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Inhibition of Iodine Transport with Perchlorate Alters the Redox Balance in Trophoblast

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A B S T R A C T

Iodine deficiency is a risk factor of preeclampsia, perchlorate (KCLO4) blockade iodine transport. Previous studies done in trophoblast cells show that that KCLO4 induce loss of differentiation and aberrant increase of migration process. Oxidative stress may contribute to endothelial dysfunction, in order to determine the effect of KCLO4 and iodine exposure on the antioxidant and oxidative stress status we use trophoblast cells. Results shown an increase in lipoperoxidation, CAT and SOD enzymatic activity in KCLO4 trophoblast cells with a P <0.05, although introphoblast cells treated with iodine after elimination of NIS blockade was observed a decrease in the activity of SOD and CAT, being significant with 100 □ M since 24 hours of treatment(P <0.001). This study show that KCLO4 cause alterations in equilibrium REDOX, however, other studies are required to corroborate the mechanisms of action of iodine in the antioxidant status.

Keywords: Iodine deficiency, perchlorate, oxidative Stress, Antioxidant Effect, Trophoblasts

1. Introduction

During pregnancy, supplementary energy is required for the growth of the fetus, placenta, and maternal tissues; this additional energy cost is accompanied by the gradual increase of oxygen consumption and its concentration may play a key role in the success or failure of pregnancy (Vaughan, 2016); Since the adequate supply of oxygen to tissues is essential for maintaining the function and physiology of cells (Smyth, 2003). Therefore, it is considered that pregnancy is a condition that exhibits a greater susceptibility to oxidative stress (Casanueva and Viteri, 2003).

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cells (Sha et al., 2015). Between 2 and 5% of the oxygen entering the respiratory chain is reduced univalent resulting in intermediates or ROS, such as the anionic radical superoxide (O_2-) and hydrogen peroxide (H_2O_2), which, during pregnancy, can be found in greater quantity due to the increase of the pregnant woman's oxygen consumption (Hansson et al., 2014). In response to increased ROS in normal pregnancy, there is an increase in antioxidant activity; However, in a pregnant woman with hypertensive pregnancy or preeclampsia, substances that participate in the reactions of the antioxidant defense systems are inactivated or deteriorated, which causes substantial increases in the production of oxidants and pro oxidants (Hansson et al., 2014). Lipoperoxidation may be evidenced by the presence of 8-isoprostane (Walsh, Wang and Jesse, 1996), which has important activities in preeclampsia and

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is a powerful vasopressor, platelet activator and mitogen (Hansson et al., 2014).

Several studies have shown that micronutrient deficiency is associated with changes in the REDOX status, contributing to endothelium dysfunction. It has been reported that iodine deficiency is a risk factor for preeclampsia, and its deficiency in gestation has been associated with increased oxidative stress and decreased antioxidant status (Gulaboglu et al., 2007; Gulaboglu et al., 2010; Olivo-Vidal et al., 2014). In addition, iodine has been reported to exert antioxidant effects by neutralizing reactive oxygen species and competing with free radicals for binding sites; Or indirectly, increasing total antioxidant capacity in serum by increasing the activity of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Venturi, 2001). A key element for the improvement of the placenta is the generation of SOD and CAT enzymes, which reduce oxidative stress, maintaining a REDOX balance (Maarten, 2004). For the above, it is of interest to determine the effect of iodine deficiency induced with perchlorate and iodine exposure in the antioxidant status by measuring SOD and CAT activity and oxidative stress in trophoblasts cells.

