

Serum deprivation affects secretory activity of cultured porcine ovarian follicles and granulosa cells and their response to hormones

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ABSTRACT: The aim of the present study is to understand the hormonal mechanisms of the effect of malnutrition on ovarian follicle functions. For this purpose, we examined the effect of malnutrition/serum deprivation, addition of metabolic hormones and gonadotropin (IGF-I, leptin and FSH) and their combination on the release of progesterone (P_4), testosterone (T), estradiol (E_2) and insulin-like growth factor I (IGF-I) by cultured whole ovarian follicles and on P_4 and IGF-I output by cultured granulosa cells isolated from porcine ovaries. It was observed that in ovarian follicles cultured with nutrients/serum addition of IGF-I reduced release of P_4 , but not of T or E_2 . Exogenous leptin reduced output of E_2 , but not of P_4 or T, and increased IGF-I output. No significant effect of FSH on release of steroid hormones by isolated follicles was found. Serum deprivation did not affect release of P_4 , but reduced output of T and E_2 , and promoted IGF-I release by cultured ovarian follicles. Addition of hormones failed to prevent the effect of malnutrition on the secretory activity of cultured ovarian follicles. In cultured granulosa cells, all the tested hormones promoted release of both P_4 and IGF-I. Food restriction/serum deprivation reduced both P_4 and IGF-I output. Additions of either IGF-I, leptin and FSH prevented the inhibitory action of malnutrition on both P_4 and IGF-I release. The present observations (1) confirm the involvement of the hormones IGF-I, leptin and FSH in the control of secretory activity of ovarian cells, (2) demonstrate, that both isolated ovarian granulosa cells and whole follicles cultured in the absence of serum nutrients could be an adequate in-vitro model for studying the effect of malnutrition on ovarian secretory functions, and (3) suggest, that malnutrition could affect ovarian functions through changes in the release of ovarian hormones.

Keywords: stress; malnutrition; IGF-I; leptin; FSH; ovarian follicles; granulosa cells

Patterns of nutrition control and synchronize reproductive cycles with optimal nutritional conditions. Malnutrition can block or prolong reproductive cycles (Crowe, 2008; Scaramuzzi and Martin, 2008). Therefore, nutritional control of reproductive processes represents not only a scientific, but also a medical and economical problem, which could be solved by understanding and manipulating the mediators of the effects of nutrition on reproduction. The mechanisms of the effect of nutrition on reproduction have however been studied insufficiently. Malnutrition can suppress GnRH/LH surges, progesterone can alter

IGF-I release and prolong or block ovulation of ovarian follicles, whilst food intake restores and promotes these processes (Crowe, 2008; Lucy, 2008; Scaramuzzi and Martin, 2008). This indicates that malnutrition inhibits reproductive processes by suppression of GnRH-gonadotropin-IGF-I-ovarian steroid hormone axis. This axis could be affected by metabolic hormones. Food restriction inhibits the release of the metabolic hormone leptin, a product of adipose and some other tissues including ovarian cells, which can affect reproduction both through the hypothalamo-hypophysial system and by direct action on gonads. Leptin is able to

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promote the release of well known promoters of ovarian cell proliferation and follicular growth, the gonadotropins FSH and LH, 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). In addition, leptin can affect gonads directly, by regulating the growth of ovarian follicles, *corpus luteum* development, suppressing ovarian cell apoptosis, activating ovarian cell proliferation and affecting the release of the steroid hormones, oxytocin, prostaglandin and IGF-I and IGFBP-3, by ovarian cells (Spicer, 2001; Sirotkin et al., 2005; Zieba 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), whose anti-apoptotic effects and stimulatory action on ovarian cell proliferation, folliculogenesis and hormone release are similar to the action of leptin and gonadotropins (Sirotkin et al., 1998, 2001, 2005; Makarevich et al., 2000; Berisha and Schams, 2005).

