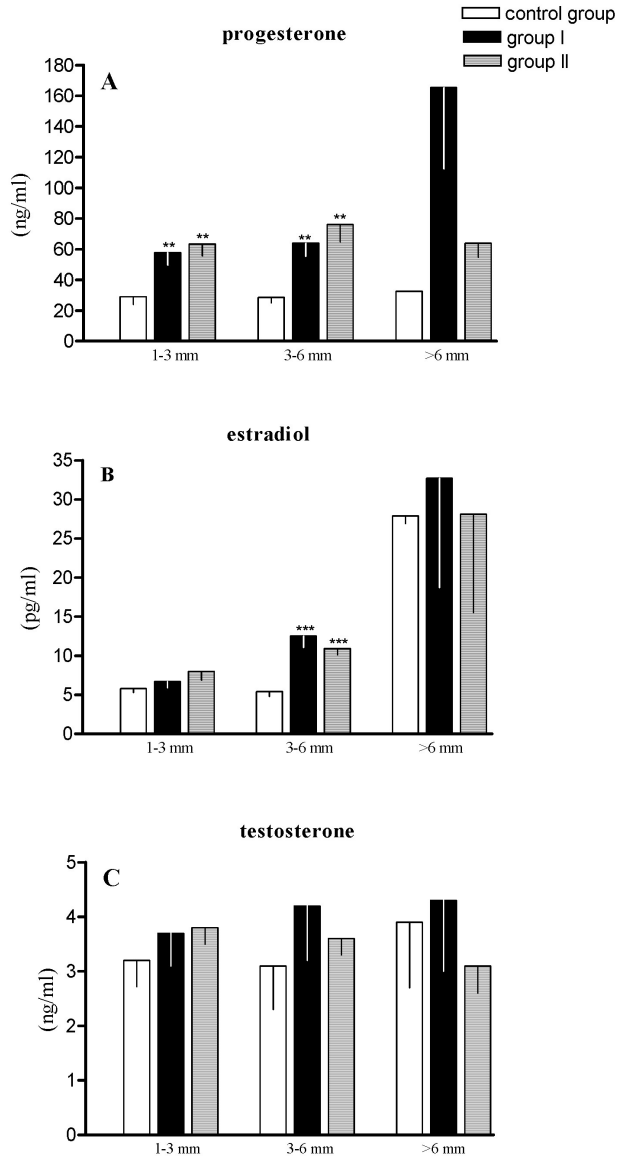

Fig. 1. Follicular fluid concentration of progesterone (A), estradiol (B) and testosterone (C) in control (group C) and androstenol-treated (groups I and II) gilts on 240 day of age. The gilts of group II were irrigated intranasally with 0.17 M zinc sulfate solution (hypoosmia). The data are presented as means ± SEM.
*** $p<0.001$,** $p<0.01$

