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Effect of two types of stress (heat shock/high temperature and malnutrition/serum deprivation) on porcine ovarian cell functions and their response to hormones

Alexander V. Sirotkin

Animal Production Research Centre, Hlohovecká 2, 951 41 Luzianky near Nitra, Slovakia

sirotkin@scpv.sk

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SUMMARY

The aim of the present study was to understand the interrelationships between stress, hormones and basic ovarian functions in the ovary. For this purpose, we compared the expression of markers of proliferation (PCNA, cyclin B1), of apoptosis (Bax, caspase-3) and secretory activity (release of progesterone, P_4, and insulin-like growth factor, IGF-I) in whole ovarian follicles and granulosa cells cultured in conditions of normal temperature (37.5°C) and feeding (with serum), high temperature (41.5°C, with serum) and malnutrition (37.5°C, without serum), with and without hormones [IGF-I, leptin and follicle-stimulating hormone (FSH)]. The expression of proliferation and apoptosis markers was evaluated by SDS PAGE–western blotting whereas radioimmunoassay (RIA) measured the release of hormones. High temperature dramatically induced a reduction in both proliferation and apoptosis markers in both ovarian follicles and granulosa cells and induced a significant increase in P_4 and IGF-I release by ovarian granulosa cells but not in P_4 secretion by ovarian follicles. Serum deprivation increased accumulation of cyclin B1 but not other markers of proliferation (PCNA) and apoptosis (Bax, caspase-3) or P_4 release in ovarian follicles. On the contrary, it inhibited the expression of apoptotic marker (Bax), release of both P_4 and IGF-I but it did not affect proliferation marker (PCNA) in granulosa cells. Adding IGF-I, leptin and FSH affected proliferation, apoptosis and secretory activity of ovarian cell functions but also prevented an inhibitory effect of high temperature on the expression of Bax and PCNA and an inhibitory action of serum deprivation on PCNA in ovarian follicles. Furthermore, treatment with these hormones prevented an inhibitory action of thermal stress on Bax, PCNA, P_4 and IGF-I in ovarian granulosa cells. The present observations (1) confirm the involvement of hormones (IGF-I, leptin and FSH) in the control of proliferation, apoptosis and secretory activity of ovarian cells, (2) demonstrate for the first time that heat stress/increased temperature can induce a reduction in ovarian cell proliferation and apoptosis and an oversecretion of ovarian hormones, (3) show that malnutrition/serum deprivation can reduce both apoptosis and secretory activity of ovarian cells, (4) demonstrate the differences in the response of granulosa and other ovarian follicular cells to stresses, and (5) are the first demonstration that hormones (IGF-I, leptin and FSH) could be used for preventing the effect of stresses on ovarian cell functions.

Key words: stress (heat shock, malnutrition), IGF-I, leptin, FSH, ovarian follicles, granulosa cells.

INTRODUCTION

Contemporarily global warming, changes in nutrition and expansion of metabolic disorders can affect human and animal reproductive processes. Ambient temperature, nutrition and other environmental factors control and synchronize reproductive cycles, although the mechanisms of such actions are studied insufficiently. It is proposed that these factors affect gonadal functions through action on its hormonal regulators and intracellular regulators of proliferation and apoptosis.

The negative effect of high ambient temperatures on reproductive processes is well documented (Putney et al., 1989; Edwards and Hansen, 1997; Lawrence et al., 2004). Hot environments can increase blood, rectal and uterine temperatures, suppress puberty (Kurowicka et al., 2006), ovarian cyclicity (Christenson, 1980), ovulation (Rozenboim et al., 2007), spermatogenesis, fertility (Kunavongkrit et al., 2005), oogenesis and embryogenesis (Putney et al., 1989; Edwards and Hansen, 1997; Beere, 2004; Lawrence et al., 2004), and reduce conception and pregnancy rates (Christenson, 1980; Putney et al., 1989) in different domestic species.

