

THE EFFECTS OF INTERLEUKIN-6 ON VIABILITY, REDOX HOMEOSTASIS AND MIGRATION CAPACITY OF HUMAN PLACENTAL CELLS JEG-3 IN CHEMICALLY INDUCED HYPOXIA

Miloš M. Matić, Ana D. Obradović*, Marija D. Milošević,
Milica G. Paunović, Branka I. Ognjanović

*University of Kragujevac, Faculty of Science, Department of Biology and Ecology,
Radoja Domanovića 12, 34000 Kragujevac, Serbia*

*Corresponding author; E-mail: ana.obradovic@pmf.kg.ac.rs

(Received April 01, 2022; Accepted April 21, 2022)

ABSTRACT. Interleukin-6 (IL-6) is a pleiotropic cytokine involved in the regulation of cell growth and differentiation exerting an important role in the immune and inflammatory response. This study aimed to evaluate the effects of three increasing concentrations of IL-6 (1 pg/mL, 5 pg/mL, and 10 pg/mL) on cell viability, redox homeostasis parameters (O_2^- , NO_2^- , glutathione) and migratory potential in human trophoblast cell line JEG-3 under chemically induced hypoxia in short-term (24 h) and long-term (72 h) exposure. The obtained results show a dose-dependent reduction of cell viability and NO levels, while the concentration of O_2^- increased. Levels of total glutathione increased in a dose-dependent manner compared to control cells, suggesting its significant antioxidative contribution in hypoxic conditions. The migratory potential of cells was significantly elevated in the two highest applied doses implying the disturbance of cell invasive homeostasis at its pathological concentrations, which could represent a risk factor in some pregnancy disorders.

Keywords: Interleukin-6; trophoblast; oxidative stress; migration.

INTRODUCTION

The migration of trophoblasts into the endometrium is a key event in the placentation process necessary for a successful pregnancy. Inadequate trophoblast invasion leads to various pregnancy disorders resulting in shallow implantation and ischemia, affecting the production of reactive oxygen species and depleting the intracellular ATP concentrations (SILVA and SERAKIDES, 2016). The earliest stages of placenta development are conducted in a hypoxic environment. Due to the crucial importance of oxidative metabolism in various aspects of cell physiology, hypoxia in human trophoblast cells can certainly induce a cellular phenotype different from the one present under normoxic conditions (JAIN *et al.*, 2014). Complex cytophysiological processes of early placentation and invasiveness of human trophoblast require strict local regulation by various molecules such as growth factors and

cytokines including interleukin-1 β , insulin growth factors, tumor necrosis factor, epidermal and hepatocyte growth factors, and others (DIMITRIADIS *et al.*, 2005).

Interleukin-6 (IL-6), the main pro-inflammatory cytokine that regulates placental processes, is produced in human endometrial stromal and epithelial cells in response to stimulation of interleukin-1 or tumor necrosis factor and has been shown to inhibit glandular cell proliferation (JAUNIAUX *et al.*, 1996). The experiments suggest the effects of IL-6 on the activation of trophoblast metalloproteinases, implying a promigratory role of this cytokine (FITZGERALD *et al.*, 2005). It was shown that IL-6 stimulates migration of the human placental HTR-8/SVneo cells (JOVANOVIĆ and VIĆOVAC, 2009). It also has been shown that IL-6 is present in higher concentrations in women with preeclampsia (AFSHARI *et al.*, 2005).

The aim of this study was to evaluate the contribution of physiological and elevated concentrations of IL-6 to certain physiological processes of human trophoblasts in hypoxic conditions. Therefore, the effects of three different concentrations (1 pg/mL, 5 pg/mL, and 10 pg/mL) of IL-6 on human trophoblast, JEG-3 cell line, were investigated. Cells were exposed to IL-6 for 24 h (short-term) and 72 h (long-term), under chemically induced hypoxia, which simulates the physiological conditions of early stages of placentation. After completing the treatments, specified tests were performed to determine the effect of IL-6 on viability, redox status parameters, and migratory potential of trophoblast cells.

MATERIALS AND METHODS

Reagents and chemicals

In this study were used followed reagents and chemicals: Dulbecco's Modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 100 μ M cobalt(II)chloride (CoCl₂), 0.4% Trypan blue, 0.25%, trypsin-EDTA, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phosphate buffered saline (PBS), nitro blue tetrazolium chloride (NBT), 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED), 0.1 M sodium nitrite (NaNO₂), 1% sulfanilamide (SAA) dissolved in phosphoric acid (H₃PO₄), 2.5% sulfosalicylic acid (SSA), phosphate-EDTA buffer (100 mM phosphate + 1 mM EDTA), 1 mM DTNB, 1 mM NADPH, 0.7 U glutathione reductase (GR), 4% paraformaldehyde, 200 mM MES buffer (2-(N-morpholino)ethanesulfonic acid), 0.1% crystal violet solution and 10% acetic acid. Interleukin-6 has been purchased from Bio-RAD. All the chemicals and reagents used in this study were of the highest commercially available purity.