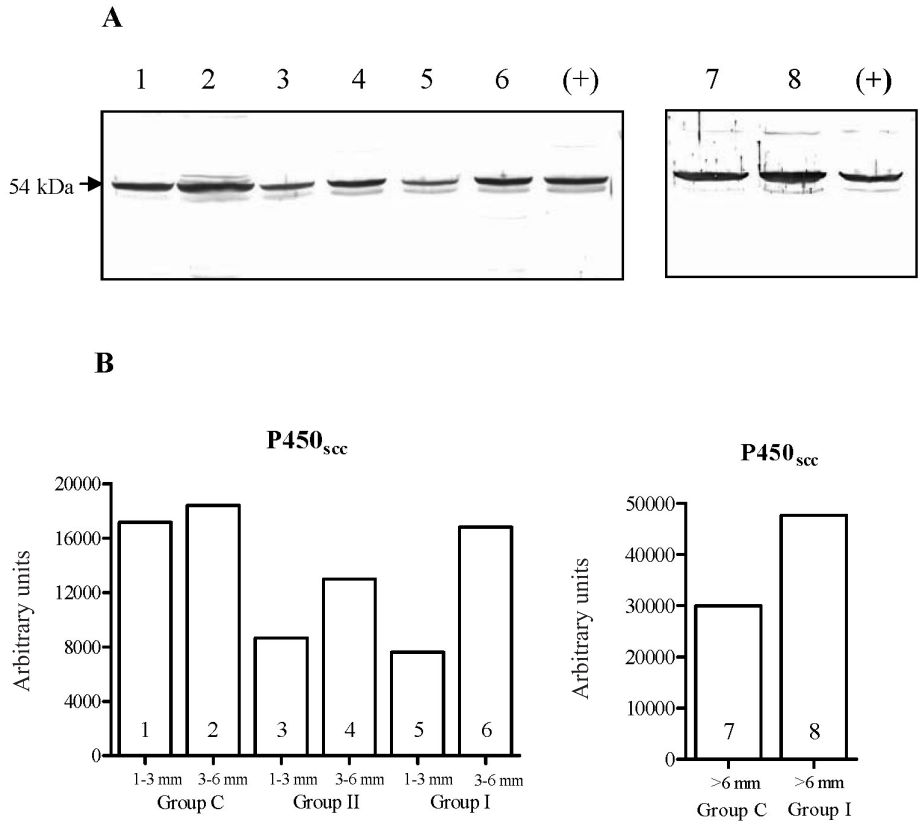

Fig. 2. Western blot of $P450_{scc}$ A/ in the follicles of control (group C) and androstenol-treated (groups I and II) gilts. Group C, the 1-3 mm follicles (lane 1); group C, the 3-6 mm follicles (lane 2); group II, the 1-3 mm follicles (lane 3); group II, the 3-6 mm follicles (lane 4); group I, the 1-3 mm follicles (lane 5); group I, the 3-6 mm follicles (lane 6); group C, the >6 mm follicles (lane 7); group I, the >6 mm follicle (lane 8); positive control (+) - (preovulatory follicles); B/ densitometric analysis of $P450_{scc}$ in arbitrary units. Data presented here are representative of three independent experiments.

Cytochrome $P450_{arom}$ immunoexpression in the 3-6 mm follicles was higher in both androstenol-treated groups than in the control group (fig. 3A, B). The higher staining intensity of the $P450_{arom}$ band was observed in the >6 mm follicles in group I as compared to the controls (fig. 3A, B).

