In Vitro Assessment of Iron Effect on Porcine Ovarian Granulosa Cells: Secretory Activity, Markers of Proliferation and Apoptosis

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Summary

It would be desirable to expand the existing general knowledge concerning direct action of metals on the ovary. Nevertheless, the results of testing of iron compound on porcine ovarian cells should be interpreted carefully because iron is an essential element which could also induce changes in cellular processes. The aim of this *in vitro* study was 1) to examine dose-dependent effects of iron on the secretory activity of porcine ovarian granulosa cells, and 2) to outline the potential intracellular mediators mediating these effects. Specifically, we evaluated the effect of iron sulphate on the release of insulin-like growth factor I (IGF-I) and progesterone, as well as the expression of markers of proliferation (cyclin B1) and apoptosis (caspase-3) in porcine ovarian granulosa cells. Concentrations of IGF-I and progesterone were determined by RIA, cyclin B1 and caspase-3 expression by immunocytochemistry (ICC). Our results show a significantly decreased IGF-I secretion by ovarian granulosa cells after iron sulphate addition at the doses 0.5 and 1.0 mg/ml. The iron sulphate additions at doses 0.17 and 1.0 mg/ml had no effect on progesterone secretion. In contrast, iron sulphate addition at doses 0.17-1.0 mg/ml resulted in stimulation of cyclin B1 and caspase-3 expression. In conclusion, the present results indicate a direct effect of iron on 1) secretion of growth factor IGF-I but not steroid hormone progesterone, 2) expression of markers of proliferation (cyclin B1), or 3) apoptosis (caspase-3) of porcine ovarian granulosa cells. These results support an idea that iron could play a regulatory role in porcine ovarian function: hormone release, proliferation and apoptosis.

Key words

Iron • IGF-I • Progesterone • Proliferation • Apoptosis • Granulosa cell

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Introduction

Environmental pollution is one of the major issues of today's world (Ishaq *et al.* 2010). Although several adverse health effects of metals have been known for a long time, exposure to metals continues (Järup 2003). However, iron is also an essential element for all living organisms (Defrère *et al.* 2008, Brard *et al.* 2006). This element is found in all kind of foods (Chase *et al.* 1994) in two chemical forms: as organic hem iron and as non-hem inorganic ferrous and ferric iron. The organic hem iron is stored mainly in liver, meat, shellfish and other animal products. The inorganic iron is found in cereals, vegetables (Reilly 2004) and other plant foods (Reilly 2004). Polluted areas are possible sources of exposure to iron besides nutrition (Mendil *et al.* 2010, Squitti *et al.* 2007, Caniglia *et al.* 1994). Main intake of iron from nutrition is through the absorption in small intestine (Kwong and Niyogi 2009, Reilly 2004). Ferric iron (Fe^{3+}) is first reduced to the ferrous (Fe^{2+}) form by the apical ferric reductase (Zhang *et al.* 2008, McKie *et*

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al. 2001), and the absorption of Fe^{2+} into the enterocytes occurs *via* the divalent metal transporter-1 (DMT1; also known as DCT1, Nramp2 or SLC11A2) (Zhang *et al.* 2008, Reilly 2004). Iron-transporting proteins are transferrin, lactoferrin, ferritin and haemosiderin. The release of iron from transferrin and its delivery to reticulocytes, hepatocytes and other cell types is brought about by the interaction with specific high-affinity transferrin receptors (TfRs) in the cell membrane, followed by receptor-mediated endocytosis and by removal of iron and release of apotransferritin (apoTf) within the cell (Reilly 2004). Iron accumulates in liver (Kojadinovic *et al.* 2007, Reilly 2004), spleen (Bires *et al.* 1995), kidneys (Kojadinovic *et al.* 2007, Bires *et al.* 1995) and uterus (Ynsa *et al.* 2004). Free iron ions are extremely toxic and capable of catalyzing many deleterious reactions in cells and tissues (Reilly 2004). Excess of iron could affect a wide range of mechanisms involved in endometriosis development (Defrère *et al.* 2008), such as oxidative stress and tissue damage (Reilly 2004) or lesion proliferation (Defrère *et al.* 2008). Proliferating cells have an absolute requirement for iron, which is delivered by transferrin with subsequent intracellular transport *via* the transferrin receptor. Transferrin plays a crucial role in the local regulation of ovarian function and it may be an important factor in the regulation of granulosa cell differentiation (Durlej *et al.* 2008). On the other hand, ovarian functions of pigs are governed by growth factors such as insulin-like growth factor-I (IGF-I), steroid hormone progesterone (Kolesarova *et al.* 2010a,b, Sirotkin *et al.* 2008) and intracellular mediators of their action (Onagbesan *et al.* 2009) by promoting granulosa cell proliferation and decreasing ovarian cell apoptosis (Mao *et al.* 2004). Cell cycle peptides, especially cyclin B1, are involved in the processes of ovarian cell proliferation, growth, and development (Kolesarova *et al.* 2010a,b, Tomanek and Chronowska 2006). On the other hand, caspase-3 plays a role in the process of cell death (Boone and Tsang 1998). As previously published, the exposure of porcine ovarian granulosa cells to metals caused various alterations in hormonal release, in the expression of proliferation- and apoptosis-related peptides (Kolesarova *et al.* 2010a,b). There is no evidence for iron effects on porcine ovarian granulosa cells in connection with growth factor IGF-I, steroid hormone progesterone, proliferation-related peptide cyclin B1 and apoptosis-related peptide caspase-3.