The mechanisms of such effects remain unknown. Heat stress, through heat shock proteins, can suppress apoptosis in non-ovarian cells (Beere, 2004; Akerfelt et al., 2007) but its effect on apoptosis and proliferation in ovarian cells is yet to be examined. These and other ovarian cell functions are under the control of steroids, peptide hormones, growth factors and prostaglandins (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005; Sirotkin et al., 2005). In ovarian cells, changes in the expression of heat shock proteins are associated with changes in the production of prostaglandins (Narayansingh et al., 2004) and receptors for steroid hormones (Salvetti et al., 2008). Furthermore, high ambient temperatures can either reduce or increase plasma follicle-stimulating hormone (FSH), prolactin, progesterone, testosterone and estradiol levels (Kurowicka et al., 2006; Rozenboim et al., 2007), reduce androstenedione and estradiol, and increase basal and luteinizing hormone (LH)-induced progesterone production by ovarian cells *in vitro* (Bridges et al., 2005; Kurowicka et al., 2006). Therefore, high temperatures can suppress ovarian functions through changes in the release of reproductive hormones, and that consequently manipulation with these hormones can neutralize the negative effect of heat environment on reproduction. Nevertheless, there is no direct evidence for the effect of heat stress on ovarian hormone release and for the ability of ovarian hormones to modify the effect of heat stress on the ovary yet.

It is proposed that heat stress can suppress gonadal functions through the reduction of food consumption (Kunavongkrit et al., 2005). Malnutrition can affect reproductive processes *via* changes

in the secretion of metabolic hormones. Food restriction reduces the release of the metabolic hormone leptin (a product of adipose and some other tissues), which can affect reproduction through the hypothalamo-hypophysial system and by direct action on gonads. Leptin is able to regulate growth of ovarian follicles, corpus luteum development, suppress ovarian cell apoptosis, activate ovarian cell proliferation and affect the release of steroid hormones, oxytocin, prostaglandin and IGF-I and IGFBP-3 by ovarian cells (Spicer, 2001; Smith et al., 2002; Barb et al., 2005; Sirotkin et al., 2005; Zieba et al., 2005). Furthermore, external factors can control reproductive processes affecting the release of gonadotropins (FSH, LH), the most known promoters of ovarian cell proliferation and follicular growth, regulators of apoptosis and stimulators of the release of ovarian steroid and peptide hormones (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005; Sirotkin et al., 2005). Both leptin (Spicer, 2001; Sirotkin et al., 2005; Zieba et al., 2005) and gonadotropins (Erickson and Danforth, 1995; Berisha and Schams, 2005) can control ovarian functions through stimulation of local production of insulin-like growth factor I (IGF-I), which anti-apoptotic effect and stimulatory action on ovarian cell proliferation, follicullogenesis and hormone release are similar to action of leptin and gonadotropins (Sirotkin et al., 1998; Sirotkin et al., 2005; Makarevich et al., 2000; Berisha and Schams, 2005).

Therefore, functional interrelationships between two kinds of stress (induced by high temperatures and malnutrition), hormones and basic ovarian cell functions (proliferation, apoptosis, secretory activity) are principally possible. It might be proposed that both kinds of stress can affect basic ovarian functions through changes in hormone release, which in turn can affect proliferation, apoptosis and secretory activity of ovarian cells. If it is true, hormones can modify and even neutralize the negative effect of stressors on ovarian functions. This hypothesis can be promising for understanding and eliminating the effect of environmental stressors (non-optimal temperature and nutrition) on reproductive functions. Nevertheless, this hypothesis requires support with direct experimental data because no effect of high temperatures, malnutrition and their combination on ovarian cell proliferation, apoptosis and release of hormones, as well as the influence of hormones on these effects of stressors have been examined yet. One of the causes of the lack of such evidence can be the absence of an adequate experimental model enabling the examination of the effect of these factors directly on ovarian cells. Such a model could be isolated ovarian cells cultured in conditions of increased temperatures (41.5°C instead of standard 37.5°C), reduced nutrition (deprivation of blood serum, an obvious nutrient of cells in culture), addition of hormones (of which the importance in controlling ovarian cell function and in mediating the effect of stress are documented) and the combination of these factors.

The general aim of the present study is to understand the interrelationships between stress, hormones and basic ovarian functions in the ovary. For this purpose, we examined the expression of markers of proliferation (PCNA, cyclin B1), of apoptosis (Bax, caspase-3) and secretory activity (release of progesterone, P_4, and IGF-I) in isolated porcine ovarian cells (whole ovarian follicles and granulosa cells). The cells cultured in conditions of normal temperature (37.5°C) and feeding (with serum), high temperature (41.5°C, with serum) and malnutrition (37.5°C, without serum), with and without hormones (IGF-I, leptin and FSH), were compared.

