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Alteration of syncytiotrophoblast mitochondria function and endothelial nitric oxide synthase expression in the placenta of rural residents

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ABSTRACT

The impact of environmental organophosphate (OP) pesticide exposure on respiratory complexes, enzymatic antioxidant defense activities, and oxidative damage markers in the syncytiotrophoblast and cytotrophoblast mitochondria was evaluated. Placental progesterone (PG) levels and endothelial nitric oxide synthase (eNOS) expression were studied. Samples from women non-exposed (control group-CG) and women living in a rural area (rural group-RG) were collected during pesticide spraying season (RG-SS) and non-spraying season (RG-NSS).

In RG-SS, the exposure biomarker placental carboxylesterase decreased and syncytiotrophoblast cytochrome *c* oxidase activity increased, while 4-hydroxynonenal levels decreased. PG levels decreased in RG-SS and in the RG. Nitric oxide synthase expression decreased in RG, RG-SS and RG-NSS. No significant changes in mitochondrial antioxidant enzyme activities were found. These results suggest that the alteration of syncytiotrophoblast mitochondrial complex IV activity and steroidogenic function may be associated to pesticide exposure. Reduction in placental PG and eNOS expression may account for low newborn weight in RG.

1. Introduction

Among modern non-persistent pesticides, the organophosphate pesticides (OP) are the most commonly used worldwide. As a result of their widespread use, OP poisoning is a major cause of morbidity and mortality, especially in developing countries [1]. Pesticides follow a dynamic fate in the environment, which includes gaining access not only to pest-specific targets but also to non-target organisms, such as human beings. Agricultural pesticide application represents a major source of human pesticide exposure. Populations residing next to crops may be exposed to pesticides via various exposure routes simultaneously [2]. In this sense, Bradman et al. (2011) [3] reported that, dietary intake as well as temporal and spatial proximity to agricultural use represent the most relevant routes among the multiple determinants of OP exposure. The OP oxon metabolites are the actual powerful inhibitors of type B-esterases [4], which include carboxylesterase (CE) [5], a sensitive indicator of environmental OP exposure [6].

Potential health effects associated with pesticide exposure during pregnancy have become a major public health concern due to high maternal and fetal sensitivity to xenobiotics [7]. Several toxic effects of prenatal OP exposure have been documented at birth, such as intrauterine growth restriction (IUGR) increased risk [8], negative correlation between OP exposure and birth weight and length [9], and gestational duration [10]. Long-term effects have been registered at later life stages, including neurobehavioral [11],

Abbreviations: α-NA, alpha-naphthyl acetate; CAT, catalase; CE, carboxylesterase; CG, control group; CT, cytotrophoblast; eNOS, endothelial nitric oxide synthase; GST, glutathione S transferase; HNE, 4-hydroxynonenal; IUGR, intrauterine growth restriction; Mn-SOD, Mn-superoxide dismutase; NO, nitric oxide; OP, organophosphate; PG, progesterone; RG, rural group; RG-NSS, non-spraying season rural group; RG-SS, spraying season rural group.

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social functioning deficits [12] as well as adverse cardiovascular risk profiles in susceptible children [13].

Placental dysfunction is the underlying mechanism of many pregnancy complications [14]. Even though it has been proposed that the placenta acts as a temporary depot of OP [15], few studies have addressed the possible OP placental toxicity. Women exposed to the OP parathion presented changes in the placental morphology, with microcalcifications, microinfarcts, and atypical characteristics of tertiary villi [16]. More recently, we have shown that environmental OP exposure modulates cytokine, arginase and ornithine decarboxylase expression in human placenta [17].

The placenta is a metabolically active organ that fulfills several physiological functions such as fetal nutrition, gas exchange, protein and hormone synthesis [18]. Placental defects in the energy provision system may impact seriously on fetal developmental processes. In fact, several lines of evidence indicate that placental mitochondrial dysfunction is associated to some pathological conditions such as IUGR [19] and placental insufficiency, probably leading fetal programming effects [20,21].

Two mitochondria types have been described in the placenta, the syncytiotrophoblast (SCT) and cytotrophoblast (CT) mitochondria [22]. It has been reported that large rounded mitochondria are observed in CT cells. In contrast, the SCT contains smaller irregular mitochondria with a condensed matrix [22,23]. Both display different morphological features and dissimilar activity levels of cytochrome P450 enzymes, that participate in progesterone (PG) synthesis, with higher activity occurring in SCT mitochondria [24].

Many experimental studies have shown that pesticide exposure exerts deleterious effects in mitochondria by disturbing oxidative balance [25,26]. Mitochondrial dysfunction in pregnant women exposed to low doses of complex pesticide mixtures was suggested by Bonvallot et al. (2013) by investigating different urinary metabolites in early pregnancy [27]. We have also reported changes in SCT mitochondrial phospholipid profile associated to OP environmental exposure in women residing next to an agricultural area [28].

Taking into consideration that the mitochondria may represent a target for OP toxicity in the placenta, in this work we analyzed several mitochondrial hallmarks in placentas from pregnant women environmentally exposed to pesticides. Alterations in mitochondrial respiratory complex activities and PG levels were addressed. Also, enzymatic antioxidant defense activities and oxidative damage marker were studied. In addition, endothelial nitric oxide synthase (eNOS) expression was assessed considering that nitric oxide (NO) modifies mitochondrial respiration [29,30] and is involved in OP toxicity [31,32]. Finally, relationships between biochemical variables and fetal and placental growth indicators were evaluated.

2. Materials and methods

2.1. Chemicals

Analytical grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Merck Laboratories (Darmstadt, Germany), and BioRad (Hercules, CA, USA).