Therefore, it might be proposed, that malnutrition can affect basic ovarian functions through changes in hormone release, which in turn can affect secretory and other functions of ovarian cells. If malnutrition affects ovarian cells through some reproductive hormones, (1) a deficit of nutrients should alter the release of hormones, and (2) treatment with these hormones should prevent or promote the effects of malnutrition. In this case, manipulation with hormones can neutralize the negative effects of malnutrition on ovarian functions. This hypothesis can be promising for understanding and eliminating the effect of non-optimal nutrition on reproductive functions. However, this notion requires support with experimental data. Nevertheless, the direct effect of malnutrition on ovarian cells, as well as the influence of hormones on the effect of malnutrition has not yet been examined. One of the reasons for the lack of such evidence may be the lack of an adequate experimental model enabling examination of the direct effect of malnutrition on ovarian cells. Such a model might be isolated ovarian cells cultured in conditions of reduced nutrition (deprivation of blood serum, an obvious nutrient of cells in culture), addition of hormones (the importance of which in the control of ovarian cell function and in mediating the effects of malnutrition are documented) and the combination of these factors.

The aim of the present study is to understand the hormonal mechanisms of the effect of malnutrition on ovarian functions. For this purpose, we examined the effect of malnutrition/serum deprivation, addition of metabolic hormones and gonadotropin (IGF-I, leptin and FSH) and their combination on the release of progesterone (P_4), testosterone (T), estradiol (E_2) and insulin-like growth factor I (IGF-I) by cultured whole ovarian follicles and on P_4 and IGF-I output by cultured granulosa cells isolated from porcine ovaries.

MATERIAL AND METHODS

Experimental design

Two series of experiments were performed. The 1st series of experiments was performed on isolated whole ovarian follicles. Here, we examined the effect of malnutrition/serum deprivation, addition of metabolic hormones and gonadotropin (IGF-I, leptin and FSH) and their combination on the release of progesterone (P_4), testosterone (T), estradiol (E_2) and insulin-like growth factor I (IGF-I) by cultured whole ovarian follicles. The 2nd series of experiments was performed on cultured ovarian granulosa cells. In this series, we examined the effect of malnutrition/serum deprivation, addition of metabolic hormones and gonadotropin (IGF-I, leptin and FSH) and their combination on the release of P_4 and IGF-I output by cultured granulosa cells isolated from porcine ovaries.

Preparation, culture and processing of granulosa cells

Granulosa cells were collected from the ovaries of non-cycling Slovakian white gilts, at 200 days of age, after slaughter at a local abattoir. They were processed and precultured as described previously (Sirotkin et al., 2001, 2008) in DMEM/F-12 1 : 1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic-antimycotic solution (all from Sigma St. Louis, USA). Granulosa cells (1×10^6 cells/ml) were cultured in 2 ml culture medium in Falcon 24 well plates (Becton Dickinson, Lincoln Park, USA). First the cells were precultured in medium at 37.5°C under 5% CO_2 in humidified air. After two days of preculture, when cells had attached to the bottom of the wells, the medium

was replaced with medium of the same composition. Cells were cultured over two days in fresh medium supplemented or not supplemented with fetal calf serum (10%, Sigma) at a temperature of +37.5°C with or without hormones. Experimental groups received biological grade recombinant human leptin (Sigma, St. Louis, USA, 100 ng/ml medium), immunological grade recombinant IGF-I (Calbiochem, Lucerne, Switzerland, 100 ng/ml) or biological grade porcine FSH (Sigma, 100 ng/ml). These doses were comparable with amounts of corresponding hormones in the blood and/or with doses used in previous *in vitro* experiments (Sirotkin, 1995; Sirotkin et al., 1998; Schams et al., 1999; Berisha and Schams, 2005). All hormones were dissolved in medium immediately before experiment. Cells were collected after 48 h of culture with and without treatments (the time of maximal response to hormones, as determined in previous experiments is not shown). Immediately after culture the culture medium was gently aspirated and frozen at –18°C before RIA to assess the secretory activity of cultured cells. After culture, cell number and viability were determined by Trypan blue staining and were counted using a haemocytometer. No statistically significant differences in these indices between the groups were observed.

