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# Effects of ghrelin and its analogues on chicken ovarian granulosa cells

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**Abstract**

The aim of these in vitro experiments was (1) to examine the effects of ghrelin on the basic functions of ovarian cells (proliferation, apoptosis, secretory activity); (2) to determine the possible involvement of the GHS-R1a receptor and PKA- and MAPK-dependent post-receptor intracellular signalling cascades; (3) to identify the active part of the 28-amino acid molecule responsible for the effects of ghrelin on ovarian cells.

We compared the effect of full-length ghrelin 1–28, a synthetic activator of GHS-R1a, GHRP6, and ghrelin molecular fragments 1–18 and 1–5 on cultured chicken ovarian cells. Indices of cell apoptosis (expression of the apoptotic peptide bax and the anti-apoptotic peptide bcl-2), proliferation (expression of proliferation-associated peptide PCNA), and expression of protein kinases (PKA and MAPK) within ovarian granulosa cells were analysed by immunocytochemistry. The secretion of progesterone ($P_4$), testosterone (T), estradiol ($E_2$) and arginine–vasotocin (AVT) by isolated ovarian follicular fragments was evaluated by RIA/EIA.

It was observed that accumulation of bax was increased by ghrelin 1–28, GHRP6 and ghrelin 1–18, but not by ghrelin 1–5. Expression of bcl-2 was suppressed by addition of ghrelin 1–28, GHRP6 and ghrelin 1–5, but promoted by ghrelin 1–18. The occurrence of PCNA was reduced by ghrelin 1–28, GHRP6, ghrelin 1–18 and ghrelin 1–5. An increase in the expression of MAPK/ERK1, 2 was observed after addition of ghrelin 1–28, GHRP6 and ghrelin 1–18, but not ghrelin 1–5. The accumulation of PKA decreased after treatment with ghrelin 1–28 and increased after treatment with GHRP6 and ghrelin 1–18 but not ghrelin 1–5.

Secretion of $P_4$ by ovarian follicular fragments was decreased after addition of ghrelin 1–28 or ghrelin 1–5 but stimulated by GHRP6 and ghrelin 1–18. Testosterone secretion was inhibited by ghrelins 1–28 and 1–18, but not by GHRP6 or ghrelin 1–5. Estradiol secretion was reduced after treatment with ghrelin 1–28 but stimulated by ghrelins 1–18 and 1–5; GHRP6 had no effect. AVT secretion was stimulated by ghrelin 1–28, GHRP6 and ghrelin 1–18, but inhibited by ghrelin 1–5.

The comparison of the effects of the four ghrelin analogues on nine parameters of ovarian cells suggest (1) a direct effect of ghrelin on basic ovarian functions—apoptosis, proliferation, steroid and peptide hormone secretion; (2) that the majority of these effects can be mediated through GHS-R1a receptors; (3) an effect of ghrelin on MAPK- and PKA-dependent intracellular mechanisms, which can potentially mediate the action of ghrelin at the post-receptor level; (4) that ghrelin residues 5–18 may be responsible for the major effects of ghrelin on the avian ovary.


*Keywords:* Ghrelin; GH secretagogue; Apoptosis (bax; bcl-2); Proliferation (PCNA); Protein kinases (MAP kinase; protein kinase A); Progesterone; Testosterone; Estradiol; Arginine–vasotocin

## 1. Introduction

The recently discovered hormone ghrelin, a peptide consisting of 28 amino acids, is a natural ligand of the

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growth hormone secretagogue receptor type 1a (GHS-R1a) which is normally produced in the stomach and other tissues. It is involved in the mediation of nutritional effects on various physiological processes. These include the secretion of GH and other hormones, food intake and energy balance. It acts both via the hypothalamo-hypophysial system and by direct control of peripheral organs [1–6].

The effects of ghrelin on cell proliferation are documented, although conflicting results on the pattern of its primary actions have been reported. Thus, ghrelin promoted proliferation of adrenocortical cells [7], pancreatic adenocarcinoma cells adipocytes [8], cardiomyocytes [9] and adrenal cells [10], whereas it suppressed proliferation of several cancer cell lines of thyroid, breast, lung and prostatic origin [11–14] as well as immature Leydig cells of the testis [6,15]. Differences in cell type and in the expression of GHS-Rs may contribute to this apparent discrepancy.

