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BRIEF REPORT

Susceptibility of placental mitochondria to oxidative stress

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Background: Two different mitochondrial fractions (MFs) have been characterized in the human placenta: the "light" and "heavy" fractions (LMF and HMF). Although these organelles are the main source of reactive oxygen species, an imbalance between their production and the rate of detoxification represents a serious threat to mitochondrial homeostasis and, in the case of the placenta, also to the fetus. The aim of this study was to evaluate the antioxidant capacity and susceptibility to oxidative stress in both types of MFs.
Methods: Human MFs were isolated from healthy donors ($n = 11$) and either incubated or not with H_2O_2. Catalase (CAT) activity, and reduced glutathione (GSH), lipid peroxidation (LP), and protein carbonylation (PC) levels were determined.
Results: H_2O_2 treatment increased LP and PC levels and decreased CAT activity. GSH levels were similar in control and treated MFs.
Conclusion: H_2O_2 caused oxidative damage in both LMF and HMF and the antioxidant system measured in these two MFs responded similarly. To the best of our knowledge, this is the first partial description of the antioxidant defense in placental HMF and LMF performed in a cell-free assay. The small number of antioxidant system parameters measured did not allow detecting differences between HMF and LMF.

KEYWORDS
mitochondria, placenta, oxidative stress, reactive oxygen species

1 | INTRODUCTION

Mitochondria are dynamic organelles involved in many physiological functions in cell development, growth and function, such as steroid hormone synthesis, cell death, and ATP supply (Ferree & Shirihai, 2012). As the result of energy metabolism, mitochondria continuously produce reactive oxygen species (ROS) as a byproduct. ROS ability to cause damage is due to their high reactivity, which allows them to alter the integrity of many biological macromolecules such as proteins, lipids, and DNA (Kowaltowski & Vercesi, 2009). The harmful effects of ROS are prevented by a coordinated network of enzymatic and nonenzymatic antioxidants. Oxidative stress is caused by both an increase in pro-oxidants and a decrease in the antioxidant capacity (Tabassum, Waseem, Parvez, & Qureshi, 2015).

Exposure to environmental stressors may induce alterations in the cellular redox balance by different interconnected mechanisms. Pesticides can directly attack mitochondria, inducing the generation of ROS or depleting the cellular antioxidant defense (Franco, Sánchez-Olea, Reyes-Reyes, & Panayiotidis, 2009), thus compromising cellular homeostasis.

The placenta is a very metabolically active organ in which mitochondria are key players in energy metabolism (Illsley, 2000). The placental syncytiotrophoblast is an uninterrupted maternal-fetal barrier composed of two plasmalemmas with an intermediate layer of syncytial cytoplasm.

Abbreviations: CAT, Catalase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HMF, heavy mitochondrial fraction; LMF, light mitochondrial fraction; LP, lipid peroxidation; MDA, Malondialdehyde; MFs, mitochondrial fractions; PC, protein carbonylation; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

In the human placenta, the syncytiotrophoblast forms the interface between the maternal and fetal systems (Holland et al., 2017). The multinucleate syncytiotrophoblast is formed by the fusion of underlying mononuclear cytotrophoblasts. It is well established that the cytotrophoblast persists until term (Ji et al., 2013).

Two different mitochondrial fractions (MFs) have been characterized in the human placenta: larger cytotrophoblast mitochondria or heavy mitochondrial fraction (HMF), and smaller syncytiotrophoblast mitochondria or light mitochondrial fraction (LMF) (Bustamante et al., 2014; Honzík et al., 2006; Martinez, Kiriakidou, & Strauss, 1997), which differ in the activity of succinate dehydrogenase, citrate synthase and cytochrome P450. As the cytotrophoblast differentiates into the syncytiotrophoblast, mitochondria become highly specialized for steroidogenesis. Syncytiotrophoblast mitochondria are smaller and more irregular in shape than cytotrophoblast mitochondria and also have atypical cristae morphology (Holland et al., 2017).

We have previously studied placental subcellular fractions of women environmentally exposed to pesticides and found changes in lipid profiles, which suggested oxidative injury of syncytiotrophoblast mitochondria (Rivero Osimani, Valdez, Guiñazú, & Magnarelli, 2016; Vera, Santa Cruz, & Magnarelli, 2012). As the transfer of pesticides from maternal circulation occurs across the syncytial membrane of the placenta, toxicants may contribute to the injury of the syncytiotrophoblast. Considering that the syncytiotrophoblast is in contact with the maternal blood, it is plausible that syncytiotrophoblast mitochondria have different susceptibility to oxidative stress than cytotrophoblast mitochondria. Therefore, the aim of this in vitro study was to evaluate the impact of H_2O_2 on the antioxidant defense and oxidative stress biomarkers in both placental MFs.