2. Methods

2. 1 Cell Culture and Reagents

Cell lines used were BeWo, obtained from the ATCC. They were proliferated in 10x1000mm cell culture plates, containing EMEM medium containing 10% FBS and 1% antibiotic, composed of penicillin 10 000 U, Streptomycin 10 mg and Amphotericin B 25 mg, at 37 ° C and in air atmosphere with 5% CO2. Cells were subculture by trypsinization with 0.25% trypsin solution, 0.53 mM EDTA (GIBCO) and resuspended in fresh medium. The human non-choriocarcinomic trophoblastic cell line BeWo cells were obtained from the American Tissue Culture Collection (ATCC). Cells were grown and maintained at 37 ºC with 5 % of CO2 in Eagle's Minimum Essential Medium (EMEM) (SIGMA, USA) containing 10 % fetal bovine serum, 1 mM of potassium iodide and 500 U/mL penicillin and streptomycin. $KCLO_4$ were obtained from Sigma-Aldrich, México. All other reagents, unless stated otherwise, were obtained from Sigma-Aldrich, México. Trophoblast cells were collected from 100-mm culture plates by trypsinization and were seeded in 96-, 24-, or 6-well sterile tissue culture plates for treatment and analysis.

2.2 Iodine deficiency induced with perchlorate

A BeWo choriocarcinomic cell line was used as a model for iodine transport by the placenta as previously reported (Manley, et al., 2005; Mitchell, Et al., 2001 and Bidart, et al., 2000), while iodine deficiency was done using 1 mM of KCLO4 to block NIS in EMEM culture medium (Manley, et al., 2005). These doses inhibit iodine transport more than 50 % in 1 h. In this study, iodine deprivation was done for 3, 6, 24, and 48 h, and then cells were washed with PBS buffer three times and then iodine was supplemented with 5–5000 µM.

2.3 ***Cell Viability Assay***

BeWo cells were seeded at a density of 2.5 × 104 cells/well in 96-well flat-bottommicroplates (Costar, USA). After 18 h, the medium was replaced by phenol red-free EMEM containing 10 % of FBS. After of iodine deprivation for 3, 6, 24, and 48 h, cells were PBS washed and then treated with 5, 50, 100, 1000, and 5000 µM of iodine (Lugol) to the corresponding wells and cultured for another 24 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well (5 mg/ml in PBS) and incubated at 37 ºC for 2 h until purple formazan crystal developed and measurement to 550 nm on an automatic microplate reader (Biotek ELX800, WI, USA). The optic density (OD) values of the treated cells were compared with the values generated from untreated control cells and reported as the percentage viability for the control.

2.4 Catalase activity

Standardization of catalase quantification was carried out by the technique of Sinha, 1972. The cell line was plated in 24-well plates at a density of 2 × 10 5 cells per well, with 1 ml of culture medium supplemented and incubated for 24 h. At the end of the incubation, the culture medium was changed (aspirated and 1 ml of fresh culture medium added) and inhibitors (1 mM KCLO4) were added. Cells were incubated at 37 ° C for 6 h and then washed with 1X PBS to remove the inhibitors. Cells were treated with different concentrations of lugol (5, 50, 100, 1,000, 5,000 µM) for 6, 24 and 48 h and disrupted by trypsinization. 50 µl of the sample was added to 1.5 ml sterile tubes and vortexed for 5 min. Thereafter, 400 µl 40 mM H 2 O 2 and immediately 2 ml of potassium dichromate (K 2 Cr 2 O 7) were added and allowed to cool to room temperature for 3 min. Subsequently, it was heated in a water bath for 10 min, allowed to cool to room temperature, 200 µl was passed to a microplate and read at 550 nm in a microplate reader (Spectra Max 190). The results are presented as the absorbance at 550 nm corrected for mg protein.

2.5 SOD activity

Quantification was carried out using the technique of Madesh and Balasubramanian, 1998. A simple micro titer plate colorimetric assay for the determination of SOD. The method involves the generation of superoxide by autoxidation with pyrogallol and the inhibition of the superoxide-dependent reduction of the tetrazolium dye MTT. In a 96-well plate cells were seeded at a density of 1x104 in each well; 24 h were incubated and the culture medium changed. Iodine inhibitors (1 mM KClO4) were added and incubated for 24 h at 37 ° C, after which time the different concentrations of I2 (5, 50, 100, 1,000 and 5,000 µM) were added. After placing the treatments were incubated for 6, 24 and 48 h. To carry out the readings, the medium was aspirated and washed with 100 µl of PBS, frozen at -80 ° C for 5 min and thawed by incubation at 37 ° C (this was done 2 times). 11.8 µl of 2 mM pyrogallol and 12 µl of MTT tetrazolium dye were added. The reaction was incubated for 10 min at room temperature in the micromixer, the formazan was dissolved by adding 76.2 µl of DMSO. Absorbance at 550 nm was measured on a microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA). The results are presented as the absorbance at 550 nm corrected for mg protein.