2.2. Antibodies

Rabbit polyclonal NOS (C-20), rabbit polyclonal β-actin (I–19), and goat polyclonal 4-HNE (P-16) antibodies purchased from Santa Cruz Biotechnology (Dallas, Texas USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG antibodies were from Sigma (St. Louis, MO, USA).

2.3. Studied population

The study included sixty-three healthy pregnant women (17–35 years old). From 2010 to 2013, forty-three women were recruited at the Sanatorio del Personal de Industrias Químicas at Cinco Saltos City, Rio Negro, a hospital located in northern Patagonia, Argentina. They were included in the rural group (RG) because they belonged to a small population living in an area surrounding fruit cultivation areas where pesticides are applied by ground-based spraying equipment during the dry season (september to december). The distance from the houses to the crops was between 50 m and 10 km. The strongest wind from west and south-west occurs during pesticide intensive application period. In the farms, aerial drift from the target area is frequent, increasing the potential environmental exposure of the rural population. In addition, they may be exposed to pesticides related to the commonly used irrigation technique of periodic flooding, as Loewy et al. (2011) [33] have reported that pesticide residues, mainly chlorpyrifos, azinphosmethyl and carbaryl, are found both in water (surface and subsurface) and soil, indicating off-site migration. Water source in the rural population was 5% from groundwater and 95% drinking water. The control group (CG) consisted of pregnant women with no history of pesticide exposure who attended the San Lucas prenatal clinic in Neuquén City (n = 20), Argentina.

Women in the third trimester of pregnancy were asked to participate in this study. They were included if they had medium income level, belonged to the same ethnic group – Hispanic – and were undergoing planned caesarean section due to previous caesarean section or fetal breech presentation. Placentas from caesarean deliveries were chosen because maternal oxidative stress is lower in a time-scheduled procedure than in women undergoing vaginal delivery [34]. Women were excluded if they smoked, suffered from a serious chronic disease or were medicated (except those included in Group A according to U. S. Food and Drug Administration), or developed a pregnancy complication (i.e., gestational diabetes, hypertension, preeclampsia). Groups were matched for reported smoking status and alcohol consumption.

At the time of recruitment, women were asked to complete a guided questionnaire including place of residence, physical characteristics, level of education, and lifestyle. Written informed consent was obtained from each participant. Information about pregnancy complications, placenta weight, the newborn morphometric parameters at birth (weight, height, head circumference), and gestational age were collected from medical records.

This study was approved by the ethical committee of the local Advisory Committee of Biomedical Research in Humans.

2.4. Sample collection

The spraying season group (RG-SS, n = 22) included tissue samples from placentas of rural residents collected from September to December, while the non-spraying season group (RG-NSS, n = 21) included those collected from April to August. Villous placental samples were collected immediately following caesarean delivery. Suitable amount of the sample was obtained from the central area of the maternal side of the placenta because the expression of various components may vary according to the location [35]. For subcellular fractioning, samples were collected in ice-cold Hepes buffer with NaCl 0.85%, pH 7.0, containing 0.11% buthylated hydroxytoluene as an antioxidant and then processed immediately. Subsamples were frozen at −20 °C for carboxylesterase (CE) activity and PG determinations.

2.5. Isolation of cytotrophoblast and syncytiotrophoblast mitochondria

Isolation of mitochondria was essentially performed according to the procedures described by Corso and Thomson [36]. Briefly, villous tissue (13 g) was washed with ice cold saline buffer and homogenized in TMS buffer (TrisHCl 20 mM; mannitol 210 mM; saccharose 70 mM, pH 7.8). The homogenate was centrifuged at 700*g* for 10 min at 4 °C. The supernatant was recovered and centrifuged at 4000*g* for 10 min at 4 °C to obtain the cytotrophoblast mitochondrial fraction. Then, the remaining supernatant was centrifuged at 16,000*g* for 15 min at 4 °C. The pellet suspended in TMS buffer corresponds to the syncytiotrophoblast mitochondrial fraction. All steps were carried out with cold solutions [28]. Isolated mitochondrial fractions were frozen at −70 °C until the assays were performed.

2.6. Progesterone assay

Progesterone (PG) levels in placental tissue were determined according to the procedures described by Feinshtein et al. (2009) [37]. Placental samples (1 g) were washed extensively (five times) and homogenized using a glass homogenizer in 3 mL of ice cold sodium chloride 0.9%. Samples were then centrifuged twice at 10,000*g* for 30 min at 4 °C. Supernatants were collected for PG determination. Placental PG was measured in supernatants by radioimmunoassay (RIA), according to the manufacturerís instructions, using commercial kits (DSL-3400 double antibody RIA; Diagnostic Systems Laboratories, Webster, TX, USA). All measurements were performed in duplicate and a mean value was considered for the calculations. Assay sensitivity was <70 fmol/tube, and inter and intra assay coefficients of variation were <10%. PG levels were expressed as ng/g placenta.

2.7. Enzyme assays

Carboxylesterase (CE) (EC 3.1.1.1) activity was measured using α-naphthyl acetate (α-NA) as described previously [38]. Placental samples (120 mg) were homogenized using a glass homogenizer in 2.5 mL of 0.1 M phosphate buffer, pH 6.5, containing 0.5% Triton X-100. Samples were then centrifuged at 20,000*g* during 10 min at 4 °C and the supernatant was collected for determination of enzymatic activity. The hydrolysis rate of α-NA was monitored at 550 nm. Enzyme activity was expressed as nmol/min × mg protein.