Isolation, culture, and processing of ovarian follicles

Non-cycling Slovakian white gilts, at 180 days of age and without visible reproductive abnormalities, were sacrificed at a local slaughterhouse. Ovarian follicles (2.5–3.5 mm dia.) were collected, processed and cultured for two days in Falcon 24 well plates (Becton Dickinson), one follicle per well/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), with and without hormones at the doses listed above and as described previously (Sirotkin et al., 1998). Immediately after culture, follicles were weighed, and the culture medium was stored at –18°C to await RIA.

Immunoassays

The concentrations of hormones were determined by RIA in 25 µl samples of incubation medium. P₄,

T, E₂ and IGF-I were assayed using RIA/IRMA kits from DSL, Webster, USA, according to the instructions of the manufacturer. The characteristics of the assays have been described previously (Sirotkin et al., 1998, 2001, 2005, 2008; Makarevich et al., 2000). 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 experimental group was represented by four culture wells with granulosa cells (2×10^6 cells per well) or six wells with ovarian follicles (one follicle per well). The data shown are means of values obtained in three separate experiments performed in different days using separate pools of ovaries. Significant differences between the groups were determined using two-way ANOVA followed by Duncan's test 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

1st series of experiments: effect of hormones, and serum deprivation on the release of hormones by cultured porcine ovarian follicles

Both serum-free and serum-supplemented medium, either before or after culture without ovarian follicles did not contain any measurable amounts of hormones: the concentrations of P₄, T, E₂ and IGF-I in these media were below the detection limit of the assays. In contrast, both serum-free and serum-supplemented medium conditioned by cultured follicles contained substantial amounts of these hormones. The accumulation/release of P₄, T, E₂ and IGF-I changed under the influence of IGF-I, leptin and FSH additions and the presence of serum in the follicle culture medium (Figure 1).

The addition of IGF-I to serum-free medium reduced the release of P₄, but not of T or E₂. Exogenous leptin reduced the output of E₂, but not of P₄ or T, and increased IGF-I output. No significant effect of FSH on the release of steroid hormones by isolated follicles was found. Serum deprivation reduced ba-