2.6 Lipoperoxidation test (TBARS method)

Cell lines were plated in 48-well plates at a density of 15x10 3 cells per well, with 500 µl of culture medium supplemented and incubated for 24 h. After that time, the culture medium was changed, inhibitors added and incubated for 6 h. Cells were treated with different concentrations of lugol 5, 50, 100, 1000, 5000µM and read at 24, 48 and 72 h. The determination of lipoperoxidation was performed by means of the TBARS test. 70µl of Tris were placed in a series of tubes, 300µl of tiobarbituric acid and 90µl of the sample were added. They were vortexed for 30 sec and left for 45 min in a water bath at 100 ° C, then cooled on ice for 2 min and 200 µl of KCl added and vortexed again for 1 min. They were allowed to incubate for 5 min at room temperature, 180 µl of the solutions were passed to a 96-well plate and read at 550 nm.

2.7 Protein determination (Bradford method)

Protein levels were measured colorimetrically by the Bradford method. This determination was made to make the correction in catalase values and in the lipoperoxidation test using the same samples. Briefly, a curve was made with BSA (Bovine Serum Albumin). 5µ of the curve and the sample were placed in each well on a microplate, 250µl of the 1x Bradford reagent was then added to each well, incubated at room temperature for 5min and read at 550nm in the microplate reader.

2.8 Statistical Analysis

The data presented in all the figures are mean ± SEM of independent experiments (n = 3 to 5). In most cases, data from different experiments were normalized to the control value before being combined for statistical analyses. Differences among several groups were determined using one-way ANOVA followed by the Student-Newman-Keuls test using GraphPad software (GraphPad Software Inc., San Diego, CA). For comparison between two groups, the t-test was used. A $P < 0.05$ was considered significant.

3. Results

3.1. Iodine affects Trophoblastic Cell Viability Inducing Oxidative Stress

In Figure 1, it is shown that1 mM $KClO_4$, dose reported that inhibit iodine uptake in BeWo cells (Manley, et al., 2005; Mitchell, et al., 2001 and Bidart, et al., 2000), results that not affect cell viability up to 6 h of treatment compared to untreated cells. However, viability increased from 24 and 48 h between $KClO_4$ cell respect to untreated control cells, being significant at $p < 0.05$ (Figure 1). In figure 2, the results of iodine deficient BeWo trophoblasts are shown, with a significant increase in liporeroxidation at 48 and 72 h compared to the control untreated cells. The treatment with different concentrations of lugol after $KClO_4$exposition, show a decrease in liporeroxidation at all concentrations after 48 h vs iodine deficient cells, this decrease can be interpreted as the reduction of ROS that were generated by iodine deficiency induced with $KClO_4$and that were neutralized by the antioxidant effect of iodine