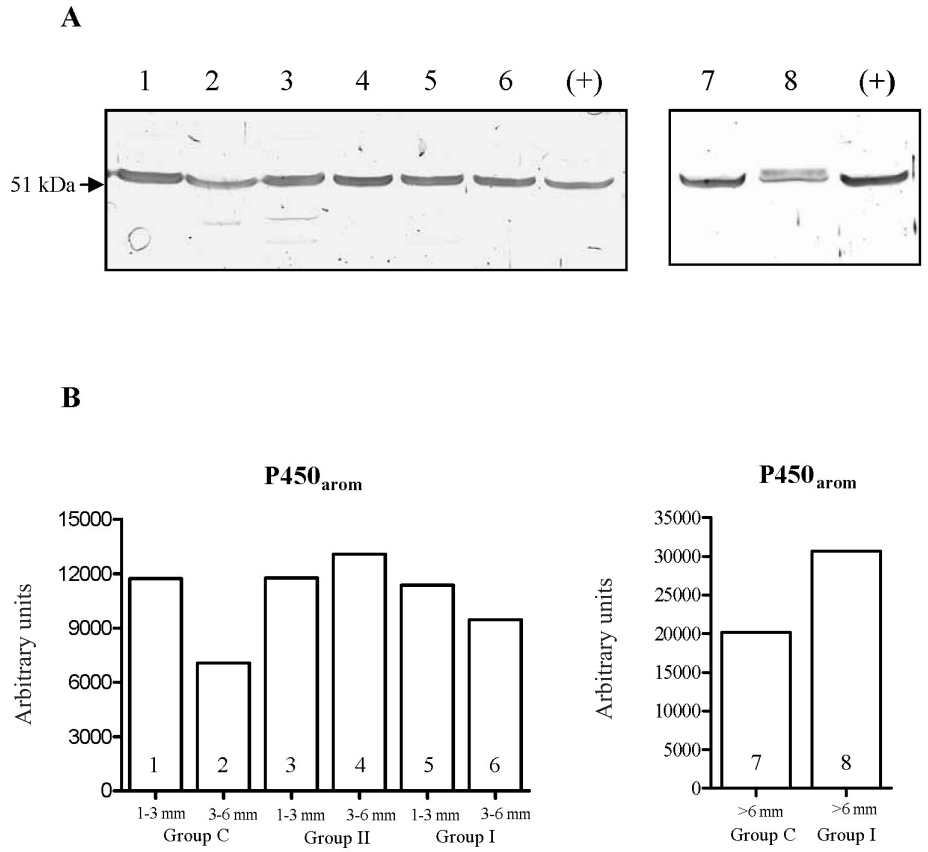

Fig. 3. Western blot of $P450_{arom}$ A/ in the follicles of control (group C) and androstenol-treated (groups I and II) gilts. Group C, the 1-3 mm follicles (lane 1); group C, the 3-6 mm follicles (lane 2); group II, the 1-3 mm follicles (lane 3); group II, the 3-6 mm follicles (lane 4); group I, the 1-3 mm follicles (lane 5); group I, the 3-6 mm follicles (lane 6); group C, the >6 mm follicles (lane 7); group I, the >6 mm follicles (lane 8); positive control (+) - (preovulatory follicles); B/ densitometric analysis of $P450_{arom}$ in arbitrary units. Data presented here are representative of three independent experiments.

Due to the lack of the sufficient amount of follicular tissues, western blot analysis of the >6 mm follicles in group II could not be performed. No immunodetectable bands for $P450_{scc}$ and $P450_{arom}$ in liver (negative control) were revealed.

DISCUSSION

The present results demonstrated that intramuscular injections of androstenol affected the development and secretory function of the ovarian follicles in gilts during sexual maturation. The ovaries of all gilts injected with boar pheromone, androstenol, contained more follicles over 3 mm in diameter than those of control group. Moreover, a lower number of 1 -6 mm atretic follicles was found in groups I and II than in the controls. The atresia index in the gilts receiving androstenol was also diminished compared to control group.

In follicular fluid of the gilts receiving androstenol, the concentration of progesterone and estradiol was significantly higher than in control gilts. The expression of cytochrome $P450_{scc}$ and $P450_{arom}$ in the follicles confirmed correct, undisturbed steroidogenesis in all classes of the follicles in both control and androstenol-injected gilts.

In general, follicular development and hormone secretion was similar in both groups injected with androstenol although in one group the olfactory function was experimentally depressed by using the method known to produce long-term anosmia in some animal species. Application of zinc sulphate solution into the nasal cavity caused extensive destruction of the neuroepithelium by coagulation necrosis and losses in olfactory behavior (anosmia) in monkey [20], rabbit [14] and mouse [6, 8]. Detailed behavioural study performed in mice [8] demonstrated that anosmia caused by intranasal irrigation with zinc sulphate solution persisted in all treated mice for at least two weeks and in 80% of them for six weeks. After one year, 85% of treated mice have recovered the olfactory function. A study on the effect of the zinc sulphate on the olfactory mucosa in gilts has not been conducted yet. However, it may be assumed that gilts respond to zinc sulphate in a similar manner as rhesus monkeys, rabbits and mice. If the irrigation of the nasal cavity of the gilts with zinc sulphate solution did not cause the anosmia, it had to produce the depression of the olfactory function i.e. hypoosmia. A lack of differences in the development and secretory function of the follicles between groups I and II (irrigation of the nasal cavity with zinc sulphate solution or with saline) suggests that

the pheromon, androstenol, injected intramuscularly did not stimulate the ovarian processes via the olfactory organ.

We assume that the effect of androstenol injections on the development and secretory function of the ovary was not caused by the androstenol's direct effect on the ovary but by its influence on the central nervous system. Normal development of the ovarian follicles, demonstrated in present study, supported this concept. The existence of the pathway for the action of androstenol through central nervous system was suggested by the results of our earlier studies [11, 23]. We have demonstrated the ability of some hypothalamic nuclei and hypophysis to accumulate androstenol supplied to the brain with arterial blood [11, 23]. In addition, our recent results confirm the existence of this pathway. We had found that the boar pheromone androstenol added into the incubation medium stimulated the secretion of GnRH from the neurones of the paraventricular nucleus and medial basal hypothalamus of prepubertal gilts [Wąsowska et al., unpublished data].

In conclusion, we postulate that intramuscular injections of boar pheromone, 5α-androstenol may change the development and secretory ovarian function of gilts during sexual maturation. It appears that the results of the present study support our earlier hypothesis [11, 12, 23] on the existence of two pathways for the action of male pheromones stimulating reproductive processes in female pigs. The pathways include a/ the olfactory pathway involving the action of boar signalling pheromones (androstenone and androstenol) on the sensory neurones of the olfactory organ, and b/ the humoral pathway involving the action of blood-derived androstenol on the brain and hypophysis. The latter mechanism may be accounted for priming effect of androstenol acting as a chemical signal. The local transfer in the perihypophyseal vascular complex from venous blood to arterial blood supplying the brain and hypophysis contributes in the humoral pathway.

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