EFFECT OF 2-METHOXYESTRADIOL ON GONADOTROPIN SECRETION AND OXIDATIVE STATUS OF PORCINE PITUITARY CELLS *IN VITRO*

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Abstract

The aim of the study was to investigate the effect of 2-methoxyestradiol (2-ME) on GnRH-induced LH and FSH secretion by porcine pituitary cells *in vitro*. Moreover, the concentrations of superoxide anion radical (O_2^-), as a possible mediator of 2-ME action, and malonyldialdehyde (MDA), as an oxidative stress indicator, were estimated. Pituitary cells were cultured in McCoy 5A medium with GnRH (positive control), with GnRH and 3.3×10^{-11} - 3.3×10^{-7} M/L of 2-ME, or with GnRH and 2.7×10^{-9} - 2.7×10^{-8} M/L of 17β -estradiol. The secretion of gonadotropins, as well as concentrations of superoxide anion and MDA were analysed after 2-72 h of the experiment. A positive correlation between 2-ME dose and FSH secretion from anterior pituitary cells ($r=0.72, 0.95, 0.92, 0.90, 0.85$, and 0.82 after 2, 6, 18, 24, 48, and 72 h, respectively) was demonstrated. In contrast, a negative correlation between 2-ME concentration and LH secretion was observed ($r=-0.82, -0.72, -0.85, -0.89, -0.93$, and -0.79 after 2, 6, 18, 24, 48, and 72 h, respectively). The concentration of O_2^- was increasing under the influence of the rising doses of 2-ME, whereas the mean level of MDA was not changing significantly.

Key words: swine, 2-methoxyestradiol, pituitary cells, FSH, LH.

2-methoxyestradiol (2-ME) is known as a biologically active, endogenous 17β -estradiol (E-2) metabolite, which is produced by sequential action of CYP450s and catechol-O-methyltransferase in porcine granulosa cells. 2-ME is released into follicular fluid and, to a lesser extent, into blood (21). Its level is augmented during the growth of follicle. There are a lot of reports showing that 2-ME can be used in experimental treatment of some types of cancer, *e.g.* angiosarcoma, gastric cancer, hepatocellular carcinoma, neuroblastoma, osteosarcoma, breast cancer, lung cancer, pancreatic cancer, and oestrogen-induced pituitary tumour (1, 20), or cardiovascular and renal disorders. According to Ricker *et al.* (18), 2-ME is a potent inhibitor of tumourigenesis. A crucial role in 2-ME-induced antitumour mechanism is played by the inhibition of angiogenesis caused by a decrease in vascular endothelial growth factor (VEGF) synthesis (2, 4), as well as antiproliferative and proapoptotic action, which is independent of α and β oestrogen receptors (13, 17). Several molecular mechanisms are involved in the process of proliferation inhibition and apoptosis, including microtubule disruption, p53 activation, upregulation of death receptor 5, inhibition of mitochondrial respiration, and the reduction of the superoxide dismutase activity (9, 15, 16). The

cardioprotective effect of 2-ME, instead, is caused by inhibition of vascular smooth muscle cell growth in arteries (22), as well as by reduction of serum cholesterol level and LDL oxidation (6). It was also reported that 2-ME induces vasodilatation by stimulation of NO synthase in artery endothelial cells, NO release, and inhibition of phenylephrine production (8).

Taking into account that 2-ME is E-2 metabolite, its use in cancer treatment, cardiovascular or renal diseases may cause the disorders in GnRH-induced gonadotropin secretion as a side effect. Accordingly, the aim of our study was to investigate the effect of 2-ME on LH and FSH secretion from porcine pituitary cells *in vitro*. Moreover, the concentrations of superoxide anion radical (O_2^-), as a possible mediator of 2-ME action, and malonyldialdehyde (MDA), as an oxidative stress indicator, were analysed.

