Short Communication

Human Adrenocortical Carcinoma (NCI-H295R) Cell Line as an *In Vitro* Cell Culture Model for Assessing the Impact of Iron on Steroidogenesis

(NCI-H295R cell line / ferrous sulphate heptahydrate / endocrine disruption / sexual steroid hormones / cell viability)

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Abstract. The aim of this *in vitro* study was to examine the dose-dependent effects of iron as a potential endocrine disruptor in relation to the release of sexual steroid hormones by a human adrenocortical carcinoma (NCI-H295R) cell line. The cells were exposed to different concentrations (3.90, 62.50, 250, 500, 1000 μM) of $FeSO_4.7H_2O$ and compared with the control group (culture medium without $FeSO_4.7H_2O$). Cell viability was measured by the metabolic activity assay. Quantification of sexual steroid production was performed by enzyme-linked immunosorbent assay. Following 48 h culture of the cells in the presence of $FeSO_4.7H_2O$, significantly ($P < 0.001$) increased production of progesterone was observed at the lowest concentration (3.90 μM) of $FeSO_4.7H_2O$, whereas the lowest release of progesterone by NCI-H295R cells was noted after addition of 1000 μM of $FeSO_4.7H_2O$, which did not elicit cytotoxic action ($P > 0.05$). Testosterone production was substantially increased at the concentrations ≤ 62.50 μM of $FeSO_4.7H_2O$. Lower levels of testosterone were recorded in the groups with higher concentrations (≥ 250 μM) of $FeSO_4.7H_2O$ ($P > 0.05$). The presented data suggest that iron has no endocrine disruptive effect on the release of sexual steroid hormones, but its toxicity may be reflected at other points of the steroidogenesis pathway.

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Abbreviations: ATCC – American Type Culture Collection, ATP – adenosine triphosphate, Ctrl – control group, CV – coefficient of variation, DMEM/F12 – Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, ED – endocrine disruptor, ELISA – enzyme-linked immunosorbent assay, Fe – iron, $FeSO_4.7H_2O$ – ferrous sulphate heptahydrate, IRPs – iron regulatory proteins, MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], MTP – microtitre plates, NCI-H295 – pluripotent adrenocortical carcinoma cell, NCI-H295R – human adrenocortical carcinoma cell line, SD – standard deviation, TfR – transferrin membrane receptor, TPP – techno plastic products, x – arithmetic mean.

Introduction

Endocrine disruptors (EDs) can strongly affect reproductive and endocrine functions in several ways, either by directly affecting hormone production through the interaction with appropriate enzymes, or through interfering with their transport to target organs to alter the natural hormone metabolism or even inactivating the function of steroidogenesis regulatory proteins (Sanderson and van den Berg, 2003).

Iron (Fe) has a widespread use and key roles in biological processes, including a bilateral role in the organ-

ism and its endocrine disrupting potential, which may lead to reproductive disorders (Lucesoli et al., 1999; Escobar-Morreale, 2012; Rossi et al., 2016; Ng et al., 2019). It participates in various physiological, regulatory and biochemical processes (Carter, 1995; Lieu et al., 2001; Gozzelino and Arosio, 2016). This trace element has a crucial role in the human body as part of metalloproteins, as well as enzymes that are associated with energetic reactions (Dev and Babitt, 2017). Iron metabolism is one of the most complex processes involving many organs and tissues, the interaction of which is critical for Fe homeostasis (Yiannikourides and Latunde-Dada, 2019). Transferrin is the primary transport protein for Fe and represents an essential Fe pool. When needed, Fe enters the cell via transferrin membrane receptor (TfR)-mediated endocytosis. The micronutrient subsequently dissociates throughout the cytosol and is taken up by ferritin, the most effective Fe storage protein. Both transferrin and ferritin are regulated by Fe regulatory proteins (IRP1/IRP2) found in the cytoplasm (Mackenzie et al., 2008). If due to genetic, lifestyle, and environmental factors transferrin is unable to effectively regulate the amount of Fe in the body, this will accumulate to toxic levels (Wang and Pantopoulos, 2011). Cellular Fe homeostasis is maintained by the IRP system. When cellular Fe levels are low, IRPs regulate expression of numerous Fe homeostasis proteins to inhibit translation of Fe transporter or storage proteins (such as ferritin and ferroportin), which leads to an increase in Fe uptake, decrease in Fe storage, and export (Muckenthaler et al., 2008). Among the numerous proteins involved in the Fe metabolism, hepcidin is the key regulator of systemic Fe levels (Yiannikourides and Latunde-Dada, 2019). At physiological levels, Fe and its compounds have not been reported to be toxic for animals and human organisms (Marzec-Wróblewska et al., 2012). Nevertheless, disturbances in the regulative absorption mechanism can appear due to pathological conditions or prolonged intake of high Fe doses. In these cases, since the capacity for storage of Fe in ferritin is exceeded, the metal is complexed to a phosphate or a hydroxide to form hemosiderin (or it is bound to proteins), and in this form it is present in the liver (Kabata-Pendias and Mukherjee, 2007). Indeed, Fe excess in the organism is associated with the metal deposition in organs throughout the body (mainly the liver, heart and endocrine glands) and relates to their specific damage. On the other hand, the most severe consequence of Fe depletion is Fe deficiency, anaemia, which is considered the most common nutrition deficiency worldwide (Clark, 2008). Moreover, it was recently suggested that Fe acts as a "double-edged sword" based on the ability to either maintain cellular homeostasis as a micronutrient or to overturn this balance as a catalyst responsible for serious structural and functional alterations (Tvrda et al., 2015).

