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Effect of Growth Hormone on Basal and LH-Stimulated Steroid Secretion by Chicken Yellow Ovarian Follicles. An *In Vitro* Study*

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The aim of the present study was to determine the effect of growth hormone (GH) on basal and LH-regulated steroid secretion by yellow hierarchical follicles before and after maturation, and the granulosa and theca layers of the largest preovulatory follicles during the ovulatory cycle in the chicken. In the first experiment, whole yellow follicles (8-12 mm, 12-18 mm, 18-24 mm and 24-30 mm) isolated from 15 and 17-18 week-old chickens were used. In the second experiment, the granulosa and theca layers of the 3 largest yellow preovulatory follicles (F3<F2<F1) were isolated from the ovary of the laying hen (age 21 weeks) at stages 22 h and 3 h before F1 ovulation. The collected follicles and fragments of the granulosa and theca layers were randomly assigned to Eagle's medium containing 0 (control), chicken GH (cGH; 10 ng/ ml), ovine LH (oLH; 10 ng/ml) or cGH+oLH (10 ng/ml+10 ng/ml) and incubated for 24 h at 38°C. Following incubation, the progesterone and estradiol levels were determined in collected medias by RIA. It was found that cGH (1) elevated basal progesterone secretion by all classes of yellow follicles, (2) decreased estradiol secretion by the largest (18-30 mm) follicles and (3) attenuated to the control level the LH-stimulated estradiol secretion by the theca layer of F3 and F2 follicles at 22 h before ovulation. These results suggest that GH directly stimulates progesterone secretion and inhibits estradiol secretion by yellow hierarchical ovarian follicles in chicken. However, the effect of GH on separated granulosa and theca layers of the preovulatory follicles is not as pronounced as on whole follicles. It cannot be excluded that GH temporarily modulates LH action in estradiol production by the theca cells of chicken preovulatory ovarian follicles.

Key words: GH, LH, estradiol, progesterone, ovary, chicken.

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The chicken ovary consists of two different populations of growing follicles: prehierarchical white (<4 mm in diameter) and yellowish (4-8 mm), and yellow preovulatory (>8-36 mm) follicles. Preovulatory follicles are arranged in a hierarchy and are identified according to size, with the largest (F1) follicle destined for the next ovulation, the second largest (F2) follicle to ovulate the following day, and so forth. In prehierarchical follicles the granulosa layer is steroidogenically incompetent whereas the theca is the source of ovarian estradiol. In hierarchical yellow follicles both layers are steroidogenically active: granulosa and theca layers produce progesterone and estradiol, respectively (HUANG *et al*. 1979; BAHR *et al*. 1983; TILLY *et al.* 1991a, b; HRABIA *et al*. 2004). Follicular steroidogenesis is principally regulated by pituitary gonadotropins and their action is modulated by numerous endocrine and locally produced in the ovary regulators. Growing evidence indicates that one of these is growth hormone (GH). A recent study revealed that the chicken ovary is an extra-pituitary site of GH synthesis and secretion as well as a GH-responsive organ. GH mRNA and protein expression were found in all tissues of the chicken ovary before and after maturation (HRABIA *et al*. 2008; AHUMADA-SOLÓRZANO *et al*. 2012) and GH receptor expression was also demonstrated in each compartment of the chicken ovary (HECK *et al*. 2003; LEBEDEVA *et al*. 2004; HRABIA *et al*. 2008). In addition, increase in chicken ovarian weight and the number of ovarian follicles as well as elevated cell proliferation and attenuated cell apoptosis in the ovary before pu-

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berty was observed after recombinant chicken GH (cGH) treatment (HRABIA *et al*. 2011). Furthermore, in the chicken, a pronounced increase in the progesterone content in the ovary just before and at the time of maturation as well as an increase in estradiol content before the first oviposition was reported after cGH injections (HRABIA *et al*. 2011). Our recently published data have shown a stimulatory effect of GH on estradiol release by intact prehierarchical (white and yellowish) follicles (HRABIA *et al*. 2012). Moreover, dose-dependent action of GH on progesterone synthesis in the primary culture of the granulosa cells of F2 preovulatory follicles and the mRNA expression of enzymes involved in progesterone synthesis, i.e. cholesterol side-chain cleavage enzyme (P450SCC) and 3β-hydroxysteroid dehydrogenase (3β-HSD) was also revealed (AHUMADA-SOLÓRZANO *et al*. 2012). These observations highlight the possibility that GH is involved in the control of ovarian steroidogenesis in chicken, however, the role of GH in the regulation of steroid synthesis is still incompletely known. Accordingly, in the present study we examine the effect of GH on progesterone and estradiol secretion by whole yellow follicles before and after maturation. Since in mammals GH may influence ovarian steroidogenesis indirectly, at least in part, by modulation of LH action (JIA *et al*. 1986; SPICER *et al*. 1992), the interrelationship between GH and LH on progesterone and estradiol secretion by isolated yellow follicles of the chicken ovary has also been evaluated. Additionally, to examine whether the effect of GH on steroid secretion changes during the ovulatory cycle, the effect of GH on basal and LH-regulated steroid secretion by the granulosa and theca layers of the three largest preovulatory follicles was analysed at two stages of the ovulatory cycle.