Cell culture and treatment

The human trophoblast cell line JEG-3, purchased from the American Type Culture Collection, is immortalized adherent cell line with expressed human chorionic gonadotropin, placental lactogen and progesterone. The effects of IL-6, a main pro-inflammatory cytokine, were examined in this study. The stock solution was prepared by diluting the initial solid substance in DMEM, forming the concentration of 1000 pg/ml. During the experiment, the concentrations of 1 pg/mL, 5 pg/mL, and 10 pg/mL were used for all assays. The used concentrations were obtained from the first stock solution by adding a certain volume of DMEM. The used concentrations approximately correspond to the level of IL-6 in endothelial cells and neutrophils isolated from women with normal pregnancy (~ 1 pg/mL) and preeclamptic pregnancy (~ 10 pg/mL) (WANG *et al.*, 2021). Hypoxic conditions were obtained by 100 μ M cobalt (II) chloride at final concentrations.

Determination of cell viability (MTT assay)

The viability of the cells was determined using a MTT assay (MOSMANN, 1983). Briefly, the cells were plated at a density of 10,000 cells in 100 μ L per well in 96-well plate with DMEM. After 24 h of incubation at a temperature of 37°C and 5% CO₂, the cells were treated with three increasing concentrations of IL-6 (1 pg/mL, 5 pg/mL, and 10 pg/mL) in a volume of 100 μ L per each well. The untreated cells (cultured only in DMEM) served as a control. Both treated and the control cells were incubated for 24 h and 72 h, after which the cell viability was determined using the MTT assay. After a period of incubation, 20 μ L of MTT (concentration of 5 mg/mL) was added to each well. MTT is a yellow tetrazolium salt that is reduced to purple formazan in the presence of mitochondrial dehydrogenase. During this reaction, which started approximately after three hours, the formed crystals were dissolved in 20 μ L of DMSO. The color formed in the reaction was measured on ELISA reader at a wavelength of 550 nm. The percentage of viable cells in treated groups were calculated in relation to the value of the control cells where viability was 100%.

Measurement of superoxide anion radical (NBT test)

Determination of intracellular superoxide anion radical (O₂⁻) was performed by the method of AUCLAIR and VOISIN (1985). This colorimetric method is based on the reduction of NBT to formazan in the presence of O₂⁻. The amount of reduced NBT is directly proportional to the concentration of O₂⁻ produced in the cells. After the treatment 20 μ L of NBT solution (5 mg/mL) was added in both treated and control cells (10,000 cells in 100 μ L per well). The plate was incubated for 45 minutes (37°C, 5% CO₂), after which the formed formazan was dissolved in 20 μ L of DMSO. The absorbances were measured by ELISA microplate reader at 550 nm. The concentrations of O₂⁻ were expressed as nmol O₂⁻/mL in 10⁵ cells.

Measurement of NO concentration (Griess method)

The spectrophotometric determination of nitrite NO₂⁻ (indicators of nitrogen monoxide levels, NO), described by Griess, is based on the diazotization reaction in which SAA and NED react with nitrate purified by nitrite unification (GRIESS, 1879). The concentration of NO₂⁻ is directly proportional to the intensity of the purple color measured on an ELISA reader at 550 nm. The cells were seeded in the wells of 96-well plate (10,000 cells in 100 μ L per well). After 24 h of incubation, the cells are attached to the surface of the well, and IL-6 dissolved in DMEM (1 pg/mL, 5 pg/mL, and 10 pg/mL) was added to each well. After treatments incubation, cells were sonicated, for 10 cycles (1 cycle per second). The obtained supernatant was poured into a new 96-well plate (50 μ L in each well), and 50 μ L of both SAA and NED (1:1) were added. During the 10 minutes of incubation (at room temperature, protected from the light sources) purple color was developed. After incubation, absorbances were measured and the nitrite concentration was expressed in μ mol NO₂⁻/mL in 10⁵ cells.

Reduced glutathione concentration

The method of BAKER *et al.* (1990) utilized to evaluate the concentration of reduced glutathione is based on the oxidation of reduced glutathione (GSH) in the presence of a sulfide reagent DTNB to form a yellow product, 5'-thio-2-nitrobenzoic acid (TNB). As in previous methods, the cells were plated in 96-well plate (10,000 cells in 100 μ L per well) and incubated for 24 h to adhere to the surface. After the initial incubation, the treatment was added and incubated for 24 h and 72 h, respectively. The treatment was then removed and 150 μ L of 2.5% (SSA) was added to each well. The cells were sonicated for 10 seconds and 50 μ L

of supernatant was poured from each well into a new plate. After that, 50 μL of the reaction mixture prepared before the analysis (1 mM DTNB dissolved in 100 mM phosphate buffer) was added. The plate was incubated in a dark for 5 minutes at room temperature, and the absorbances were measured on ELISA reader at 405 nm. The concentration of reduced glutathione was expressed in $\mu\text{mol}/\text{mL}$ in 10^5 cells.

Total glutathione concentration

Determination of total glutathione is based on the oxidation of the reduced glutathione using the DTNB reagent to form a yellow TNB product (BEUTLER, 1975). The method was performed on cells placed in a 96-well plate (10,000 cells in 100 μL per well). After period of incubation with treatment, the supernatant was aspirated, 150 μL of 2.5% SSA was added, and the plate was sonicated. After sonication, 50 μL of supernatant reacted with 50 μL of the reaction mixture (1 mM DTNB, 1 mM NADPH, and 0.7 U glutathione reductase in 100 mM phosphate buffer). After 5 minutes of incubation in the dark, at room temperature, the absorbances were measured on ELISA reader, and the concentration of total glutathione was expressed as $\mu\text{mol}/\text{mL}$ in 10^5 cells.

