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PMSG influence on oestrogen secretion by porcine granulosa cells from ovarian follicles and cysts in vitro

Wpływ PMSG na wydzielanie estrogenów przez komórki ziarniste pęcherzyków i torbieli jajnikowych macior *in vitro*

SUMMARY

The aim of the study was a comparison of oestrogen secretion from the granulosa cells isolated from porcine ovarian follicles and follicular cysts in the control conditions and under the influence of PMSG (pregnant mare serum gonadotropin). The ovaries were isolated from the 5–6 years old sows, weighting on an average 283.5 ±21.7 kg, in the slaughterhouse. The granulosa cells were isolated from 6–7 mm in diameter ovarian follicles and 9–10 mm in diameter follicular cysts, and after that cultured in DMEM/F 12 (1:1) medium without gonadotropins or with 0.1, 1, 10 and 100 IU/l of PMSG, respectively. After 24 h of culture the media were collected to analyse the concentration of oestrogens (17β-oestradiol – E-2, oestrone – E-1) by high-pressure liquid chromatography (HPLC, Beckman 125 SM) method with UV detection. In the control conditions, significantly ($P \leq 0.05$) lower secretion of steroid hormones from the cystic cells was found (E-2: 2,44 ±0.45 pg, E-1: 2,46 ±0.23 pg / 5×10^5 cells/24 h) in comparison to the cells from follicles (E-2: 9,8 ±0.93 pg, E-1: 9,24 ±1.15 pg /5×10^5 cells/ 24 h). The introduction of PMSG to the culture medium, both in the case of granulosa cells from follicles and cysts resulted in significant ($P \leq 0.05$) increase in oestrogen secretion. This augmentation was in positive correlation with PMSG doses. After the 24 h period of follicle-derived granulosa cells culture with the highest dose of PMSG (100 IU) 3.9-fold increase in the quantity of secreted E-2 and 3.8-fold increase of E-1 in comparison to control was found. At the same conditions the secretion of E-2 and E-1 from cyst cells was 8.9 and 6.7-fold higher than in control. Apart from the lower quantity of steroid hormones secreted from the granulosa cells isolated from cysts in control conditions, their response to PMSG was significantly higher in comparison to the cells isolated from ovarian follicles.

Key words: PMSG, 17β-oestradiol, oestrone, granulosa cells *in vitro*, ovarian follicle, follicular cyst, sow

INTRODUCTION

Single and multiple follicular cysts compose about 6.2 % of fertility disturbances in female pigs [Heinonen *et al.* 1998]. One of the ways of the experimental cyst induction in sows is gonadotropin, especially PMSG (pregnant mare serum gonadotropin) treatment [Hall *et al.* 1993, Fitko *et al.* 1996]. However, PMSG as a gonadotropin with FSH and LH activity, also exerts many positive reproductive effects. Low doses of PMSG, especially in combination with hCG, are used for induction of oestrous in gilts and anoestrous sows after weaning [Britt *et al.* 1989, Bates *et al.* 1991, Huhn *et al.* 1996]. PMSG can stimulate the follicular growth and increase the maximal follicle diameter [Miller *et al.* 1999]. This gonadotropin also reverses the inhibition of follicle development resulting from pituitary desensitisation and additionally promotes it by inhibition of granulosa cell apoptosis [Liu *et al.* 2003]. Because this gonadotropin can both promote the development of normal ovarian follicles and induce the cyst formation, it was interesting to find out the difference in its effect on secretory activity of normal follicular and cystic granulosa cells. The answer to this question is especially important in relation to possible mechanisms of cyst formation and therapy using this gonadotropin.

Therefore, the aims of this work were:

– to study the disparity in oestrogen secretion by granulosa cells isolated from porcine ovarian follicles and follicular cysts *in vitro* without hormonal stimulation;

– to compare the PMSG effect on oestrogen secretion from the granulosa cells derived from porcine ovarian follicles and follicular cysts *in vitro*.