Direct effects of ghrelin in the control of apoptosis have been described. Again, the picture is controversial as ghrelin was demonstrated to prevent apoptosis in cardiomyoctes [3,16,17], adipocytes [8], endothelial [17] and adrenal [10] cells, but to be pro-apoptotic in endothelial [17], aldosteroma and adrenocarcinoma-derived cells [18].

In addition to regulating cell death and proliferation, ghrelin could be involved in the control of the secretory activity of the pituitary and some peripheral organs. In the pituitary it is a potent secretagogue for GH and, to a lesser extent, of ACTH and prolactin. It is also an inhibitor of LH secretion [5,6]. Besides its action as a secretagogue at the hypophysial level, ghrelin has been reported to inhibit insulin secretion [19–21] and to stimulate glucagon [19,21,22], but not somatostatin [19] secretion by the pancreas. Furthermore, it was able to inhibit testosterone secretion and the expression levels of several key steroidogenic enzymes in rat testis [6,23], whereas no effect on adrenal steroidogenesis has been demonstrated [15].

The effects of all known hormones are mediated by specific receptors and postreceptor signalling cascades. The best known are the signalling cascades including mitogen-activated protein kinases (MAPK) and protein kinase A (PKA; [24,25]). PKA can regulate the secretory activity of mammalian ovarian cells [27–30]. MAPK are involved in the control of mammalian [28,30,31] and avian [32] ovarian secretory activity. Both PKA [27–30], and MAPK [32] not only regulate ovarian secretory activity, but also mediate the action of hormones and growth factors. The intracellular mechanisms of action of ghrelin on non-reproductive tissue may include ghrelin receptor GHS-R1a and post-receptor signalling mechanisms dependent on PKA [21,33–36], and MAPK [7,37,38]. It remains unknown whether the hypothesized action of ghrelin on ovarian function is mediated through GHS-R1a and post-receptor MAPK- and PKA-dependent intracellular mechanisms.

It is not fully understood which part of the 28-amino acid ghrelin molecule is responsible for its biological effects. Addressing this question could help in understanding interrelationships between hormone structure and effect, as well as elucidating the activity of truncated hormone analogues. The latter may be more biologically active and economical to use than the natural hormone. For example, it was previously established that a ghrelin molecular fragment consisting of the first 23, 18, 14, 10 and even five or four residues retained the ability of native ghrelin to bind to GHS R1a and to elevate $Ca2+$ level in HEK-293 cells [39]. The search for the active part of ghrelin molecule continues and there is yet be a published comparison of the activities of native ghrelin and its fragments on ovarian cells. Thus it is still unknown, whether and how ghrelin affects the basic functions of ovarian cells (proliferation, apoptosis, secretory activity), whether its effects on the ovary are mediated by GHS-R1a receptor and by PKA- and MAPK-dependent post-receptor intracellular signalling cascades, and which is the active part of the 28-amino acid molecule. To approach these questions, we have carried out some in vitro experiments comparing the effects of native ghrelin 1–28, synthetic activator of GHS-R1a, GHRP6, and ghrelin molecular fragments 1–18 and 1–5 on chicken ovarian cells. Indices of cell apoptosis (expression of the apoptotic peptide bax and anti-apoptotic peptide bcl-2), proliferation (expression of proliferation-associated peptide PCNA) and expression of protein kinases (PKA and MAPK) have been analysed in avian ovarian granulosa cells, together with the secretion of progesterone ($P_4$), testosterone (T), estradiol ($E_2$) and arginine–vasotocin (AVT) by isolated ovarian follicular fragments.

## 2. Material and methods

### *2.1. Preparation, culture and processing of ovarian cells*

White Leghorn hens (LSL) about 6 months old, with an egg laying rate of more than 95%, were held under standard conditions at the Experimental Station of the Institute of Animal Science on a 12L:12D photoperiod (illumination 8.00 a.m. to 8.00 p.m.). Birds were decapitated between 9.00 and 11.00 a.m. and the largest

(F1–F2) follicles were isolated from the ovary. The stage of folliculogenesis was determined by recording the time of the last oviposition and by the size and the position of the next ovarian follicle. Fragments of follicular wall were isolated as described previously [32]. Fragments from F1 and F2 follicles were pooled in each experiment. After washing three times in sterile culture medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution, all from Sigma, St. Louis, USA), these fragments were cultured with treatments for 96 h in 2 ml culture medium in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA) at 38.5 °C under 5% $CO_2$ in humidified air. This protocol provides maximal accumulation of ovarian hormones and a good response to hormones [32].