Material and Methods

Animals

Experiments were performed on Hy-Line Brown hens (n=62) at the age of 15 and 17-18 (Exp. 1) and 21 (Exp. 2) weeks, caged individually under a light schedule of 14L:10D (light on at 0800 h) with free access to commercial food and water. In laying hens, individual lay patterns were monitored daily. Based on recording of oviposition time, cloacal palpation and autopsy, it was found that ovulation occurred about 5 min after oviposition of the previous egg in the series. All procedures were carried out according to research protocols approved by the local animal ethics committee in Kraków, Poland (No. 50/OP/2004).

Reagents

Bovine serum albumin (BSA), antibiotic antimycotic solution 100× (Sigma, St Louis, MO, USA), Eagle'a medium (Laboratory of Sera and Vaccines, Lublin, Poland), ovine LH (oLH; NIADDK-oLH-26; Dr. A.F. Parlow, National Hormone and Pituitary Program, USA), progesterone – DSL-3900 and estradiol – DSL-43100 kits (Diagnostic Systems Laboratories, Inc., Webster, Tex, USA), recombinant cGH was prepared as described by PACZOSKA-ELIASIEWICZ *et al*. (2006) and purchased from Protein Laboratories Rehovot (Rehovot, Israel).

Incubation procedure

Experiment 1

In the first experiment, to examine the effect of GH on basal and LH-stimulated steroid secretion by whole, yellow ovarian follicles in the chicken during maturation, the hens were decapitated about 2.5 weeks before the predicted onset of egg laying (n=24) and after the first or second oviposition (n=24). Yellow follicles were isolated from the ovaries and divided according to diameter into 4 groups: 8-12 mm, 12-18 mm, 18-24 mm and 24-30 mm. Whole follicles were incubated according to the method of YU *et al*. (1992) for 24 h at 38°C and atmosphere of 95% air and 5% CO_2 in Eagle'a medium supplemented with 0.05% bovine serum albumin, 2 μl/ml antibiotic-antimycotic solution (10000 units penicillin, 10 mg streptomycin and 25 μg amphoteracin B/ml) (control) or with the addition of recombinant cGH at a dose of 10 ng/ml, oLH at a dose of 10 ng/ml or with both hormones i.e. cGH and oLH (10 ng/ml + 10 ng/ml). Follicles of diameter 8-12 mm were incubated in 1 ml of medium in a 24-well multidish and each larger follicle in a glass container in a volume of incubation medium calculated as previously in YU *et al*. (1992): Vmedium = weight of follicle/ yolk density ($0.781 g \cdot cm^{-3}$). The dose od cGH and oLH was applied after our earlier *in vitro* study (HRABIA *et al*. 2012; SECHMAN *et al*. 2009, 2011, 2014). After incubation, the medium was collected and stored frozen for further steroid hormones analysis. The secretion of estradiol and progesterone was expressed per follicle per 24 h.

Experiment 2

To examine whether the effect of GH on steroid secretion depends on the stage of the ovulatory cycle, in a separate experiment hens were decapitated 22 h (n=7) and 3 h (n=7) before expected ovulation and the ovaries were dissected. The largest yellow preovulatory follicles F3-F1 (F3<F2<F1) were isolated from the ovary and the granulosa and theca layers were separated. Each layer was divided into 4 equal pieces, placed in separate wells of a 24-well multidish and incubated in 1 ml of Eagle'a medium supplemented as in the previous experiment with BSA, antibiotic-antimycotic solution and hormones. Following 24 h incubation as above, the medium was collected for steroid analysis and tis-

sue for protein determination by Lowry's method. The estradiol concentration was determined in medium after the theca layer culture. The progesterone concentration was measured in medium after the granulosa layer culture. The secretion of steroids was expressed per milligram of protein per 24 h.