2 | METHODS

2.1 | Participants and tissues

To minimize the oxidative stress caused by vaginal delivery (Veerbeek, Tissot Van Patot, Burton, & Yung, 2015), placentas were collected through scheduled cesarean section and immediately placed at 4 °C for transportation ($n = 11$). The women health status and inclusion/exclusion criteria were checked by the medical staff. Women were included if they had medium income level and belonged to the same ethnic group—Hispanic. Women with arterial hypertension, gestational diabetes, or thyroid or cardiac diseases, women on medication (except those included in Group A according to the U.S. Food & Drug Administration), consuming alcohol or drugs, and women with serious pregnancy complications such as eclampsia, preeclampsia or any other chronic condition were excluded. The average age of participants was 29.6 ± 3.8 years old. Participants showed no underweight, overweight, obesity or pregnancy complications. Fetal weight was of 3500.0 ± 380.0. This study was performed with full ethical permission of the local Advisory Committee of Biomedical Research in Humans, which approved the study protocol. All the participants provided written consents.

2.2 | Isolation of human placental MFs

HMF and LMF were isolated as previously described by Martinez, Kiriakidou & Strauss (1997). Within 2 hr after the cesarean section, placentas were placed in ice-cold saline and dissected from different cotyledons, excluding the chorionic and basal plates. Chorionic villi were harvested from the central region and tissue was dissected 0.5–1 cm deep from placental tissue and freed from the connective tissue. Tissue was homogenized in ice-cold lysis solution containing 20 mM Tris, 210 mM mannitol, and 70 mM sucrose (pH = 7.8) and filtered through a nylon sieve. The filtrated homogenate was centrifuged at 700 ×g for 10 min and the supernatant was recovered and centrifuged at 9750 ×g for 15 min. To remove red blood cells, pellets were washed for 30 s with distilled water and resuspended in 40 mM Tris, 420 mM mannitol, and 140 mM sucrose (pH = 7.8). HMF and LMF were obtained by centrifugation for 15 min at 4,000 ×g and 16,000 ×g, respectively. Finally, they were stored at −80 °C. Reagents were from Sigma Chemical Co., St Louis, MO.

2.3 | H_2O_2 treatment

From each placenta, 1 mg/mL of HMF and LMF mitochondrial protein were separately incubated for 1 h at 37 °C, using three different H_2O_2 concentrations: 1, 5, and 10 mM. Control groups were treated in the same way but using saline instead of H_2O_2.

2.4 | Enzymatic determinations

Catalase (CAT) activity was determined according to the method of Watson, Skepper, Jauniaux, and Burton (1998) with minor modifications (Chiapella, Genti-Raimondi, & Magnarelli, 2014). The rate of disappearance of H_2O_2 was followed spectrophotometrically at 240 nm for 2 min (37 °C). Assays were performed in 50 mM PBS (pH = 7.0) containing 0.1% Triton X-100 (v/v) and 12 mM H_2O_2. CAT activity was determined at the linear range of response with respect to the substrate concentration. Baseline absorbance was controlled to be stable and 10 μL of HMF or LMF was added to initiate the reaction. CAT activity was expressed as mUI/mg protein, using an extinction coefficient of 50 mM^{-1} cm^{-1}.

2.5 | Glutathione (GSH) determination

Mitochondrial GSH levels were determined as described by Tietze (1969). HMF and LMF suspensions were mixed with

10% of Trichloroacetic acid (TCA) 1:1(v/v) and centrifuged at 10,000 ×*g* for 10 min at 4 °C. Then, the supernatants (0.2 mL) were mixed with 1 mL of 1.5 mM 5,5′-Dithio-bis (2-nitrobenzoic acid) and 0.25 M PBS (pH = 8.0) and then incubated for 20 min at room temperature. The absorbance of the samples was measured against the blank at 412 nm. GSH levels were calculated from a standard calibration curve with pure GSH as a standard. Results are expressed as nmol of GSH/mg protein.

2.6 | Oxidative damage

Lipid peroxidation (LP) was estimated using the Thiobarbituric acid (TBA) test to measure Malondialdehyde (MDA), an end-product of fatty acid peroxidation, as described in Chiapella, Genti-Raimondi & Magnarelli (2014). Briefly, 0.5 mL of mitochondrial suspension was mixed with 1 mL of 20% TCA (v/v), 1.5 mL of 0.67% TBA, and 0.17 mL of 10 mM EDTA. Samples were heated to boiling point for 15 min and then ice-cooled for 5 min. Next, 2.5 mL of n-Butanol was added for lipid extraction followed by mechanical mixing for 10 min. Samples were then centrifuged for 10 min at 4,000 ×*g* and the lipid soluble phase extracted. Absorbance was measured at 532 nm. MDA content was determined using a standard curve of MDA. Results are expressed as nmoles of MDA/mg protein.