Reports concerning the effects of Fe in the area of steroid hormone biosynthesis pathway are relatively scarce, and therefore, this *in vitro* study examined the effects of Fe (in the form of ferrous sulphate heptahydrate – $FeSO_4.7H_2O$) as a potential ED in relation to the release of sexual steroid hormones by the NCI-H295R cell line. This cell line was derived from pluripotent adrenocortical carcinoma (NCI-H295) cells. The NCI-H295R cell line has physiological characteristics of zonally undifferentiated human foetal adrenal cells, and represents a unique *in vitro* model system having the ability to produce all the steroid hormones found in the adult adrenal cortex and gonads, thus allowing testing the effects of diverse EDs on both corticosteroid synthesis together with production of sexual steroid hormones (Gazdar et al., 1990).

Material and Methods

Cell culture

The NCI-H295R cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in a Good Laboratory Practice certified laboratory (National Institute of Chemical Safety, Budapest, Hungary; OGYI/31762-9/2010) according to previously established and specifically validated protocols (Zhang et al., 2005; Hecker et al., 2006; OECD, 2011). After initiation of the NCI-H295R culture from the original ATCC batch, cells were cultured for five passages, and these cells were split and frozen down in liquid nitrogen (−196 °C). The cells for the experiments were cultured for a minimum of five additional passages using new NCI-H295R batches from frozen stocks prior to initiation of the exposure studies. The cells were grown in 75 cm² plastic cell culture flasks (TPP – Techno Plastic Products AG, Trasadingen, Switzerland) in an incubator under standard conditions (37 °C and 5.0 % CO_2). Subsequently, the cells were grown in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12; Sigma-Aldrich, St. Louis, MO) supplemented with 1.2 g/l sodium bicarbonate (Sigma-Aldrich), 5.0 ml/l of ITS+Premix (BD Bioscience, San Jose, CA) and 12.5 ml/l of BD Nu-Serum (BD Bioscience). The medium was changed 2–3 times per week and cells were detached from the flasks for sub-culturing using sterile 0.25 % trypsin-EDTA (Sigma-Aldrich). After trypsinization, cells were plated at the appropriate density to obtain 90–100 % confluence. The cell number was determined using a haemocytometer and adjusted with culture medium to a final concentration of 300,000 cells/ml. The cell suspensions were plated (with a final volume of 1.0 ml/well) into sterile plastic 24-well plates (TPP, Grainer, Frickenhausen, Germany) for estimation of sexual steroid hormones (with 50–60 % confluence of cells). For cytotoxicity evaluation, the cells (100 μl/well) were seeded into 96-well microtitre plates (MTP, Grainer). The seeded plates were incubated at 37 °C and 5.0 % CO_2 for 24 h to allow the cells to attach to the wells (Knazicka et al., 2013).