Determination of migratory potential by Transwell method

The *Transwell* migration assay described by CHEN (2005) was used to evaluate the changes in the migratory potential of trophoblast cells in response to various stimuli. Cell migration capacity was determined by the ability of cells to pass the pores (8 μm in size) of polycarbonate membranes placed at the bottom of transwell chambers. After 72 h of exposure to investigated treatments, the cells were trypsinized, resuspended in DMEM, and seeded on the upper surface of the *Transwell* chambers (100,000 living cells in 500 μL per chamber). The lower chambers contained 750 μL of DMEM with dissolved IL-6 in used concentrations, while the chambers filled with the control cells contained 750 μL of DMEM with 10% FBS. After 6 h of incubation at 37°C, the cells from the upper surface of the filter were completely removed by gentle wiping. Other migrating cells were fixed for 20 minutes at room temperature in 4% paraformaldehyde and stained with 0.1% crystal violet within 10 minutes. The washed and dried membranes were then cut and placed in 96-well plate. After the addition of 10% acetic acid, the absorbances were measured on ELISA reader at 595 nm. The migration index was calculated as the absorbance ratio of the treated group divided by the absorbance of the control group and multiplied by 100 to calculate the percentage.

Statistical analyses

All experiments were performed in triplicate for all the used methods. All data were evaluated using IBM-SPSS 23 software for Windows (SPSS Inc., Chicago, IL, USA). The data were presented as a mean \pm standard error (S.E.M). The statistical significance was determined using Paired-Samples – T test. The level of statistical significance was set at $p < 0.05$.

RESULTS

This study investigated the effect of three different concentrations (1 pg/mL, 5 pg/mL, and 10 pg/mL) of IL-6 on human trophoblast, JEG-3 cell line. Cells were exposed to IL-6 for 24 h (short-term) and 72 h (long-term), under chemically induced hypoxia, which simulates the physiological conditions of placentation. After completing the treatments, the effect of IL-

6 on cell viability, redox homeostasis parameters and migratory potential of trophoblasts were determined.

The effects of IL-6 on cell viability

The effects of different IL-6 concentrations on cell viability under hypoxic conditions were assessed using the MTT test. The results of cell viability after 24 h and 72 h of exposure to investigated treatments were presented in Fig. 1. Obtained results showed that all applied treatments after 24 h caused a decrease in cell viability, especially in higher concentrations (5 pg/mL and 10 pg/mL), where the cell viability was significantly reduced compared to control cells. After 72 h of treatments with all three concentrations of IL-6, the viability of exposed cells was significantly diminished compared to control cells. In addition, cell viability after 72 h was further reduced than after 24 h of exposing to examined treatments, indicating time dependence. Also, a dose-dependent reduction in the viability of treated cells was observed, and accordingly, 10 pg/ml concentration of IL-6 was singled out as the concentration with the most intense cytotoxic effect.

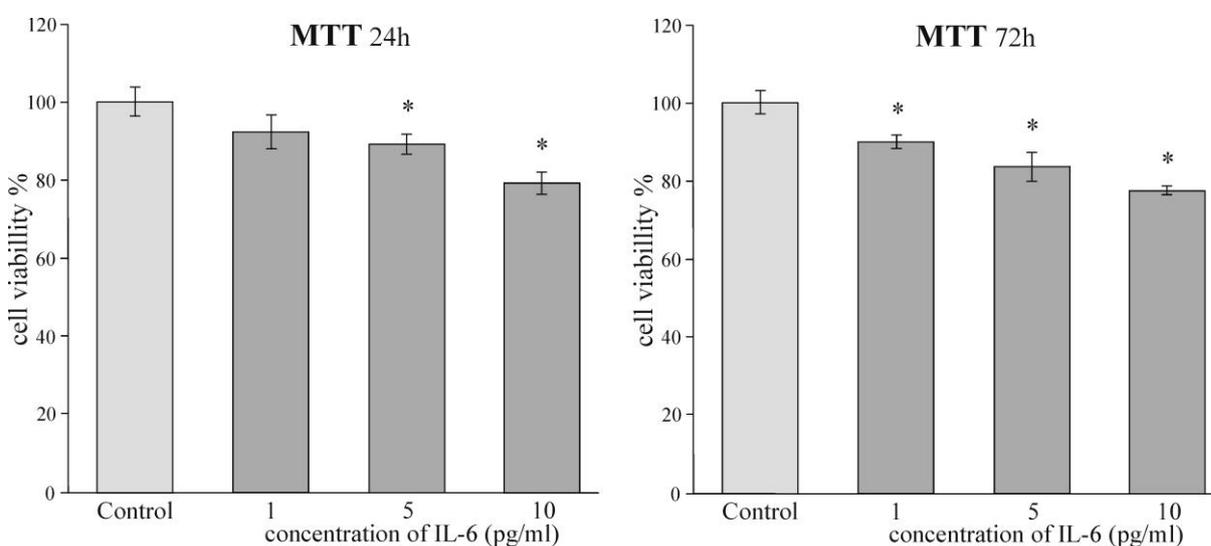


Figure 1. The effects of IL-6 on JEG-3 cell viability after 24 h and 72 h of treatment under chemically induced hypoxia.

Results are presented as the mean of three independent experiments \pm standard error
* $p < 0.05$ relative to control.