MATERIALS AND METHODS

Experimental design

Experimental design is presented in Table 1. Two series of experiments were performed. In the first series of experiments, we examined proliferation (accumulation of PCNA and cyclin B1), apoptosis (expression of Bax and caspase-3) and secretory activity (release of P_4) of isolated whole ovarian follicles cultured in

Table 1. Experimental design

Series number	Parameters of experiments			
	Objects of analysis	Temperature (°C)	Presence of serum	Addition of hormones
1	Proliferation (PCNA, cyclin B1), apoptosis (Bax, caspase-3) and secretory activity (release of P_4) of cells of whole ovarian follicles	37.5	Serum	None IGF-I Leptin FSH
		41.5	Serum	None IGF-I Leptin FSH
		37.5	No serum	None IGF-I Leptin FSH
2	Proliferation (PCNA), apoptosis (Bax) and secretory activity (release of P_4 and IGF-I) of ovarian granulose cells	37.5	Serum	None IGF-I Leptin FSH
		41.5	Serum	None IGF-I Leptin FSH
		37.5	No serum	None IGF-I Leptin FSH

IGF-I, insulin-like growth factor; FSH, follicle-stimulating hormone.

conditions of normal (37.5°C) or high (41.5°C) temperatures (with and without serum) and of normal (37.5°C) temperature without serum. All the follicles were cultured either with or without hormones, IGF-I, leptin and FSH. In the second series of experiments, we examined proliferation (accumulation of PCNA), apoptosis (expression of Bax) and secretory activity (release of P_4 and IGF-I) of isolated ovarian granulosa cells cultured in the same conditions as follicles in the first series of experiments.

Preparation, culture and processing of granulosa cells

Granulosa cells were collected from the ovaries of non-cycling Slovakian white gilts (*Sus scrofa domestica* L., Suidae, Artiodactyla), 200 days of age, after slaughter at a local abattoir. They were processed and pre-cultured as described previously (Sirotkin et al., 2008) in DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution (all from Sigma Chemical Co., St Louis, MO, USA). Granulosa cells (1×10^6 cells ml^{-1}) were cultured in 2 ml culture medium in Falcon 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA). First, the cells were pre-cultured in medium at 37°C under 5% CO_2 in humidified air. After two days of pre-culture, when cells attached to the bottom of the wells, the medium was replaced with medium of the same composition. Cells were cultured for two days in fresh medium supplemented or not supplemented with fetal calf serum (10%, Sigma Chemical Co.) in temperatures 37.5°C or 41.5°C, with or without hormones. Experimental groups received biological grade recombinant human leptin (Sigma Chemical Co., 100 ng ml^{-1} medium), immunological grade recombinant IGF-I (Calbiochem, Lucerne, Switzerland, 100 ng ml^{-1} medium) or biological grade porcine FSH (Sigma Chemical Co., 100 ng ml^{-1} medium). These doses were comparable with amounts of corresponding hormones in the blood and/or with doses used in previous *in vitro* experiments (Sirotkin et al., 1998; Berisha and Schams, 2005). All hormones were dissolved in medium immediately before the experiment. Cells were collected after 48 h of culture with and without treatments (the time of maximal response to hormones, as was determined in previous experiments, not shown). Immediately after culture, cells were lyzed in ice-cold lysis buffer (1% Triton X-100, 0.5% Igepal NP-40, 5 mmol l^{-1} EDTA, 20 μg ml^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotonin, 10 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 10 mmol l^{-1} sodium ortovanadate in PBS, pH 7.5, all from Sigma Chemical Co., 50 μg well^{-1}) and frozen at –18°C to await SDS PAGE–western blotting for evaluation markers of proliferation and apoptosis, while the culture medium was gently aspirated and frozen at –18°C to await RIA to assess the secretory activity of cultured cells. After culture, cell number and viability were determined by Trypan Blue staining and counting by hemocytometer. No statistically significant differences in these indices between the groups were observed.