The activities of complexes I–III, II–III, and IV were determined spectrophotometrically at 30 °C. Mitochondrial membranes were obtained from mitochondrial fractions and suspended in 62.5 mM phosphate buffer (pH 7.4) with 0.025 mM cytochrome c^{+3} and 1 mM sodium azide and different substrates for each enzymatic complex. For NADH-cytochrome c reductase (complexes I–III) and succinate-cytochrome c reductase (complexes II–III) activities, mitochondrial membranes were supplemented with 0.2 mM NADH or 35 mM succinate as substrates, respectively. The enzymatic activity at 550 nm was calculated using the extinction coefficient ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) and expressed as nmol of reduced cytochrome c/mg protein. Cytochrome c oxidase (complex IV) activity was determined in 100 mM phosphate buffer with 0.1 mM cytochrome c^{+2}. The rate of cytochrome c oxidation was determined at 550 nm and expressed as nmol of oxidized cytochrome c/min × mg protein [39].

Catalase (CAT) (EC 1.11.1.6) was determined by recording the continuous decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm at 25 °C [40] and calculated using the extinction coefficient ($\varepsilon = 40\ M^{-1}\ cm^{-1}$). Mitochondrial membrane samples were diluted in sodium phosphate buffer (50 mM, pH 7.0) containing 0.1% Triton X-100 and incubated for 5 min at 4 °C. Enzyme activity was expressed as μmol/min × mg protein.

Mn-superoxide dismutase (Mn-SOD) (EC 1.15.1.1) activity was determined by the adrenochrome spectrophotometric assay [41] at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine (pH 10). Enzyme activity was expressed as USOD/min × mg protein.

Glutathione S transferase (GST) (EC 2.5.1.13) activity was determined spectrophotometrically at 340 nm according to Habig et al. (1974) [42]. Mitochondrial membranes were diluted in sodium phosphate buffer (0.1 M, pH 6.5) containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in acetonitrile and 1.2 mM glutathione as substrates in a final volume of 1 mL. The activity was expressed as CDNB conjugate (nmoles/min × mg protein) using the extinction coefficient ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). Activity values were corrected for spontaneous chemical reaction between the two substrates.

The measurements of all enzyme activities were carried out with a UV/vis 1603 Shimadzu Spectrophotometer (UV-16030). Linear conditions for all enzyme activities were previously adjusted. All measurements were performed in triplicate and a mean value was considered for the calculations. Total protein content was quantified by the Lowry method [43].

2.8. Western blot analysis

Nine randomly chosen placenta samples per group were homogenized (500 mg) in an ice bath using 1 mL RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 9% SDS, 5% DTT, 1 mM sodium orthovanadate, 10 μg PMSF, 30 μg aprotinin). Equal amounts of protein (90 μg) were diluted in SDS sample buffer, boiled at 100 °C for 5 min, loaded onto a 10% SDS-PAGE gel and run at 150 V for 1 h. After migration, proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories) at 100 V for 1 h. The membrane was blocked in Tris buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4 containing 0.2% Tween 20 and 5% non-fat dry milk, washed, and incubated overnight with the following primary antibodies: anti-eNOS (1:1000), anti-HNE (1:2000), and anti-β-actin (1:3000). After washing, the blots were incubated with peroxidase-conjugated secondary anti-rabbit antibodies (1:5000) for 1 h at room temperature. Protein antibody complexes were visualized by an enhanced chemiluminescence detection system. Actin protein was used as an internal standard.

2.9. Statistical analysis

Data were grouped and analyzed in two ways: according to the place of residence (RG and CG) and stratifying the RG according to the spraying season (RG-NSS, RG-SS, and CG).

Power calculations to determine the required sample size was based on available data of the analytical variables were performed with Fisher method (1935) [44] with the statistical program InfoStat. Comparison between CG and RG was performed by Student*ít* test. Comparison among CG, RG-NSS and RG-SS was performed using one-way ANOVA followed by the post-hoc Tukey's multiple comparison test. Statistical significance was assumed as $p < 0.05$. Categorical variables (Tables 1 and 2) were compared using the Pearson's chi-squared test (χ^2). The associations between analytical variables and morphometric parameters were estimated by calculating Pearson's correlation coefficient.

3. Results

3.1. Population characteristics

The socio-demographic characteristics of the recruited women are presented in Table 1. The three groups studied showed similar parity, level of education, nutritional state, and smoking status.

Table 1
Demographic characteristics of the studied groups.

Variable	CG(n = 20)	RG-NSS(n = 21)	RG-SS(n = 22)
Age (years)[a]	32.1 ± 2.9	24.7 ± 5.2	29.7 ± 4.3
Parity	1.6 ± 0.8	0.9 ± 1.0	1.4 ± 1.1
Level of education (%)			
Greater	17	11	8
Secondary complete	61	56	42
Secondary incomplete	22	33	50
No education/primary incomplete	0	0	0
Body-mass index			
Normal	66.6	90	88.8
Smoking status (%)			
Passive smoker	10.5	5.5	13.0
Groundwater consumption (%)	0	5.2	5.5
Alcoholconsumption (%)	0	0	0
Indoor pesticides use (%)[b]	10.5	31.6	64.7

The results were expressed as mean ± SD or as percentage when indicate. Parity means the number of times that a woman has given birth, with a viable gestational age.
[a] $p = 0.0001$ for non-spraying season group (RG-NSS) compared with control group (CG), $p = 0.0014$ for RG-NSS compared with spraying season group RG-SS. One way ANOVA followed by Tukey's Multiple Comparison Test.
[b] $p = 0.0037$ for RG-SS compared with CG by chi-square test.