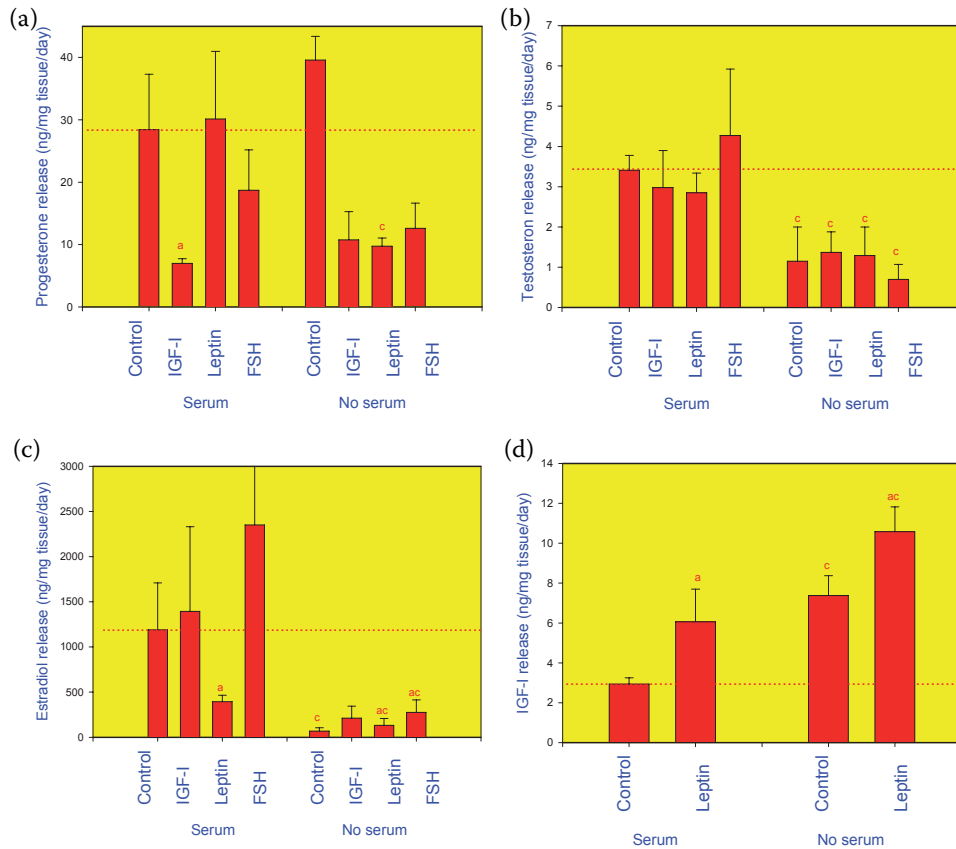


Figure 1. Release of progesterone (a), testosterone (b), estradiol (c) and insulin-like growth factor I (d) by porcine ovarian follicles cultured with and without serum and with and without hormones IGF-I, leptin and FSH. Values are means \pm S.E.M. obtained in three separate experiments. Indications of significant differences between the groups: (a) effect of hormones, shows a significant ($P < 0.05$) difference between corresponding groups of cells cultured with and without hormones (c) effect of serum deprivation, shows significant ($P < 0.05$) differences between corresponding group of cells cultured with and without serum

sal and/or hormone-induced P_4 , T , E_2 , release, but promoted IGF-I release by cultured ovarian follicles. The addition of hormones failed to prevent the effects of malnutrition on the secretory activity

of cultured ovarian follicles, and serum deprivation did not substantially affect the pattern of hormonal action on P_4 , T or IGF-I release, but in some cases it modified effect of hormones on E_2 output.

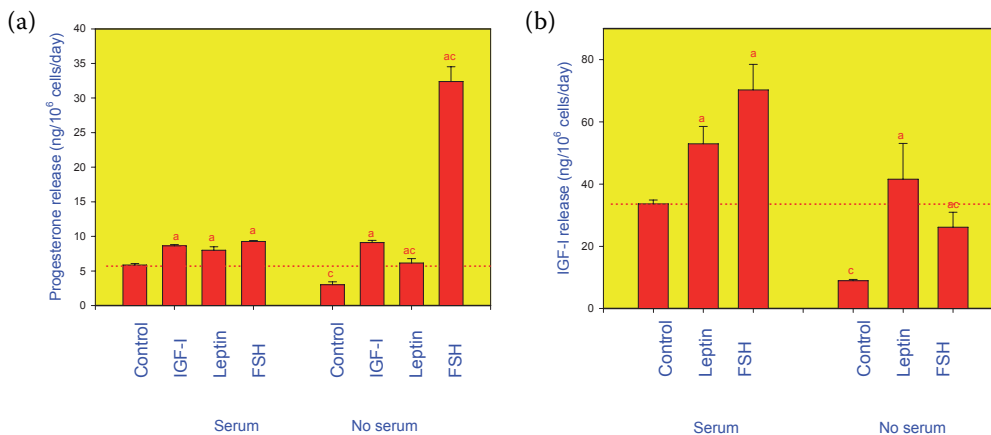


Figure 2. Release of progesterone (a) and insulin-like growth factor I (b) by porcine ovarian granulosa cells cultured with and without serum and with and without hormones IGF-I, leptin and FSH. Legends as in Figure 1

2nd series of experiments: the effect of hormones and serum deprivation on the release of hormones by cultured porcine ovarian granulosa cells

The release of both P_4 and IGF-I by granulosa cells into the culture medium was also detected. In granulosa cells cultured in serum-supplemented medium all hormonal additions significantly increased the release of both P_4 and IGF-I. Serum deprivation reduced both P_4 and IGF-I output by granulosa cells cultured without exogenous hormones. The addition of either IGF-I, leptin or FSH prevented the inhibitory actions of malnutrition on both P_4 and IGF-I release. Moreover, serum deprivation substantially promoted effect of FSH, but not of other hormones, on P_4 , but not on IGF-I secretion (Figure 2).