The aim of this *in vitro* study was to examine dose-dependent effects of iron treatment on the secretory activity of porcine ovarian granulosa cells and to outline the potential intracellular mediators which mediate these effects. Specifically, to evaluate the iron effect on the release of IGF-I and progesterone, as well as on the expression of markers of proliferation (cyclin B1) and apoptosis (caspase-3) in porcine ovarian granulosa cells.

Materials and Methods

Preparation, culture and processing of granulosa cells from ovaries

Ovaries of non-cycling pre-pubertal Slovakian White gilts aged 100-120 days were obtained after slaughter at the Experimental Station of the Animal Production Research Centre Nitra. Conditions of their care, manipulations, and use corresponded to the instruction of EC no. 178/2002 and related EC documents, which were approved by the local ethics commission. Porcine ovaries obtained from healthy gilts without visible reproductive abnormalities were transported to the laboratory at 4 ºC and washed in sterile physiological solution. Follicular fluid was aspirated from 3-5 mm antral follicles. Granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10 % fetal calf serum (BioWhittaker™) and 1 % antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration of 10^6 cells per ml (determined by hemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1 ml per well) for radioimmunoanalysis (RIA) or Lab-Tek 16-welled chamber slides (Nunc Inc., International, Naperville, USA, 100 µl per well) for immunocytochemistry (Kolesarova *et al.* 2010ab). Both well plates and chamber slides were incubated at 37.5 °C and 5 % CO_2 in humidified air until a 75 % confluent monolayer was formed (5-7 days) (Sirotkin *et al.* 2003, 2004, Kolesarova *et al.* 2010a,b). At this time point, the medium (1 ml per well plates or 200 µl medium in 16-welled chamber slide cells) was renewed and luteinizing ovarian granulosa cells in culture media (Channing and Tasfriri 1977) were incubated for 18 h with the same supplements (10 % fetal calf serum, 1 % antibiotic-antimycotic solution) and with or without iron sulphate ($FeSO_4.7H_2O$) at the doses 0.17, 0.33, 0.5 and 1.0 mg/ml (Table 1). After 18 h the culture media from

well plates were collected for RIA, wells from chamber slides were washed in ice-cold PBS (pH 7.5). Cells were fixed for 1 h at room temperature in 4 % paraformaldehyde, dehydrated in alcohols (70, 80, 96 %; 10 min each) and stored in 96 % alcohol at –4 °C until immunocytochemical analysis.

Table 1. Iron concentrations used in the study.

Concentrations of $FeSO_4 . 7 H_2O$ ($mg.ml^{-1}$)
0
1.0
0.5
0.33
0.17

Maximum used dose: 1.0 mg $FeSO_4.7H_2O$ ml^{-1} corresponds to 0.2008 mg $Fe.ml^{-1}$.

Immunoassay

Concentrations of IGF-I and progesterone were determined in 25-100 µl incubation medium by RIA. These substances were assayed using RIA kits (Immunotech SAS, Marseille Cedex, France) according to the manufacturer's instructions (Kolesarova *et al.* 2010ab, Massanyi *et al.* 2000, Makarevich and Sirotkin 1999). All RIA were validated for use in samples of culture medium. RIA assay sensitivity for IGF-I was 2 ng/ml. Inter- and intra-assay coefficients of variation did not exceed 6.8 %, and 6.3 %, respectively. RIA assay sensitivity for P_4 was 0.05 ng/ml. Inter- and intra-assay coefficients of variation did not exceed 9.0 % and 5.8 %, respectively.