Isolation, culture and processing of ovarian follicles

Non-cycling Slovakian white gilts, 180 days of age and without visible reproductive abnormalities, were killed at a local abattoir. Ovarian follicles (2.5–3.5 mm diameter) were collected, processed and cultured for two days in Falcon 24-well plates (Becton Dickinson), 1 follicle per well per 2 ml culture medium DME/F-12 1:1 mixture supplemented with 1% antibiotic–antimycotic solution, with or without 10% heat-inactivated fetal calf serum (all from Sigma Chemical Co.), with or without hormones at doses listed above and as described previously (Sirotkin et al., 1998). Immediately after culture, follicles were weighed and stored at –18°C. Thereafter the frozen follicles were lyzed by three repeated cycles of thawing, pipetting and freezing (5 min each) followed by lysis in lysis buffer, as was described for granulosa cells, and subjected to SDS PAGE–western blotting. The number and viability of cells within the follicles were not determined, although markers of cell proliferation, apoptosis and secretory activity were determined. The culture medium was stored at –18°C to await RIA.

Protein gel electrophoresis and immunoblotting

Frozen lysates of ovarian tissue were mixed 1:1 with electrophoretic buffer (0.0625 mol l^{-1} Tris-base, 2% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, 0.003% Bromophenol Blue; all from Sigma Chemical Co.), boiled at 95°C for 3 min and subjected to SDS–polyacrylamide gel electrophoresis in 4% and 10% stacking and resolving gels, respectively, at 25 mA constant current according to Laemmli (Laemmli, 1970). The samples were then transferred to Porablot PVDF membranes (Macherey-Nagel, Duren, Germany) using a semi-dry trans-blotter (Bio-Rad Labs, Richmond, WA, USA). Endogenous peroxidase in samples was quenched by incubation in 3% H_2O_2 for 15 min. Non-specific binding of antiserum was prevented by incubation in 5% blot-qualified BSA (Amersham plc, Little Clafton, UK) in TTBS (20 nmol l^{-1} Tris-base, 137 nmol l^{-1} NaCl, 0.1% Tween-20). Blocked membranes were probed with mouse monoclonal antibodies against the PCNA, cyclin B1, Bax, caspase-3 and loading control protein GAPDH (binds corresponding antigens of human, mouse and rat origin; dilution 1:250; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were then incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sevac, Prague, Czech Republic) and visualized using Super-Signal West-Pico luminescent substrate (Pierce, Rockford, IL, USA) and ECL Hyper-film (Amersham). Incubation medium without cells was used as a negative (blank) control. Molecular weights of fractions were evaluated using a molecular weight calibration kit (18, 24, 45 and 67 kD; ICN Biomedicals Inc., Irvine, CA, USA). To check for possible variability in protein loading, both the control and molecular weight standards were loaded twice into the gel in different lanes. No significant differences in expression of these replicates were detected.

Immunoassays

Concentrations of hormones were determined by RIA in 25 μl samples of incubation medium. P_4 and IGF-I were assayed using RIA/IRMA kits from DSL (Webster, TX, USA) according to the manufacturer's instructions. Antiserum against P_4 cross-reacted less than 0.001% to cortisol, corticosterone, cortisol, androstenediol, pregnenolone, oestradiol and testosterone. Sensitivity of the assay was 0.12 ng ml^{-1}, intra- and inter-assay coefficients of variation did not exceed 13.0% and 8.0%, respectively. The cross-reactivity of antiserum against IGF-I was less than 1.9% to IGF-II, less than 0.01% to insulin, proinsulin and less than 0.001% to EGF, oxytocin and P_4. Sensitivity of the assay was 0.3 ng ml^{-1}, the maximal intra- and inter-assay coefficients of variation were 3.4% and 8.2%, respectively. All RIAs were validated for use in samples of culture medium by dilution tests.

Statistics

Each experiment was performed on ovaries obtained from 15–20 animals each. Each experimental group was represented by four culture wells with granulosa cells (2×10^6 cells per well) or six wells with ovarian follicles (1 follicle per well). The data shown are means of values obtained in three separate experiments performed on different days using separate pools of ovaries. The samples intended

for RIA were analyzed separately. The corresponding samples intended for SDS PAGE–western blotting (total 12 samples of granulosa cells or 18 samples of follicles per treatment obtained in three experiments) were pooled before processing. Significant differences between the experiments were determined using two-way analysis of variance (ANOVA), followed by Student's *t*-test to detect significant differences between treatment and control groups by using Sigma Plot 9.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences from control at $P<0.05$ were considered as significant.

