In vitro Assessment of Iron on Porcine Ovarian Granulosa Cells: secretory activity, markers of proliferation and apoptosis

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SUMMARY

The obtained data could 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 but on the other hand it could induce changes in cellular processes. The general objective of this *in vitro* study was to first examine dose–dependent effects of iron on the secretory activity of porcine ovarian granulosa cells, secondly to outline the potential intracellular mediators that mediate these effects. Specifically, we evaluated the effect of $FeSO_4.7H_2O$ 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 Fe addition at the doses 0.5 and 1.0 mg/ml. The iron additions at doses 0.17 and 1.0 mg/ml had no effect on progesterone secretion. In contrast, iron addition at doses 0.17 and 1.0 mg/ml, resulted in stimulation of cyclin B1 and caspase -3 expression. In conclusion, the present results indicate, (1) a direct effect of Fe on secretion of growth factor IGF-I but not steroid hormone progesterone, (2) expression of markers of proliferation (cyclin B1) and (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.

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 (Jarüp, 2003). At the same time, iron (Fe) 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 haem iron, and as non–haem inorganic ferrous and ferric iron. The organic haem 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). Possible sources of exposure to Fe besides nutrition are polluted areas (Mendil *et al.*, 2010; Squitti *et al.*, 2007; Caniglia *et al.*, 1994). Main intake of Fe from nutrition is through absorption in small intestine (Kwong *et al.*, 2009; Reilly, 2004). Ferric iron (Fe^{3+}) is the first reduced to the ferrous (Fe^{2+}) form by the apical ferric reductase (Zhang *et al.*, 2008; McKie *et al.*, 2001), and the absorption of Fe^{2+} into the enterocytes occurs via the divalent metal transporter–1 (DMT1; also known as DCT1, Nramp2 and SLC11A2) (Zhang *et al.*, 2008; Reilly, 2004). Iron–transporting proteins are transferrin, lactoferrin, ferritin and haemosiderin. The Fe release from transferrin and its delivery to reticulocytes, hepatocytes and other cell types are brought about by 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 in uterine tissue (Ynsa *et al.*, 2004). Free iron ions are extremely toxic, and capable of catalysing 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 Fe, 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). Caspase-3, on the other hand, plays 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 alternations in hormonal release, in the expression of proliferation- and apoptosis-related peptides (Kolesarova *et al.*, 2010a,b). There is no evidence for Fe involvement 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 general objective 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 Fe effect on the release of insulin-like growth factor I (IGF-I) and steroid hormone progesterone, 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 pubertal Slovakian White gilts at the ages of 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 4ºC 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 haemocytometer). 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 the 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; Sirotkin *et al.*, 2004; Kolesarova *et al.*, 2010a,b). At this 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 18 h with the same supplements (10 % fetal calf serum, 1 % antibiotic–

antimycotic solution) and with or without chemical substance ferrous sulphate ($FeSO_4.7H_2O$) at the doses 0.17, 0.33, 0.5 and 1.0 mg/ml (Tab. 1). After 18h the culture media from wells 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 to await immunocytochemical analysis.

Immunoassay

Concentrations of IGF–I and P_4 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

Signalling substances within granulosa cells plated on chamber slides were detected using immunocytochemistry according to a previous study (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

directed by the manufacturer at a dilution of 1:500. Visualisation of the primary antibody binding sites was achieved with a secondary rabbit polyclonal antibody against mouse IGs, labelled 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 (DAKO, Carpinteria, CA, USA) mounting medium. 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 of four Chamber-slides 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 (n = 3) 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 Fe at 0.5 and 1.0 mg/ml (Fig. 1), while progesterone output was not affected by Fe 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, 4). The presence of some proliferation - and apoptosis-associated substances in the cells was affected by Fe treatment (Fig. 3). Cyclin B1 expression was increased by Fe additions (at all concentrations; Fig. 3). Similarly an increase in the expression of caspase–3 was observed after Fe addition (at all concentrations; Fig. 4).