Immunocytochemistry

Signaling substances within granulosa cells plated on chamber slides were detected using immuno-cytochemistry according to Osborn and Isenberg (1994). The ImmunoCruz Staining System and primary mouse monoclonal antibodies against cyclin B1 and caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as described by the manufacturer at a dilution of 1:500. Visualization of the primary antibody binding sites was achieved with a secondary rabbit polyclonal antibody against mouse IGs, labeled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10 %). Chamber slides stained with peroxidase/DAB reagent were mounted with Glycergel mounting medium (DAKO, Carpinteria, CA, USA). The presence of each peptide was determined by light microscopy (Kolesarova *et al.* 2010a,b).

Statistical analysis

Each experimental group was represented by four culture wells or four chamber slide wells. The proportions of cells containing specific imunoreactivity were calculated from inspection of at least 1000 cells per chamber. Assays of hormone levels in the incubation media were performed in duplicate. The data shown are means of values obtained in three separate experiments each obtained from 10 to 12 animals. The samples intended for RIA or immunocytochemistry were processed separately. The rates of substance secretion were calculated per mg tissue per day. Significant differences between the control and experimental groups were evaluated by using two-way ANOVA, paired t-test or chi-square (χ^2) test using statistical software Sigma Plot 11.0 (Jandel, Corte Madera, USA). The data are expressed as means ± SEM. Differences from control at $P<0.05$ were considered as significant.

Results

Secretion of IGF-I by ovarian granulosa cells was decreased after addition of iron at 0.5 and 1.0 mg/ml (Fig. 1), while progesterone output was not affected by iron addition (Fig. 2). The occurrence of proliferation (cyclin B1)- and apoptosis (caspase-3)-associated markers within porcine ovarian granulosa cells were demonstrated by immunocytochemistry (Figs 3 and 4). The presence of some proliferation- and apoptosis-associated substances in the cells was affected by iron treatment (Fig. 3). Cyclin B1 expression was increased by iron additions (at all concentrations; Fig. 3). Similarly an increase in the expression of caspase-3 was observed after iron addition (at all concentrations; Fig. 4).

Discussion

Results of this study extend our previous observation on the secretory activity, as well as on markers of proliferation and apoptosis in porcine ovarian granulosa cells after metal additions (Kolesarova *et al.* 2009, 2010a,b). The effect of metals ions on secretion of growth

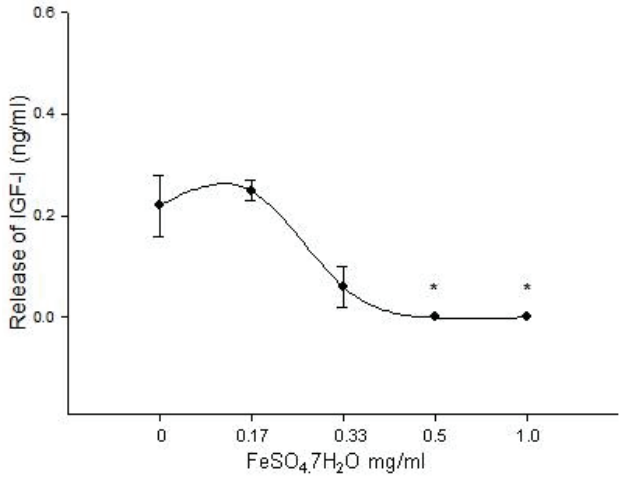

Fig. 1. Effect of iron on IGF-I release by porcine ovarian granulosa cells. The data shown are means of values obtained in three separate experiments each obtained from 10 to 12 animals. * Significant differences from control P<0.05 were evaluated by paired t-test.