RESULTS

First series of experiments: effect of hormones, high temperature and serum deprivation on the expression of proliferation- and apoptosis-related substances and the release of P_4 and IGF-I by cultured porcine ovarian follicles

SDS PAGE–western blotting (Fig. 1) demonstrated the presence of substances related to apoptosis (Bax, caspase-3) and proliferation (PCNA, cyclin B1) in cultured porcine ovarian follicles. Caspase-3 and cyclin B1 were presented by two well-expressed fractions whereas Bax and PCNA were expressed much less, as single fractions. Follicles cultured at high temperature (41.5°C, with serum) contained substantially less caspase-3 (but not of Bax), PCNA and cyclin B1 than follicles cultured at normal temperature (37.5°C, with serum). Serum deprivation (37.5°C) did not affect the markers of apoptosis but a visible decrease in PCNA and an increase in cyclin B1 in follicles cultured without serum were observed. In each group, hormones (IGF-I, leptin but not FSH) were able to increase the accumulation of caspase-3, cyclin B1 and PCNA in follicles. Pattern of response of PCNA (but not of other parameters) to hormonal treatments depended on temperature and presence of serum.

RIA (Fig. 2) showed the release of P_4 by cultured ovarian follicles. Exposure to high temperature, but not serum deprivation, stimulated P_4 release. IGF-I and leptin inhibited, but FSH stimulated, this process in normal condition (37.5°C, with serum). In high temperatures, leptin, on the contrary, stimulated and FSH inhibited P_4 output. Serum deprivation induced a stimulatory effect of IGF-I, prevented an inhibitory effect of leptin but did not modify FSH action on P_4 release.

Second series of experiments: effect of hormones, high temperature and serum deprivation on the expression of proliferation- and apoptosis-related substances and release of P_4 and IGF-I by cultured porcine ovarian granulosa cells

Presence of Bax and PCNA in cultured porcine granulosa cells was demonstrated by SDS PAGE–western blotting (Fig. 3). Comparison of cells cultured without hormones showed that high temperature induced full disappearance in both Bax and PCNA. Serum deprivation reduced accumulation of Bax but not PCNA. No substantial effect of hormonal treatments on these parameters in granulosa cells was observed, although in conditions of high temperature both leptin and FSH increased Bax accumulation.

Release of both P_4 and IGF-I by cultured granulosa cells was revealed (Fig. 4). Exposure to high temperature significantly increased whereas serum deprivation decreased output of both P_4 and IGF-I. In normal conditions all the tested hormones significantly promoted the release of both P_4 and IGF-I. Exposure to high temperatures or serum deprivation did not modify the effect of hormones to P_4 release,

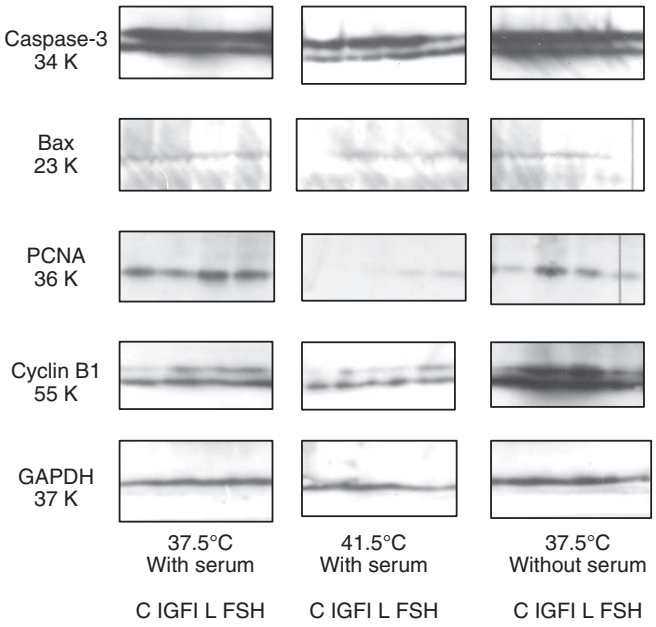

Fig. 1. Accumulation of apoptosis-related substances (Bax, caspase-3), proliferation-related substances (PCNA, cyclin B1) and housekeeping protein GAPDH in porcine ovarian follicles cultured at normal conditions (37.5°C, with serum), at high temperature (41.5°C with serum) and in conditions of malnutrition (37.5°C without serum), with and without hormones, i.e. insulin-like growth factor (IGF-I), leptin (L) and follicle-stimulating hormone (FSH) (SDS PAGE–western blotting). Samples from the corresponding groups obtained in different experiments were pooled before electrophoresis. Molecular weights of fractions are indicated left. C, control.