Fig. 1

Fig. 2

Fig. 3

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 factor IGF-I and steroid hormone P_4, (Kolesarova *et al.*, 2010a,b), expression of proliferation- (cyclin B1) and apoptosis (caspase-3)- associated peptides (Kolesarova *et al.*, 2010a,b) and human cellular processes (Stawarz *et al.*, 2009) were described in the previous studies. The effects of iron on the secretory activity, markers of proliferation (cycline B1) and apoptosis (caspase-3) are unknown. Our results, together with our previous observations (Kolesarova et al., 2010a,b) demonstrate a direct effect of metal on ovarian function.

First, Fe is shown to be a potential regulator of ovarian secretory activity. There was a significant decrease in the secretion of growth factor IGF-I after Fe 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), Fe 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 Fe intakes – 50 or 10 mg Fe/kg (groups Fe50 and Fe10, respectively) (Ceppi *et al.*, 1994). Different pattern of influence of Fe on IGF-I in comparison with present study could be due to varied animal species, biological material and in different experiments *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, addition of Fe did not changed progesterone secretion by ovarian granulosa cells. This data reflect our previous results (Kolesarova et al., 2010b) on the secretion P_4 by porcine after cobalt treatment.

Second, the significant Fe-induced expression of cyclin B1 as a marker of proliferation (Wyllie *et al.*, 1998), suggest that Fe could be involved in proliferation of ovarian cells. Our present finding confirms 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. Regulation of gene transcription provides critical development, cell cycle and cell–type–specific controls on iron metabolism (Reilly, 2004). Iron may be

201 involved in regulation of cell cycle through cyclin B1 as it was described in our previous
202 studies (Kolesarova et al., 2010a,b).
203 Third, Fe is considered as a regulator of apoptosis, because it suppressed the expression of
204 caspase-3 as we concluded in our previous reports on the involvement of caspase-3 in
205 mediating metal (lead, cobalt) action on porcine ovarian granulosa cells (Kolesarova et al.,
206 2010a,b). Our previous studies show the ability of metal additions to promote both
207 proliferation and apoptosis, (Kolesarova *et al.*, 2010a,b). These results suggest, that Fe
208 could play a regulatory role in the turnover of cells within the ovary and therefore activate
209 ovarian remodelling. However, excess of iron can result in toxicity and is associated with
210 pathological disorders (Defrère *et al.*, 2007; Carriquiriborde *et al.*, 2004). Iron plays an
211 important role in oxidative stress mechanisms (Defrère *et al.*, 2008; García–Fernández *et*
212 *al.*, 2005) and produce the deleterious hydroxyl radical (*OH) which peroxides lipid
213 membranes and damages DNA (García–Fernández *et al.*, 2005). Excess of Fe
214 accumulation can result in toxicity and may be one of the factors contributing to the
215 development of endometriosis (Defrère *et al.*, 2008) and cancerous endometrial tissues
216 (Yaman *et al.*, 2007). On the other hand, moderate iron deficiency is associated with
217 increased susceptibility to chemically induced breast carcinogenesis (Grill *et al.*, 2001).
218 Deprivation of Fe, an essential micro–nutrient, by chelation is known to inhibit
219 proliferation of several human cancers but its potential in ovarian cancer treatment remains
220 unknown. Growth of tumor cells was inhibited by Fe chelators *in vitro* and *in vivo* (Taetle
221 *et al.*, 1989). Brard *et al.* (2006) have evaluated the anti–proliferative and cytotoxic
222 activities of iron chelators to human and rat ovarian cancer cells. Cell cycle analysis
223 showed a G0/G1– and S–phase block with increased apoptosis. Increase in caspase–3, –8,

and –9 activities were associated with apoptosis. Organometallic compound iron (III)–salophene Fe–SP is a patent 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 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 general knowledge concerning direct action of metals on the ovary. Nevertheless, the results of testing of iron on porcine ovarian cells should be interpreted carefully because iron is an essential element but on the other hand it could induce changes in cellular processes. In conclusion, our results indicate, (1) a direct effect of Fe on secretion of growth factor IGF-I but not steroid hormone progesterone, (2) expression of markers of proliferation (cyclin B1) and (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.