Granulosa cells were gently scraped by lancet from the inner surface of washed ovarian follicles and washed three times by centrifugation and resuspension in culture medium. They were cultured in 0.3 ml of medium in Lab-Tek chamber slides (Nunc Inc., Naperville, USA) at $10^6$ cells/ml (determined by haemocytometer) in the same medium as that described for fragments of follicular wall at 38.5 °C under 5% $CO_2$ in humidified air. After 4 days of pre-culture (when the cells reached 50–60% confluent monolayer), the medium was replaced with fresh medium with or without treatment, and the cells were cultured in fresh medium for 2 days.

Treatments included recombinant human ghrelin 1–28 (Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg; Phoenix Europe GmBH, Karlsruhe, Germany), synthetic GHRP6 (Bachem, Bubendorf, Switzerland), recombinant ghrelin 1–18 (Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-$NH_2$; Peptides International Inc., Louisville, Kentucky, USA) and recombinant ghrelin 1–5 (Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-$NH_2$; Peptides International Inc.). All preparations were of research grade. All peptides were added in concentrations 0, 1, 10 or 100 nM. Two substances were tested in each experiment. The preparations were dissolved in culture medium immediately before the experiment. Controls intended for RIA/EIA included not only follicular fragments cultured without treatments, but also incubation medium cultured without ovarian tissue (assay blank).

Immediately after culture the medium conditioned by follicular fragments was gently aspirated from culture wells and frozen at −18 °C until assayed by RIA or EIA. All follicular fragments were weighed. Chamber slides with monolayers of granulosa cells were washed three times in ice-cold PBS, fixed for 20 min in 4% paraformaldehyde in PBS, washed in PBS (2× 5 min), ethanol (70%, 5 min; 80%, 10 min; 96%, 2× 10 min; 100%, 10 min) and kept in 100% ethanol at −22 °C to await immunocytochemical analysis. In addition, after culture, before fixation of cells in Chamber slides, cell concentration and viability were determined by trypan blue staining and counting on haemocytometer. No statistically significant differences in these indices were observed between the groups.

### 2.2. Immunocytochemical analysis

Signalling substances within granulosa cells plated on chamber slides were detected by immunocytochemistry [40]. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against bax, bcl-2, PCNA, MAPK/ERK1, 2 and PKA (which cross-react with corresponding rat, human, chicken and yeast substances; all from Santa Cruz Biotechnology Inc., Santa Cruz, USA; dilution 1:100) were used as directed by the manufacturer. Corresponding secondary antibody from the ImmunoCruz Staining System or secondary goat IgG labeled with horseradish peroxidase (Santa Cruz; dilution 1:1000) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany; 10%) were used for the visualization of primary antibody. The specificity of primary antibodies and molecular weights of ligands were confirmed prior to experiment by Western blotting [32]. Cells treated with secondary antibody and DAB but omitting the primary antibody were used as negative controls. The presence of specific immunoreactivity in cells was determined by light microscopy.

### 2.3. Immunoassay

Levels of $P_4$, T, $E_2$ and AVT were determined in 25–100 μl of incubation medium by RIA and EIA, previously validated for use in culture medium, using antisera against steroids produced in the Institute of Animal Science, Neustadt, Germany and against AVT produced in the Max-Plank Institute for Physiological and Clinical Research, Bad Nauheim, Germany [41]. Progesterone concentrations were measured by using EIA as described previously [42]. Rabbit antiserum against $P_4$ was obtained from Research Institute for Animal Production, Schoonoord, The Netherlands. It cross-reacted ≤0.1% with estradiol, dihydrotestosterone, testosterone and 17 beta hydroxyprogesterone. Sensitivity was 12.5 pg/ml. Inter- and intra-assay coefficients of variation did not exceed 3.3% and 3.0%, respectively. Testosterone was assayed by using EIA according to Münster [43]. Sensi-