Steroid hormone concentration measurement

Progesterone and estradiol concentrations in collected medias were measured radioimmunologically using commercially available kits (DSL). The detection limits of progesterone and estradiol assay were 120 pg/ml and 11 pg/ml and the mean recoveries were 96.3% and 94.2%, respectively. The intra- and interassay coefficients of variation for progesterone were 6.6% and 11.7%, and for estradiol 3.3% and 7.3%, respectively. The cross reactivities of progesterone antiserum with pregnenolone, testosterone and cortisol was undetectable. In the case of estradiol antibodies, they exhibited 3.4% cross-reactivity with estron and 0.75% with estriol.

Statistical analysis

Data were analysed statistically by two-way ANOVA followed by Duncan's multiple range test. Log transformations were performed as needed to maintain homogeneity of variance. Student's *t* test or the Mann-Whitney test was applied for comparison of two means. Values are expressed as the mean ± SEM from 6-12 (Exp. 1) or 7 (Exp. 2) ovaries and considered significantly different at $P<0.05$. Analyses were performed using Sigma Stat 2.03 (Systat Softwere GmbH, Berlin, Germany).

Results

Effect of cGH on basal and oLH-regulated steroid secretion *in vitro* by chicken yellow ovarian follicles during maturation (Experiment 1)

Secretion of steroids by ovarian follicles determined at the age of 15 and 17-18 weeks, i.e. 2.5 weeks before and just after the onset of egg laying did not differ significantly. Thus, the results for particular population of the follicles from both ages were added together and expressed as mean ± SEM from 6-16 determinations.

Table 1 shows the effects of cGH, oLH and their combination on progesterone secretion. The concentration of progesterone in medium from yellow follicles <12 mm was under the sensitivity of the method. Progesterone secre-

Table 1

Effects of cGH, oLH and their combination on progesterone secretion (ng/follicle/24h) by chicken intact yellow ovarian follicles during maturation. Each value represents the mean ± SEM of 6-16 determinations. *P<0.05, **P<0.01, ***P<0.001 – compared to control group. nd – not detected

Follicles	Group			
	control	cGH	oLH	cGH + oLH
8-12 mm	nd	nd	nd	nd
12-18 mm	0.41 ± 0.04	1.77 ± 0.33*	0.47 ± 0.06	0.48 ± 0.07
18-24 mm	2.08 ± 0.30	3.99 ± 0.58**	5.94 ± 0.96***	9.22 ± 2.10***
24-30 mm	2.74 ± 0.49	24.7 ± 8.80**	13.5 ± 5.59**	48.2 ± 17.80*

Table 2

Effects of cGH, oLH and their combination on estradiol secretion (pg/follicle/24h) by chicken intact yellow ovarian follicles during maturation. Each value represents the mean ± SEM of 6-16 determinations. *P<0.05, **P<0.01, ***P<0.001 – compared to control group

Follicles	Group			
	control	cGH	oLH	cGH + oLH
8-12 mm	293 ± 35.3	254 ± 34.1	440 ± 38.2*	514 ± 59.1*
12-18 mm	841 ± 109	705 ± 100	1129 ± 155	1244 ± 189
18-24 mm	923 ± 122	454 ± 77.0*	786 ± 139	753 ± 230
24-30 mm	164 ± 37.6	68 ± 12.0*	124 ± 27.1	580 ± 139*

tion (ng/follicle/24 h) by yellow follicles >12-30 mm gradually increased along with follicle development. Chicken GH (10 ng/ml) increased ($P<0.05$, $P<0.01$) basal progesterone secretion by all sizes of follicles. Ovine LH at a dose of 10 ng/ml increased ($P<0.01$, $P<0.001$) progesterone release by follicles 18-30 mm. Treatment of the follicles with a combination of cGH + oLH stimulated ($P<0.05$, $P<0.001$) basal progesterone secretion by follicles 18-30 mm. As compared to the individual effect of oLH, combined treatment with cGH + oLH tended to intensify progesterone secretion, but the increase was statistically insignificant ($P<0.05$).

In the control group estradiol secretion (pg/follicle/24h) increased along with follicle size enlargement from 8-12 mm to 12-24 mm and next decreased by follicles 24-30 mm (Table 2). cGH did not alter estradiol secretion by follicles 8-18 mm, but reduced ($P<0.05$) estradiol secretion by 18-30 mm follicles. oLH added to the medium increased ($P<0.05$) estradiol release by follicles 8-12 mm, while it did not affect estradiol secretion by larger yellow follicles. Co-treatment with cGH + oLH elevated ($P<0.05$) estradiol secretion by follicles 8-12 mm and 24-30 mm (Table 2).