Protein oxidation was estimated using 2,4Diniphenylhydrazine (DNPH) to measure protein carbonyl content according to Fagan, Sleczka, and Sohar (1999). Briefly, proteins from 0.5 mL of mitochondrial suspension were precipitated using 0.5 mL of 6.5% TCA and centrifuged at 4,000 ×*g* for 10 min. Thereafter, samples and blanks were incubated for 1 h with 1 mL of DNPH (0.1%) or 2 N HCl, respectively, and then washed three times with 1 mL of ethanol/ethyl acetate 1:1 (v/v). Proteins were resuspended in 8 M Urea, 20 mM Tris, and 20 mM EDTA (pH = 8) and absorbance measured at 370 nm. Protein carbonyl levels were expressed as nmol/mg protein by using a molar absorbance coefficient of 21,000 M^{-1} cm^{-1}.

2.7 | Protein determination

Protein content was determined according to the Lowry method, using bovine serum albumin as a standard. In samples where protein oxidation was measured, serum albumin standard was prepared in a buffer containing 8 M Urea, 20 mM Tris and 20 mM EDTA (pH = 8).

2.8 | Data analyses

Results are presented as means ±standard error of the mean (SEM). Data were analyzed by analysis of variance (ANOVA) with a split plot design. Tukey's test was applied to evaluate differences between the H_2O_2-treated and control groups. The interaction between "MF type" and "H_2O_2 concentration during the incubation" was studied. Differences were considered statistically significant when $p \leq .05$.

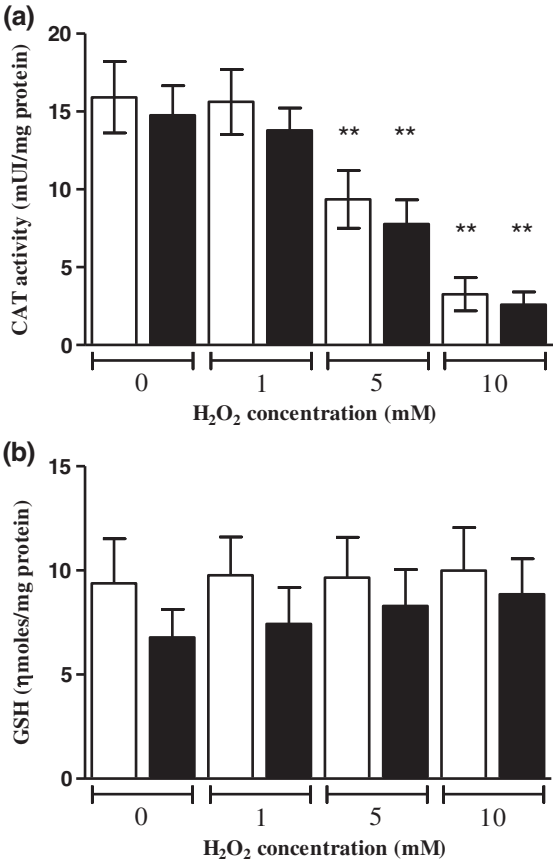

FIGURE 1 (a). Catalase (CAT) activity of the placental heavy and light mitochondrial fractions. White bars correspond to light mitochondrial fraction and black bars to heavy mitochondrial fraction. Mean ± SEM ($n = 9$). (b) Reduced glutathione (GSH) levels. Differences with respect to the control group: $^{**}p < .01$. NS: No significant differences

3 | RESULTS

H_2O_2 treatment elicited a significant decrease in CAT activity of both HMF and LMF (Figure 1a). At 5 and 10 mM of H_2O_2, CAT activity decreased 50–60 and 80% as compared to the control ($p < .001$). MFs treated with 10 mM of H_2O_2 showed a significant decrease in CAT activity as compared with those treated with 1 or 5 mM ($p < .01$). GSH content was similar in control, HMF and LMF (Figure 1b).

All H_2O_2 concentrations tested induced a significant increase in MDA levels in both placental MFs (Figure 2a). In comparison to the control group, exposure to 1 mM of H_2O_2 caused a 20% increase in MDA levels ($p < .05$), while exposure to 5 and 10 mM of H_2O_2 increased MDA levels by 40 and 60%, respectively ($p < .01$). MDA levels were significantly higher in the groups treated with 5 and 10 mM of H_2O_2 than in those treated with 1 mM of H_2O_2 ($p < .01$).