Table 2
Morphometrical parameters of newborns and placenta.

Variable	CG (n = 20)	RG-NSS(n = 21)	RG-SS (n = 22)
Neonate weight (g)[a,b]	3716 ± 580.6	3425 ± 381.3	3361 ± 274.8
Newborn height (cm)[a]	49.05 ± 2.78	49.70 ± 2.59	48.07 ± 2.73
Head circumference (cm)[a]	36.91 ± 2.27	35.95 ± 1.86	36.10 ± 2.43
Placental weight (g)	736.5 ± 101.5	689.3 ± 137.3	674.4 ± 98.26
Placental weight/neonate weight ratio	0.201 ± 0.02	0.200 ± 0.03	0.195 ± 0.06
Gestational age (weeks)	38.63 ± 1.34	38.62 ± 0.80	38.67 ± 0.73

Data are expressed as mean ± SD or as percentages when correspond.
[a] Data were corrected by gestational age and sex.
[b] $p = 0.024$ for spraying season group (RG-SS) compared with control group (CG). One way ANOVA followed by Tukey's Multiple Comparison Test.

None of the participants reported alcohol consumption during pregnancy. However, within the RG, mean age of RG-NSS was lower than that of RG-SS ($p = 0.0014$). Also, RG-NSS had a lower mean age than CG ($p = 0.0001$). Indoor pest control, which may indicate an additional chemical exposure source, was significantly higher in RG-SS than in CG ($p = 0.0037$).

Also, the socio-demographic analysis was performed considering the residence place (RG vs CG). Age and the risk factor for chemical exposure (indoor pest control utilization) were significantly different ($p = 0.0005$ and $p = 0.007$, respectively) between groups.

3.2. Morphometric characteristics of the newborn and the placenta

Newborn morphometric parameters (height, head circumference and gestational age) and placenta weight were similar among groups (Table 2). However, the mean neonate weight was significantly lower in RG-SS (difference of 9.6%) with respect to the CG ($p = 0.024$). Also the mean neonate weight appeared to be different between RG-NSS and the CG (difference of 7.8%); this difference was not significant with a p value of 0.063. The morphometric characteristics analysis was also performed considering the residence place (RG vs CG). Differences in mean neonate weight between groups (RG vs CG) were highly significant ($p = 0.0069$) (data not shown).

Additionally, the placenta weight to neonate weight ratio (pw/nw), an indicator of placental functional efficiency [45], showed no significant changes among groups. The pw/nw ratio of all groups studied were within the 5th and 95th percentiles of published values at 37–42 gestational weeks [46].

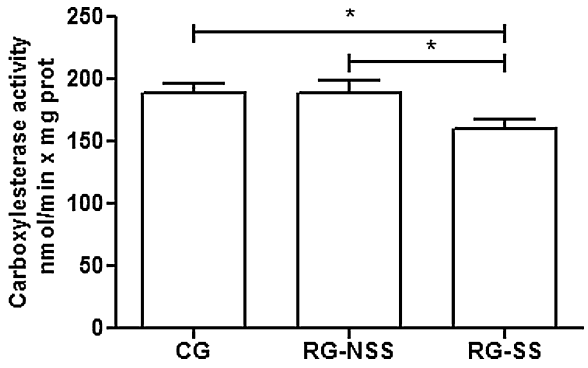

Fig. 1. Carboxylesterase activity. Enzyme activity was assessed in the placentas from the control group (CG) and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons. Results are expressed as nmol hydrolyzed substrate (α-naphthyl acetate)/min x mg protein. The graph shows the mean activity ± SEM. Difference between groups was evaluated with one-way ANOVA, followed by Tukey's multiple comparison test. *$p = 0.045$.

3.3. Placental carboxylesterase activity

Carboxylesterase (CE) activity was evaluated in order to determine placental OP exposure. CE inhibition represents a biomarker of anticholinesterase pesticide exposure, as CE is a secondary target of oxon toxicity [47] and the inhibition persists for days to weeks in animal tissues [48]. As shown in Fig. 1, rural placental samples collected during SS showed a significant decrease in CE enzyme activity compared to those collected in NSS (15%, $p = 0.045$) or to the CG samples (19%, $p = 0.045$).

