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MODULATORY EFFECT OF GHRELIN IN PREPUBERTAL PORCINE OVARIAN FOLLICLES

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Ghrelin is a novel growth hormone-releasing peptide, originally identified in rat stomach as an endogenous ligand of the growth hormone secretagogue receptor. Ghrelin is an important regulator of growth hormone secretion, food intake, and reproductive function. This study investigates whether or not ghrelin can modulate prepubertal pig ovary function, which was measured as ovarian estradiol secretion, aromatase activity, cell proliferation, and apoptosis. To investigate this, ovarian cells were co-cultured with four different doses of ghrelin (100, 250, 500, and 1000 pg/ml) for 48 h. Culture media samples were collected, and estradiol levels were determined, while aromatase expression was measured in the cultured cells. Cell apoptosis was measured by determination of caspase-3 activity, DNA fragmentation and TUNEL assay. Ghrelin in 250 and 500 pg/ml doses stimulated estradiol secretion. At all doses ghrelin stimulated aromatase activity and protein expression. Moreover, ghrelin increased cell proliferation and decreased apoptosis. This study provides novel evidence that ghrelin has a modulatory effect in the ovary. We suggest two mechanisms that explain how ghrelin acts on estradiol secretion: 1) ghrelin directly influences aromatase activity and protein expression; 2) ghrelin stimulates cell proliferation and antiapoptotic actions.

Key words: *prepubertal ovarian follicles, ghrelin, estradiol, aromatase, proliferation, apoptosis*

INTRODUCTION

Ghrelin, a novel peptide of 28 amino acids, was recently isolated from rat stomach as a ligand of the growth hormone secretagogue receptor (GHS-R) (1). Two subtypes of the receptor have been identified: the fully functional GHSR-1a and the biologically inactive GHSR-1b (2, 3). Ghrelin is a multifaceted hormone that plays

an important role in the regulation of growth hormone (GH) secretion, food intake, and energy balance in vertebrates. Evidence has been provided that ghrelin is expressed in some endocrine tissues, such as human or rat pancreatic islets and pituitary glands. In these endocrine tissues, it stimulates insulin release and influence of GH, adrenocorticotropic hormone (ACTH), and prolactin secretion (4).

Data published in recent years has shown an important interrelationship between gonadal function and energy homeostasis (5). Similar to the adipocyte-driven hormone leptin, ghrelin is a regulator of homeostasis, food intake, and energy balance. As such, ghrelin may also play a role in the regulation of reproductive function. Ghrelin and ghrelin receptors are present in many peripheral tissues, including the human ovary (6, 7). Recent studies suggest that ghrelin may participate in the control of key aspects of reproductive function (8-12). These studies showed a persistent expression of the ghrelin gene in the rat ovary throughout the estrous cycle, although relative ghrelin mRNA levels varied depending on the phase of the estrous cycle. The lowest levels of ghrelin mRNA were observed in the proestrus phase, whereas peak expression was observed in the diestrous d1 phase, *i.e.*, during the luteal phase of the cycle (13). These authors suggested that ghrelin might operate as an autocrine or paracrine regulator of ovarian physiology. Of particular note, follicular GHS-R1a immunoreactivity has been shown in both granulosa and theca layers of healthy antral follicles (7). The presence of both the ghrelin ligand and the active form of the ghrelin receptor within the human ovary points to the importance role of this molecule in ovarian function. Until now there have been no studies which investigated the mechanism of ghrelin in the pig ovary. To better define the direct modulatory action of ghrelin in ovarian follicles, we examined: 1) the role of ghrelin on estradiol secretion and aromatase activity and 2) the effect of ghrelin on cell proliferation and apoptosis.

MATERIAL AND METHODS

Reagents

M199, trypsin, fetal bovine serum (FBS, heat-inactivated), antibiotic/antimycotic solution (100x), Trypan blue, TRIS, Na-deoxycholate, Nonidet NP-40, sodium dodecyl sulfate SDS, protease inhibitor (EDTA-free), DTT, Tween 20, bromophenol blue, dibenzylfluorescein (DBF), NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and human ghrelin were obtained from Sigma (Chemical Co. St. Louis, MO, USA). Human ghrelin was utilized in this experiment because porcine ghrelin was not readily available at the onset of this experiment. Human ghrelin differs from porcine ghrelin by three amino acids (1).