Effect of cGH on basal and oLH-stimulated progesterone and estradiol secretion in vitro by the granulosa and theca layers of yellow preovulatory follicles at two stages of the ovulatory cycles (Experiment 2)

During passage of the follicle from F3 to F1 position, progesterone secretion (μg/mg protein/24h) at the stage 22 h before F1 ovulation increased ($P<0.05$) from 2.25 ± 0.20 to 4.45 ± 0.73 (Fig.1a), whereas 3 h before ovulation from 3.31 ± 0.46 to 14.5 ± 1.86 (Fig.1b). Progesterone secretion by the granulosa layer of examined follicles in the presence of cGH in the medium was not changed at either stage. oLH elevated ($P<0.01$) progesterone secretion by the granulosa layer of F3, F2 and F1, respectively by 688%, 689% and 817% at 22 h before ovulation (Fig.1a) and by follicles F3 and F2 by 761% and 608% at 3 h before ovulation (Fig.1b). Co-treatment of the granulosa layer with both hormones stimulated ($P<0.01$) progesterone secretion by F3-F1 at 22 h before ovulation by 762%, 677%

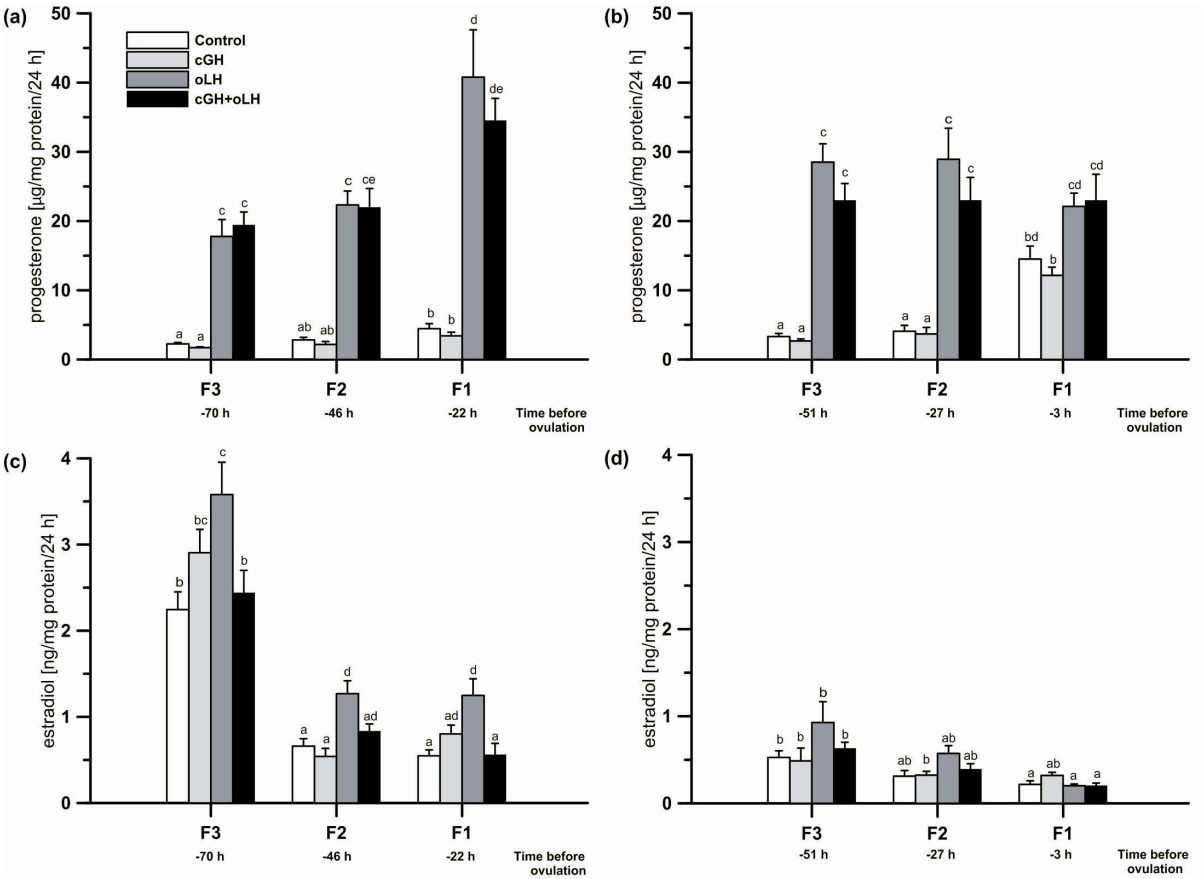

Fig. 1. *In vitro* effect of cGH on basal and oLH-stimulated progesterone secretion by granulosa layer (a, b), and on estradiol secretion by the theca layer (c, d) of preovulatory F3-F1 (F3<F2<F1) chicken ovarian follicles isolated at 22 h (a, c) and 3 h (b, d) before F1 ovulation. Each value represents the mean ± SEM from 7 determinations. Values marked with different letters differ significantly ($P<0.05$).

and 676%, respectively (Fig.1a), and by follicles F3 and F2 at the 3 h stage by 594% and 523%, respectively (Fig.1b). Combined treatment with cGH + oLH did not differ significantly in comparison to the individual effect of oLH (Fig. 1a, b).

Along with follicle enlargement from the F3 to F1, estradiol secretion (ng/mg protein/24h) at stage 22 h before F1 ovulation decreased ($P<0.05$, $P<0.01$) from 2.24 ± 0.20 to 0.55 ± 0.07 (Fig. 1c) and at stage 3 h before ovulation from 0.53 ± 0.07 to 0.22 ± 0.04 (Fig. 1d). cGH had no effect on basal estradiol secretion by the theca layer of any of the examined follicles at either stage of the ovulatory cycle (Fig. 1c, d). oLH stimulated ($P<0.05$) estradiol secretion by the theca layer of F3, F2 and F1 follicles at 22 h before F1 ovulation by 59%, 92% and 128%, respectively (Fig. 1c). At stage 22 h, the amount of estradiol secreted by the theca layer of F3 and F1 follicles in the presence of cGH + oLH was lower ($P<0.05$) by 32% and 55%, respectively, than in the presence of oLH alone (Fig. 1c).

Discussion