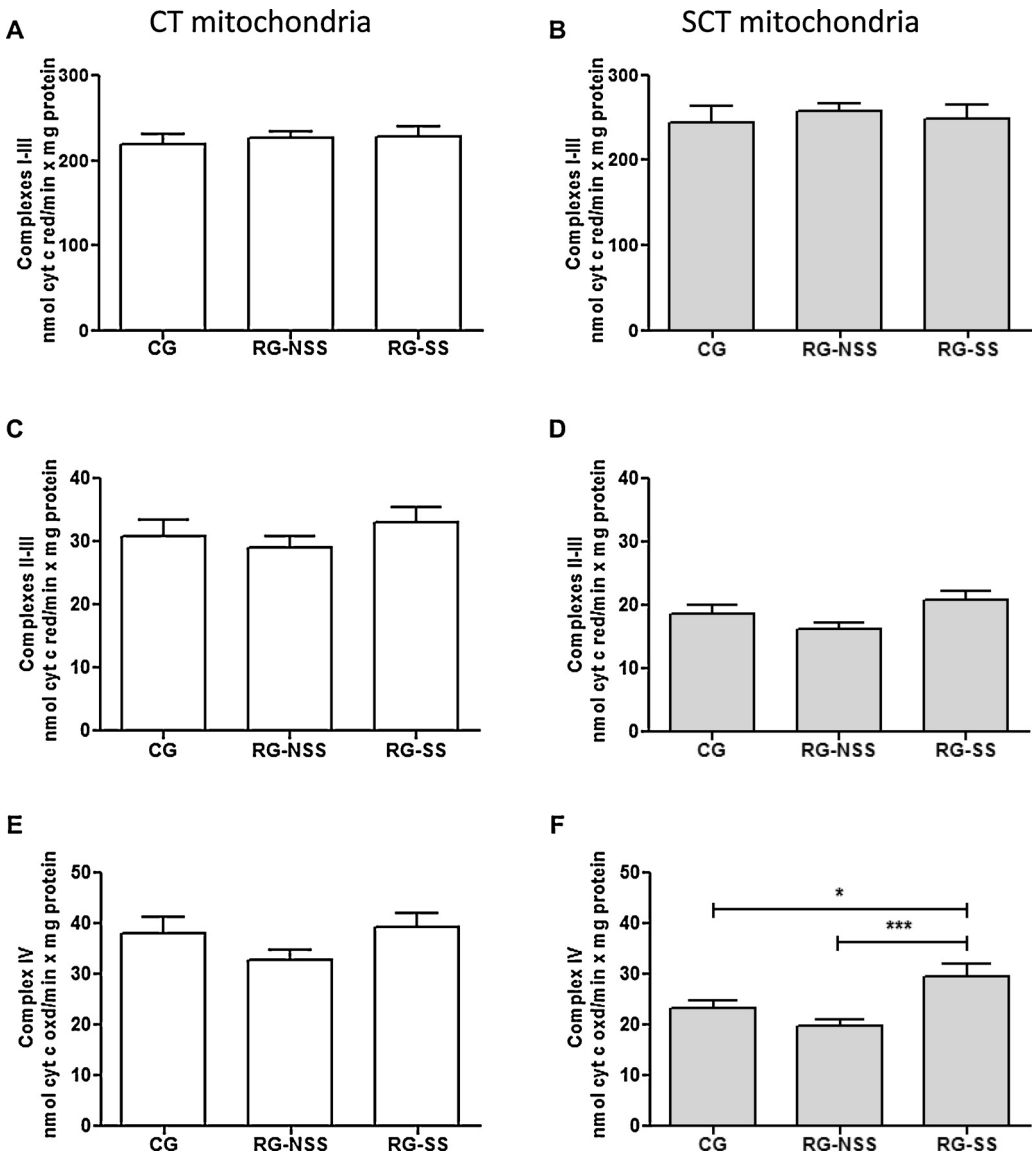

Fig. 2. Mitochondrial respiratory complex activities in cytotrophoblasts and syncytiotrophoblasts. Complexes I–III (A, B); complexes II–III (C, D) and complex IV (E, F) activities were assessed in cytotrophoblast (CT) and syncytiotrophoblast (SCT) mitochondria derived from the control group (CG); and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons. Results are expressed as nmol cytochrome *c* (reduced or oxidized)/min x mg protein. The graphs show the mean activity ± SEM. Difference between groups was evaluated with one-way ANOVA followed by Tukey's Multiple Comparison Test. $^{*}p = 0.049$; $^{***}p = 0{,}0009$.

When CE activity analysis was performed in accordance with the place of residence (RG vs CG), non-significant differences were seen in CE activity (data not shown).

3.4. Respiratory chain complex activities

As mitochondrial bioenergetics is a target for pesticide toxicity [26,49], the activity of the respiratory chain complexes I–III, II–III, and IV was analyzed.

No significant changes in the respiratory chain complex activities were observed in the CT mitochondrial fraction among groups (Fig. 2A, C, and E). SCT mitochondrial respiratory complex activities (I–III, II–III) showed non-significant changes (Fig. 2B and D). However, activity of complex IV (Fig. 2F) was significantly higher in RG-SS than in CG and RG-NSS, $p = 0.049$ and $p = 0.0009$; respectively.

When the analysis was performed in accordance with to the place of residence (RG vs CG) non-significant differences were seen (data not shown).

3.5. Mitochondrial oxidative status

In order to determine whether mitochondrial oxidative balance was altered in the studied groups, the levels of a toxic product of lipid peroxidation (HNE) and the activities of CAT, Mn-SOD, and GST were evaluated. These enzymes were chosen since they are important for controlling chemical and oxidative stress [50].

HNE content in CT (Fig. 3A) and SCT (Fig. 3B) mitochondria was analyzed by Western blot. Up to 7 and 6 anti-HNE-positive protein bands of varying densities could be distinguished in CT and SCT, respectively. Band density comparison of the immunopositive bands showed no changes among groups in CT mitochondria. The density of HNE immunopositive bands in SCT mitochondria was significantly lower ($p = 0.015$) in RG-SS than in CG.

The antioxidant defense enzyme activity of neither mitochondrial type was affected in RG or CG (Fig. 4).

When analyses were performed in accordance with the place of residence (RG vs CG) non-significant differences were seen (data not shown).