Cell cultures

Prepubertal porcine ovaries were obtained from a local abattoir and transported to the laboratory. Approximately 1.5 h elapsed from slaughter to arrival at the laboratory. Follicles (4-5 mm in diameter) were excised from ovaries. Granulosa cells (Gc) and theca interna cells (Tc) from

the same follicles were subsequently prepared according to the technique described by Stoklosowa *et al*. (14, 15). After isolation, Gc and Tc were collected and resuspended in M199 supplemented with 10 % FBS. For co-culture experiments, the viability of granulosa and theca cells was determined by the Trypan blue exclusion test; cells were subsequently plated in Nunc 96-well tissue culture plates at a concentration of 6 x 10^4 and 1.5 x 10^4 cells/well, respectively. Therefore, the *ratio* of both types of cells was comparable to that observed *in vivo* (Gc:Tc = 4:1) (14). All cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2/95 % O_2.

Experimental procedure

Cells were initially cultured in the absence of ghrelin for 24 h to allow for cell attachment. After 24 h, the culture media were discarded, and fresh culture media (M199 supplemented with 5 % FBS) were added to the control culture, whereas to the four experimental groups ghrelin was added at the following concentrations: 100, 250, 500, and 1000 pg/ml. The experimental ghrelin concentrations were chosen based on the work of Rigamonti *et al* (16).

Estradiol secretion analysis

After 48 h of incubation, the culture media were collected and frozen for estradiol determination. The concentrations of 17β-estradiol (E_2) in the media were determined by EIA (Enzyme Immuno Assay) using commercially available kits (DiaMetra, Italy) according to the manufacturer's instructions. All samples were run in duplicate. The E_2 assay sensitivity was 5 pg/ml, and the inter- and intra-run precision had coefficients of variation of 7.25 % and 6.81 %, respectively. Every treatment was conducted in quadruplicate (four wells each), and each experiment was repeated three times.

Aromatase activity measurement

After 48 h of incubation, cells were collected and frozen to assess their aromatase activity. Aromatase catalyzes aromatization of androgens to estrogens and is a key enzyme in estrogen biosynthesis. We estimated the activity of aromatase using the fluorometric substrate dibenzylfluorescein (DBF). The DBF fluorescence assay was performed on 96-well plates according to Stresser *et al*. (17) Cells were thawed at room temperature for 10 min and then lysed with Tris-HCl (0.5 M, pH 7.5). Next, a solution composed of 2.6 mM NADP, 6.6 mM glucose-6 phosphate and 0.4 U of glucose-6-phosphate dehydrogenase solution/ml was added to each well. The reaction mixtures were incubated for 15 min at 37°C, after which time, the fluorometric substrate DBF (2 μM) in acetonitrile was added to each well. The reaction was stopped with 2 M NaOH after 30 min. The fluorescent DBF metabolite, fluorescein, was measured at an excitation wavelength of 485 nm and emission wavelength of 538 nm using a fluorometric plate reader (Bio-tek Instruments). Measurements were expressed as RFUs (Relative Fluorescence Units).

Immunoblotting

After 48 h, the culture medium was removed, and cells were lysed in 50 μl of ice-cold lysis buffer containing 50 mM Tris HCl (pH 7.5) 100 nM NaCl, 0.5 % Na-deoxycholate, 0.5 % Nonidet NP-40, 0.5 % sodium dodecyl sulfate SDS and protease inhibitor (EDTA-free). Lysates were sonicated and centrifuged at 10 000 x g at 4°C for 20 min. Supernatants were collected and stored at -20°C and later used for immunoblotting analyses. Twenty μg of protein was reconstituted in the appropriate amount of sample buffer (125 nM Tris pH 6.6, 4 % SDS, 25 % glycerol, 4 mM EDTA, 20 mM DTT, and 0.01 % bromophenol blue). The samples were separated by 7.5 % SDS-polyacrylamide gel electrophoresis in a BIO-RAD Mini-Protean II Electrophoresis Cell. Next, the proteins were transferred to