To the authors' knowledge, this is the first study that clearly demonstrates the *in vitro* effect of GH on basal and LH-regulated progesterone and estradiol secretion by yellow chicken ovarian follicles at different maturation stages.

First, in the *in vitro* experiment we demonstrated the effect of cGH on progesterone and estradiol secretion by intact yellow follicles of different classes isolated from the chicken ovary before and just after maturation. It was found that progesterone secretion (ng/follicles/24 h) by yellow follicles increased along with their progressing maturation, whereas estradiol secretion by yellow follicles (pg/follicles/24 h) was as follows: 12-18 mm>18-24 mm>8-12 mm≥24-30 mm. Profiles of examined steroid secretion by follicles were in agreement with previous observations (YU *et al*. 1992; HRABIA *et al*. 2004; SECHMAN *et al*. 2009, 2011). cGH present in the incubation medium augmented basal progesterone secretion by all classes of yellow follicles, whereas it attenuated estradiol secretion by the largest (18-30 mm) follicles. In a recent *in vivo* study in which chickens were injected with cGH during maturation, we observed an elevated content of progesterone in the ovary just before and at the time of maturation and increased content of estradiol before the onset of egg laying (HRABIA *et al.* 2011). Furthermore, we revealed stimulation of estradiol secretion by whole prehierarchical ovarian follicles (1-8 mm) after cGH treatment (HRABIA *et al*. 2012). Taking into consideration the former and present results, we conclude that in the chicken ovary GH promotes progesterone production, whereas its effect on estradiol synthesis depends on follicular maturation; it stimulates the production of this steroid in small follicles and inhibits it in large follicles. oLH added to the medium elevated progesterone secretion by large (18-30 mm) yellow follicles and estradiol release by small (8-12 mm) yellow follicles. A stimulating effect of oLH on progesterone and estradiol secretion by preovulatory yellow follicles was also found in previous studies (ROBINSON & ETCHES 1986; SECHMAN *et al*. 2011, 2014). cGH did not affect the oLH-stimulated progesterone secretion by the examined follicles. Only in the case of yellow large follicles (18-30 mm), a tendency for a synergistic effect of cGH +oLH on progesterone secretion was observed. Strong stimulatory action of cGH +oLH on estradiol secretion by the largest yellow follicles was surprising and difficult to explain since oLH alone did not change and cGH decreased its secretion.