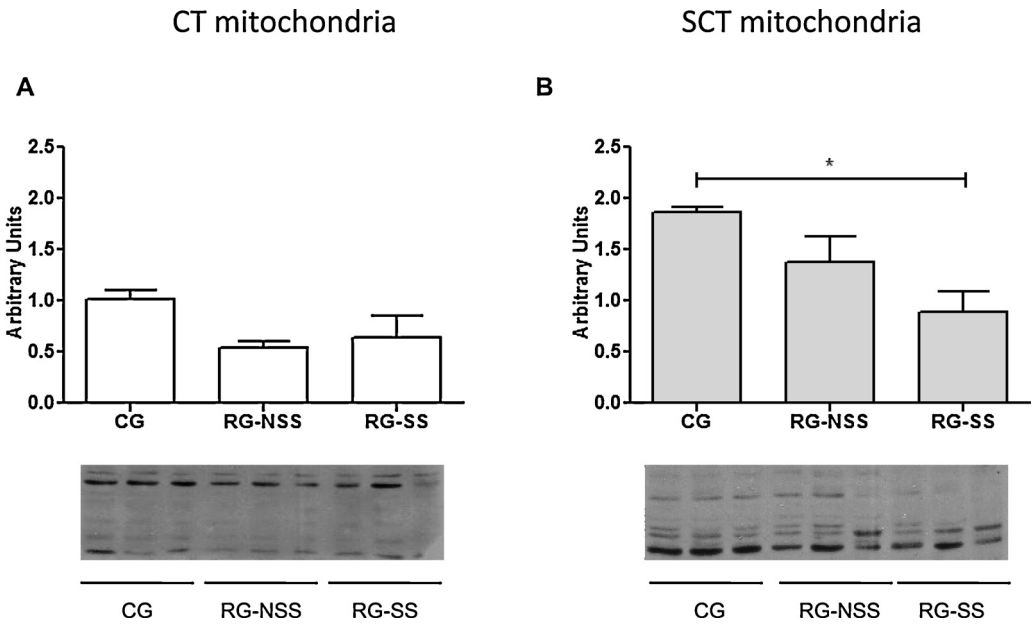

Fig. 3. 4-hydroxy-2-nonenal (HNE)-modified proteins in cytotrophoblast and syncytiotrophoblast mitochondria. Samples derived from the control group (CG); and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons were tested. HNE modified proteins were assayed by western blot, in cytotrophoblast (CT) (A) and syncytiotrophoblast (SCT) (B) mitochondria. Equal amounts of protein (90 μg) were tested. Results shown are representative of 3 independent experiments. Band relative intensity was estimated by standardization with densitometry analysis (Gel Pro Analyzer 3.2 program). Difference between groups was evaluated with one-way ANOVA followed by Tukey's multiple comparison test. $^{*}p = 0.015$.

3.6. Placental progesterone levels

Taking into consideration that PG synthesis occurs in SCT inner mitochondrial membrane [51], and that SCT mitochondria derived from rural RG-SS showed an alteration in complex IV activity, we examined whether there was a difference in PG placental levels. Fig. 5A shows that the PG content in placentas that were collected in RG-SS decreased 25% compared to CG PG levels, being significantly lower ($p = 0.035$).

When the analysis was performed in accordance with the place of residence (RG vs CG) significant differences ($p = 0.014$) were seen (Fig. 5B).

3.7. eNOS expression

After the first trimester, there is an increase and redistribution eNOS expression, mainly to SCT and endothelial cells [52]. Since pesticides are transferred from maternal circulation across the endothelial–syncytial membrane of the placenta and eNOS may represent a target for OP induced toxicity [17], its expression was analyzed. A significant reduction in eNOS expression was observed in RG-SS and RG-NSS when compared to CG. $p = 0.006$; $p = 0.00111$, respectively (Fig. 6).

When the analysis was performed in accordance to the place of residence (RG vs CG) significant differences ($p = 0.0002$) were seen (data not shown).

3.8. Associations between variables

Possible associations among analytical variables, and between analytical variables with placental or newborn morphometric parameters, were studied. The analysis revealed a significant positive relationship between placental PG levels and neonate weight ($p = 0.001$, $r^2 = 0.48$), and between placental PG levels and placental weight in CG ($p = 0.045$, $r^2 = 0.22$).

4. Discussion

Pregnant women are daily exposed to a wide range of xenobiotics. Toxicant sources are as different as maternal life styles, medication or occupational/environmental exposures [53]. Some toxicants may temporarily be deposited [15], or accumulated in the placenta, potentially affecting its development or function. Understanding the underlying mechanism of pesticide toxicity in the placenta, may provide the basis to predict aspects of developmental toxicity [54]. Mitochondrial toxicity has been widely studied in adults, although there is relatively little information within the context of human pregnancy, particularly in the *intra utero* xenobiotic exposure scenario [55].

Many OP rapidly degrade in the environment. As a result levels often fall below detectable thresholds within hours. By contrast, esterase inhibition following OP exposure can persist from days to weeks [56]. In this sense, CE is a secondary target [47] inhibited by OP-oxon binding, which is considered as a sensitive indicator of environmental exposure [6]. Moreover, CE inhibition can be important in cumulative toxicity resulting from the exposure to multiple anticholinesterase pesticides [57]. In this study, differential effects were observed for CE activity (Fig. 1) regarding the sampling period in the rural population, samples collected in SS presented significantly lower CE activity than NSS or CG samples, in accordance with our previous reports [17,28,58]. Results suggest that in SS OP compounds reach the placenta and are bioactivated to the oxon form.