MATERIALS AND METHODS

The ovaries were obtained from the 5–6 years old cyclic (n = 6) and infertile (n = 5) sows, weighing on an average 283.5 ±21.7 kg, in the slaughterhouse. They were collected into Dulbecco Modified Eagle Medium (DMEM)/F 12 (1:1) contained 0.1% of BSA and transported to the laboratory within 1 h after slaughter. The granulosa cells were isolated from 6–7 mm in diameter ovarian follicles and 9–10 mm in diameter follicular cysts. After aspiration of follicular fluid, each follicle and follicular cyst was sliced open and granulosa cells were removed by gently scrapping the interior surface of the follicle wall. The cell suspensions were filtered and then centrifuged at 300 × g for 10 min. The obtained pellet was resuspended in the medium. The number of cells was estimated by their calculation using Bűrker table. The viability of the cells, determined using the trypan blue (0,4%) method, was between 90–93%. The granulosa cells were plated at a density of 5×10^5 cells per well on 24-well plates. After that the cells were cultured for the first 72 h in DMEM/F 12 (1:1) medium, supplemented with 5% of fetal calf serum, testosterone (10^{-7} M) and gentamicin (20 μg/ml) in a humified incubator at 37°C in the atmospheric air with 5% CO_2 [Khalid *et al.* 2000, Saasson *et al.* 2002]. After the attachment, the cells were cultured in serum free – DMEM/F 12 medium (1:1) without gonadotropins (control) or with 0.1, 1, 10 and 100 IU/l of PMSG, respectively. The proliferation of cells was estimated using MTT test, based on the reduction of tetrazolium salt into a blue formazan by mitochondrial dehydrogenase of viable cells. After 24 h of the culture with PMSG, the medium was collected to analyse the concentration of secreted oestrogens (17β-oestradiol – E-2, oestrone – E-1). The steroids were extracted from medium with dichloromethane and analysed by high-performance liquid chromatography (HPLC, Beckman, Gold System, USA). The analysis employed a reverse-phase analytical column (250 × 4 mm, 5 μm, LiChrospher 100, Merck, Germany). The mobile phase, consisting of 0.25% orthophosphoric acid and acetonitrile, was pumped at 0.8 ml/min (125 SM, Beckman). The separation of hormones was performed in a gradient of acetonitrile (40–100% in 20 min). The UV detection

at 220 nm (DAD 168, Beckman) was used. Oestrogen secretion was expressed as a concentration of hormone, which was released to the culture medium by about 5×10^5 granulosa cells during 24 h.

Statistical analysis of the obtained results was performed using Excel 97. The results are expressed as a mean and standard deviation ($x \pm SD$). Comparisons between the series of the results were performed using the Student's *t*-test. Statistica 5.0 was used for the regression analysis.

RESULTS

17β-oestradiol (E-2)

The secretion of E-2 from porcine cystic cells was significantly (P≤0.05) lower (2,44 ±0.45 pg/ 5×10^5 cells/24 h) than from follicular cells (9,8 ±0.93 pg/ 5×10^5 cells/24 h) in control conditions (without PMSG) – Fig. 1. Additional confirmation of the reduced secretory activity of cystic cells was the lower number of secretory granules inside the cystic in comparison to follicular cells (Fig. 2). The introduction of PMSG to the culture medium, resulted in the dose-dependent enhancement (P≤0.05) of E-2 secretion, both from cystic and follicular cells (Fig. 1, 3).

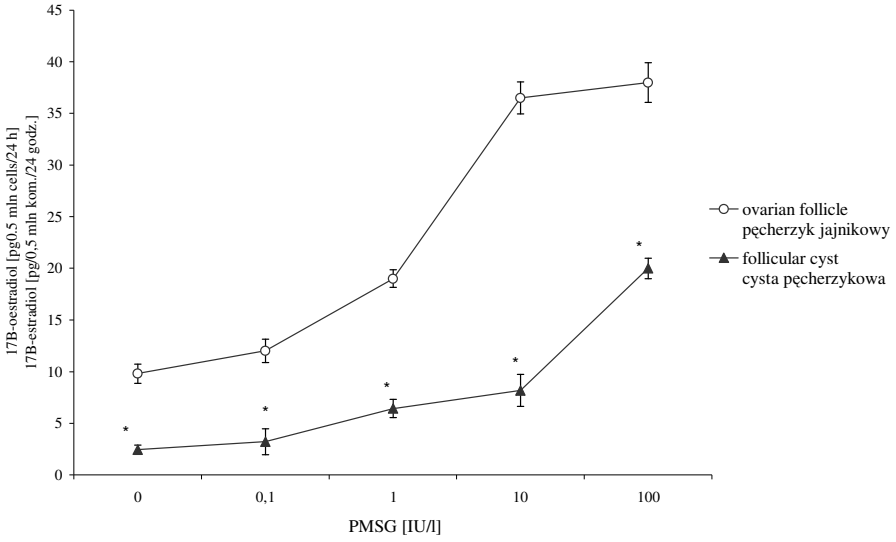

Fig. 1. The effect of PMSG on 17β-oestradiol secretion from porcine granulosa cells isolated from ovarian follicles and follicular cysts *in vitro*
* A significant (P≤0.05) difference in comparison to the value obtained for the granulosa cells from follicle