nitrocellulose membranes using a BIO-RAD Mini Trans-Blott apparatus. Following the transfer, membranes were washed, and non-specific binding sites were blocked with 5 % dried milk and 0.2 % Tween 20 in 0.02 M TBS for 2 h. Then, the membranes were incubated overnight with anti-aromatase antibody (mouse anti-human cytochrome P450 aromatase, MCA2077S, AbD Serotec Ltd., UK) diluted at 1:200 in TBS/Tween at 4°C. After incubation with the primary antibody, the membranes were washed with TBS and 0.02 % Tween 20 and incubated for 2 h with horseradish peroxidase-conjugated antibody (anti-mouse IgG-HRP, P-0447, DakoCytomation, Denmark) diluted at 1:500 in TBS/Tween. To control for the variable amounts of protein that were loaded onto each of the gels, the membranes were stripped and re-probed with anti-β-actin antibody. Signals were developed by chemiluminescence (ECL) using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology) and visualized with the use of PhosphoImager FujiLas 1000. The immunoreactive bands were quantified by an image analyzer (Image Gauge V 4.0; Fuji Photo Film Co., LTD).

Alamar Blue assay

The Alamar Blue assay (BioSource Int., USA) measures the proliferation of different cell types. The dye used in this assay is an oxidation-reduction indicator and detects the metabolic activity of cells. Cellular metabolism induces a chemical reduction of the Alamar Blue medium. This assay is based on the quantitative metabolic conversion of the non-fluorescent blue resazurin to fluorescent pink resorufin by living cells. After 48 h of incubation, the Alamar Blue stock solution was aseptically added to the wells in amounts equal to 10 % of the incubation volume. In the cultures, the resazurin reduction reaction was determined after 5 h of Alamar Blue incubation by measuring the absorbance using using FLUOROmicroplate reader (Bio-tek Instruments) at wavelengths 570 nm and 600 nm.

Caspase - 3 activity

After 48 h of incubation, cells were lysed with caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 % CHAPS, 1 mM EDTA, 10 % glycerol and 10 mM DTT) and then cell lysates were incubated with caspase-3 colorimetric substrate - Ac DEVD-pNA-(7-amino-4methyl coumarin, Sigma). The reaction mixtures were incubated at 37°C for 1 h, and the absorbance was measured at 405 nm using micro plate reader (Bio-tek Instruments).

DNA fragmentation measurement

DNA fragmentation was determined using a Cellular DNA fragmentation ELISA kit (Roche Molecular Biochemicals) by following the manufacturer's instructions. This assay allows for the quantitative detection of bromodeoxyuridine (BrdU)-labeled DNA fragments. Cells were pulsed with 10 ml of 100 mM BrdU solution per well during the last 18 h of Con A-stimulation. At 72 h after the initial stimulation, the 96 well plates were centrifuged, and the cells were denatured and then incubated for 120 min with 1:100 diluted mouse anti-BrdU mAbs conjugated to peroxidase. After removing antibody conjugate, substrate solution was added for 20 min. This reaction was stopped by adding 1 M H_2SO_4 solution. The absorbance was measured at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader (Bio-tek Instruments). One hundred ml of culture medium with or without BrdU was used as blanks.

TUNEL assay

Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit (BioCAT, USA) was used to labele DNA breaks to detect apoptotic cells. The cells were cultured on sterile slides for 24 h. After this

incubation, the cells were fixed in 3 % paraformaldehyde, washed PBS, and incubated in proteinase K and 30 % H_2O_2. The cells were washed in PBS again and then incubated in a DNA-labeling solution for 4 hr at 37°C. At the end of the incubation period, the cells were washed twice in Rinse Buffer and then incubated in antibody solution (anti-BrdU biotin mAb). The cells were incubated in diaminobenzidine (DAB) and methyl green to detect TUNEL-positive and TUNEL-negative cells. In each culture, cell morphology and percentage of TUNEL-positive cells were determined by visual evaluation. TUNEL-positive and TUNEL-negative cell numbers were determined using the Nikon Eclipse E200 light microscope.