The effects of IL-6 on redox status parameters

The effect of short-term and long-term exposure of JEG-3 cells to different concentrations of IL-6 in hypoxic conditions on redox status parameters was monitored and obtained results were given in Figs. 2 and 3. In order to obtain more comprehensive data of the influence of the examined treatments on the redox homeostasis of trophoblasts, as established indicators of oxidative stress, the concentrations of the following parameters were determined: $O_2^{\cdot-}$, NO, GSH and total GSH. Values are expressed in nmol/ml in 10^5 cells and $\mu\text{mol/ml}$ in 10^5 cells.

The data presented in Fig. 2 shows the concentrations of $O_2^{\cdot-}$ and NO after 24 h and 72 h of exposure of JEG-3 cell to three doses of IL-6 in hypoxic conditions. The results show that the production of $O_2^{\cdot-}$ (Fig. 2A) was significantly increased compared to control after short-time exposure to all three concentrations of IL-6, while the greatest production of $O_2^{\cdot-}$ was noticed after treatment with a concentration of 1 pg/mL. After 72 h of all treatments, a

significant increase in the production of $O_2^{\cdot-}$ compared to control cells was also observed, however, the most intensive production of this parameter was induced by a 5 $\mu\text{g}/\text{mL}$ concentration of IL-6.

The obtained results show that after 24 h of treatment with IL-6 in hypoxic conditions, the concentration of NO_2^- (as an indicator of NO) was significantly increased only after treatment with the highest concentration of IL-6 (10 $\mu\text{g}/\text{mL}$) (shown in Fig. 2B). The remaining two applied concentrations of IL-6 provoked a decrease in the concentration of NO compared to the control cells. A decrease in NO concentration was observed in JEG-3 cells even after long-term exposure to all three examined IL-6 concentrations compared to the control cells. The obtained values were approximately 60% lower than the control values. In fact, the most intensive decrease in NO concentration was induced by the lowest applied concentration of IL-6 (1 $\mu\text{g}/\text{mL}$), while the highest applied concentration of IL-6 (10 $\mu\text{g}/\text{mL}$), compared to the other two applied doses, had the mildest effect on the decrease in NO concentration.