Rys. 1. Wpływ PMSG na wydzielanie 17β-estradiolu przez komórki ziarniste pochodzące z pęcherzyków jajnikowych i torbieli pęcherzykowych macior *in vitro*
* Różnica istotna statystycznie (p≤0,05) w porównaniu z wartością otrzymaną dla komórek ziarnistych pochodzących z pęcherzyka jajnikowego

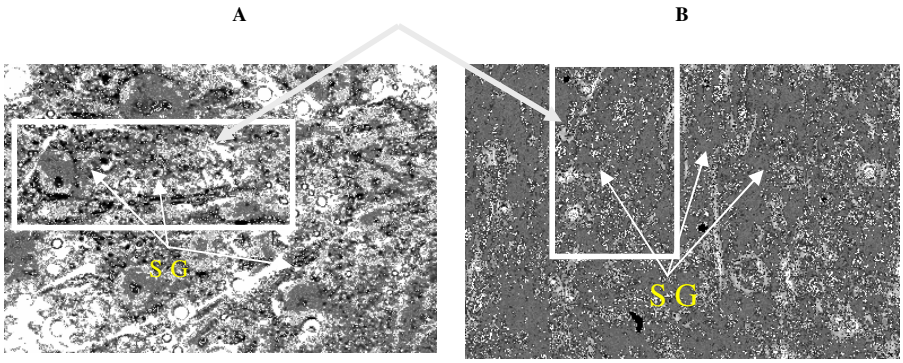

Fig. 2. Control culture (without PMSG) of granulosa cells isolated from: A – ovarian follicle; B – follicular cyst; SG – secretory granules; single granulosa cell in the centre of the marked area

Rys. 2. Kontrolna (bez PMSG) hodowla komórek ziarnistych pochodzących z: A – pęcherzyka jajnikowego; B – torbieli pęcherzykowej; SG – ziarnistości wydzielnicze; pojedyncza komórka ziarnista pośrodku zaznaczonego pola

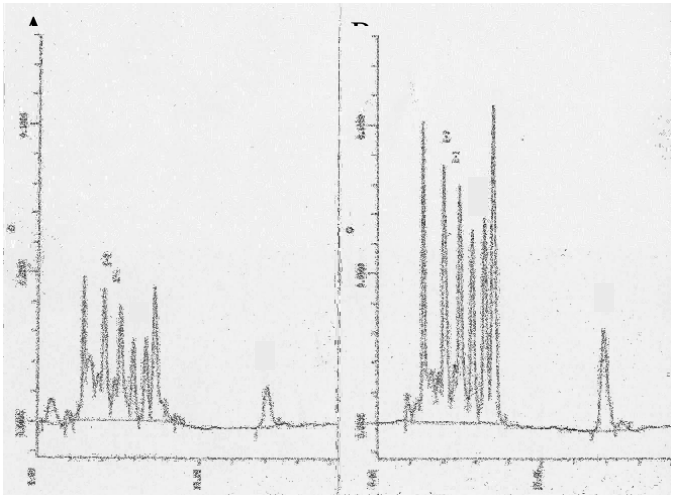

Fig. 3. HPLC separation of the steroid hormones (E-2 – 17β-oestradiol, E-1 – oestrone) secreted by granulosa cells isolated from follicular cyst: A. in control culture (without PMSG); B – during 24 h period of culture with 100 IU/l PMSG

Rys. 3. Rozdział hormonów sterydowych (E-2 – 17β-estradiol, E-1 – estron) wydzielonych przez komórki ziarniste cyst pęcherzykowych macior: A – w hodowli kontrolnej (bez PMSG); B – podczas 24 godz. hodowli z PMSG w stężeniu 100 IU/l; metodą HPLC

Both in the cystic and follicular cell culture, a positive correlation between the quantity of secreted E-2 and PMSG concentration was found. In the case of cystic cells, the correlation coefficient was higher (r = 0.96) than in the case of follicular cells (r = 0.69) (Fig. 4). Moreover, the cystic cells were more sensitive to PMSG influence than follicular cells. After the introduction of PMSG to the cystic cells culture in the highest dose (100 IU/l), the E-2 secretion was 8.9-fold higher in comparison to the non-stimulated culture, whereas in the case of follicular cells the same dose of PMSG caused only a 3.9-fold increment in E-2 secretion.