Carbonyl content was measured as a marker of protein oxidation. H_2O_2 treatment significantly increased protein

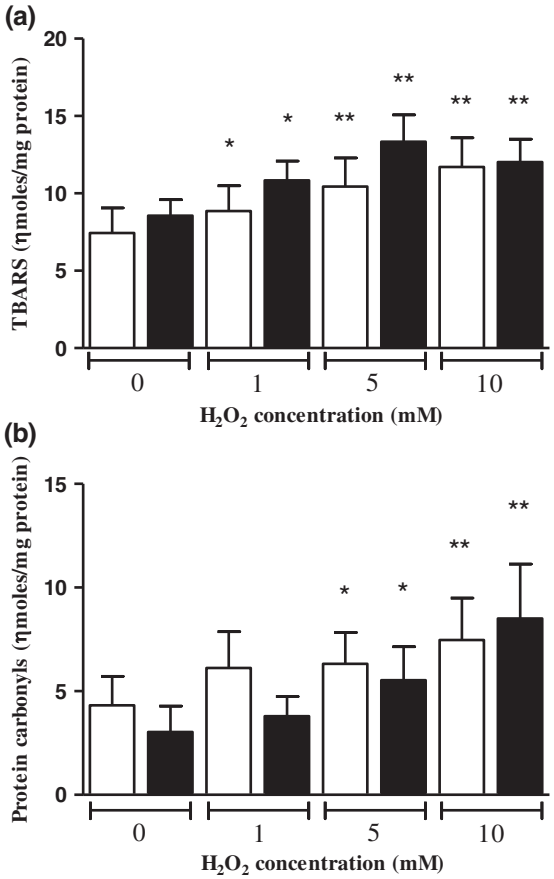

FIGURE 2 (a) MDA levels of the placental heavy and light mitochondrial fractions. (b) Protein carbonyls of the placental heavy and light mitochondrial fractions. White bars correspond to light mitochondrial fraction and black bars to heavy mitochondrial fraction. Mean ± SEM ($n = 9$). Differences with respect to the control group: *$p < .05$; **$p < .01$

oxidation in both HMF and LMF (Figure 2b). Exposure to 5 mM of H_2O_2 increased carbonyl levels by 46% in LMF and by 82% in HMF ($p < .05$) in comparison to the control groups. Consistently, LMF and HMF treated with 10 mM of H_2O_2 showed a 73 and 180% increase in protein oxidation, respectively ($p < .01$). Carbonyl levels were significantly higher in the group treated with 10 mM of H_2O_2 than in the group treated with 1 mM ($p < .05$). In general, no significant differences were found between HMF and LMF.

4 | DISCUSSION

The effects of H_2O_2 exposure on human placental MFs were investigated by measuring both components of the antioxidant system and oxidative stress markers. To the best of our knowledge, this is the first partial description of the antioxidant defense in placental HMF and LMF performed in a cell-free assay. H_2O_2 treatment decreased CAT activity, although we do not discard that this result could be partly explained by a loss of protein activity due to peroxidation in the treated samples. The GSH content of mitochondria has been previously found to be between 5 and 14 nmol GSH/mg protein (Wahlländer, Soboll, Sies, Linke, & Müller, 1979). These results are similar to ours, but we found no changes in the GSH levels, suggesting that GSH would not be a key player in H_2O_2 detoxification of placental mitochondria. Considering its low affinity for H_2O_2 (Halliwell, 1995), one might speculate that GSH may be acting mainly as an electron donor for the antioxidant enzymes.

Our data indicate that exposure of human placental MFs to H_2O_2 results in oxidative damage of both HMF and LMF, since lipid and protein oxidation were increased. Lipids were more sensitive to oxidative damage than proteins, since MDA levels were significantly increased at 1 mM H_2O_2, while protein carbonyls were not. Oxidative stress in placental mitochondria plays a causative role in the "acute fatty liver of pregnancy" (Natarajan et al., 2010), and, in preeclamptic placentas, the accumulation of HNE-modified proteins is much more evident in mitochondria than in the cytosol, indicating that LP occurs mainly in this organelle (Shibata et al., 2003).

Although high levels of ROS during embryonic, fetal and placental development are a feature of pregnancy, oxidative stress has emerged as a likely promoter of several pregnancy-related disorders with an increased risk of developing altered health outcomes in adulthood, as part of the Developmental Origins of Health and Disease (Li, Zhang, Pan, Xu, & Sun, 2017; Poston & Raijmakers, 2004; Wigle et al., 2008). In this context, both lipid and protein oxidation in placental mitochondria could have potentially deleterious effects for both the mother and the fetus.

In conclusion, our results suggest that increased levels of ROS affect mitochondrial components and that the antioxidant system of the placental LMF and HMF respond similarly to the concentrations of H_2O_2 assayed. The small number of antioxidant system parameters measured did not allow detecting differences between HMF and LMF.

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CONFLICTS OF INTEREST

No conflicts of interest to declare.

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