Statistical analysis

Each treatment consisted of four groups and was repeated three times. Thus, the experiment was replicated a total of 12 times. Since the variations between the experiments were small, those 12 results were averaged and analyzed by one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) multiple range test. All data (n = 12) are expressed as the mean ± SEM.

RESULTS

Effect of ghrelin on estradiol secretion

In control cultures, the estradiol secretion into the medium during the 24 h of culture was 10.22 ± 0.39 pg/ml (*Fig. 1*). A significant increase in estradiol secretion was noted after the addition of 250 and 500 pg/ml of ghrelin. The amounts of estradiol in the experimental media were 12.14, and 14.93 pg/ml, respectively *vs*. 10.22 pg/ml in the control culture ($p<0.05$, $p<0.001$).

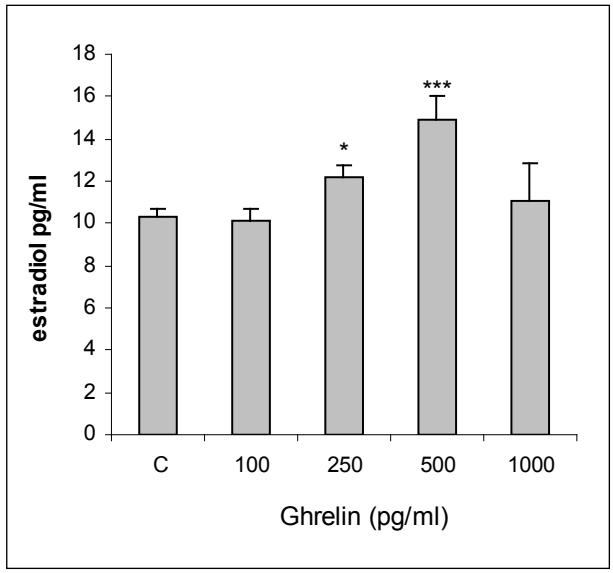

Fig. 1. Effect of ghrelin on estradiol secretion by co-cultured granulosa and theca cells. Each point represents the mean ± S.E.M. of results of three independent experiments, each of which consisted of four replicates per treatment group. All means marked with *($p<0.05$), ***($p<0.001$), are significantly different from the control (C).

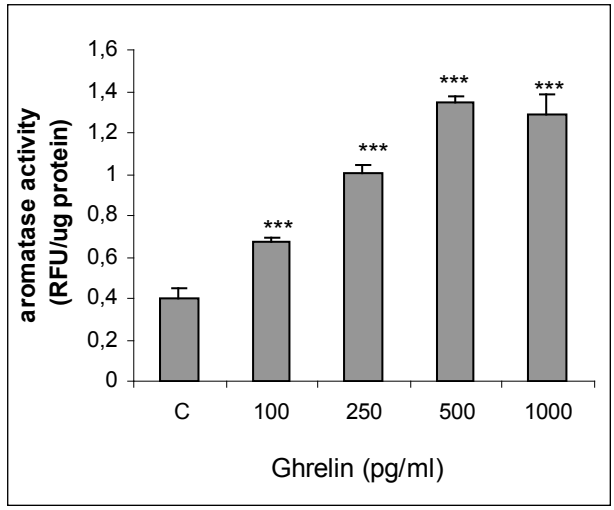

Fig. 2. Effect of ghrelin on aromatase activity. Aromatase activity was measured using the fluorescent substrate DBF. Each point represents the mean ± S.E.M. of results of three independent experiments, each of which consisted of four replicates per treatment group. All means marked with ***($p<0.001$) are significantly different from the control (C).