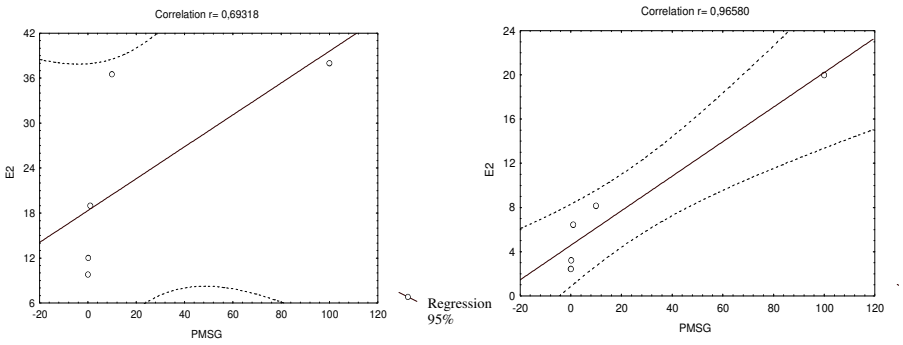

Fig. 4. A graph of linear regression for relationship between PMSG doses and the quantity of 17β-oestradiol (E-2) secreted from: A – follicular granulosa cells; B – granulosa cells isolated from follicular cysts

Rys. 4. Wykres regresji liniowej obrazujący zależność pomiędzy dawkami PMSG i ilością 17β-estradiolu wydzielonego przez: A – komórki ziarniste pęcherzyka jajnikowego; B – komórki ziarniste wyizolowane z cyst pęcherzykowych

Oestrone (E-1)

In the control conditions, the mean quantity of E-1 secreted from cystic cells (2.46 ±0.23 pg/ 5×10^5 cells/24 h) was 3.75-fold lower than in a case of follicular cells (9.24 ±1.15 pg./5×10^5 cells/ 24 h) (Fig. 5). All used PMSG doses caused increment ($P\leq0.05$) in E-1 secretion from both of the examined types of cells (Fig. 3, 5).

This increase was in positive correlation with PMSG doses both in the culture of granulosa cells from follicles (r = 0.63) and ovarian cysts (r = 0.97). The response to PMSG was higher in the case of cysitc cells, which under influence of 100 IU/l PMSG secreted 6.7-fold more E-1 than in control. The response of follicular cells to the same dose of PMSG was 3.8-higher in comparison to the control.

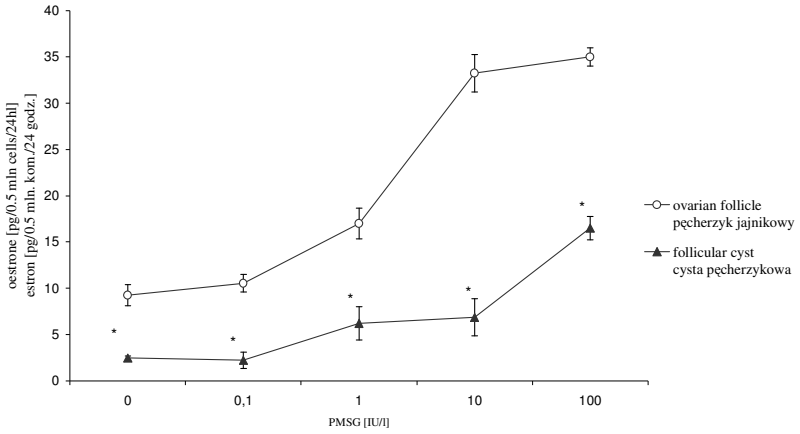

Fig. 5. The effect of PMSG on oestrone secretion from porcine granulosa cells isolated from ovarian follicles and follicular cysts *in vitro*
* A significant (P≤0.05) difference in comparison to the value obtained for the granulosa cells from follicle
Rys. 5. Wpływ PMSG na wydzielanie estronu przez komórki ziarniste pochodzące z pęcherzyków jajnikowych i torbieli pęcherzykowych macior *in vitro*
* Różnica istotna statystycznie (p≤0,05) w porównaniu z wartością otrzymaną dla komórek ziarnistych pochodzących z pęcherzyka jajnikowego

DISCUSSION

As a result of our experiment we found lower basal secretion of E-2 from porcine cystic granulosa cells in comparison to follicular cells *in vitro*. These data are at variance with the well established and obtained *in vivo* results. According to some authors [Hamilton et al. 1995, Calder et al. 2001], follicular cysts produce more E-2 than preovulatory follicles. As a consequence, the plasma E-2 concentration is usually higher in the affected than in normal females. However, it has to be pointed out that cystic cells used in our experiment were more sensitive to gonadotropin (PMSG) stimulation, than cells isolated from ovarian follicle. It may be a reason of higher plasma oestrogen level in the affected in comparison to healthy sows, especially that *in vivo* also greater concentration of LH is reported for females with ovarian follicular cyst [Hamilton et al. 1995].