tivity was 10 pg/ml. The antiserum cross-reacted ≤96% with dihydrotestosterone, ≤3% with androstenedione, ≤0.01% with progesterone and estradiol, ≤0.02% with cortisol and ≤0.001% with corticosterone. Inter- and intra-assay coefficients of variation were 12.3% and 6.8%, respectively. Estradiol concentrations were evaluated by using EIA according to Münster [43]. Sensitivity was 5 pg/ml. The cross-reactivity of the E antiserum was <2% to estrone, ≤0.3% to estriol, ≤0.004% to testosterone and ≤0.0001% to progesterone and cortisol. Inter- and intra-assay coefficients of variation did not exceed 16.6% and 11.7%, respectively. AVT was determined by using RIA according to Gray and Simon [41]. Sensitivity was 0.3 pg/ml. The antiserum cross-reacted ≤1.0% with mesotocin and angiotensin II. Inter- and intra-assay coefficients of variation did not exceed 8.8% and 7.2%, respectively.

### 2.4. Statistics

Each experimental group was represented by six culture wells with follicular fragments or by four slide chambers with granulosa cells. Assays of hormones in incubation medium were performed in duplicate. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate pools of ovaries, each obtained from 10 to 12 animals. The samples intended for RIA/EIA were processed separately. The values of blank controls were subtracted from the value determined by RIA/EIA in cell-conditioned medium to exclude any non-specific background (less than 17% of total values). The rates of hormone secretion were calculated per mg tissue per day. The proportion of cells containing each analysed substance was calculated following immunocytochemical analysis by counting at least 1000 cells per chamber slide well. Significant differences between the experiments were evaluated using one-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Duncan's multiple range test. Differences from control where $P < 0.05$ were considered as significant.

## 3. Results

The occurrence of apoptosis (bax, bcl-2)-, proliferation (PCNA)-associated markers, protein kinases (MAPK/ERK1, 2, PKA) within chicken ovarian granulosa cells was demonstrated by immunocytochemistry (Figs. 1 and 2). The presence of some apoptosis- and proliferation-associated substances in the cells is shown in Fig. 1. RIA/EIA showed the secretion of $P_4$, T, $E_2$ and AVT by cultured ovarian fragments (Fig. 3).

These parameters were influenced by additions of ghrelin 1–28, GHRP6, ghrelin 1–18 and ghrelin 1–5. Although there were substantial differences in the initial values of these parameters between individual experiments, the pattern of influence of ghrelin and its analogues was similar in all the replicates. Accumulation of bax was increased by ghrelin 1–28 (at 100 nM), GHRP6 (at 10 or 100 nM), ghrelin 1–18 (at 1, 10 or 100 nM), but not by ghrelin 1–5 (Fig. 2A). Expression of bcl-2 was suppressed by addition of ghrelin 1–28 (at 1, 10 or 100 nM) and ghrelin 1–5, whilst ghrelin 1–18 promoted it (at 10 or 100 nM; Fig. 2B). The occurrence of PCNA was increased by either ghrelin 1–28, GHRP6, ghrelins 1–18 and 1–5 (at all concentrations; Fig. 2C). An increase in the expression of MAP/ERK1, 2 kinase was observed after addition of ghrelin 1–28 (100 nM), GHRP6 (100 nM), ghrelin 1–18 (10 or 100 nM), but not of ghrelin 1–5 (Fig. 2D). Accumulation of PKA decreased after treatment with ghrelin 1–28 (100 nM), but GHRP6 and ghrelin 1–18 (10 or 100 nM) increased it (at all concentrations). Ghrelin 1–5 did not affect this parameter (Fig. 2E).