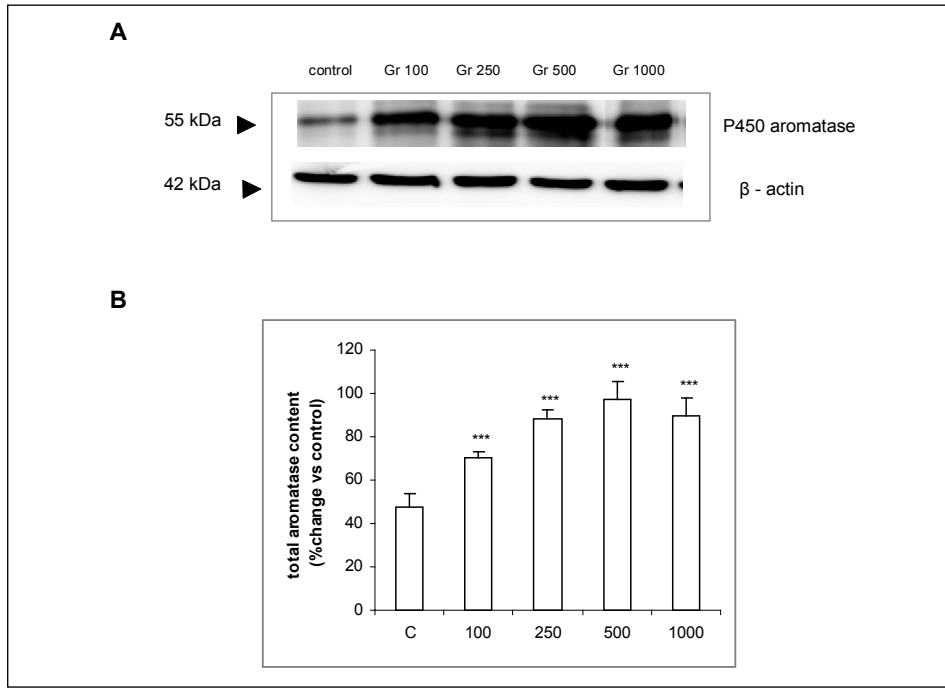

Fig. 3. Effect of ghrelin on expression of the aromatase protein. (A) Representative Western blot analysis of aromatase protein levels in co-cultured granulosa and theca cells without treatment (C), and ghrelin (Gr) treated cells. Blots were stripped and re-probed with anti actin antibody to control the amounts of protein loading. (B) Densitometric analysis of total aromatase content, expressed as percent changes with respect to the control group, which has been set at 100 %. Each bar represents the mean ±S.E.M. of the three independent experiments. All means marked with ***($p<0.001$) are significantly different from the control (C).

Direct effect of ghrelin on aromatase activity

The aromatase activity was determined by the fluorescence measurement of fluorescein as described in Materials and Methods. In cells exposed to 100, 250, 500, or 1000 pg/ml of ghrelin, aromatase activity was increased by 1.6-; 2.5-; 3.3- and 3.2-fold, respectively ($p < 0.001$) (*Fig. 2*).

Effect of ghrelin on aromatase protein expression

The immunoblot analysis of aromatase protein expression showed a strong band in all cells treated with ghrelin (*Fig. 3A*). Additionally, densitometric analysis showed that in cells exposed to 100, 250, 500, or 1000 pg/ml of ghrelin, aromatase protein was increased by 1.4-; 1.8-; 2.0- and 1.8-fold, respectively ($p < 0.001$) (*Fig. 3B*).

The action of ghrelin on cell proliferation

Cell proliferation was determined using an Alamar Blue assay. In cells exposed to 100, 250, and 500 pg/ml of ghrelin, cell proliferation was increased by 1.4-; 1.4-; and 1.6-fold, respectively ($p<0.05$) (*Fig. 4*). At a higher dose, 1000 pg/ml, ghrelin had no effect on cell proliferation.

The effect of ghrelin on cell apoptosis

In all investigated doses (100, 250, 500, and 1000 pg/ml), ghrelin significantly decreased caspase-3 activity (*Fig. 5A*) and DNA fragmentation (*Fig. 5B*) ($p<0.05$, $p<0.01$, $p<0.001$). The TUNEL assay showed that the number of TUNEL-positive cells was decreased after ghrelin treatment as compared to control cells (*Fig. 5C, Fig. 6*).