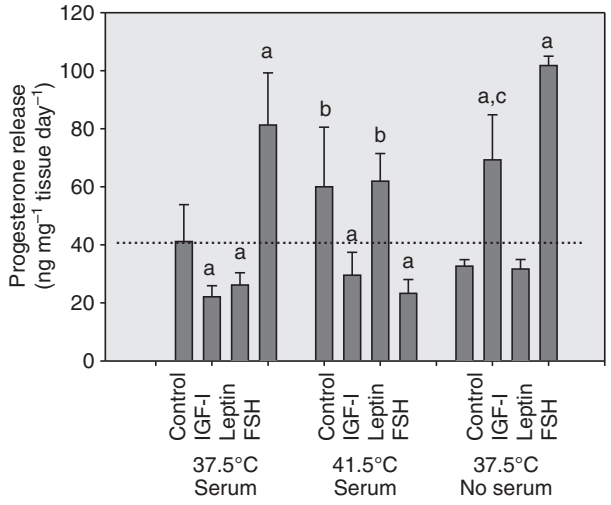

Fig. 2. Release of progesterone by porcine ovarian follicles cultured at normal conditions (37.5°C, with serum), at high temperature (41.5°C with serum) and in conditions of malnutrition (37.5°C without serum), with and without hormones, i.e. insulin-like growth factor (IGF-I), leptin (L) and follicle-stimulating hormone (FSH) (RIA). Values are means + s.e.m. obtained in three separate experiments. Indications of significant differences between the groups: (a) effect of hormones, shows a significant ($P<0.05$) difference between corresponding groups of cells cultured with or without hormones, (b) effect of high temperature, shows significant ($P<0.05$) differences between corresponding group of cells cultured at normal (37.5°C) and high (41.5°C) temperatures in the presence of serum, (c) effect of serum deprivation, shows significant ($P<0.05$) differences between corresponding group of cells cultured with or without serum at normal (37.5°C) temperature. The dotted line indicates the control level.

although in high temperatures, leptin effect was not expressed. High temperature, but not serum deprivation, prevented a stimulatory effect of hormones on IGF-I release.

DISCUSSION

Presence of proliferation- and apoptosis-related substances as well as the release of P_4 and IGF-I by porcine ovarian follicles and granulosa cells correspond with our previous observations (Sirotkin et al., 1998; Sirotkin et al., 2008).

This is the first comparison of these substances in whole ovarian follicles and granulosa cells. It demonstrated that some molecules are present in whole follicles and granulosa cells, not only in different amounts but also in different forms; PCNA, like GAPDH, in ovarian follicles were present by two fractions of similar molecular weights whereas in granulosa cells only a single fraction of these substances occurred. It suggests that granulosa cells and other compartments of the ovarian follicle can contain signalling substances in different forms. Although causes of such differences remain to be studied, it suggests different chemical modifications and probably different biological significance of these substances in different parts of ovarian follicle.

Our observations of hormones-induced changes in accumulation of proliferation- and apoptosis-related substances and of hormone secretion in both whole follicles and granulosa cells confirm previous reports on the involvement of IGF-I (Erickson and Danforth, 1995; Sirotkin et al., 1998; Sirotkin et al., 2005; Makarevich et al., 2000; Berisha and Schams, 2005), leptin (Spicer, 2001; Smith et al., 2002; Barb et al., 2005; Sirotkin et al., 2005; Zieba et al., 2005) and FSH (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005) in the control of these processes in the ovary. Some of the effects of hormones could be primary but other effects could be secondary. For example, both FSH (Erickson and Danforth, 1995; Berisha and Schams, 2005) and leptin (Sirotkin et al., 2005) can affect ovarian function through stimulation of ovarian IGF-I release. Furthermore, substantial differences in the action of these hormones on whole ovaries and granulosa cells suggest different functional roles of these hormones in controlling basic functions of different compartments of ovarian follicles and corresponding differences in responsibility and sensitivity of these compartments to hormonal stimuli.

