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**Original article**

# Effects of prolactin on estrogen, cAMP and oxytocin secretion by porcine granulosa cells *in vitro*

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**Summary** — Granulosa cells were isolated from ovaries (without pre-ovulatory follicles or corpora lutea) of cycling gilts slaughtered at 8 months of age. They were cultured in the presence or absence of exogenous porcine prolactin (1, 10, 1 000, 10 000, or 100 000 pmol/ml medium). The secretion of estrogen, cAMP and oxytocin into the incubation medium was analysed by radioimmunoassay. It was found that prolactin (10, 1 000, 10 000 and 100 000 pmol/ml) inhibited estrogen secretion ($p < 0.001$) and stimulated cAMP output ($p < 0.001$, $p < 0.001$, not significant, and $p < 0.01$, respectively). Small doses of prolactin (1 and 10 pmol/ml) decreased ($p < 0.001$, $p < 0.05$), whilst high doses (1 000, 10 000 and 100 000 pmol/ml) increased ($p < 0.001$) oxytocin secretion by granulosa cell culture. The data suggest a direct action of prolactin on porcine ovarian cyclic nucleotides, steroid and nonapeptide hormone release.

**prolactin / estradiol / cAMP / oxytocin / ovary**

**Résumé — Effets de la prolactine sur la sécrétion d'œstrogène, d'AMPc et d'ocytocine par les cellules de la granulosa porcine *in vitro*.** *Les cellules de la granulosa ont été isolées d'ovaires (sans follicules préovulatoires, ni corps jaunes) de truies cycliques abattues à l'âge de 8 mois. Elles ont été cultivées en présence ou en absence de prolactine (PRL) porcine exogène (1, 10, 1 000, 10 000 ou 100 000 pmol/ml de milieu). La sécrétion d'œstrogène, d'AMPc et d'ocytocine dans le milieu d'incubation a été analysée par RIA. La PRL (10, 1 000, 10 000 et 100 000 pmol/ml) inhibe la sécrétion d'œstrogène ($p < 0,001$) et stimule la production d'AMPc ($p < 0,001$ ; $p < 0,001$ ; non significatif et $p < 0,01$ respectivement). Des petites doses de PRL (1 et 10 pmol/ml) décroissent et des doses supérieures (1 000, 10 000 et 100 000 pmol/ml) augmentent ($p < 0,001$) la sécrétion d'ocytocine par les cellules de la granulosa en culture. Ces résultats suggèrent une action directe de prolactine sur la sécrétion de nucléotides cycliques, de stéroïdes et d'ocytocine par l'ovaire porcin.*

***prolactine / œstradiol / AMPc / ocytocine / ovaire***

## INTRODUCTION

There is data to suggest that prolactin (PRL) plays an important role in the regulation of reproductive cyclicity, lactational infertility and hyperprolactinemia-associated disfunctions of the gonads. In particular, PRL receptors have been demonstrated in granulosa and luteal cells in the pig (Dusza and Tilton, 1990) and in ovarian follicles in the hamster (Roy *et al,* 1987) but not in bovine ovarian follicles (Bevers *et al,* 1988). It was demonstrated that PRL can directly inhibit the function of premature ovarian follicles, stimulate luteinization of ovarian cells and development of the corpus luteum (see reviews by McNeilly *et al,* 1982; Murphy and Rajkumar, 1985; Tsafriri, 1988; Dusza and Tilton, 1990). However, information on the direct action of PRL is limited almost entirely to an influence on ovarian steroidogenesis and some of the data is contradictory. In particular, an artifical increase in blood PRL concentration failed to change steroid levels in bovine (Bevers *et al,* 1988), ovine and porcine (Dusza, 1989; Dusza and Tilton, 1990) plasma. On the other hand, PRL inhibited steroid production by rat ovaries *in vivo* (Kalison *et al,* 1985, Adashi and Resnik, 1987), by perfused human ovaries (Demura *et al,* 1982), by human (Sato *et al,* 1985), pig (Rajkumar *et al,* 1988) and rat (Adashi and Resnik, 1987; Gitay-Goren *et al,* 1989a,b) granulosa cells, and by rat theca-interstitial cells (Magoffin and Erikson, 1982; McNeilly, 1984). Aromatase activity in isolated hen ovarian follicles (Zadworny *et al,* 1989) and rat granulosa cells (Dorrington and Gore-Langton, 1982; Wang and Chan, 1982; Gitay-Goren *et al,* 1989 a,b; Krasnow *et al,* 1990) was also inhibited. In other reports, PRL stimulated progesterone production by rat (Wang and Chan, 1982) and porcine (Einspanier *et al,* 1986) granulosa cell cultures.