The current work demonstrates that placental mitochondria show alterations in rural residents. Placentas from the RG collected during SS showed an increased complex IV activity in SCT. The dynamic nature of mitochondria in adapting to stress and metabolic changes is well known. In fact, the selective up-regulation of mitochondria complexes I, IV, and V in mice liver after intragastric alcohol feeding has been reported [59]. Also, increased gene expression of cytochrome c oxidase subunit 1 has been reported in fish exposed to the fungicide copper sulphate [60]. The increase in complex IV activity observed in RG-SS, may indicate a compensatory

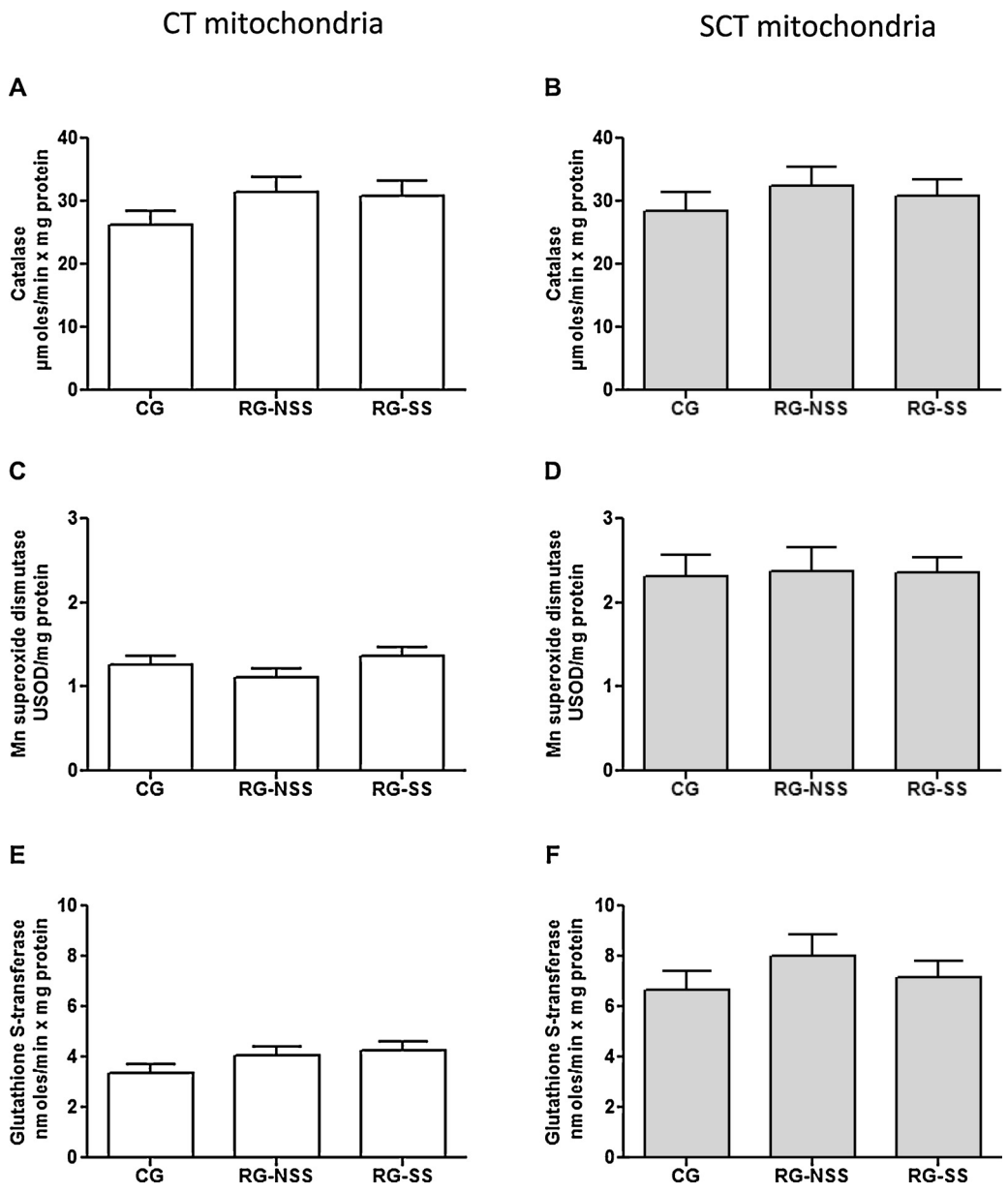

Fig. 4. Antioxidant defense enzyme activity in cytotrophoblast and syncytiotrophoblast mitochondria. Catalase (CAT), Mn-superoxide dismutase (Mn-SOD) and glutathione S- transferase (GST) enzyme activities were assayed in cytotrophoblast (CT) and syncytiotrophoblast (SCT) mitochondria, derived from the control group (CG); and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons. CAT activity (A, B) is expressed as μmol/min x mg protein. Mn-SOD activity (C, D), is expressed as USOD/min x mg protein. GST activity (E, F), is expressed as nmoles/min x mg protein. Data are represented as mean ± SEM. Difference between groups was evaluated by one-way ANOVA followed by a Tukey's multiple comparison tests.

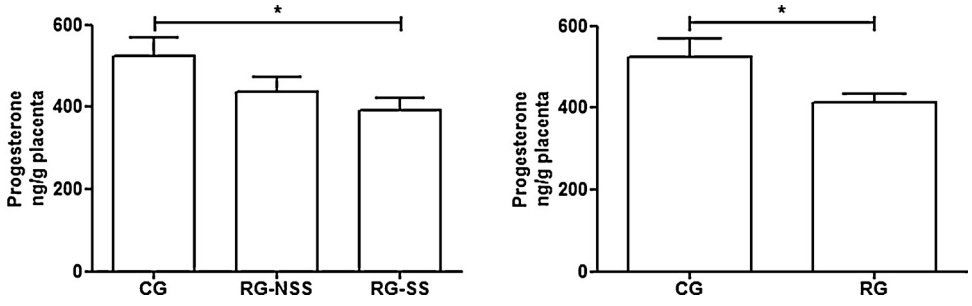

Fig. 5. Progesterone levels in placental villi. Progesterone levels were assessed in the placentas from the control group (CG); and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons. Results are expressed as ng progesterone/g placenta. The graph shows the mean ± SEM. Difference among CG, RG-NSS and RG-SS was evaluated by one-way ANOVA, followed by Tukey's multiple comparison test.*$p = 0.035$. Difference between CG and RG was evaluated by Student's t-test *$p = 0.014$.