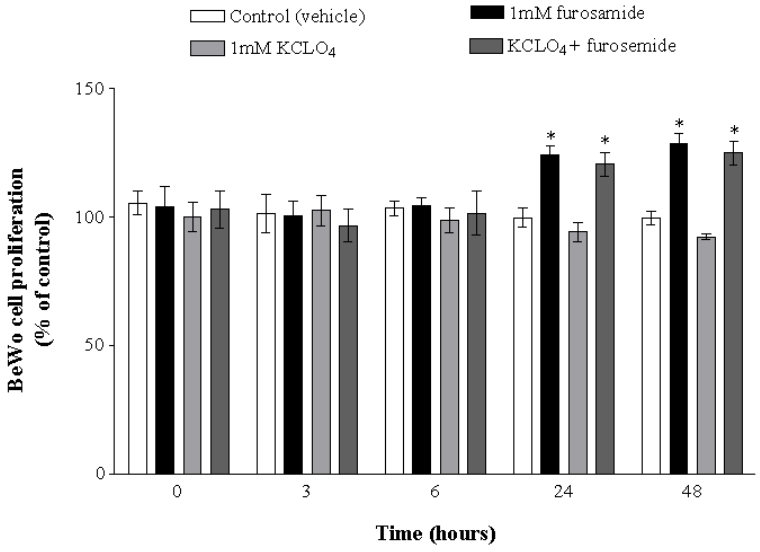

Figure 1.Viability of BeWo cell lines affected by iodine deprivation. The BeWo cells were deprived of iodine with $KClO_4$ (1 mM) and furosemide (1 mM) for 0-48 h. Viability of BeWo cells after iodine deprivation. Data are expressed as a percentage of the control and the mean ± SD (significant at * $p < 0.05$ vs untreated cells) is plotted for both n = 6 cell lines.

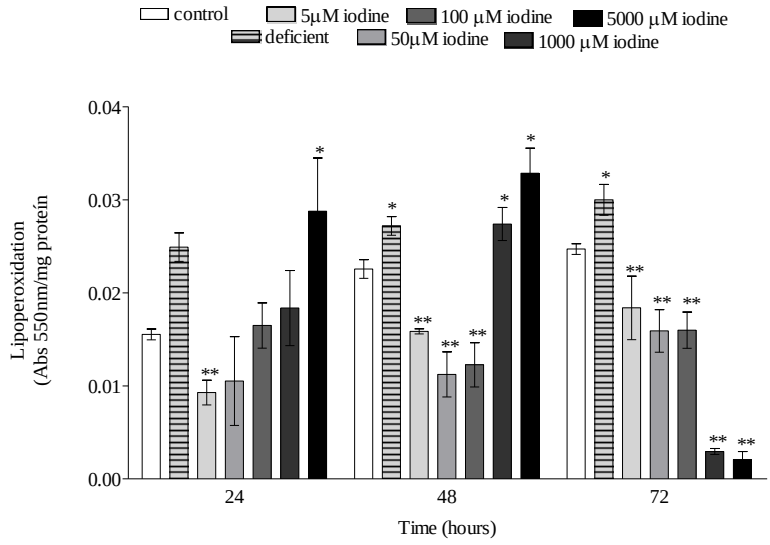

Figure 2. Effect of iodine deficiency and exposure on lipoperoxidation of human trophoblasts. Values presented as the mean ± SE; * P <0.05 vs control cells, ** p <0.001 vs iodine deficient cells in their respective times. (n = 5)

3.2 Effect of KCLO exposition and Iodine Treatment on Antioxidant Enzymatic Status

In figure 3, the results of catalase activity on iodine deficient trophoblasts exposed with $KClO_4$, show a significant increase in catalase activity at 24 and 48 h in iodine deficient trophoblasts compared to control trophoblasts, p <0.05. In the trophoblasts supplemented with iodine (after $KClO_4$exposition) it is observed that the catalase activity decreases, observing a dose-response and time-dependent effect, when compared with the iodine-deficient trophoblasts (p <0.05). At doses of 5 to 100 µM iodine, a decrease in catalase activity is observed at levels comparable to control trophoblasts.The results obtained are shown in figure 4, where it is observed that iodine deficient trophoblasts with$KClO_4$ significantly increase the SOD enzyme activity, relative to the control trophoblast (non-$KClO_4$ exposed), at 24 and 48 h P <0.05). On the other hand, iodine supplementation significantly decreased SOD enzyme activity at 24 and 48 h of treatment with concentrations of 5, 50, 100, 1,000 µM with respect to $KClO_4$exposed trophoblasts (p <0.05)