PRL stimulated LH receptor formation (Murphy and Rajkumar, 1985; Adashi and Resnik, 1987), lipoprotein uptake (Murphy and Rajkumar, 1985; Rajkumar *et al,* 1988) and progesterone (Grinwich *et al,* 1983; Murphy and Rajkumar *et al,* 1988) and progesterone (Grinwich *et al,* 1983; Murphy and Rajkumar, 1985) but not androgen (Gregoraszczuk, 1985) release from porcine luteal cells. PRL also activated lipoprotein utilization and progesterone production by porcine granulosa cells luteinized in culture (Chedreese *et al,* 1988). In rat luteal cells, PRL stimulated progesterone, testosterone and estradiol release (Kalison *et al,* 1985; Menon *et al,* 1985; Murphy and Rajkumar, 1985, Gitay-Goren *et al,* 1989a). Enhanced progesterone output was observed after PRL treatment in human corpus luteum slices in culture (Hunter, 1989). In other *in vitro* experiments PRL either had no effect on porcine luteal cell progesterone production (Gregoraszczuk, 1985) or it was inhibitory (Grazul, 1985; Einspanier *et al,* 1986). Thus, although most of the available data suggest that PRL inhibits steroidogenesis in follicular tissue and stimulates it in luteal tissue there is no concensus regarding its overall effect on the ovary.

In addition to steroid hormones, the ovary produces other biologically active substances including the nonapetide hormone oxytocin and the cyclic nucleotide cAMP, which are also potent regulators of ovarian function (Guraya, 1985; Hansel and Dowd, 1986; Richards and Hedin, 1988; Tsafriri, 1988; Wathes, 1989). There is little information concerning PRL's influence on the non-steroidal ovarian substances. Gitay-Goren *et al* (1989a,b) reported that PRL inhibited cAMP release from rat granulosa cells in culture, in contrast to its effect on mammary tissue (Rillema *et al,* 1988). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine abolished the PRL effect indicating that PRL may activate phosphodiesterase within ovarian cells. In rat luteal cell culture the same authors observed PRL-induced stimulation of cAMP accumulation.

The effects of PRL on the ovarian cells of other species have not been investigated.

The aim of the present studies was to examine the role of PRL in the regulation of estrogen, cAMP and oxytocin secretion by porcine granulosa cells in culture.