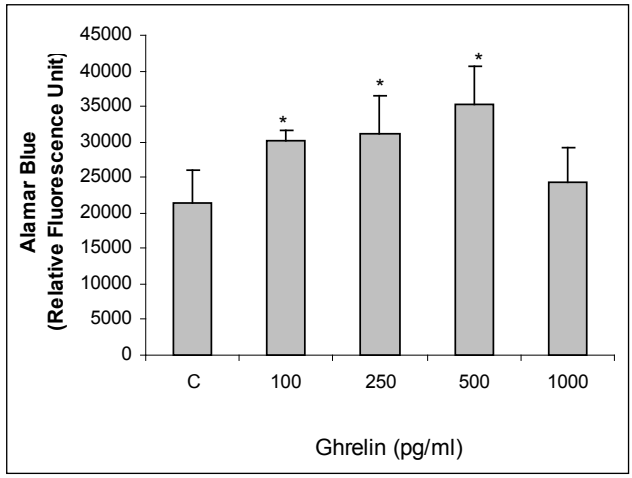

Fig. 4. Effect of ghrelin on cell proliferation. Cell proliferation was measured using Alamar Blue assay. Each point represents the mean ± S.E.M. of results of three independent experiments, each of which consisted of four replicates per treatment group. All means marked with *($p<0.05$) are significantly different from the control (C).

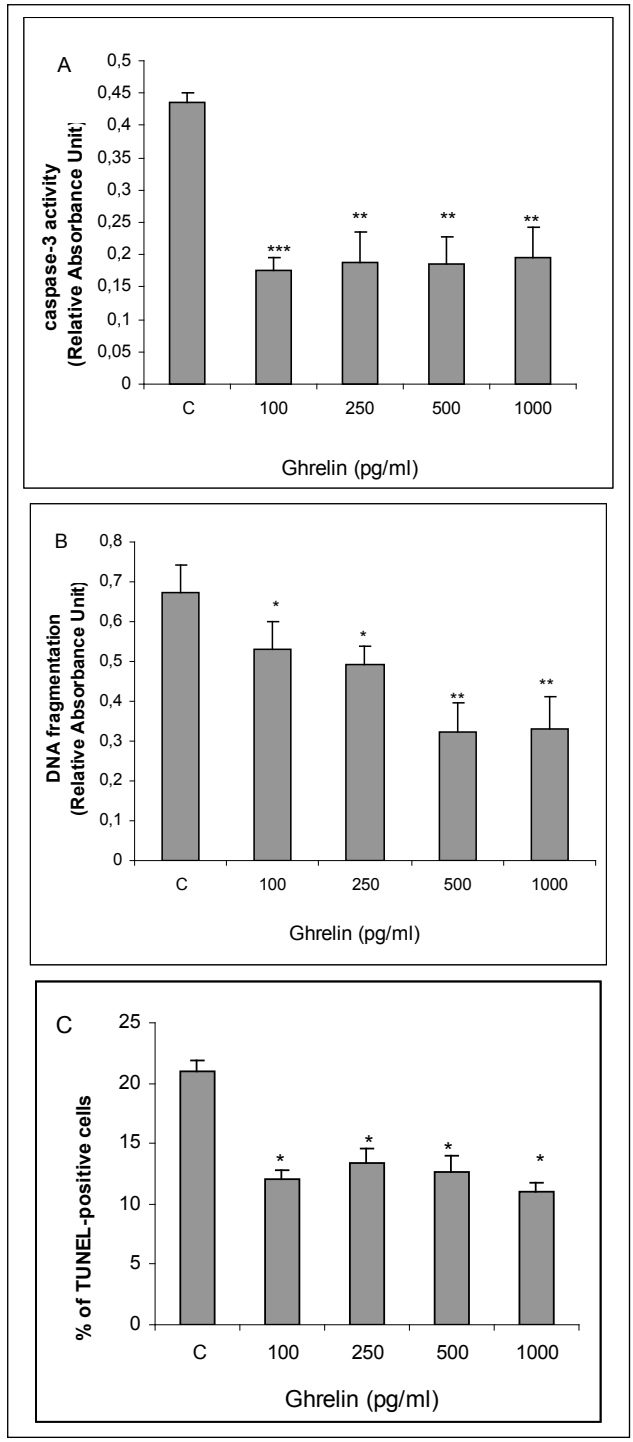

Fig. 5. Effect of ghrelin on cell apoptosis. Cell apoptosis was measured using (A) caspase - 3 activity, (B) DNA fragmentation and (C) TUNEL assay. All means marked with *($p<0.05$), **(0.01), ***($p<0.001$), are significantly different from the control (C).