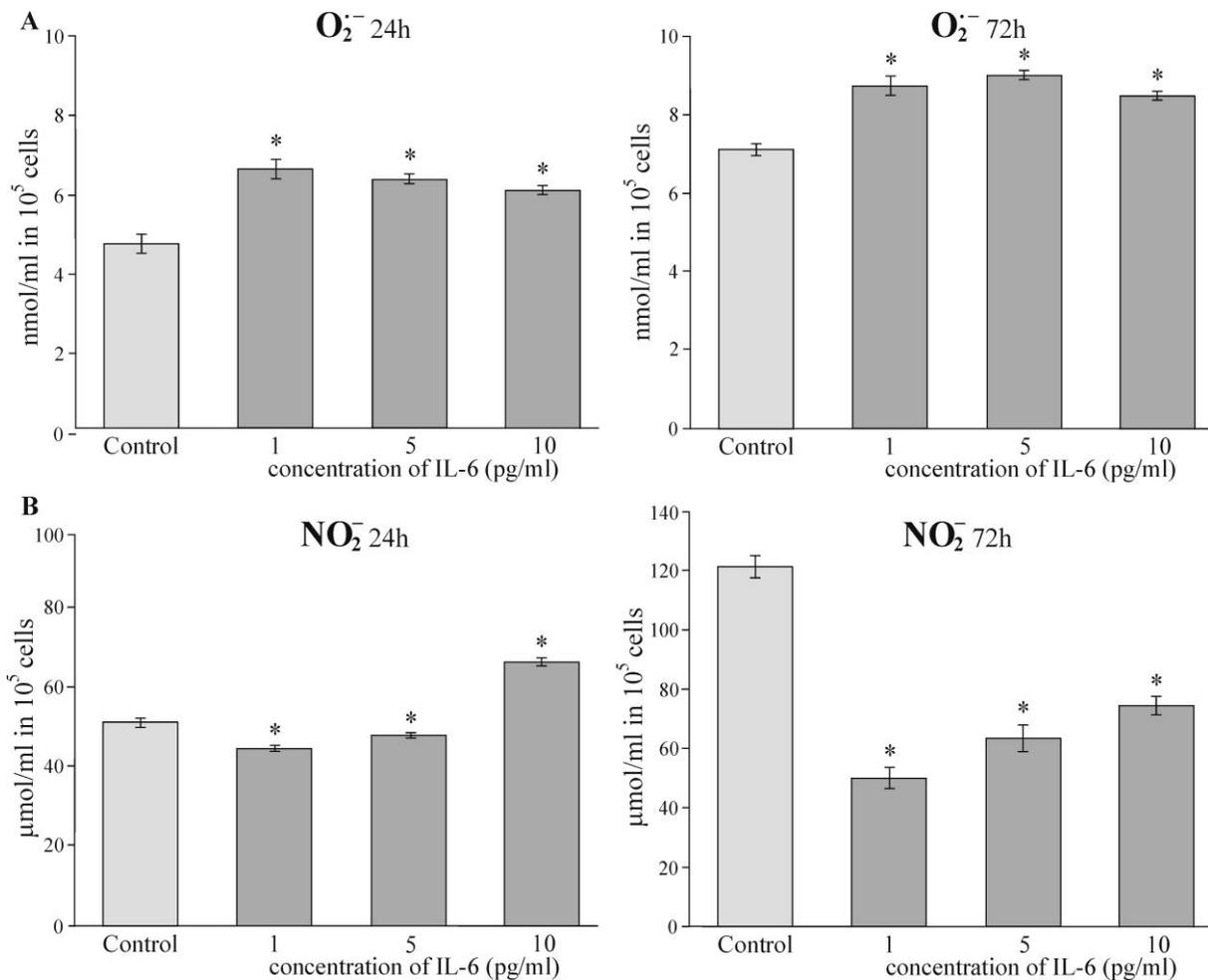


Figure 2. The effects of IL-6 on concentration of $O_2^{\cdot-}$ (A) and NO_2^- (B) in JEG-3 cells after 24 h and 72 h of treatment under chemically induced hypoxia.

Results are presented as the mean of three independent experiments \pm standard error

* $p < 0.05$ relative to control.

The data presented in Fig. 3 shows the effects of the investigated treatments on reduced and total glutathione levels in trophoblasts. After 24 h of exposure of JEG-3 cells to all IL-6 concentrations under hypoxic conditions, a reduction in GSH level (Fig. 3A) was noticed, with a statistically significant decrease after treatments with 1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$

IL-6 compared to control cells. The lowest production of GSH was measured in cells treated with 1 pg/mL IL-6, while the other two applied doses of IL-6 (5 pg/mL and 10 pg/mL) induced a slighter decrease in the level of the mentioned parameter than the previously indicated dose. After 72 h of the incubation period, the same trend in the level of GSH was observed. Namely, the decrease in GSH concentration was inversely proportional to the applied doses of IL-6. Once again, the lowest dose of IL-6 induced the strongest decrease in GSH concentration, while the higher doses exhibit a dose-dependent increase in GSH production compared to lowest dose (1 pg/mL).

Further, the level of total GSH after short-term exposure to all investigated concentrations of IL-6 was significantly higher compared to the level measured in control cells. The increase in total GSH level was dose-dependent, accordingly, the IL-6 in the concentration of 10 pg/ml had the strongest effect. Similar results were obtained after the long-term incubation period. A significant increase in the level of total GSH was reported after all conducted treatments, and that increase was dose dependent. In addition, IL-6 in the highest used concentration (10 pg/mL), caused an approximate doubling of the value of total GSH compared to the level measured in the control.