Material and Methods

The pituitary glands were obtained at slaughter from 4-5- year-old sows (Polish Large White breed) with active healthy ovaries during the follicular phase of the oestrous cycle. Isolation of cells was carried out

through the digestion of the pituitary with 0.25% trypsin solution. Suspension of cells in trypsin combined with the preparatory medium (DMEM supplemented with 0.1% BSA, 0.08% glucose, 0.59% HEPES, and gentamycin in the final concentration of 20 µg/mL) was centrifuged (1,200 rpm for 10 min.). The sedimented cells were washed twice and finally cultured in McCoy 5A medium containing 2.5% foetal calf serum, 10% horse serum, mixture of amino acids and vitamins, 0.59 % HEPES, gentamycin (20 µg/mL), and adjusted to pH 7.4 (5, 11, 12). One millilitre of dispersed cell suspension (5×10^5 cells/mL) was transferred to each well of the 24-well culture plate and incubated for 96 h at 37°C under the atmosphere of 5% CO₂. After attachment to the dishes, the cells were washed and finally incubated with McCoy 5A medium without hormones (negative control), with GnRH (4×10^{-9} M/L) (positive control), with GnRH (4×10^{-9} M/L) and 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , or 10^{-7} M/L of 2-ME (Sigma Chemical Co., USA), respectively, and with GnRH (4×10^{-9} M/L) and 10^{-9} or 10^{-8} M/L of E-2 (Sigma Chemical Co., USA), respectively. Each sample was performed in duplicate.

After 2, 6, 18, 24, 48, and 72 h of incubation, the media were collected for analysis of LH, FSH, superoxide anion radical (O₂⁻) and MDA concentrations. In parallel, proliferation index (PI) of cells treated with 2-ME and E-2 was determined. The results were used for calculation of LH and FSH secretion. Assessment of cell proliferation was based on the reduction of the tetrazolium salt (MTT) into a blue formazan. Both control and experimental cultures were pulsed with 15 µl of MTT (for 3 h at 37°C) and then solubilised with SDS overnight. The optical density (OD) of the formed blue formazan was measured by ELISA microplate reader at the wavelength of 600 nm. LH concentration in culture medium was determined using LH [¹²⁵I] IRMA KIT (Orion Diagnostica, Spectria, Finland), whereas FSH level using FSH [¹²⁵I] IRMA KIT (Orion Diagnostica, Spectria, Finland). Gonadotropin secretion was expressed as a concentration (IU/L) of given hormone (LH or FSH), which was released into the culture medium by 5×10^5 cells during 2, 6, 18, 24, 48, and 72 h, respectively.

Medium concentration of MDA was measured spectrophotometrically. Samples of 0.25 ml medium were mixed with 1.25 ml of trichloroacetic acid (TCA) and 0.75 ml of thiobarbituric acid and thereafter heated for 20 min in a boiling water bath. After cooling of the sample to the room temperature, 2 ml of n-butanol was added and the mixture was shaken vigorously for 3 min, and centrifuged 10 min at 1,500 x g. After the transfer of upper n-butanol layer to the glass cuvette, its absorbance was measured at 532 nm. Concentrations of MDA were calculated on the basis of the standard curve obtained using malondialdehyde bis-dimethylacetal.

Superoxide anion radical was measured by Confer method. The collected medium was immediately mixed with 0.1% nitroblue tetrazolium (NBT, Sigma) solution and incubated at room temperature for 10 min. Thereafter, absorbance was read at 545 nm. Nanomoles of superoxide anion radical produced over the

incubation period were calculated using the extinction coefficient 21.1 nmol.

The obtained results were calculated using Statistica 5.0 PL and expressed as a mean and standard deviation ($\bar{x} \pm \text{SD}$). Comparisons between the control and experimental cultures were performed using analysis of variance and the paired *t*-tests. Differences were considered as significant at $P \leq 0.05$ or $P \leq 0.001$.