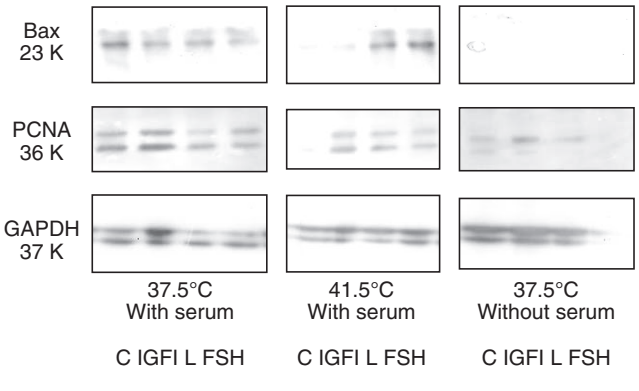

Fig. 3. Accumulation of apoptosis-related substances (Bax), proliferation-related substance (PCNA) and housekeeping protein GAPDH in porcine ovarian granulosa cells cultured at normal conditions (37.5°C, with serum), at high temperature (41.5°C with serum) and in conditions of malnutrition (37.5°C, without serum), with and without hormones, i.e. insulin-like growth factor (IGF-I), leptin (L) and follicle-stimulating hormone (FSH) (SDS PAGE–western blotting). Legend as in Fig. 1. C, control.

In our experiments, high temperature dramatically induced a reduction in both proliferation and apoptosis markers in both ovarian follicles and granulosa cells and a significant increase in P_4 and IGF-I release by ovarian granulosa cells but not in P_4 secretion by ovarian follicles. The reduction in ovarian cell proliferation and/or turnover and oversecretion of ovarian hormones can be causes of negative effects of heat stress on reproductive functions described previously (Christenson, 1980; Putney et al., 1989; Edwards and Hansen, 1997; Beere, 2004; Lawrence et al., 2004; Kunavongkrit et al., 2005; Rozenboim et al., 2007). They are in line with previous reports (Bridges et al., 2005; Kurowicka et al., 2006; Rozenboim et al., 2007) on the ability of heat stress to induce abnormal basal release of steroid hormones and to promote stimulatory effect of gonadotropin on steroid hormones output by cultured ovarian cells. Our observations represent the first data on the direct effect of high temperature on the proliferation and apoptosis of ovarian cells. Furthermore, they confirm that heat stress can induce inadequate release of both steroid hormone and growth factor by ovarian cells.

Serum deprivation, in contrast to high temperature, did not suppress the proliferation (PCNA) and apoptosis (Bax, caspase-3) markers and