Secretion of $P_4$ by ovarian follicular fragments was decreased after addition of ghrelin 1–28 or ghrelin 1–5

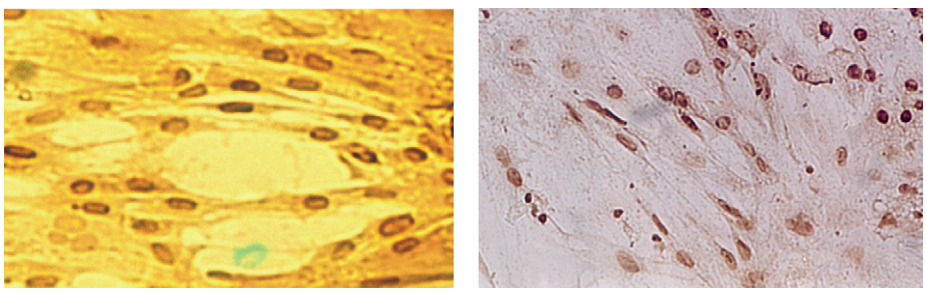

Fig. 1. Presence of immunoreactive proliferation-associated peptide PCNA (left) and apoptosis-associated peptide bax (right, brown-yellow staining) in cultured chicken ovarian granulosa cells. Immunocytochemistry using secondary antibody labeled with horseradish peroxidase and DAB. Magnification 400×.