In vitro exposure

After 24 h attachment period, the cell culture medium was removed from the plates and replaced with new medium supplemented with 3.90, 62.50, 250, 500, and 1000 μM $FeSO_4.7H_2O$ ($\geq$ 99 %; Sigma-Aldrich), respectively. Cell cultures were set in 24 and 96-well plates (MTP, Grainer). Following treatment, the cells were maintained for 48 h. The experimental groups (exposed to different concentrations of $FeSO_4.7H_2O$) with the control group (Ctrl) (culture medium without $FeSO_4.7H_2O$) were compared.

Cell viability

The viability of the cells exposed to $FeSO_4.7H_2O$ was evaluated by the metabolic activity (MTT) assay (Mosmann, 1983). This colorimetric assay measures the conversion of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan particles by the mitochondrial succinate dehydrogenase enzyme of intact mitochondria of living cells. Formazan was measured spectrophotometrically. Following termination of $FeSO_4.7H_2O$ exposure, the cells were stained with MTT (Sigma-Aldrich) at a final concentration of 0.2 mg/ml. After 2 h incubation (37 °C and 5.0% CO_2), the cells and the formazan crystals were dissolved in 150 μl of acidified (0.08 M hydrochlorid acid) isopropanol (CentralChem, Bratislava, Slovak Republic). The absorbance was determined at a measuring wavelength of 570 nm against 620 nm as a reference by a microplate reader (Anthos MultiRead 400, Eugendorf, Austria). The data were expressed in percentage of the control group (i.e., absorbance of formazan from cells not exposed to $FeSO_4.7H_2O$).

Hormone measurement

At the end of 48 h $FeSO_4.7H_2O$ exposure, aliquots of the culture medium were removed from the 24-well cell culture plates and after centrifugation, the supernatant was collected and frozen at −80 °C until sexual steroid hormone measurements. Enzyme-linked immunosorbent assay (ELISA) was used for quantification of progesterone and testosterone (Dialab GmbH, Wiener Neudorf, Austria) directly from the aliquots of the medium. According to the manufacturer's data, the sensitivity of progesterone assay was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation were $\leq$ 4.0 % and $\leq$ 9.3 %, respectively. The sensitivity of testosterone assay was 0.075 ng/ml, and the intra- and inter-assay coefficients of variation were 4.6 % and 7.5 %, respectively. Cross-reactivity with 5α-dihydrotestosterone was 16.0 %. The absorbance was determined at a wavelength of 450 nm using a microplate reader and the data were evaluated by WinRead 2.30 computer software. In order to verify the performance of the NCI-H295R cells, a quality control plate was run in parallel to each NCI-H295R assay in accordance with the OECD guideline (2011). Forscolin, prochloraz and aminoglutethimide

(Sigma-Aldrich) dissolved in 0.1% dimethyl sulphoxide were used as positive controls.

Statistical analysis

The data were statistically analysed using the GraphPad Prism 3.02 program (GraphPad Software Incorporated, San Diego, CA). Descriptive statistical characteristics – arithmetic mean (x), minimum, maximum, standard deviation ($\pm$ SD), and coefficient of variation (CV) were evaluated. Homogeneity of variance was assessed by Bartlett's test. One-way analysis of variance and Dunnett's multiple comparison tests were used for statistical evaluations. The level of significance was set at *** ($P < 0.001$); ** ($P < 0.01$) and * ($P < 0.05$). Three independent experiments were performed.