Our results show that PMSG significantly stimulates the secretion of oestrogens (17β-oestradiol and oestrone) both from follicular and cystic granulosa cells *in vitro*. This stimulating effect of PMSG on secretion of oestrogens (especially E-2) by follicular cells may play a key role in the mechanism of cyst induction using this gonadotropin. It is known that LH surge is necessary to ovulation. This rapid change in LH secretion is due to surge in GnRH in response to elevated plasma E-2 concentration [Karsch *et al*. 1997]. E-2 can activate oestrogen receptors on cells in the mediobasal hypothalamus [Blache *et al*.

1991, Caraty *et al*. 1998] and by this way induce GnRH surge. But the rapid increase in E-2 secretion from granulosa cells, especially following by long-term high circulating E-2 concentration, can downregulate the expression of oestrogen receptors in the lateral mediobasal hypothalamus and by this way prevent the induction of GnRH surge in response to E-2 [Gűmen and Wiltbank 2002].

One of the ways of ovarian cyst therapy is using exogenous gonadotropins, among others, PMSG. Our results show that PMSG significantly enhances oestrogen secretion from cystic granulosa cells. It is known that the initial GnRH/LH surge can be induced by high E-2. However, E-2 induction of the subsequent surge requires exposition to progesterone, which can reinitiate sensitivity to E-2 by an increase in the number of oestrogen receptors on the cells of mediobasal hypothalamus [Blache *et al.* 1991, Hewitt and Korach 2000, Gűmen and Wiltbank 2002]. Thereby, in the case of PMSG-induced follicular cyst, repeated administration of this hormone seems to be ineffective, because it can only intensify E-2 resistance in hypothalamus by an increase in the level of circulating E-2.

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STRESZCZENIE

Celem pracy było porównanie wydzielania hormonów sterydowych przez komórki ziarniste pęcherzyków i torbieli pęcherzykowych macior pod wpływem PMSG (gonadotropina surowicy źrebnej klaczy) w warunkach *in vitro*. Jajniki pobierano od macior w wieku 5–6 lat, o średniej masie ciała 283,5 ±21,7 kg. Komórki ziarniste izolowano z pęcherzyków o średnicy 6–7 mm i cyst pęcherzykowych o średnicy 9–10 mm, a następnie hodowano w podłożu DMEM/F12 (1:1) bez gonadotropin (kontrola) oraz odpowiednio z 0,1; 1; 10 i 100 IU/l PMSG. Po 24 godz. zbierano podłoże hodowlane w celu analizy ilości wydzielonych estrogenów (17β-estradiol – E-2, estron – E-1). Stężenie hormonów w podłożu analizowano metodą wysokosprawnej chromatografii cieczowej (HPLC, Beckman 125 SM) z detekcją UV. W warunkach kontrolnych stwierdzono znacząco ($p \leq 0,05$) niższe wydzielanie estrogenów przez komórki ziarniste pochodzące z torbieli (E-2: 2,44 ±0,45 pg, E-1: 2,46 ±0,23 pg/5×10^5 komórek/24godz.) w porównaniu z komórkami wyizolowanymi z pęcherzyków (E-2: 9,8 ±0,93 pg, E-1: 9,24 ± 1,15 pg/5×10^5 komórek/24godz.). Wprowadzenie PMSG do hodowli, zarówno w przypadku komórek pochodzących z pęcherzyków, jak i z cyst spowodowało istotny ($p \leq 0,05$) i dodatnio skorelowany z dawką gonadotropiny wzrost sekrecji estrogenów. Po 24 godz. hodowli z najwyższą z zastosowanych dawek PMSG (100 IU) stwierdzono 3,9-krotny wzrost ilości wydzielonego E-2 oraz 3,8-krotny wzrost ilości E-1 przez komórki pęcherzyka w stosunku do kontroli. W tych samych warunkach eksperymentalnych wydzielanie E-2 przez komórki wyizolowane z torbieli zwiększyło się 8,9-krotnie, zaś E-1 6,7-krotnie. Pomimo mniejszej ilości sterydów wydzielanych w warunkach kontrolnych przez komórki ziarniste izolowane z torbieli jajnikowych, ich odpowiedź stymulowana przez PMSG była istotnie wyższa w porównaniu z komórkami izolowanymi z pęcherzyków.

Słowa kluczowe: PMSG, 17β-estradiol, estron, komórki ziarniste *in vitro*, pęcherzyk jajnikowy, cysta pęcherzykowa, maciora