## MATERIALS AND METHODS

### *Preparation and culture of granulosa cells*

Ovaries in the late and middle follicular phase of the estrous cycle were obtained from Slovakian white gilts, 8 months of age without visible reproductive abnormalities, at a local slaughterhouse. Ovaries with preovulatory or hemorrhagic follicles were excluded. One hour after killing, the contents of 2–5 mm diameter follicles were aseptically aspirated with a syringe. Granulosa cells were separated from follicular fluid by 5 min centrifugation at 200 *g* and resuspended in sterile TCM 199 (Sigma, St Louis, USA) supplemented with 5% bovine fetal serum (Institute of Veterinary Medicine, Brno, Czechia). The sequence of centrifugation and resuspension was carried out 3 times. After the final centrifugation, granulosa cells were resuspended in incubation medium TCM 199 supplemented with 10% bovine fetal serum, 10 mIU/ml insulin (Lečiva, Prague, Czechia), 200 μg/ml pyruvate Na (Serva, Heidelberg, FGR), 600 μg/ml lactate Ca (Serva, Heidelberg, FRG) 50 mg/ml gentamicin (Pharmachim, Sophia, Bulgaria). A 2 ml portion of suspension (concentration $10^6$ cells/ml) was transferred to cell-cult plate wells (Sterilin Ltd, Feltham, England) and incubated at 38.5°C in 5% $CO_2$ in humidified air. Cell viability (determined by trypsan blue stain) was 75–85%. After 2 d culture, when practically all the cells were attached to the bottom of the wells, the medium was replaced. PRL was added to the medium of experimental groups to final concentrations of 1, 10, 100, 1 000, 10 000, or 100 000 pmol/ml. USDA pPRL B-1, a pure PRL preparation of analytical grade for radioimmunoassay (RIA) standards, was kindly given by Dr AF Parlow (Harbor–UCLA Medical Center, Torrance, USA) to Dr GV Marinchenko (Research Institute of Animal Breeding and Genetics, St Petersburg, Pushkin, Russia) and was used in all experiments. The preparation was dissolved immediately before the addition to the medium. After 2 d culture, the medium was gently aspirated with a syringe and frozen at −40°C to await hormone analysis. Counting the cells at this point revealed an approximately 2-fold increase in cell number. Viability varied between 55 and 65%. No statistically significant influence of PRL treatment on cell number or viability was observed. In an initial experiment, medium samples were collected after 2 d culture in the absence of cells so as to determine any background hormone concentration.

### *Radioimmunoassays*

Estrogen, cAMP and oxytocin concentrations were determined in duplicate by RIA without extraction.

Estrogen concentrations were measured with commercial kits for estradiol measurement of the Institute for Radioecology and Application of Nuclear Techniques (URVJT, Kosice, CFSR) following the manufacturers' instructions. The sensitivity of determination was 0.025 pmol/ml. The cross-reaction of the antiserum was 25% with estron, 1.84% with estriol, and less than 0.001% with 20-OH progesterone, androstenedione, testosterone, cortisol and cortisone. The inter- and intraassay coefficients of variation did not exceed 3 and 4%, respectively.

cAMP concentrations were determined using RIA kits from the Institute for Research, Production and Application of Radioisotopes (UVVVR, Prague, CSFR). The sensitivity was 0.05 fmol/ml. The cross-reaction of the antiserum with cGMP was less than 0.01% and with AMP, ADP and ATP less than 0.003%. The inter- and intraassay coefficients of variation did not exceed 7 and 9%, respectively.

Oxytocin levels were measured by kits from UVVVR. The sensitivity was 1.2 fmol/ml. The antiserum cross-reacted at less than 0.005% with arginine-vasopressin, 0.04% with lysine-vasopressin, 17% with arginine-vasotocin and 22.6% with desamino-oxytocin. Interassay coefficient of variation varied between 11 and 12%, and the intraassay coefficient of variation did not exceed 9%.

### *Statistics*

The data presented are the values obtained from 3 separate experiments, each using a pool of granulosa cells isolated from 30–40 ovaries. Each

experimental group was represented by 4 culture wells. Since the serum-supplemented medium contained small amounts of estradiol, cAMP and oxytocin (table I), the pre-culture medium for each experimental group was used as a background control. Rates of estrogen, cAMP and oxytocin secretion were calculated to $10^6$ viable cells/d. Significant differences between the groups were evaluated by ANOVA, followed by Duncan's test for individual significant differences.