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Table 1 Iron concentration used in the the study

$FeSO_4.7H_2O$ (ml)	Medium (ml)	Dilution rate	Concentrations of $FeSO_4.7H_2O$ ($mg.ml^{-1}$)
0	1	0:1	0
1	0	1:0	1.0
0.5	0.5	1:1	0.5
0.33	0.67	1:2	0.33
0.17	0.83	1:5	0.17

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

FIGURE CAPTIONS

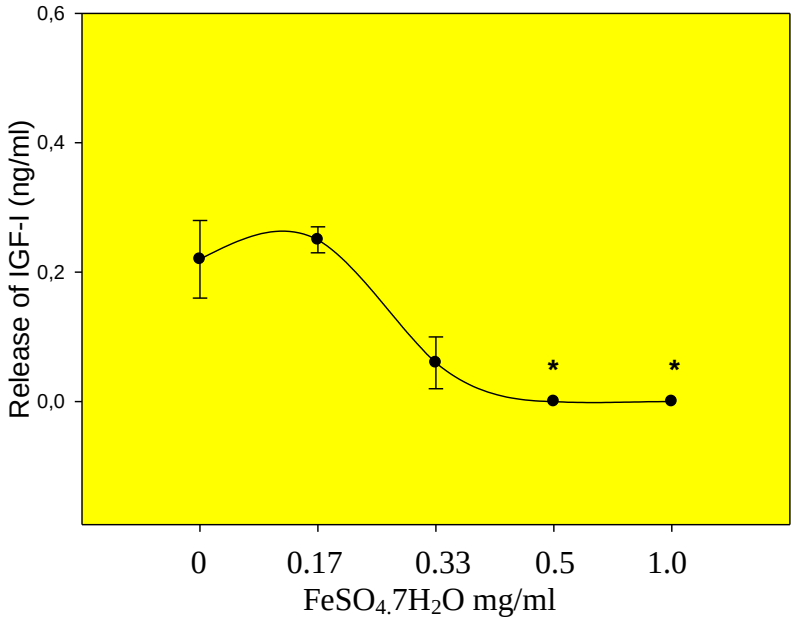
Figure 1. Effect of iron on IGF–I release by porcine ovarian granulosa cells. The data shown are means of values obtained in three separate experiments (n = 3) each obtained from 10 to 12 animals. *Significant differences from control $P<0.05$ were evaluated by paired t–test. RIA.

Figure 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 (n = 3) each obtained from 10 to 12 animals. Non-significant differences from control $P>0.05$ were evaluated by paired t–test. RIA.

Figure 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 (n = 3) each obtained from 10 to 12 animals. *Significant differences from control $P<0.05$ were evaluated by chi–square (χ^2) test. Immunocytochemistry.

Figure 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 (n = 3) each obtained from 10 to 12 animals. *Significant differences from control $P<0.05$ were evaluated by chi–square (χ^2) test. Immunocytochemistry.

Fig. 1


0,6
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Release of IGF-I (ng/ml)
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0
0.17
0.33
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1.0
FeSO4.7H2O mg/ml

Fig. 2

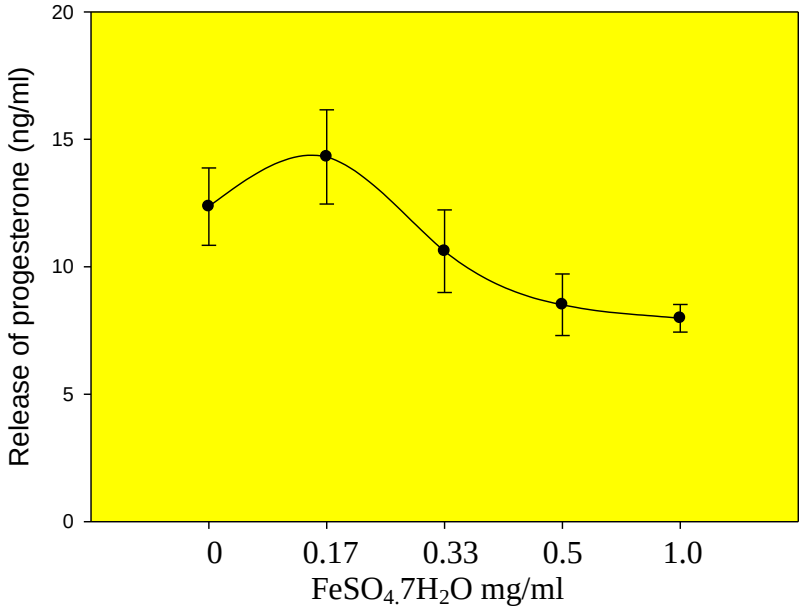

20
15
10
5
0
Release of progesterone (ng/ml)
0
0.17
0.33
0.5
1.0
FeSO4.7H2O mg/ml