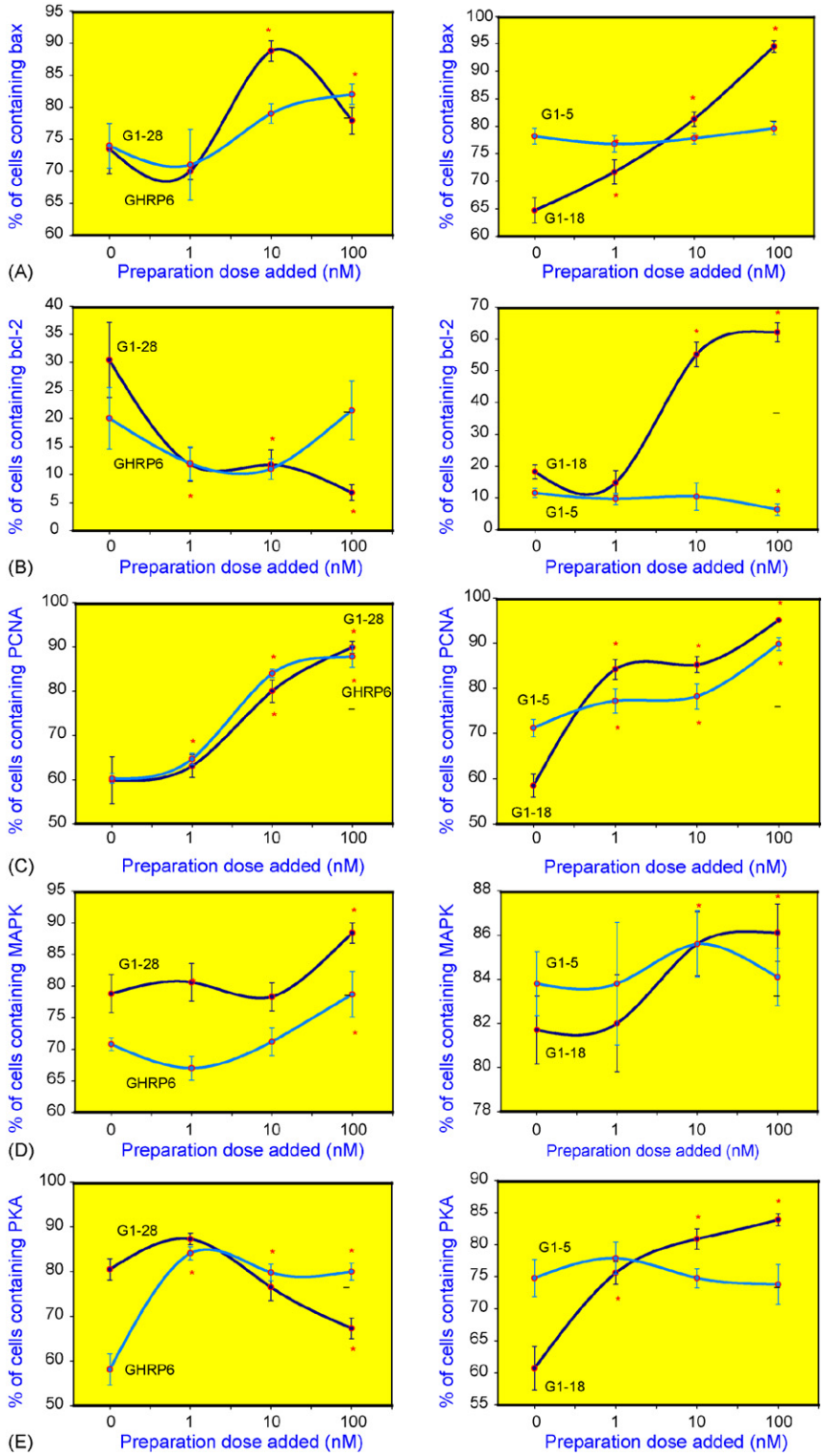


Fig. 2. Effect of ghrelin 1–28, GHRP6 (left), ghrelin 1–18 and ghrelin 1–5 (right) on the expression of apoptosis-associated peptide bax (A); anti-apoptotic peptide bcl-2 (B); proliferation-associated antigen PCNA (C); protein kinases MAPK/ERK1, 2 (D); PKA (E) in cultured chicken granulosa cells. Data represent the proportion of cells containing immunoreactivity and are means ± S.E.M.; *, significant differences compared to control (cells cultured without treatment). Immunocytochemistry using secondary antibody labeled with horseradish peroxidase and DAB.