## RESULTS

Initial experiments (table I) showed that significant quantities of estrogen, cAMP and oxytocin accumulate in the medium during the culture of granulosa cells. After 2 d, the concentrations of these substances in the medium exceeded initial levels by 2.4–2.6 times ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively). Addition of PRL influenced the secretion of substances as follows. PRL doses of 10 nmol/ml and above significantly ($p < 0.001$) reduced estrogen release (fig 1). PRL doses of 10 and 1 000 pmol/ml significantly ($p < 0.001$) increased cyclic nucleotide CAMP accumulation (fig 2). Higher PRL doses produced a lower level of stimulation and the effect at 10 000 pmol/ml was not significant. Low PRL doses (1 or 10 pmol/ml) decreased oxytocin release ($p < 0.001$ and $p < 0.05$ respectively) whilst doses of 1 000 pmol/ml and above were highly stimulatory ($p < 0.01$) (fig 3).

**Table I.** Estrogen, cAMP and oxytocin concentrations in porcine granulosa cell incubation medium before and after 2 d of culture.

| *Substance analyzed* | *Concentration* | | |
|---|---|---|---|
| | *Before culture* | *After 2 d culture* | *Differences* |
| Estrogen (pmol/ml) | 0.86 ± 0.28 | 2.22 ± 0.48 | $p < 0.001$ |
| cAMP (fmol/ml) | 0.34 ± 0.04 | 0.80 ± 0.04 | $p < 0.001$ |
| Oxytocin (fmol/ml) | 19.68 ± 0.62 | 48.50 ± 11.42 | $p < 0.001$ |

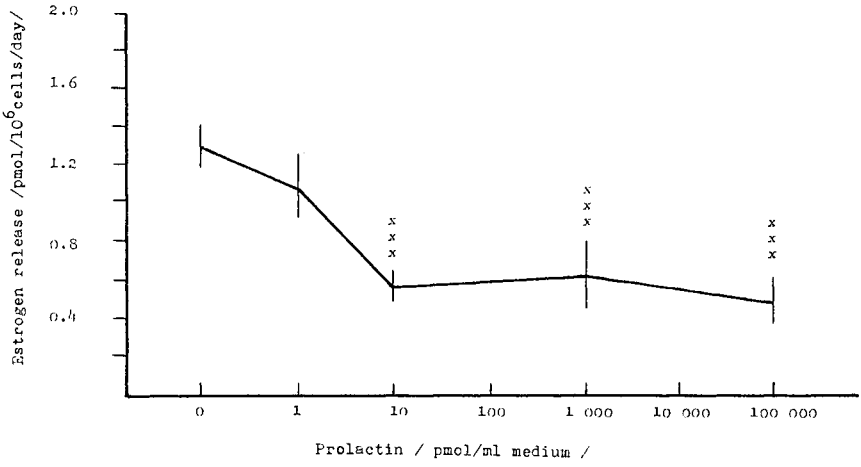


**Fig 1.** Effect of prolactin on estradiol-17β secretion by porcine granulosa cells *in vitro*. Values are means ± SEM. xxx: highly significant difference ($p < 0.001$) compared with control (without prolactin addition).