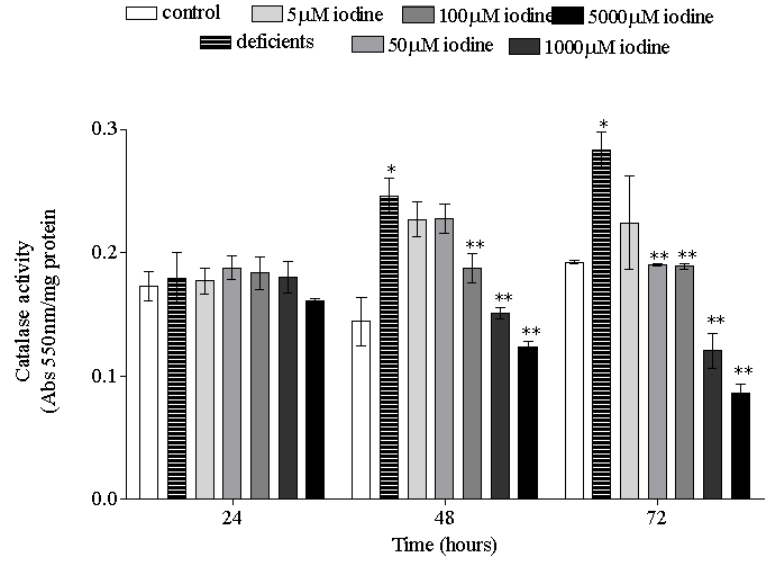

Figure 3. Effect of iodine deficiency and exposure on catalase activity. BeWo cells deficient in iodine; Were supplemented with 5, 10, 50, 100, 500, 1,000 and 5,000 µM iodine. After 24 h of treatment, catalase activity was measured according to the methodology. Values presented as mean ± SD; * P <0.05, ** p <0.001 at their respective times. (n = 4)

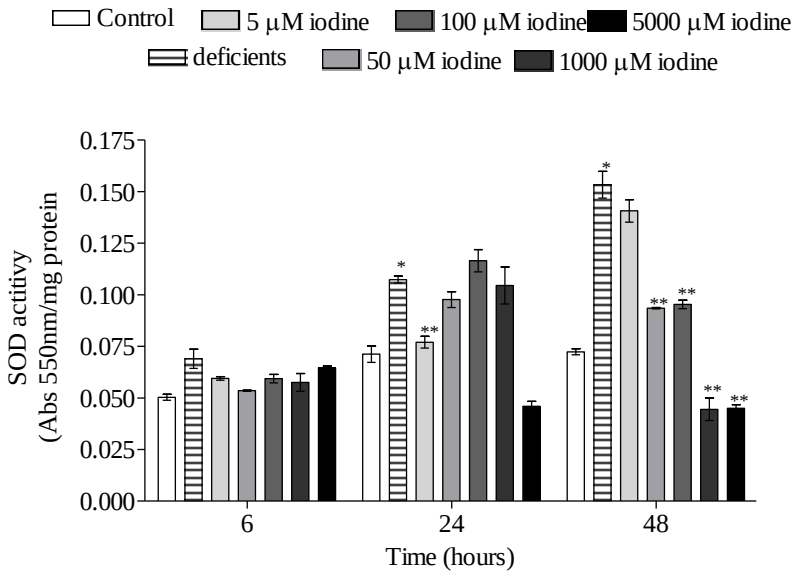

Figure 4. Effect of iodine deficiency and exposure on superoxide dismutase activity. BeWo cells deficient in iodine. Were supplemented with 5, 10, 50, 100, 500, 1,000 and 5,000μM iodine. After 24 h of treatment, SOD activity was measured according to the methodology. Values presented as mean ± SD; * P <0.05, ** p <0.001. (n = 4)