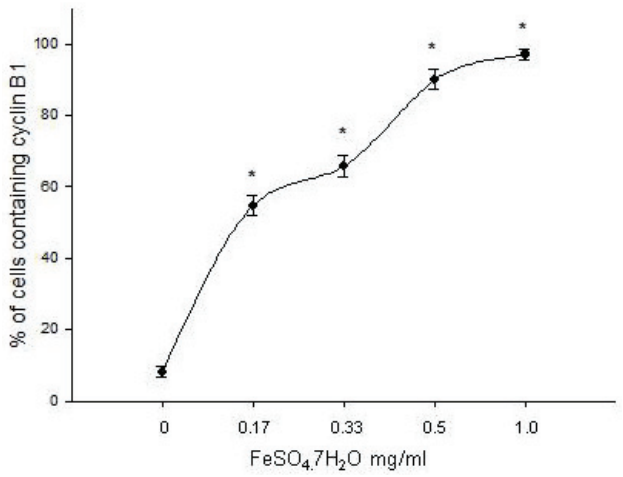

Fig. 3. Effect of iron on cyclin B1 expression in porcine ovarian granulosa cells. Control represents culture medium without iron addition. The data shown are means of values obtained in three separate experiments each obtained from 10 to 12 animals. * Significant differences from control P<0.05 were evaluated by chi-square (χ^2) test.

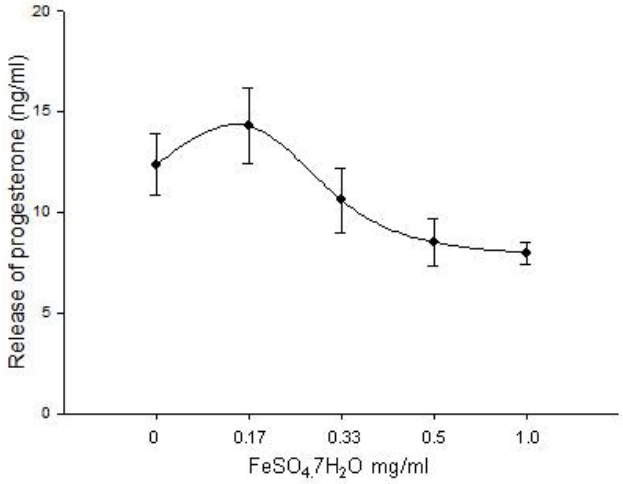

Fig. 2. Effect of iron on progesterone release by porcine ovarian granulosa cells. Control represents culture medium without iron addition. The data shown are means of values obtained in three separate experiments each obtained from 10 to 12 animals. Non-significant differences from control P>0.05 were evaluated by paired t-test.

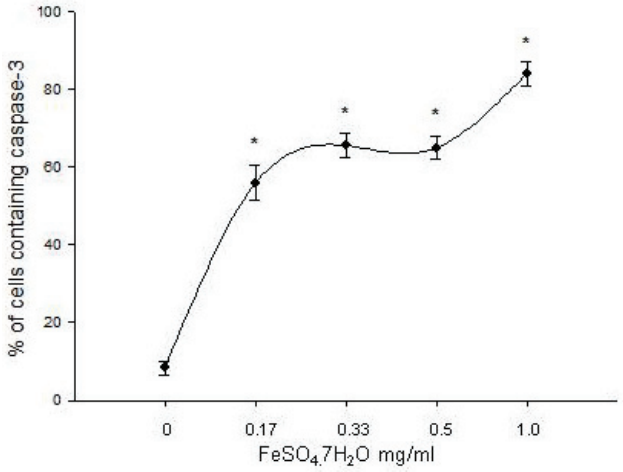

Fig. 4. Effect of iron on caspase-3 expression in porcine ovarian granulosa cells. Control represents culture medium without iron addition. The data shown are means of values obtained in three separate experiments each obtained from 10 to 12 animals. * Significant differences from control P<0.05 were evaluated by chi-square (χ^2) test.

factor IGF-I and steroid hormone progesterone, (Kolesarova *et al.* 2010a,b), on the expression of cyclin B1 and caspase-3 (Kolesarova *et al.* 2010a,b) and human cellular processes (Stawarz *et al.* 2009) were described in previous studies. However, the effects of iron on the secretory activity, markers of proliferation (cyclin B1) and apoptosis (caspase-3) in porcine ovarian granulosa cells were unknown. Present results, together with our previous observations (Kolesarova *et al.* 2010a,b), demonstrate a direct effect of certain metal ions on ovarian function.