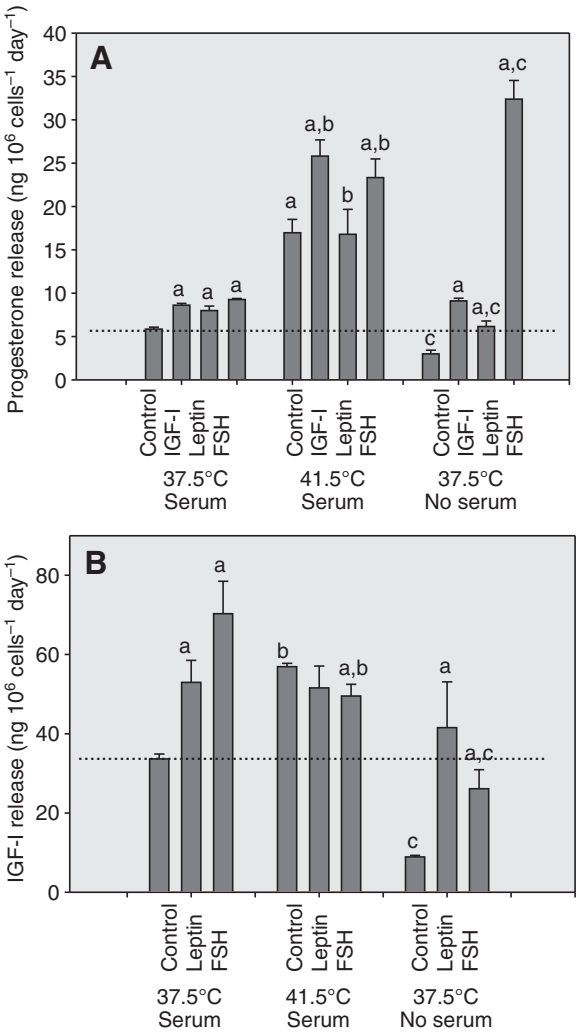

Fig. 4. Release of progesterone (A) and insulin-like growth factor (IGF-I) (B) by porcine ovarian granulosa cells cultured at normal conditions (37.5°C, with serum), at high temperature (41.5°C, with serum) and in conditions of malnutrition (37.5°C, without serum), with and without hormones, i.e. IGF-I, leptin (L) and follicle-stimulating hormone (FSH) (RIA). Legend as in Fig. 1. The dotted line indicates the control level.

P_4 release in ovarian follicles. Moreover, it increased accumulation of another proliferation marker, cyclin B1, in ovarian follicles. On the contrary, in granulosa cells serum deprivation inhibited expression of apoptotic marker, release of both P_4 and IGF-I not affecting proliferation marker. This is the first demonstration of the effect of serum deprivation on basic ovarian cell functions. If serum deprivation could be considered as an adequate model of cell malnutrition, it demonstrates that a deficiency in nutrients could inhibit the release of steroid hormone and growth factor but, in some cases, promote multiplication of ovarian cells by stimulating their proliferation and inhibiting apoptosis.

Our observations demonstrated the different response of whole ovarian follicles and granulosa cells to both high temperature and serum deprivation. Although not all of the analyzed parameters were measured in both ovarian follicles and granulosa cells, it was shown that granulosa cells were affected by both heat stress and malnutrition whereas ovarian follicles were affected mainly only by high temperatures. This may demonstrate that heat stress affects all of the compartments of the ovarian follicle, but the main target of nutritional stress are granulosa cells, whereas the theca cells can protect granulosa cells against nutritional stress probably by providing by necessary nutrients.

Comparison of the effects of high temperatures and serum deprivation on granulosa cells demonstrate that both kinds of stress have similar inhibitory influence of proliferation and apoptosis. It confirms the hypothesis of Kunavongkrit et al. that at least part of the negative effects of high temperatures on reproductive functions could be mediated by malnutrition (Kunavongkrit et al., 2005). However, opposite effects of these stresses on the release of hormones by granulosa cells suggest relatively independent regulation of ovarian secretory activity by external temperature and nutrition.

It is to be noted that the methodological approaches used in our short-term experiments enabled the detection of stress-induced changes in accumulation of some early apoptosis and proliferation markers, but not in number of cells or their death rate, which could occur by longer exposure of cells to stress.

In our experiments, treatments of ovarian cells with IGF-I, leptin and FSH were able not only to affect main ovarian cell functions but also modify the effect of both heat and nutritional stresses on these functions. Treatment with these hormones was able to prevent inhibitory effect of high temperature on the expression of Bax and PCNA and an inhibitory action of serum deprivation on PCNA in ovarian follicles. Furthermore, treatment with these hormones prevented inhibitory action of thermal stress on Bax, PCNA, P_4 and IGF-I in ovarian granulosa cells. The ability of hormones to control basic ovarian functions, to be influenced by stresses and to prevent effect of stress on ovarian functions suggest that stresses can influence ovarian cell functions *via* changes in hormone release. If this hypothesis would be supported by further *in vitro* and *in vivo* studies, it is possible that hormones, together with other indexes, could be used for the assessment of potential effects of stress on reproductive functions and for the elimination of negative effects of stresses on reproductive functions in animal production, veterinary and human medicine.

Taken together, our observations (1) confirm the involvement of hormones (IGF-I, leptin and FSH) in the control of proliferation, apoptosis and secretory activity of ovarian cells, (2) demonstrate for the first time that heat stress/increased temperature can induce a reduction in ovarian cell proliferation and apoptosis and oversecretion of ovarian hormones, (3) show that malnutrition/serum deprivation can reduce both apoptosis and secretory activity of ovarian cells, (3) demonstrate the differences in response of granulosa and other ovarian follicular cells to stresses, and (4) are the first demonstration that hormones IGF-I, leptin and FSH could be used for preventing the effect of stresses on ovarian cell functions.

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