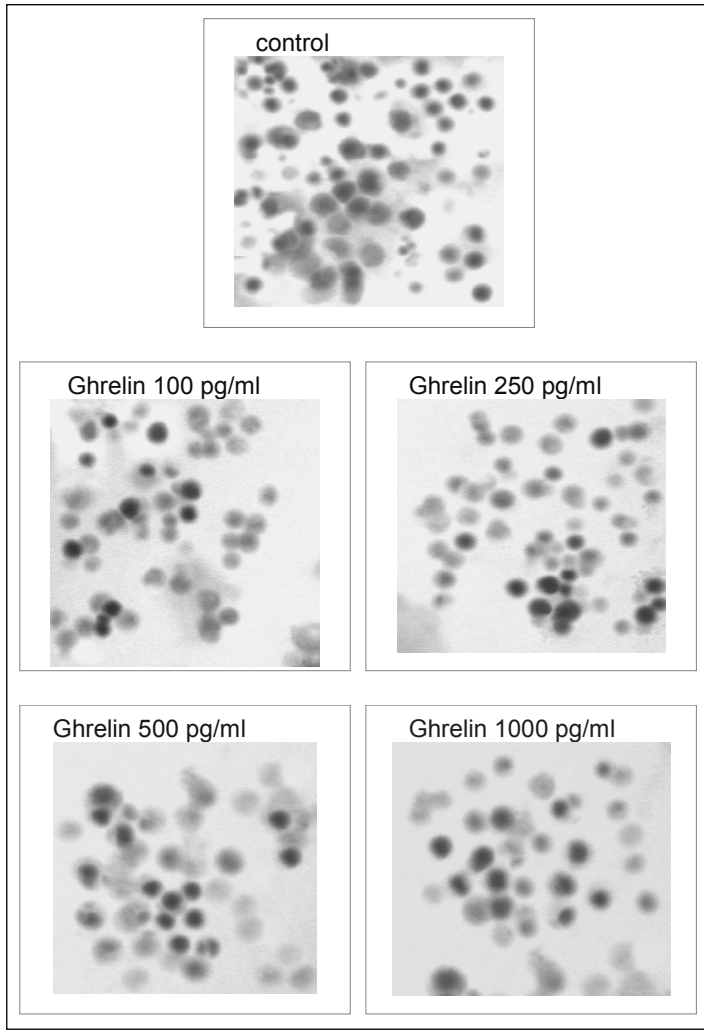

Fig. 6. Effect of ghrelin on cell apoptosis. The pictures shown TUNEL-positive cells.

DISCUSSION

This study showed for the first time that ghrelin could act directly on the prepubertal pig ovary to increase estradiol secretion. A significant increase in estradiol secretion was noted after the addition of 250, and 500 pg/ml of ghrelin. The results presented here are not in agreement with the data of Sirotkin and Grossmann (18) who showed a decrease of estradiol secretion in chicken ovarian cells following ghrelin treatment. Viani *et al*. (19) showed an inhibitory effect of ghrelin on estradiol and progesterone secretion by human granulosa-lutein cells collected from women with infertility due to uni-or bilateral tubal impotency. It

is difficult to compare these investigations because different species were used in these experiments. In addition, Viani *et al*. (19) used monolayers of granulosa-lutein cell cultures, while we used cocultures of theca and granulosa cells in our experiment, creating more physiological conditions. The most important difference between our experiments was that we used follicles collected from prepubertal animals, while the granulosa cells were collected from women treated with gonadotropine hormones before the cells were recovered.