Results

The effect of 2-methoxyestradiol on FSH secretion from porcine pituitary cells. The average secretion of FSH amounted to 8.72 ± 2.67 IU/L/ 5×10^5 cells during the whole time of the experiment (0–72 h) in the negative control culture, whereas in the positive control it was 16.75 ± 4.76 IU/L/ 5×10^5 cells. The addition of 10^{-11} – 10^{-9} M/L of 2-ME to culture medium did not affect FSH secretion significantly in comparison to the positive control. Treatment of the cells with 10^{-8} and 10^{-7} M/L of 2-ME resulted in a significant ($P \leq 0.05$) increase in FSH secretion starting from 48 h or 6 h of exposition, respectively. After 72 h, FSH secretion under the influence of 10^{-8} and 10^{-7} M/L of 2-ME reached the maximum values (31.81 ± 2.31 and 34.47 ± 1.95 IU/L/ 5×10^5 cells/72 h, respectively). It was significantly ($P \leq 0.001$) higher compared to positive control (23.64 ± 1.36 IU/L/ 5×10^5 cells/72 h). Comparison of the effect of the same doses of 2-ME and E-2 (10^{-9} and 10^{-8} M/L) showed the significantly ($P \leq 0.001$) lower stimulation of FSH secretion under the influence of 2-ME than E-2 (42.57 ± 2.45 IU/L/ 5×10^5 cells/72 h and 44.25 ± 3.55 IU/L/ 5×10^5 cells/72 h, respectively) (Fig. 1A). The positive correlation between 2-ME concentration in culture medium and FSH secretion from anterior pituitary cells ($r = 0.72$, $r = 0.95$, $r = 0.92$, $r = 0.90$, $r = 0.85$, and $r = 0.82$ after 2, 6, 18, 24, 48, and 72 h, respectively) was found (Fig. 2A).

The effect of 2-methoxyestradiol on LH secretion from porcine pituitary cells. The average secretion of LH amounted to 54.03 ± 28.86 IU/L/ 5×10^5 cells during the whole time of the experiment (0–72 h) in the negative control culture, whereas in the positive control it was 91.55 ± 39.92 IU/L/ 5×10^5 cells. The influence of 2-ME on LH secretion depended on time and dose of 2-ME used. The introduction of 2-ME in concentration of 10^{-11} , 10^{-10} , 10^{-9} , and 10^{-8} M/L did not affect significantly LH secretion in comparison to the positive control; however, it was subtly increased in cell cultures treated with abovementioned doses of 2-ME after 2, 6, 18, and 24 h. After 72 h, LH secretion was insignificantly decreased under the influence of 10^{-9} and 10^{-8} M/L concentrations (Fig. 1B). The highest concentration of 2-ME (10^{-7} M/L) significantly ($P \leq 0.05$) reduced LH secretion from porcine pituitary cells starting from 18 h of the experiment. Comparison of the effect of the same doses of 2-ME and E-2 (10^{-9} and 10^{-8} M/L) showed the significantly lower stimulation of LH secretion under the influence of 2-ME than E-2 after 2, 6, 18, and 24 h. After 72 h, instead, an opposite effect of

10^{-9} and 10^{-8} M/L of 2-ME and E-2 on LH secretion was observed (Fig. 1B). The negative correlation between 2-ME dose and LH secretion from anterior pituitary cells ($r = -0.82$, $r = -0.72$, $r = -0.85$, $r = -0.89$, $r = -0.93$, and $r = -0.79$ after 2, 6, 18, 24, 48, and 72 h, respectively) was found (Fig. 2B).

Influence of 2-ME on superoxide anion radical and MDA concentration in the culture medium. A

A.

B.

Fig. 1. The effect of 2-methoxyestradiol on FSH (A) and LH (B) secretion from porcine pituitary cells *in vitro* (a, b – significant difference in comparison to positive control ($P \leq 0.05$ or $P \leq 0.001$, respectively)).

positive linear correlation between 2-ME and mean superoxide anion radical concentration was found ($r = 0.72$) (Fig. 3). The concurrent analysis of relationship between 2-ME and mean MDA concentration during the experiment (0-72 h) showed a negative correlation ($r = -0.45$). However, the mean level of MDA was not changing in a significant way (Table 1).

Fig. 2. Relationship between 2-methoxyestradiol (2-ME) and FSH (A) or LH (B) secretion from porcine pituitary cells *in vitro* after 72 h of incubation.

Fig. 3. Relationship between 2-methoxyestradiol (2-ME) (M/L) and concentration of superoxide anion radical (nM/mL) produced by porcine pituitary cells *in vitro*.