Fig. 3

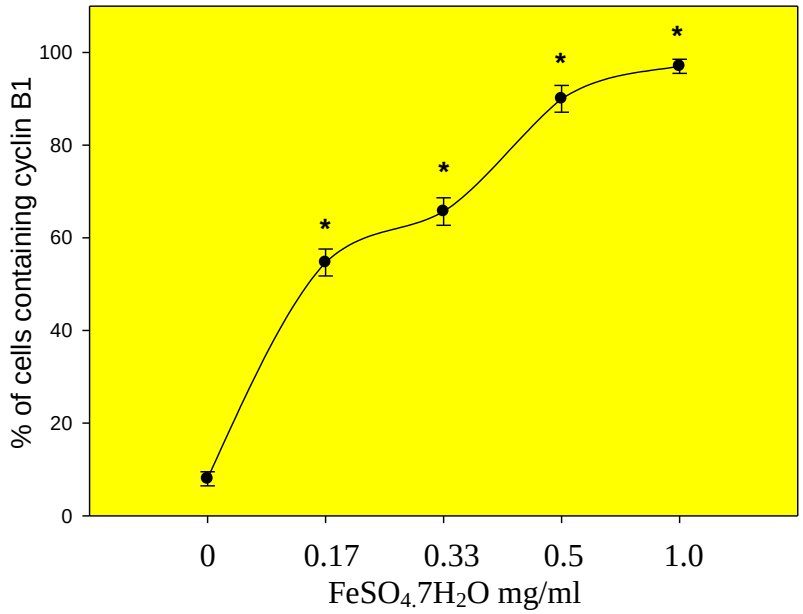
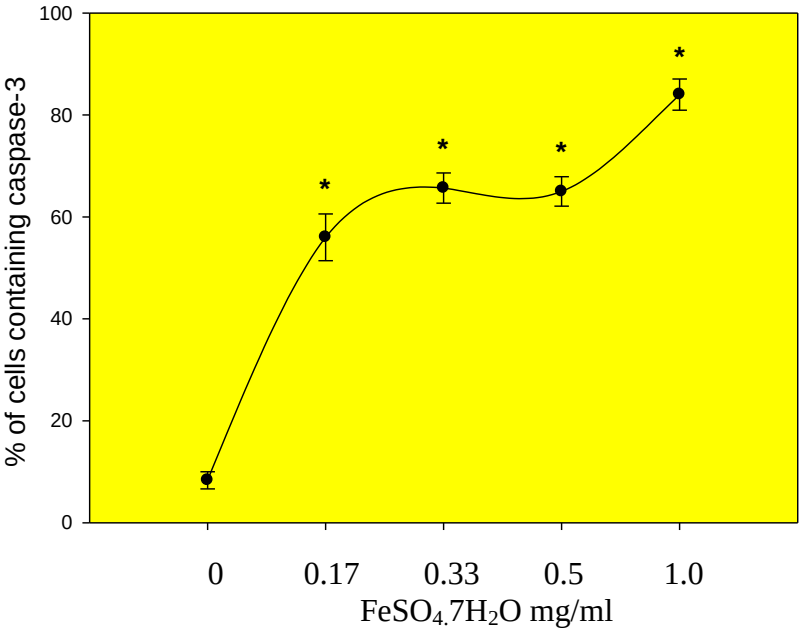

% of cells containing cyclin B1
100
80
60
40
20
0
*
*
*
*
*
0
0.17
0.33
0.5
1.0
FeSO4.7H2O mg/ml

Fig. 4


% of cells containing caspase-3
100
80
60
40
20
0
*
*
*
*
*
0
0.17
0.33
0.5
1.0
FeSO4.7H2O mg/ml