Participation of GH in the regulation of steroidogenesis in the chicken ovary may be related to its effect on mRNA synthesis and/ or activity of enzymes involved in this process. It was found that in porcine granulosa cells GH increased expression of cholesterol side-chain cleavage enzyme (P450scc) mRNA, an enzyme which converts cholesterol to pregnenolone (XU *et al*. 1997). In the ovary of women (TAPANAINEN *et al*. 1992), pig (RAK & GREGORASZCZUK 2008) and seatrout (SINGH & THOMAS 1993) GH changes P450aromatase activity. Increased activity of 3β-HSD in human (TAPANAINEN *et al*. 1992) and porcine (GREGORASZCZUK *et al*. 2000) ovaries after GH treatment was also observed. However, in the chicken, the granulosa cells of F2 follicles cultured in a medium supplemented with GH did not change 3β-HSD mRNA expression, whereas it increased the expression of P450scc mRNA, a rate-limiting enzyme during progesterone synthesis (AHUMADA-SOLÓRZANO *et al*. 2012). Thus, it can not be excluded that the stimulating action of GH on progesterone secretion by intact follicles noted also in the current investigation may reflect its effect on P450scc mRNA expression.

Second, to examine whether the effect of cGH on progesterone and estradiol secretion changes during the ovulatory cycle of the laying hen, the three largest yellow follicles F3-F1 were isolated 22 h and 3 h before F1 ovulation. Analysis of *in vitro* progesterone secretion by the granulosa layer and estradiol secretion by the theca layer showed that during the final phase of follicular maturation the progesterone secretion increased, whereas estradiol decreased. These results were in agreement with previous findings on isolated layers of the follicular wall of hen ovarian follicles (MARRONE & HERTELENDY 1983; HRABIA *et al*. 2004; SECHMAN *et al*. 2014). Steroid secretion by the granulosa and

theca layers of F3-F1 correlated with the examined steroid concentrations in the tissues during the ovulatory cycle (BAHR *et al*. 1983; ETCHES & DUKE 1984; KATO *et al*. 1995) and activity of key enzymes in progesterone and estradiol synthesis – 3β-HSD (ARMSTRONG 1985; NITTA *et al*. 1993; SECHMAN *et al*. 2014) and P450aromatase (KATO *et al*. 1995; SECHMAN *et al*. 2014), respectively. oLH stimulated progesterone secretion by the granulosa layer of F3-F1 follicles at 22 h and F3-F2 follicles 3 h before ovulation as well as estradiol secretion by the theca layer of F3-F1 follicles at 22 h but not 3 h before ovulation. A lack of secretory response of follicles, especially the theca cells, to oLH 3 h before ovulation is probably the result of decreased expression of LH receptors. ZHANG *et al*. (1997) observed reduced LH receptor mRNA expression in the theca layer along with follicle maturation. The presence of cGH in the incubation medium did not change basal and stimulated by oLH progesterone release by the granulosa layer and basal estradiol secretion by the theca layer of preovulatory follicles at both stages of the ovulatory cycle. Contrary to the present results, AHUMADA-SOLÓRZANO *et al.* (2012) demonstrated a stimulatory, dose-dependent effect of GH on progesterone secretion by cultured chicken granulosa cells of F2 follicles. This difference could be a result of different experimental conditions such as stage at which follicles were isolated, duration of incubation, applied culture medium and method of progesterone determination. Importantly, recombinant cGH reversed LH-stimulated estradiol secretion to control level by the theca layer of F3 and F1 follicles at 22 h before ovulation. These results indicate that GH does not have a direct effect but modulates LH action on estradiol secretion by the theca layer of preovulatory ovarian follicles. This suggestion may be additionally supported by the increased concentration of plasma GH in the hen observed around the time of ovulation (HARVEY *et al*. 1979), i.e. after LH surge as well as the increase in the concentration of gonadotropin receptors after GH treatment in cultured granulosa cells of rat (JIA *et al*. 1986) or its reduction in the pig (SPICER *et al*. 1992). Moreover, changes in GH and GH receptor expression in the granulosa and theca layers of preovulatory follicles of chicken (LEBEDEVA *et al*. 2004; HRABIA *et al.* 2008) suggest auto- and/ or paracrine GH action.

These results suggest that GH directly stimulates progesterone secretion and inhibits estradiol secretion by yellow hierarchical ovarian follicles in chicken. However, the effect of GH on separated granulosa and theca layers of the preovulatory follicles is not as pronounced as on whole follicles. It cannot be excluded that GH temporarily modulates LH action in estradiol production by the theca cells of chicken preovulatory ovarian follicles. Further experiments to clarify the mechanism of GH and LH interaction in the regulation of ovarian steroidogenesis in chicken are in progress.

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