Results and Discussion

In the present *in vitro* study, the viability of cells (Fig. 1) increased substantially ($P < 0.001$) at the lowest concentration (3.90 μM) of $FeSO_4.7H_2O$ in comparison with the control group. It can be postulated that this concentration could stimulate cell proliferation through oxidation-reduction reactions. Generally, cancer cells exhibit an increased demand for intracellular Fe, because the metal has a pivotal role in cellular homeostasis as a substrate or cofactor of enzymes that participate in cell proliferation (Steegmann-Olmedillas, 2011). Furthermore, Fe is needed for formation of Fe-sulphur clusters in mitochondrial succinate dehydrogenase (Eid et al., 2017). Probably, at the lowest concentration, Fe provides proper activity of this enzyme via the nicotinamide adenine dinucleotide-cytochrome oxidase systems and protects cells. The biological systems are equipped with a myriad assortment of mechanisms that regulate Fe homeostasis and serve to prevent insufficiency or toxicity of the metal (Eid et al., 2017). Our results of the MTT assay did not confirm the cytotoxic effect of Fe. The lowest viability of cells was found after addition of the highest concentration (1000 μM) of $FeSO_4.7H_2O$ ($P > 0.05$). This observation points to another possible mechanism of its toxicity that could be reflected at other points of the steroidogenesis pathway. Equally, the experimental study by Ng and Liu (1990) confirmed that Fe (tested up to a concentration of 100 μM $FeCl_2$) had no deleterious effect on the cell viability and hormone-induced steroidogenesis of Leydig cells and cells in the adrenal gland. The toxic effects of Fe on cells were described in another study by Bauckman et al. (2015). Ovarian carcinoma cell lines treated with 250 μmol/l of non-transferrin-bound Fe during 24 h induced mitochondrial damage, reduced expression of outer mitochondrial membrane proteins, increased reactive oxygen species levels, and reduced cell viability.

The results of this study extend our knowledge about effects of several heavy metals on steroidogenesis pathways in the NCI-H295R cell line. Hence, they contribute to the establishment of this cellular model system for
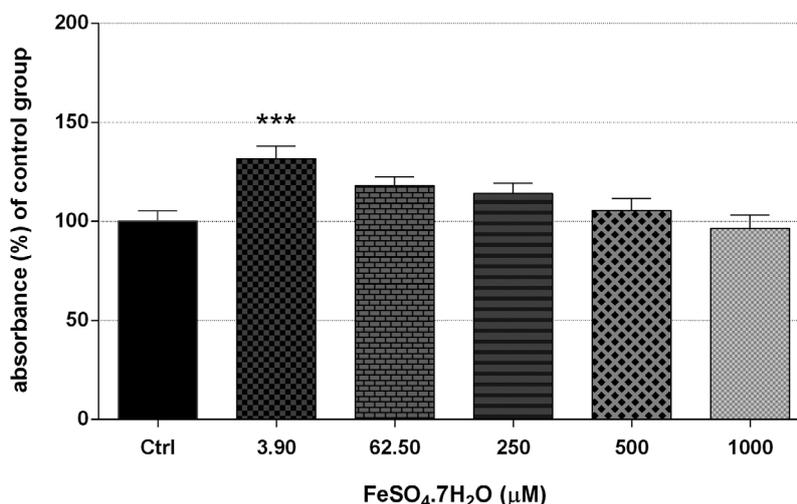
Fig. 1. Viability of the NCI-H295R cell line in culture after 48 h of $FeSO_4.7H_2O$ exposure
The cytotoxicity was assessed using the MTT assay following $FeSO_4.7H_2O$ exposure. The bar values represent the arithmetic mean ($\pm$ SD) absorbance in % of control (untreated) group (Ctrl) determined in three independent experiments. The number of replicate wells was 8–15 at each point. An increase in absorbance reflects an increase in cell viability. The statistical difference between the values of Ctrl and treated cells was indicated by asterisks – *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$).

the study of cell behaviour after exposure to various metals. Our previous results revealed significantly decreased viability of the NCI-H295R cell line after exposure to mercury ($\geq 25\ \mu M$ of $HgCl_2$; $P < 0.05$) (Knazicka et al., 2013), cadmium (1.90–62.50 μM of $CdCl_2$; $P < 0.01$) (Knazicka et al., 2015), nickel ($\geq 125\ \mu M$ of $NiCl_2$; $P > 0.05$) (Lukac et al., 2020) and copper (3.90–1000 μM of $CuSO_4.5H_2O$; $P < 0.001$) (Bilcikova et al., 2020), which elicited cytotoxic action. It is reasonable to believe that even exposure to low concentrations of some metals may be sufficient to affect the steroidogenic pathway. However, our results here demonstrate that $FeSO_4.7H_2O$ has a beneficial action at the lowest concentration tested (3.90 μM). This may be explained by the unique position of Fe among other biometals, and the transferrin cycle and regulation of Fe homeostasis acts to keep the amount of free ferrous iron (Fe^{2+}) at the lowest possible level. In cancer cells, upregulation of Fe regulatory proteins including transferrin, TfR-1 and ferritin has been found to further induce the level of accessible Fe ion pool (called labile Fe pool), which acts as a crossroads of metabolic pathways of Fe-associated physiological or pathological processes (Jamnogkan et al., 2017; Wang et al., 2019). Recently, Weber et al. (2020) suggested Fe homeostasis as a key function of cancer cell viability and proliferation independent of metabolic and adenosine triphosphate (ATP)-associated processes. Moreover, their findings demonstrated that Fe supplementation protects cells from cytotoxic death upon release of degradative enzymes or heavy metals and restores mitochondrial function even in the condition of lysosomal dysfunction. This indicates the special role of Fe homeostasis, uptake and regulation in cancer cells.