Knowledge concerning the relationship between the expression of ghrelin in the pig ovary, serum estradiol levels and aromatase activity is scarce. In our study, ghrelin strongly stimulated aromatase activity, the key enzyme in estradiol synthesis. Estrogens are biosynthesized from androgens by the cytochrome P450 aromatase. Cytochrome P450 aromatase expression was detected in the granulosa layer of follicles ≥ 6 mm diameter. In pigs, aromatase expression was also found in the theca layer of large follicles (20). A very recent study has also shown that female rats treated with ghrelin at puberty displayed normal estrous cycling and were fertile (21). Estrogen may also regulate ghrelin secretion by a positive feedback mechanism. Evidence for this is that the plasma ghrelin concentrations during the follicular phase of the menstrual cycle in women are greater than in men (22). On the other hand, the animal studies have shown that estrogen is negative involved in the regulation of ghrelin secretion. Estrogen administration decreased the number of ghrelin-producing cells, ghrelin mRNA levels in the stomach, and plasma ghrelin levels in ovariectomized rats. In addition, ghrelin and estrogen receptor immunoreactivity were present in the same cells, suggesting that estrogen may have a direct effect on ghrelin expression (23).

Does ghrelin influence cell apoptosis? Our data suggest an antiapoptotic action of ghrelin in the prepubertal ovary. These results have been confirmed by three independent methods: caspase-3 activity, DNA fragmentation and TUNEL assay. Ghrelin in all doses used decrease caspase-3 activity. Caspase-3 is activated in the final stages of cellular death. In the ovary, caspase-3 is known to be involved in the process of follicular cell atresia, and many studies have shown an important role of caspase-3 in executing granulosa cell apoptosis (24, 25). Moreover, the rates of DNA fragmentation, as estimated by detection of bromodeoxyuridine labeled DNA fragments and TUNEL assay, were to decrease by ghrelin. The anti-apoptotic action of ghrelin has also been reported in cardiomiocytes (26, 27), adipocytes (28), and endothelial cells (29). Sirotkin *et al*. (30) demonstrated decreased expression of caspase-3 and TUNEL-positive cells in chicken granulosa cells. Pro-apoptotic effects of ghrelin has been also describe in literature, such as aldosteroma and adrenocarcinoma derived cells (31) and endothelial cells (29). We suggest that stimulatory action of ghrelin on estradiol secretion is due to its antiapoptotic effect in the ovarian follicles. This hypothesis seems to be correct in light of data that suggest a direct interference of estrogens with apoptotic processes. Recently, estradiol was found to prevent caspase-6-mediated neuronal cell death, probably by inducting a caspase inhibitory factor

(CIF) through a receptor-mediated nongenomic pathway (32). Another example in support of this hypothesis is the stimulation of the expression of antiapoptotic proteins, such as Bcl-2 or Bcl-X_L. These antiapoptotic proteins prevent cytochrome c efflux from mitochondria into the cytoplasm, which inhibits caspase-dependent apoptotic cell death (33).

The effect of ghrelin on cell proliferation was also determined in this study. Decreased several markers of apoptosis: caspase-3 activity, DNA fragmentation and TUNEL positive cells paralleled the stimulatory action of ghrelin on cell proliferation. This strongly suggests that ghrelin could promote ovarian cell division. This observation is in line with previous reports on the stimulatory effect of ghrelin on cell proliferation in non-ovarian cell types, such as adipocytes (26) and cardiomiocytes (34). Nevertheless, the anti-proliferative effect of ghrelin has been described in other cell types, such as cancer cell lines of breast, prostatic orgin (35, 36) as well as in immature Leydig cells of the testis (37). Sirotkin *et al*. (30) showed that ghrelin has a stimulatory action on the expression levels of PCNA (marker of G1-phase and S-phase of the cell cycle) and cyclin B1 (marker of G2 phase of mitosis) in both granulose cells and lysates of whole ovarian chicken follicular walls.

Taken together, our results demonstrate a direct regulatory action of ghrelin on key cellular functions, such as proliferation, apoptosis and estradiol secretion. We suggest two mechanisms of ghrelin action in the ovary: 1) ghrelin directly influences aromatase activity and protein expression; 2) ghrelin stimulates cell proliferation and antiapoptotic actions. In light of this finding, the next step is to elucidate the mechanism of ghrelin action in the ovary.

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Conflicts of interest statement: None declared.

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