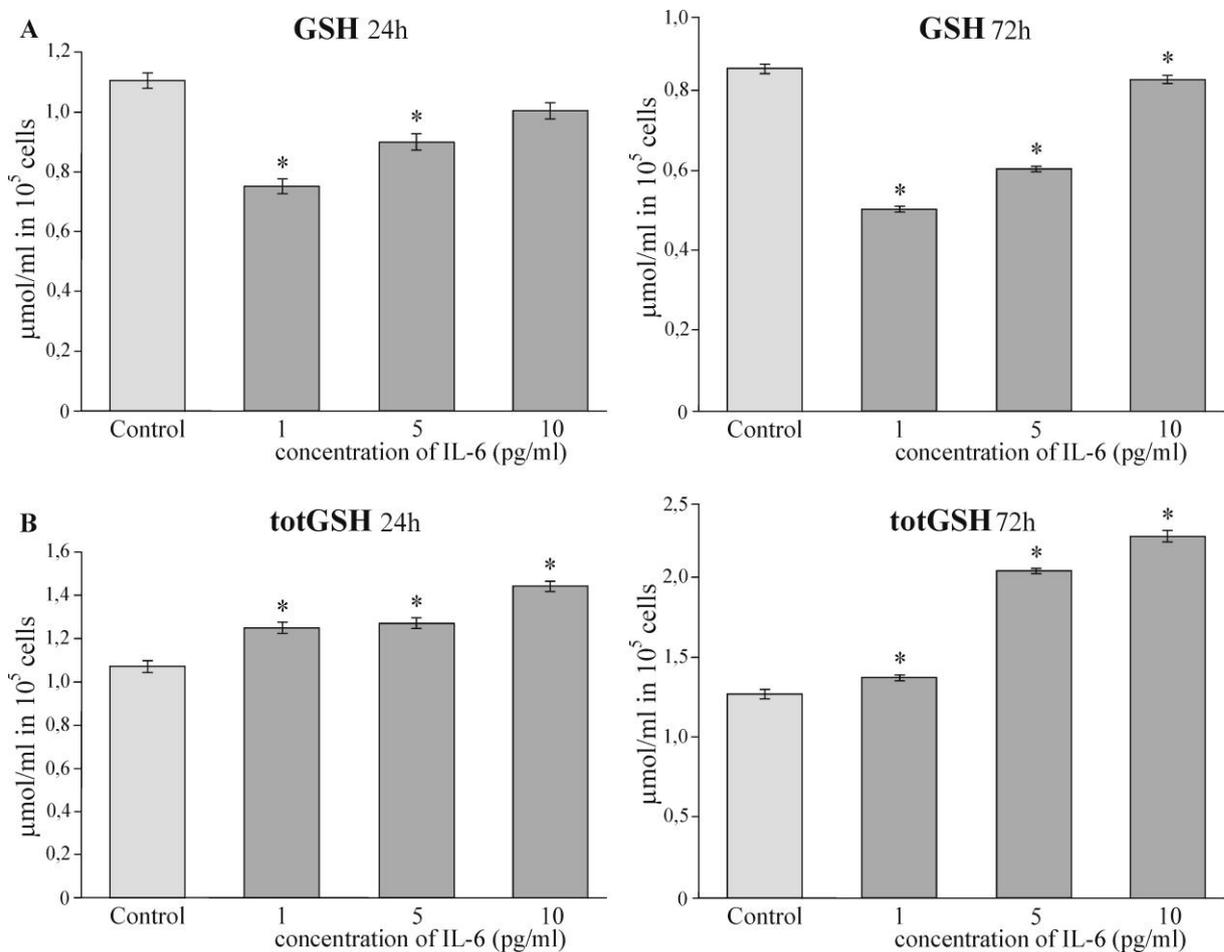


Figure 3. The effects of IL-6 on concentration of GSH (A) and totGSH (B) in JEG-3 cells after 24 h and 72 h of treatment under chemically induced hypoxia.

Results are presented as the mean of three independent experiments \pm standard error

* $p < 0.05$ relative to control.

The effects of IL-6 on the cell migratory potential

The influence of IL-6 in three different doses on JEG-3 cell migratory potential under hypoxic conditions was recorded after 72 h of exposure of the cells to investigated treatments. Obtained results are presented in Fig. 4. As seen, the index of cell migration was exponentially rising in a manner of the applied dose. Lower doses (1 pg/mL and 5 pg/mL) induced a significantly increased index of migration, which was approximately 15% higher than in control cells, while a higher applied dose (10 pg/mL) exhibited remarkable promigratory potentials on JEG-3 cells compared to control cells. The migration index, measured after the mentioned treatment, was increased by approximately 50% compared to the control values.

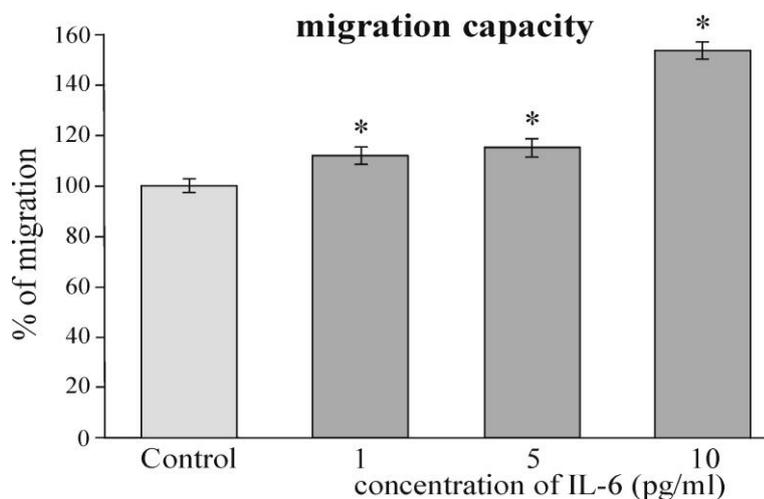


Figure 4. The effects of IL-6 on migratory potential of JEG-3 cells after 72 h of treatment under chemically induced hypoxia.

Results are presented as the mean of three independent experiments \pm standard error

* $p < 0.05$ relative to control.

DISCUSSION

Hypoxic conditions represent a physiologically optimal environment for placenta formation and many processes that take place in the placenta have certain oxygen adjustments (JAMES *et al.*, 2012). To establish an environment that physiologically resembles *in vivo* conditions, we conducted the study in chemically induced hypoxia. The effects of different concentrations of IL-6 on the viability, redox status, and migratory behavior of human trophoblast cell line JEG-3 were tested.

As IL-6 is mainly a pro-inflammatory cytokine, it seems that depending on the applied concentration, it can induce or suppress inflammatory processes, leading to different effects on the target tissues, including the effects on cell viability (TANAKA and KISHIMOTO, 2014). The obtained results of MTT assay show that IL-6 decreases the viability of JEG-3 cells under hypoxic conditions in doses of 5 pg/mL and 10 pg/mL, suggesting that elevated levels of IL-6 can induce apoptosis or decrease oxygen consumption in human trophoblasts.

Reactive oxygen and nitrogen species (ROS and RNS) regulate cellular homeostasis and act as one of the key modulators of cellular dysfunction contributing to the pathophysiology of the diseases. Reactive oxygen species are generally considered to mediate acute inflammation (FORRESTER *et al.*, 2018). Oxidative stress is a state of disturbed balance of the antioxidant protection system activity and production of ROS/RNS. In the state of oxidative

stress, oxidation of various biomacromolecules such as DNA, proteins and lipids occurs (AMES *et al.*, 1993). $O_2^{\cdot-}$ as one of the most reactive radical species, is very harmful to cell biomacromolecules, and by activating certain signaling pathways can trigger apoptosis. However, ROS also act as signaling molecules in the maintenance of various physiological functions and represents an essential regulator of cell metabolism and oxidative homeostasis, especially in the state of hypoxia (SCHIEBER and CHANDEL, 2014). Since IL-6 exerts pro-inflammatory response, it was expected that its elevated levels could increase the production of $O_2^{\cdot-}$, that could exert different effects on trophoblast cells. Interleukin-6 treatment under hypoxic conditions induced slightly increased values of $O_2^{\cdot-}$ compared to the control. All three applied concentrations elevated the production of $O_2^{\cdot-}$, while prolonged treatment exerted stronger increase compared to 24 h incubation. Taking in account the obtained cell viability data, we can suggest that IL-6 in non-pathological concentrations (1 pg/mL) may be of physiological benefit, contributing to the maintenance of optimal oxygen species levels in the state of hypoxia. Higher applied doses (pathological) provoked stronger $O_2^{\cdot-}$ production, as well as the significant drop in cell viability implying their potential deteriorating role on trophoblast cell homeostasis in hypoxia.