First, iron is shown to be a potent regulator of ovarian secretory activity. There was a significant decrease in the secretion of growth factor IGF-I after iron addition. These results are in accordance with our previous data about effect of cobalt addition on secretory activity of porcine ovarian granulosa cells (Kolesarova *et al.* 2010b). Similarly to cobalt addition (Kolesarova *et al.* 2010b), iron application decreased secretion of IGF-I by porcine ovarian granulosa cells. In contrast, IGF-I concentrations in the blood of calves were not changed by different iron intakes – 50 or 10 mg Fe/kg (Ceppi *et al.* 1994). Different pattern of influence of iron on IGF-I in comparison with

present study could be due to different animal species, biological material and experimental conditions *in vivo* and *in vitro*. On the other hand, iron deficiency lowers the level of progesterone during estrus of rats (38 % reduction) (Grill *et al.* 2001). The progesterone-AAG (α1-acid glycoprotein, orosomucoid) interaction was inhibited by Fe^{2+} (Kerkay and Westphal 1969), while in our *in vitro* study, the addition of iron did not changed progesterone secretion by ovarian granulosa cells. This data reflect our previous results (Kolesarova *et al.* 2010b) on the secretion progesterone by porcine after cobalt sulphate treatment (at doses 0.17-1.0 mg/ml).

Second, the significant iron-induced expression of cyclin B1 as a marker of proliferation (Wyllie *et al.* 1998), suggest that iron could be involved in proliferation of ovarian cells. Our present findings confirm our previous data (Kolesarova *et al.* 2010a,b) about influence of some metals (lead and cobalt) on the cyclin B1 expression in porcine ovarian granulosa cells. Iron homeostasis is maintained by a combination of sensory and regulatory networks that modulate the expression of proteins of iron metabolism at the transcriptional and/or post-transcriptional levels (Reilly 2004). Iron may also be involved in the regulation of cell cycle through cyclin B1 as it was described in our previous studies (Kolesarova *et al.* 2010a,b).

Third, iron is considered as a regulator of apoptosis, because it suppressed the expression of caspase-3. This we also concluded in our previous experiments on the involvement of caspase-3 in mediating metal (lead, cobalt) action on porcine ovarian granulosa cells (Kolesarova *et al.* 2010a,b). Our previous studies showed the ability of metal ions to promote both proliferation and apoptosis (Kolesarova *et al.* 2010a,b). The present results suggest that iron could play a regulatory role in the turnover of cells within the ovary and therefore activate ovarian remodeling. However, excess of iron can result in toxicity and is associated with pathological disorders (Defrère *et al.* 2007, Carriquiriborde *et al.* 2004). Iron plays an important role in oxidative stress mechanisms (Defrère *et al.* 2008, García-Fernández *et al.* 2005), producing the deleterious hydroxyl radical which peroxides lipid membranes and damages DNA (García-Fernández *et al.* 2005). Excess iron accumulation can result in toxicity and may be one of the factors contributing to the development of endometriosis (Defrère *et al.* 2008) and cancerous endometrial tissues (Yaman *et al.* 2007). On the other hand, moderate iron deficiency is associated with increased susceptibility to chemically induced breast carcinogenesis (Grill *et al.* 2001). Deprivation of iron, an essential micronutrient, by chelation is known to inhibit proliferation of several human cancers, but its potential in ovarian cancer treatment remains unknown. Growth of tumor cells was inhibited by iron chelators *in vitro* and *in vivo* (Taetle *et al.* 1989). Brard *et al.* (2006) have evaluated the antiproliferative and cytotoxic activities of iron chelators to human and rat ovarian cancer cells. Cell cycle analysis showed a G0/G1- and S-phase block with increased apoptosis. Increased activities of caspase-3, -8, and -9 were associated with apoptosis. Organometallic compound iron (III)-salophene (Fe-SP) is a growth-suppressing agent *in vitro* for cell lines derived from ovarian cancer and a potential therapeutic drug to treat such tumors *in vivo.* Fe-SP treatment led to the activation of markers of the extrinsic (caspase-8) and intrinsic (caspase-9) pathway of apoptosis as well as of executioner caspase-3 (Lange *et al.* 2008). Our results demonstrate that iron may be involved in the regulation of the expression of marker of cytoplasmic apoptosis, such as caspase-3 in porcine ovarian granulosa cells. The obtained data could expand the existing knowledge concerning direct action of metal ions on the ovary. Nevertheless, our results concerning iron effects on porcine ovarian cells should be interpreted carefully because iron is an essential element, which could induce changes in cellular processes. In conclusion, our results indicate, 1) a direct effect of iron on secretion of growth factor IGF-I but not on progesterone secretion, 2) expression of marker of proliferation (cyclin B1) and 3) marker of apoptosis (caspase-3) in porcine ovarian granulosa cells.

These results support an idea that iron could play a regulatory role in porcine ovarian function: hormone release, proliferation and apoptosis.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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