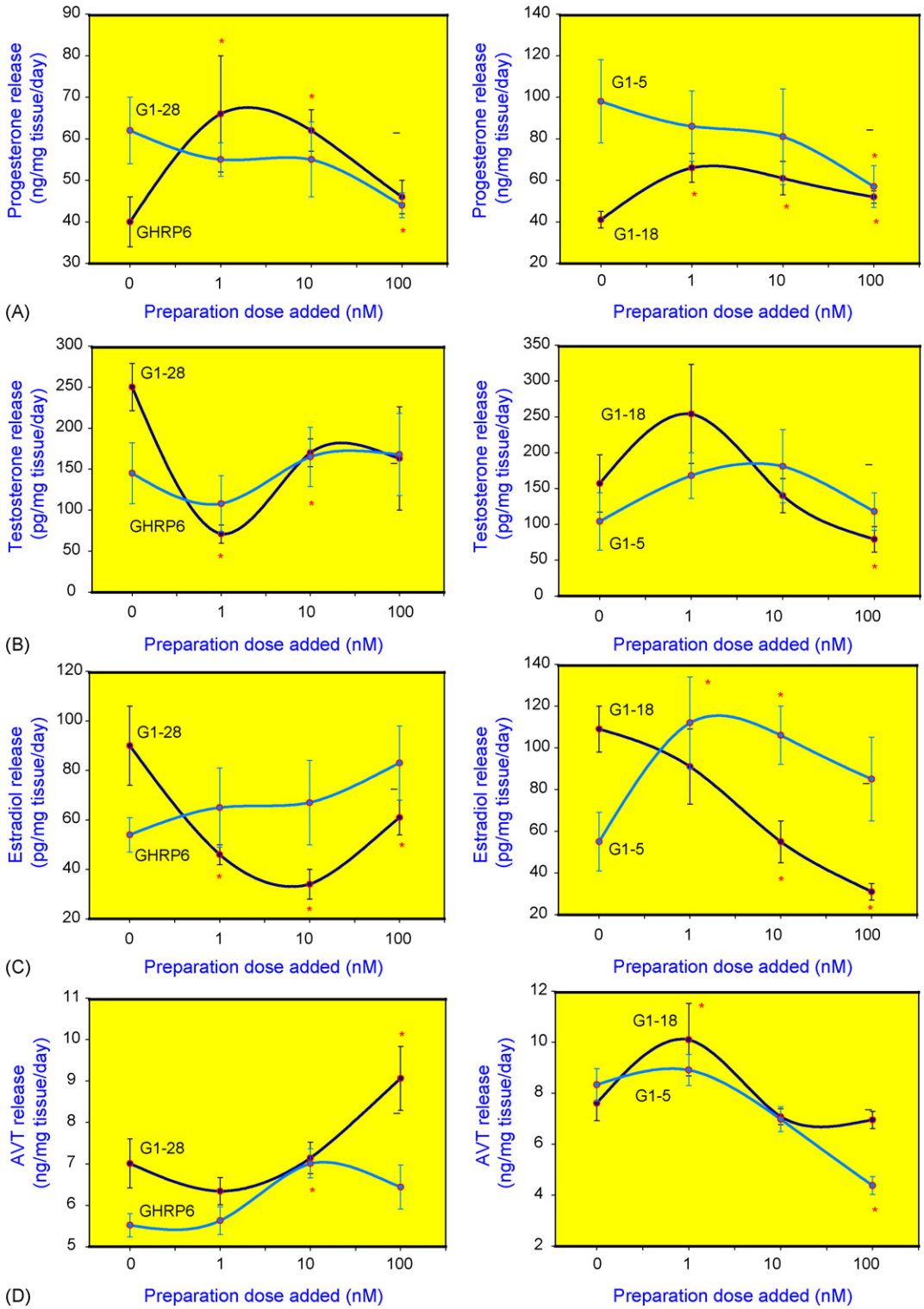


Fig. 3. Effect of ghrelin 1–28, GHRP6 (left), ghrelin 1–18 and ghrelin 1–5 (right) on the on the release of progesterone (A); testosterone (B); estradiol (C); AVT (D) from fragments of follicular wall isolated from F1-F2 chicken ovarian follicles. Values are means ± S.E.M.; *, significant difference ($P < 0.05$) compared to control (medium without treatment).

Table 1
Summary of effects of ghrelin 1–28, GHRP6, ghrelins 1–18 and 1–5 on different parameters of cultured chicken ovarian cells

| Treatment | Expression of intracellular substances | | | | | Release of hormones | | | |
|---|---|---|---|---|---|---|---|---|---|
| | bax | bcl-2 | PCNA | MAPK | PKA | $P_4$ | T | $E_2$ | AVT |
| Ghrelin 1–28 | + | − | + | + | − | − | − | − | + |
| GHRP6 | + | − | + | + | + | + | 0 | 0 | + |
| Ghrelin 1–18 | + | + | + | + | + | + | − | − | + |
| Ghrelin 1–5 | 0 | − | + | 0 | 0 | − | 0 | + | − |

Pattern of effect: +, stimulation ($P < 0.05$); −, inhibition ($P < 0.05$); 0, no significant effect.

(both at 100 nM), whilst GHRP6 (at 1 and 10 nM) and ghrelin 1–18 (at all concentrations) promoted it (Fig. 3A). Testosterone output was inhibited by ghrelin 1–28 (at all concentrations), ghrelin 1–18 (at 100 nM), but not by GHRP6 or ghrelin 1–5 (Fig. 3B). Estradiol secretion was reduced after treatment with ghrelin 1–28 (at all concentrations) and ghrelin 1–18 (at 10 or 100 nM), whilst ghrelin 1–5 promoted it (at 1 or 10 nM); GHRP6 had no effect (Fig. 3C). AVT secretion was stimulated by ghrelin 1–28 (100 nM), GHRP6 (10 nM) or ghrelin 1–18 (1 nM), but ghrelin 1–5 inhibited it (at 100 nM; Fig. 3D). These observations are summarized in Table 1.

## 4. Discussion

### *4.1. Does ghrelin affect ovarian function?*

Our results, despite some variability in measured parameters, demonstrate a direct influence of ghrelin on chicken ovarian function. First, they suggest a pro-apoptotic action of ghrelin on ovarian cells. This can be realized in two ways—through promotion of the pro-apoptotic peptide bax and by suppression of its inhibitor, anti-apoptotic protein bcl-2 [44,45]. Second, the substantial ghrelin-induced accumulation of PCNA, markers of S-phase of mitosis [46,47] suggest that ghrelin can promote proliferation of ovarian cells. This hypothesis is supported by ghrelin-induced accumulation of MAPK/ERK1, 2, which is usually expressed in proliferating cells [25,46,47]. The ability of ghrelin to promote both proliferation and apoptosis suggest that it might activate turnover of the cells within the ovary and therefore activate ovarian remodeling. Third, ghrelin is shown to be a regulator of ovarian secretory activity: it suppressed the secretion of steroid hormones ($P_4$, T and $E_2$) and promoted output of the nonapeptide hormone AVT. Since these hormones are markers and regulators of reproductive processes, including ovarian follicular growth and remodeling [48,49], it is possible that the stimulatory action of ghrelin on both apoptosis and proliferation is mediated by changes in local hormone production.