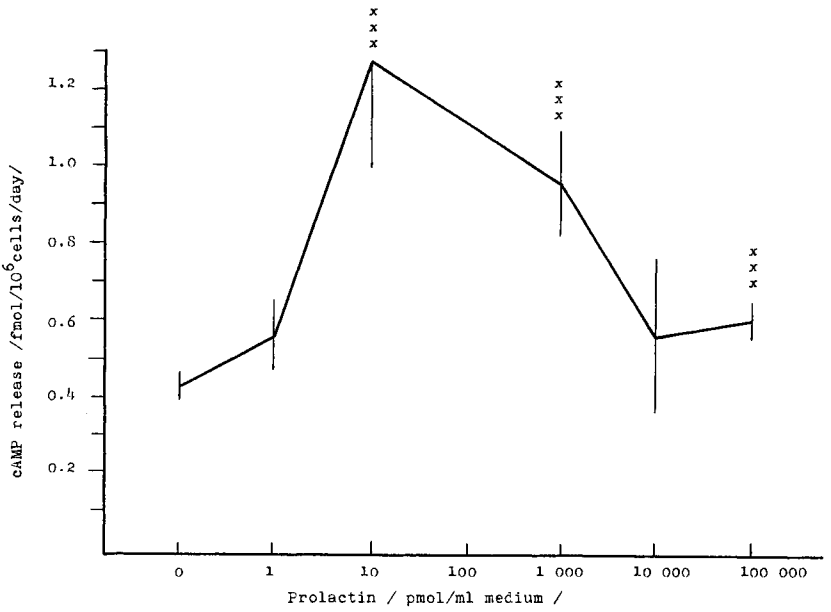


**Fig 2.** Effect of prolactin on cAMP release by porcine granulosa cells *in vitro*. Values are means ± SEM. xx: $p < 0.01$; xxx: $p < 0.001$ compared with control (without prolactin addition).

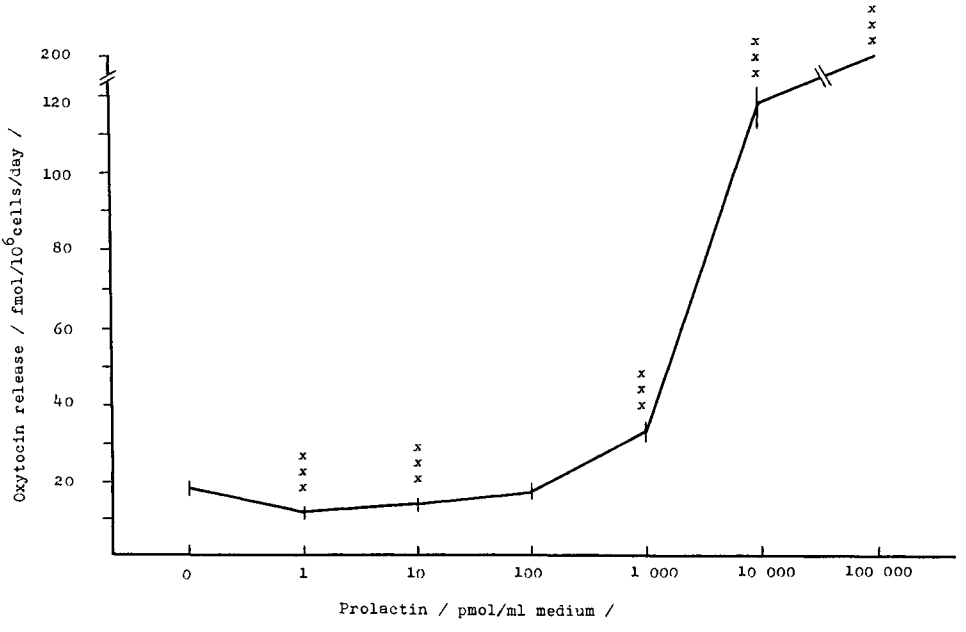


**Fig 3.** Effect of prolactin on oxytocin release by porcine granulosa cells *in vitro*. Values are means ± SEM. x: $p < 0.05$; xxx: $p < 0.001$ compared with control (without prolactin addition).