Based on the presented results, we can conclude that IL-6 in hypoxia has a slight inhibitory effect on NO production during prolonged treatment, while after short-term treatment there is an increase in the concentration of 10 pg/mL. All concentrations induced a statistically significant reduction in NO production compared to the control, and a concentration of 1 pg/mL caused as much as 60% lower production. There is great interest in the role of NO in establishing a normal pregnancy. It is thought to play a major role in establishing the invasive trophoblast phenotype and their role in the formation of spiral arteries. Inhibition of NOS that directly affects NO production causes inhibition of invasion and preeclampsia (CARTWRIGHT *et al.*, 1999). Reduced nitric oxide production may be of physiological importance in some stages of placental formation, by limiting excessive trophoblast motility and allowing adequate endometrial colonization. However, the prolonged effect of IL-6 may be a risk factor for preeclampsia.

In addition to the oxidative stress parameters monitored in this study, antioxidant protection that regulates and reduces the harmful effects of radical species is also important. An important parameter of antioxidant protection is the glutathione, a tripeptide presents in the cell in two forms, reduced (GSH) and oxidized (GSSG) (BIRBEN *et al.*, 2012). Although GSH and GSSG occur in tissues, GSH is far more prevalent. As much as 95% of total glutathione (GSH + GSSG) is GSH (ANDERSON, 1985). Our results indicate that in hypoxic conditions IL-6 has a significant contribution in increasing the antioxidant potential of trophoblasts, which is an important factor in maintaining oxidative homeostasis during hypoxia. GSH is known as a molecule that scavenges $O_2^{\cdot-}$ neutralizing it and thereby turning itself into a disulfide form (GSSG) (HALLIWELL and GUTTERIDGE, 2015). The obtained glutathione values partially correspond to the changes in the production of superoxide anion radicals after treatment in hypoxia. This fact is supported by the results obtained in this study, which show significantly reduced GSH in the treated cells compared to the control, while the concentration of totGSH was increased. Based on these results, we can conclude that the GSH level was reduced due to its role in neutralizing the overproduction of $O_2^{\cdot-}$, which was also shown in this study. In addition, the increased level of totGSH implies its *de novo* synthesis in order to protect trophoblasts from possible oxidative damage.

Our study showed a statistically significant effect on the migratory potential of trophoblasts of this cell line at all concentrations, with the most stimulating effect at highest concentration (10 pg/mL). The rise in migration capacity after IL-6 may be correlated with the reduced nitric oxide production registered in our study. Nitric oxide is an important regu-

lator of cell migration in many examined cell lines, and could act in antimigratory fashion (ZHOU *et al.*, 2016). Since IL-6 inhibits the production of nitric oxide in hypoxic conditions, maintaining the moderate cell migration is a possible physiological mechanism of proper placentation during early pregnancy, given that excessive cell migration potential can cause similar abnormalities in placental function. In future studies, it would be indicative to examine the effect of IL-6 on the expression and distribution of cell adhesion proteins, as well as on the activation and secretion of metalloproteinases as key enzymes responsible for human trophoblast invasion and migration.

These results show that IL-6 has a significant effect on trophoblast migration processes. In the early stages of pregnancy, it may be physiologically important in limiting trophoblastic migration, but its elevated concentrations in certain stages of placental development may contribute to the pathogenesis of preeclampsia and other disorders caused by inadequate trophoblast motility and invasiveness.

Acknowledgments

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia [451-03-68/2022-14/200122].

References:

- [1] AFSHARI, J.T., GHOMIAN, N., SHAMELI, A., FAHMIDEHKAR, M.A., MAHAJER, E., KHOSHNAVAZ, R., EMADZADEH, M. (2005): Determination of interleukin-6 and tumor necrosis factor-alpha concentrations in Iranian-Khorasanian patients with preeclampsia. *BMC Pregnancy Childbirth* **5**: 14. doi: 10.1186/1471-2393-5-14
- [2] AMES, B.N., SHIGENAGA, M.K., HAGEN, T.M. (1993): Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America* **90** (17): 7915–7922. doi: 10.1073/pnas.90.17.7915
- [3] ANDERSON, M.E. (1985): Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology* **113**: 548–555. doi: 10.1016/s0076-6879(85)13073-9
- [4] AUCLAIR, C., VOISIN, E. (1985): Nitroblue tetrazolium reduction. In: Greenwald, R.A., (ed) *CRC Handbook of methods for oxygen radical research*. Boca Raton, Florida, USA, CRC Press: 123–132.
- [5] BAKER, D., WUESTEHUBE, L., SCHEKMAN, R., BOTSTEIN, D., SEGEV, N. (1990): GTP-binding Ypt1 protein and Ca²⁺ function independently in a cell-free protein transport reaction. *Proceedings of the National Academy of Sciences of the United States of America* **87** (1): 355–359. doi: 10.1073/pnas.87.1.355
- [6] BEUTLER, E. (1975): Reduced Glutathione (GSH). In: Beutler, E. (ed) *Red Cell Metabolism: A Manual of Biochemical Methods.*, New York, NY, USA, Grune and Stratton: 112–114.
- [7] BIRBEN, E., SAHINER, U.M., SACKESSEN, C., ERZURUM, S., KALAYCI, O. (2012): Oxidative Stress and Antioxidant Defense. *World Allergy Organization Journal* **5** (1): 9–19. doi: 10.1097/WOX.0b013e3182439613