4. Discussion

With these results it is observed that iodine deficiency induced with the exposition to $KClO_4$it is show a REDOX imbalance. Was observed an increase of ROS and SOD activity to neutralize them and prevent their toxic damages to the cell. When treated with iodine, it was observed that SOD activity decreased, suggesting that iodine has antioxidant effects. REDOX imbalance during gestation is related to complications of pregnancy, such as preeclampsia, which is the result of endothelial dysfunction and endothelial cell damage mediated by free radicals (Damayanti-Chakraborty, 2013). In the present study it was observed that iodine-deficient trophoblasts had an increase in lipoperoxidation from the first 24 hours, with this increase being significant with respect to the control ($p < 0.05$). Likewise, in this trial, it was observed that this process can be reversed if it is supplemented to the trophoblast with doses of 5-100 μM iodine.In addition, our results are consistent with that reported in the literature in pregnant women with iodine deficiency, which showed an increase in ROS in contrast to low levels in pregnant women with normal iodine levels (Olivo-Vidal et al., 2014). In our study, ROS increased in iodine deficient trophoblasts at 24, 48 and 72 h, unlike trophoblasts supplemented with iodine at concentrations of 5 to 100 mM iodine, in which the percentage of iodine ROS decreased, being significant for the concentration of 100 μM iodine at 24 h of treatment ($p < 0.001$vs iodine deficient cells).On the other hand, our data show another interesting effect, since with high doses of iodine (500, 1,000 and 5,000 μM), increase ROS, as well as the loss of cellular proliferation. These in vitro effects can be explained by the fact that iodine is able to react with proteins, lipids and nucleic acids that could determine the loss of cell and mitochondrial membrane integrity with the generation of ROS and lipid peroxidation and induce death (Pereira et al., 1990). In this regard, in thyroid cells, excess iodide at millimolar doses induces apoptosis due to ROS increase (Vitale et al., 2000). In our study with higher doses starting at 500 μM, a cytotoxic effect was observed in the presence of decreased trophoblastic proliferation. However, it is required another research to determine the type of cell death involved.

With regard to antioxidant enzymes, an increase in the enzymatic activity of catalase and superoxide dismutase from the first 24 h of treatment was observed in iodine deficient trophoblasts; which contrasts with that reported in pregnant women with iodine deficiency, where there is a decrease in the activity of both enzymes (Olivo-Vidal et al., 2014). This could be due to the difference of models, since in pregnant the levels of catalase and SOD were analyzed in blood, and our study was in vitro using protein extract of the trophoblasts.

There is some evidence for the antioxidant effects of iodine in vitro, for example, in iodine deficient rats exposed to potassium iodide reported an antioxidant increase in the retina (Walsh et al., 1996). In vivo studies, an increase in the enzymatic activity of catalase and glutathione peroxidase was demonstrated in patients with type II diabetes mellitus, after drinking brines with iodine (Moser et al., 1991). In agreement with our results, since the induction of iodine deficiency, an increase of ROS and the antioxidant enzymes catalase and SOD, this increase of the antioxidant enzymes in order to neutralize the formation of superoxide and hydrogen peroxide. However, by adding iodine, antioxidant and ROS enzymes decrease, suggesting and supporting the hypothesis of the role of iodine as a direct antioxidant (Venturi and Venturi, 2009).

According to the results obtained in this study, it is demonstrated that iodine treatment significantly decreases levels of oxidative stress, also decreasing catalase and SOD activity, in contrast to that reported by Moser et al., 1994 and Winkler et al., 2000, who demonstrated that iodine supplementation increases the activity of the enzyme catalase in serum of patients with diabetes mellitus II. These data suggest that *in vitro* iodine would behave as a direct antioxidant by neutralizing ROS, whereas *in vivo* the activity of antioxidant enzymes such as catalase would be increasing. Suggesting that iodine helps

REDOX balance; However, it is necessary to analyze the mechanisms of action involved.
Gulaboglu et al., 2007, Aceves et al., 2005 and Venturi and Venturi, 2009 proposed the hypothesis of an antioxidant role of iodine in tissues outside the thyroid, which corroborates with the results obtained, since to supplement with iodine to trophoblastic cells, we observed a decrease in oxidative stress levels, and decreased activity of antioxidant enzymes catalase and SOD, supporting the hypothesis that iodine is acting as a direct antioxidant.

5. Conclusion

The present study shows that iodine blockinginduced with $KClO_4$significantly increases the levels of oxidative stress and increase the activity of antioxidant enzymes, as a response system to increase ROS concentration present. In the trophoblast cells treated with iodine after eliminate $KClO_4$, the levels of antioxidant enzymes decrease with respect to those deficient, reaching levels similar to those in the control at concentrations of 100 µM iodine, this suggest that iodine helps to maintain REDOX balance. Iodine deficiency induced a REDOX imbalance promoting an increase in lipoperoxidation, which could contribute to endothelial dysfunction. All this data suggest that iodine levels are important to maintain an antioxidant balance during gestation; however other studies are required to corroborate the mechanisms of action of iodine.

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