Our presented data suggest a direct impact of Fe on the steroid-producing NCI-H295R cells and subsequent changes in hormonal release. Following 48 h culture of the cells, significantly ($P < 0.001$) increased progesterone production was observed at the lowest concentration (3.90 μM) of $FeSO_4.7H_2O$ (35.33 $\pm$ 17.28 ng/ml) in comparison with the control group (17.48 $\pm$ 6.44 ng/ml). The lowest release of progesterone by the NCI-H295R cell line was noted at the highest concentration (1000 μM) of $FeSO_4.7H_2O$ (12.61 $\pm$ 5.86 ng/ml), which, however, did not elicit cytotoxic action ($P > 0.05$) (Table 1). Testosterone production was substantially increased at low concentrations (3.90 to 62.50 μM) of $FeSO_4.7H_2O$. Lower levels of testosterone were recorded in the groups with higher concentrations ($\geq 250\ \mu M$) of $FeSO_4.7H_2O$ ($P > 0.05$), whereas this decline was more prominent in comparison with that of progesterone (Table 2). The lowest release of testosterone by the NCI-H295R cell line was detected at the highest concentration (1000 μM) of $FeSO_4.7H_2O$ (1.40 $\pm$ 0.40 ng/ml) in comparison with the control group (2.68 $\pm$ 1.95 ng/ml). Altogether, the findings of the present *in vitro* study suggest that Fe has no endocrine disruptive effect on the release of sexual steroid hormones involved in the regulation of reproductive processes. However, testosterone release seemed to be more vulnerable than progesterone when NCI-H295R cells were exposed to $FeSO_4.7H_2O$, suggesting multiple sites of action of this metal in the steroidogenesis pathway. It may be assumed that the effect of enzymatic action of 17β-hydroxysteroid dehydrogenase is more sensitive, which further results in the decreased release of testosterone in comparison to progesterone. Additional studies are required to define the precise molecular mechanism of action of Fe on the

Table 1. Effect of 48 h FeSO₄.7H₂O exposure on the release of progesterone (ng/ml) by the NCI-H295R cell line

Groups	Control Ctrl	3.90 E	62.50 D	250 C	500 B	1000 A
FeSO4.7H2O (µM)						
x (ng/ml)	17.48	35.33***	24.93	18.16	16.86	12.61
minimum	5.96	14.80	6.74	6.96	5.11	8.43
maximum	25.43	59.54	36.21	26.17	27.21	19.31
± SD	6.44	17.28	11.72	7.96	8.00	5.86
CV (%)	36.85	48.91	47.00	43.80	47.44	46.48

Ctrl – control group; CV (%) – coefficient of variation; ± SD – standard deviation; x – arithmetic mean. The level of significance was set at *** ($P < 0.001$).

Table 2. Effect of 48 h FeSO₄.7H₂O exposure on the release of testosterone (ng/ml) by the NCI-H295R cell line

Groups	Control Ctrl	3.90 E	62.50 D	250 C	500 B	1000 A
FeSO₄.7H₂O (µM)						
x (ng/ml)	2.68	3.30	3.02	2.30	2.39	1.40
minimum	1.16	1.66	0.75	0.46	1.04	0.94
maximum	7.20	5.75	5.38	4.45	3.82	1.90
± SD	1.95	1.52	1.82	1.43	1.00	0.40
CV (%)	72.76	45.96	60.09	62.22	41.54	28.19

Ctrl – control group; CV (%) – coefficient of variation; ± SD – standard deviation; x – arithmetic mean. The statistical difference between the values of Ctrl and treated cells was not recorded ($P > 0.05$).

sexual steroid production and their metabolites, whose production is dependent on steroidogenic enzymes.

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