- [8] CARTWRIGHT, J.E., HOLDEN, D.P., WHITLEY, G.S.J. (1999): Hepatocyte growth factor regulates human trophoblast motility and invasion: a role for nitric oxide. *British Journal of Pharmacology* **128** (1): 181–189. doi: 10.1038/sj.bjp.0702757
- [9] CHEN, H.C. (2005): Boyden chamber assay. *Methods in Molecular Biology* **294**: 15–22. doi: 10.1385/1-59259-860-9:015
- [10] DIMITRIADIS, E., WHITE, C.A., JONES, R.L., SALAMONSEN, L.A. (2005): Cytokines, chemokines and growth factors in endometrium related to implantation. *Human Reproduction Update* **11** (6): 613–630. doi: 10.1093/humupd/dmi023
- [11] FITZGERALD, J.S., BUSCH, S., WENGENMAYER, T., FOERSTER, K., DE LA MOTTE, T., POEHLMANN, T.G., MARKERT, U.R. (2005): Signal transduction in trophoblast invasion. In: Markert, U.R. (ed): *Immunology of gametes and embryo implantation*. Basel, Karger **88**: 181–199.
- [12] FORRESTER, S.J., KIKUCHI, D.S., HERNANDES, M.S., XU, Q., GRIENDLING, K.K. (2018): Reactive Oxygen Species in Metabolic and Inflammatory Signaling. *Circulation Research* **122** (6): 877–902. doi: 10.1161/CIRCRESAHA.117.311401.
- [13] GRIESS, P. (1879): Bemerkungen zu der Abhandlung der HH. Weselky und Benedikt Ueber einige Azoverbindungen. *Berichte der deutschen chemischen Gesellschaft* **12** (1): 426–428. doi: 10.1002/cber.187901201117
- [14] HALLIWELL, B., GUTTERIDGE, J.M.C. (2015): Antioxidant defences synthesized in vivo. In: Halliwell, B., Gutteridge, J.M.C. (eds) *Free Radicals in Biology and Medicine, 5th edn*. USA, Oxford University Press: 77–151.
- [15] JAMES, J.L., CARTER, A.M., CHAMLEY, L.W. (2012): Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta*. **33** (5): 327–34. doi: 10.1016/j.placenta.2012.01.020
- [16] JAUNIAUX, E., GULBIS, B., SCHANDENE, L., COLLETTE, J., HUSTIN, J. (1996): Molecular interactions during pregnancy: Distribution of interleukin-6 in maternal and embryonic tissues during first trimester. *Molecular Human Reproduction* **2** (4): 239–243. doi: 10.1093/molehr/2.4.239
- [17] JAIN, A., SCHNEIDER, H., ALIYEV, E., SOYDEMIR, F., BAUMANN, M., SURBEK, D., HEDIGER, M., BROWNBILL, P., ALBRECHT, C. (2014): Hypoxic treatment of human dual placental perfusion induces a preeclampsia-like inflammatory response. *Laboratory investigation* **94** (8): 873–880. doi: 10.1038/labinvest.2014.76.
- [18] JOVANOVIĆ, M., VIĆOVAC, L. (2009): Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. *Placenta* **30** (4): 320–328. doi: 10.1016/j.placenta.2009.01.013
- [19] MOSMANN, T. (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** (1–2): 55–63. doi: 10.1016/0022-1759(83)90303-4
- [20] SCHIEBER, M., CHANDEL, N.S. (2014): ROS function in redox signaling and oxidative stress. *Current biology* **24** (10): 453–462. doi: 10.1016/j.cub.2014.03.034
- [21] SILVA, J.F., SERAKIDES, R. (2016): Intrauterine trophoblast migration: A comparative view of humans and rodents. *Cell Adhesion & Migration* **10** (1–2): 88–110. doi: 10.1080/19336918.2015.1120397

- [22] TANAKA T., KISHIMOTO T. (2014): The biology and medical implications of interleukin-6. *Cancer Immunology Research* **2** (4): 288–94. doi: 10.1158/2326-6066.CIR-14-0022.
- [23] WANG, Y., GU, Y., ALEXANDER, J.S., LEWIS, D.F. (2021): Preeclampsia Status Controls Interleukin-6 and Soluble IL-6 Receptor Release from Neutrophils and Endothelial Cells: Relevance to Increased Inflammatory Responses. *Pathophysiology* **28** (2): 202–211. doi: 10.3390/pathophysiology28020013.
- [24] ZHOU, L., ZHANG, H., WU, J. (2016): Effects of nitric oxide on the biological behavior of HepG2 human hepatocellular carcinoma cells. *Experimental and Therapeutic Medicine* **11**: 1875–1880. doi: 10.3892/etm.2016.312