### *4.2. Does ghrelin affect ovarian cells through the growth secretagogue receptor GHS-R1a?*

A comparison of the effects of ghrelin 1–28 and GHRP6, a synthetic activator of GHS-R1a, demonstrates similarity for parameters (bax, bcl-2, PCNA, MAPK, AVT). This suggests a common mechanism of action. Since both these substances act in non-ovarian cells through activation of GHS-R1a, it may be suggested that ghrelin's action on ovarian cell proliferation, apoptosis and secretion of nonapeptide hormones is also mediated through GHS-R1a. On the other hand, the different pattern of ghrelin 1–28 and GHRP6 action on other parameters (PKA and steroid hormones) indirectly indicates that ghrelin controls those functions through receptors other than GHS-R1a. The existence of different types of ghrelin receptors has been proposed previously for mammals [4,5] and chicken [50]. Nevertheless, it remains to be possible, that different pattern of ghrelin 1–28 and GHRP6 action on PKA and steroid hormones could be due to differences in stage of development of follicles obtained from different pools of ovaries.

### *4.3. Does ghrelin affect ovarian cells through post-receptor MAPK- and PKA-dependent mechanisms?*

The stimulatory influence of ghrelin 1–28 on accumulation of MAPK and its inhibitory action on expression of PKA indirectly indicates that there are at least two, relatively independent, post-receptor signalling pathways mediating ghrelin's action on ovarian cells. This agrees with available reports on the involvement of PKA [21,33–37] and MAPK [7,10,37,38] in mediating ghrelin action on non-ovarian cells. Detailed studies, including analysis of protein kinase activity, phosphorylation etc. and other processes, after treat-

ment of cells with ghrelin and protein kinase blockers or stimulators are required in order to address this question.

### 4.4. What is the active part of ghrelin molecule?

Comparison of effects of full-length ghrelin 1–28 and its truncated analog 1–18, despite some differences in control values, shows the similarity of their effects on bax, PCNA, MAPK, T, $E_2$ and AVT (i.e. on 2/3 of analysed parameters). It suggests that the active part of the molecule is localized between amino acids 1 and 18. The intact 18–28 residues are probably necessary for its remaining effects (on bcl-2, PKA and $P_4$). These observations indicate, that different parts of the ghrelin molecule could be responsible for different ghrelin effects, although the major ghrelin action is probably associated with its 1–18 residues.

The comparison of the effects of full-length ghrelin molecule and its 1–5 fragment shows only 1/3 coincidence (on bcl-2, PCNA and $P_4$). This indicates, that truncation of the ghrelin molecule up to five residues produces a loss of basic activity on the ovary.

The retention of basic ghrelin activity after truncation to 1–18 residues but loss at 1–5 residues suggests that the most important part of the molecule is represented by between 5 and 18 residues. From a practical viewpoint, the data of this comparative analysis shows that a truncated 1–18 analogue or even a 5–18 fragment could be sufficient to regulate the majority of ovarian functions. This hypothesis could be verified by further studies of effects of truncated ghrelin analogue with residues 5–18 only.

The obtained data could expand the existing general knowledge concerning direct action of ghrelin on the ovary. Nevertheless, the results of testing of human ghrelin analogues on chicken ovarian cells should be interpreted carefully because of some differences in ghrelin molecule [51], ghrelin receptor [50] and ghrelin effects on feeding [52,53] but not on release of GH and corticosterone [51,54] in chicken and mammals.

Taken together, the present comparison of the effects of four ghrelin analogues on nine parameters of ovarian cell function indicate (1) a direct effect on apoptosis, proliferation, steroid and peptide hormone secretion; (2) that the majority of these effects can be mediated by GHS-R1a receptors; (3) that MAPK- and PKA-dependent intracellular mechanisms potentially mediate post-receptor activity; (4) that residues 5–18 of ghrelin molecules are probably responsible for the majority of its effects on the ovary.

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