## DISCUSSION

The results presented in table I are consistent with available data (Guraya, 1985; Wathes, 1989) concerning the ability of ovarian cells to release estradiol, cAMP and oxytocin *in vitro.* The significant inhibition of porcine granulosa estrogen output under the influence of PRL (fig 2) is in agreement with other reports on the suppressive effect of PRL on estradiol release (Dorrington and Gore-Langton, 1982; Wang and Chan, 1982; Gitay-Goren *et al,* 1989ab; Krasnow *et al,* 1990), particularly in hen (Zadworny *et al,* 1989) and human (Demura *et al,* 1982) ovarian follicular cells. It has been shown (Ledwitz-Rigby, 1987; Einspanier *et al,* 1991) that, as in other mammals (Guraya, 1985), the luteinization of porcine follicles *in vivo* and *in vitro* is associated with a decrease in estradiol release and with the subsequent stimulation of progesterone output. Figure 1 may suggest that PRL stimulated the luteinization of our granulosa cells in culture. Together with the information in the *Introduction* about PRL-induced inhibition of the production of other steroids by ovarian follicular cells, our results may support the hypothesis (McNeilly *et al,* 1982; Murphy and Rajkumar, 1985; Dusza and Tilton, 1990) that during corpus luteum development and lactation a high blood PRL level can inhibit follicular development, steroidogenesis and ovulation and maintain corpus luteum function.

These effects may be mediated by cAMP-dependent intracellular mechanisms. In rat follicular cells, PRL-induced inhibition of progesterone release was accompanied by stimulation, and PRL-activated progesterone secretion in rat luteocytes by reduction of cAMP accumulation in the culture medium (Gitay-Goren *et al,* 1989a,b). In the granulosa cells, PRL prevented the steroidogenic effects of forskolin (a stimulator of cAMP production) (Krasnow *et al,* 1990), but not of 3-isobutyl-1-methylxanthine (a blocker of phosphodiesterase) (Gitay-Goren *et al,* 1989b). In other words PRL can act at the level of cAMP degradation rather than on cAMP synthesis. In our experiments, as with rat luteal cells, treatment with PRL resulted in cAMP stimulation. The PRL effect had biphasic characteristics (fig 2). The mechanism and biological significance of this phenomenon remains unknown but it may confirm available data (Gitay-Goren *et al,* 1989ab; Krasnow *et al,* 1990) about cAMP involvement in the PRL action on the ovary. It is to be noted that the effects of PRL on other target tissues, for example on mammary gland, are probably not mediated by cAMP (Rillema *et al,* 1988).

The observed PRL-induced changes in ovarian cAMP release may be associated with the regulation of the secretion of steroids or of other substances, for example, oxytocin. In our experiments a significant PRL biphasic effect on oxytocin secretion was observed: low PRL doses inhibited, and high doses stimulated nonapeptide hormone output (fig 3). It is however important to note that the stimulatory effect of prolactin was observed only at supraphysiological doses ($\geq$ 1 000 pmol/ml). In the available literature we failed to find any reports concerning PRL influence on ovarian oxytocin. The cause and significance of such biphasic PRL effects on oxytocin release remain to be found. One can suggest that the stimulation of oxytocin release under the influence of high doses of PRL may reflect a luteotropic role of PRL; in ruminants the luteinization of granulosa cells is associated with the increase of oxytocin produciton (Wathes, 1989; Wathes *et al,* 1992). It cannot be excluded that the PRL-stimulated oxytocin release is due to the stimulation of cAMP production since the cAMP analogue dbcAMP or 3-isobutyl-1-methylxanthine can stimulate oxytocin release by bovine granulosa cells in culture (Wathes, 1989).

Thus the present observations, together with published reports, suggest the hypo-

thesis that the first step in the action of PRL on ovarian cells includes the stimulation of cAMP formation and/or the inhibition of cAMP catabolism. The second step may be a cAMP-induced luteinization of the cell including specific changes in steroidogenesis (inhibition of estrogen and activation of progesterone secretion) and the stimulation of oxytocin release. On the other hand, a feedback effect of oxytocin (Nitray and Sirotkin, 1992) and steroid hormones (Sirotkin and Nitray, 1993) on cAMP release, as well as a reciprocal influence of steroids on oxytocin (Wathes, 1989; Sirotkin and Nitray, 1992) can also occur.

The fine interrelationships between ovarian cyclic nucleotides, steroid and nonapeptide hormones require further investigation, but these data suggest that PRL influences all these 3 classes of biologically active substances, playing an important role in the regulation of ovarian function.

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