DISCUSSION

The release of P_4 , T, E_2 and IGF-I by both porcine ovarian follicles and granulosa cells into the culture medium observed in the present experiments corresponds with our previous observations (Sirotkin et al., 1998, 2001, 2008).

Our observations of hormone-induced changes in the secretory activity of both whole follicles and granulosa cells confirms previous reports on the involvement of IGF-I (Erickson and Danforth, 1995; Sirotkin et al., 1998, 2005; Makarevich et al., 2000; Berisha and Schams, 2005), leptin (Spicer, 2001; Sirotkin et al., 2001, 2005; Zieba et al., 2005) and FSH (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005) in the control of steroid hormone and IGF-I release by the ovary. Some of the effects of the 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 the stimulation of ovarian IGF-I release. Furthermore, substantial differences in the actions 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 the sensitivity of these compartments to hormonal stimuli.

In our experiments, ovarian follicles cultured without serum produced similar amounts of P_4 , less T and E_2 , but more IGF-I, than the follicles cultured

in a serum-supplemented medium. Granulosa cells cultured without serum exhibited reduced release of both P_4 and IGF-I. This suggests that malnutrition can reduce the release of steroid hormones and alters the release of IGF-I by ovarian cells. Different effects of serum deprivation on P_4 and IGF-I output by granulosa cells and ovarian follicles (containing both granulosa and theca cells) suggests different nutritional control of these hormones in different compartments of the ovarian follicle. Furthermore, the influence of serum deprivation on P_4 release by granulosa cells, but not by follicles, demonstrates that the monolayer of granulosa cells could be, at least in respect to P_4 , more sensitive to deficit of nutrients than whole follicles. The reason for this might be a higher level of endogenous nutrients in numerous layers of cells of the follicular wall and in follicular fluid. Furthermore, it suggests different mechanisms of action of serum deprivation on steroidogenesis in whole follicles and in granulosa cells. In ovarian follicles it might reduce the activity of 17-hydroxysteroid dehydrogenase-3 (17-HSD3), the key enzymes of production of androgens from progestagens, and of aromatase, which converts androgens to estrogens. In granulosa cells serum deprivation could also inhibit 3-hydroxysteroid dehydrogenase/⁵ 4 isomerase (3-HSD), an enzyme important for the synthesis of both progesterone and testosterone.

The action of serum deprivation on ovarian steroid hormones and IGF-I may be the first clue that the negative effects of malnutrition on reproduction might be mediated by abnormal release of these hormones and/or their regulators, the important roles of which in the control of ovarian functions are well documented (Hillier, 1991; Erickson and Danforth, 1995; Berisha and Schams, 2005). The second line of evidence could be ability of IGF-I, leptin and FSH to prevent the effects of malnutrition on the secretory activity of cultured granulosa cells. It suggests that a deficit of these hormones could be a hormonal signal for a deficit of nutrients for the reproductive system. It is known that food intake and the development of adipose tissue activate production of leptin, an activator of both FSH and IGF-I release, which in turn are known promoters of reproductive processes (Spicer, 2001; Sirotkin et al., 2005; Zieba et al., 2005). From a practical viewpoint, our observations suggest, that IGF-I, leptin, FSH or their regulators could be potentially used not only for the control of basal reproductive functions, but also for prevention or neutraliza-

tion of the negative effects of malnutrition on these processes in animal production and medicine.

In our experiments, whole ovarian follicles were less sensitive not only to serum deprivation, but also to the protective action of exogenous hormones, than granulosa cells. This suggests that both ovarian follicles and granulosa cells could be used for such investigations; granulosa cells could be a more suitable model for studying the effects of both nutrition and nutrition-related reproductive hormones.

Taken together, our results (1) confirm the involvement of the hormones IGF-I, leptin and FSH in the control of the secretory activity of ovarian cells; (2) demonstrate that both isolated ovarian granulosa cells and whole follicles cultured in the absence of serum nutrients could be an adequate *in vitro* model for studying the effects of malnutrition on ovarian secretory functions; and (3) suggest that malnutrition could affect ovarian functions through changes in ovarian hormone release.

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