Table 1
Effect of 2-ME on mean MDA concentration ($\bar{x} \pm \text{SD}$) in culture medium

	Negative control	Positive control	2-ME (10^{-11} M/L)	2-ME (10^{-10} M/L)	2-ME (10^{-9} M/L)	2-ME (10^{-8} M/L)	2-ME (10^{-7} M/L)	E-2 (10^{-9} M/L)	E-2 (10^{-8} M/L)
MDA [nM/mL]	0.55 ± 0.12	0.60 ± 0.25	0.60 ± 0.19	0.55 ± 0.20	0.70 ± 0.26	0.65 ± 0.26	0.55 ± 0.07	0.55 ± 0.07	0.55 ± 0.14

Discussion

The use of 2-methoxyestradiol is suggested in the treatment of many types of cancer or cardiovascular and renal diseases (1, 2, 6, 16-18, 20, 22). Apart from its widely described auto- and paracrine effect on granulosa, theca, or oocyte cells, it can act also in an endocrine fashion on the pituitary, changing gonadotropin secretion, as a side effect. The obtained results showed that 2-ME affects secretion of gonadotropins from porcine pituitary cells *in vitro* in a concentration-dependent way. Increasing dose of 2-ME caused enhancement in FSH secretion, but compared to the E-2 this effect was less marked. Positive correlation

between 2-ME and FSH confirmed the stimulating effect 2-ME on FSH secretion from pituitary cells. In contrast to FSH, correlation between secretion of LH and dose of 2-ME was negative. It was also observed that 2-ME at relatively low concentration stimulated pituitary cells to secretion of luteinising hormone, while addition of 10^{-7} of 2-ME resulted in a decrease in LH secretion. The exact mechanism of action of 2-ME is still unclear. However, the observed relationships could be connected with dual action of 2-ME, which was dependent on its concentration. It was noted that 2-ME at low concentration had a mitogenic influence on ER-positive (oestrogen receptor positive) cells, which was related to 2-ME's residual oestrogen activity, whereas high

concentration of 2-ME exhibited mainly antiproliferative action. This effect was dependent on concentration of 2-ME and type of cells (ER positive or negative). 2-ME acted only as an antiproliferative (but not mitogenic) factor on ER-negative cells (14). Additionally, reports about mechanisms of methoxyestradiol action are ambiguous. According to studies of Spicer *et al.* (21), 2-ME stimulated production of LH and acted by binding to oestrogen receptor, but this affinity compared to oestradiol, was very low, in both: α and β form. In contrast, Shang *et al.* (19) noted that 2-ME could act by different receptor or by other undiscovered way. It is important to emphasise that it is difficult to confront entirely the results of our study with the literature data, because to our knowledge, no other reports on the effect of 2-ME on gonadotropin secretion by porcine pituitary cells have appeared so far.

Basini *et al.* (3) found that 2-ME inhibits $O_2^{\cdot -}$ generation but stimulates the activity of superoxide dismutase (SOD) in porcine granulosa cells. These data are incompatible with the findings of Das *et al.* (7) who showed that 2-ME causes death of tumour cells by the increase in superoxide anion radical generation. Additionally, Huang *et al.* (10) reported that 2-ME increases superoxide production by inhibiting the activity of cellular superoxide dismutases. In our experiment, the correlation between 2-ME and $O_2^{\cdot -}$ level was also positive, whereas MDA level did not change significantly under the influence of various concentrations of methoxyestradiol. Supposedly, the observed augmented level of $O_2^{\cdot -}$ did not cause a marked oxidative stress and lipid peroxidation in porcine pituitary cells due to relatively short time (2-72 h) of exposition to 2-ME.

The obtained results suggest that methoxyestradiol used in high concentration for the treatment of sows may decrease LH secretion and as a consequence cause some reproductive disorders, both during follicular phase of the oestrous cycle (*e.g.* delay or inhibition of ovulation resulting in ovarian cyst development) and pregnancy (abortion due to premature regression of gestational corpus luteum). Simultaneously, it did not exert the suppressive effect on FSH secretion and presumably, should not cause FSH-mediated disturbances in ovarian follicle selection and early follicular development. However, the further studies are necessary to find the reason of difference in FSH and LH secretion under the influence of high concentrations of 2-ME.

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