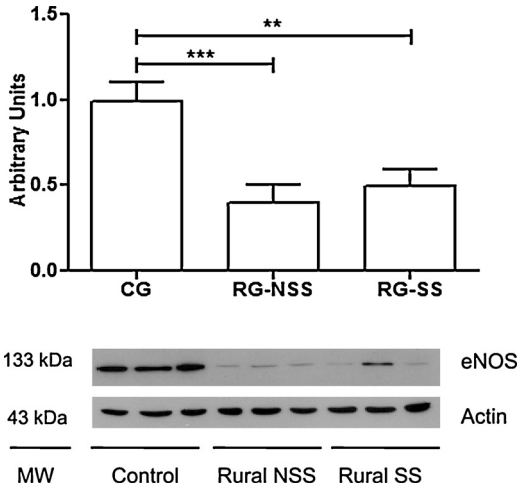

Fig. 6. Endothelial nitric oxide synthase expression. The expression of endothelial nitric oxide synthase (eNOS) was assessed in the placentas lysates from the control group (CG); and the rural group (RG) during non-spraying (NSS) and spraying (SS) seasons. eNOS expression was assayed by western blot. Equal amounts of protein (90 μg) were tested. Results shown are representative of 3 independent experiments. Band relative intensity was estimated by standardization with densitometry analysis (Gel Pro Analyzer 3.2 program). Actin was used as loading control. Right panels show the molecular weight markers (kDa). Difference between groups was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. $^{**}p = 0.0061$; $^{***}p = 0.0011$.

response in SCT mitochondria, however complex IV protein expression was no addressed. Cytochrome c oxidase, the rate-limiting enzyme of the respiratory chain [61], is a major player in the energy equilibrium of organisms, contributing to ATP balance and affecting upstream production of ROS [62]. Though ATP production was not analyzed in the current work, increased cytochrome c oxidase activity may represent an adaptive response to maintain ATP levels. In accordance with this hypothesis, we have previously shown that placentas collected in SS developed adaptive changes compatible with tissue repair [17,28]. However, Mando et al. (2014) [19] suggested that higher mitochondrial functionality of cytotrophoblast respiratory chain complexes and the increased placental oxygen consumption found in placentas of IUGR fetuses might represent a limiting step in fetal growth restriction, preventing adequate oxygen delivery to the fetus.

The transfer of pesticides from maternal circulation occurs across the endothelial–syncytial membrane of the placenta. This may partially explain why the SCT mitochondria but not the CT mitochondria showed this compensatory response. Previous studies with rural residents of this agricultural area showed changes in the phospholipid profile of the SCT mitochondria but not in the CT mitochondria [28]. Moreover, cardiolipin which has been shown to be specifically required for optimal functioning of cytochrome c oxidase [61], increased in SCT mitochondria of women exposed to OP [28].

Mitochondrial dysfunction and oxidative stress are closely intertwined. Irrespective of the state of other complexes, an increased mitochondrial ROS production and cellular toxicity, is induced in most cases of cytochrome c oxidase deficiency [30]. Some experimental studies have shown that OP-induced oxidative stress is caused by the inactivation of mitochondrial respiratory complexes [26,63]. An increase in cytochrome c oxidase activity and a decrease in HNE adducts levels, a hallmark of lipid peroxidation, was observed in SCT mitochondria derived from RG-SS. In addition, no changes in the mitochondrial antioxidant enzymes Mn-SOD, CAT, and GST were found among groups. In agreement, we recently reported that enzymatic and non-enzymatic defense systems, oxidative stress biomarkers and nuclear factor erythroid 2-related factor levels showed no changes in placental tissues from rural population environmentally exposed to OP [58]. It has been reported that complex IV activity can be modulated by HNE levels [64] and nitric oxide (NO) [29,30]. We observed an increase in complex IV activity with a concomitant decrease in endogenous HNE levels in the mitochondria, which was also accompanied with a decreased eNOS expression. Interestingly, eNOS strong immune reactivity has been reported in the SCT [65,66].

SCT mitochondria metabolism involves two major pathways: energy production and PG synthesis, both being closely related [51]. In the current work, placental PG concentration decreased in the SS group, as well as in the rural group as a whole, with respect to CG, suggesting an alteration of the SCT endocrine function. Chemicals can alter hormone concentration in blood and tissues. In fact, we have reported changes in cortisol and PG levels in blood of women living in this agricultural area and exposed to OP during first and second pregnancy trimesters [67]. PG has been shown to have an inhibitory effect on myometrial contractions, preventing preterm birth and spontaneous abortion in early pregnancy [68], thus it is used in the treatment of threatened abortion and prevention of recurrent miscarriage [69]. Remarkably, despite the small study sample a p value of 0.055 was found between CG and RG in pregnancy complications. These complications were: premature birth threat (0% control vs. 12% rural), threat of miscarriage (0% control vs. 7% rural) and premature rupture of membrane (0% control vs. 5% rural). In agreement, an association between farm residence and higher risk for spontaneous abortion has been suggested [70]. Furthermore, in women chronically exposed to OP in Mexico, an association between maternal PON1 polymorphisms and the increased risk of miscarriage has been reported [71].

A trophic action of PG has been described in rat placenta [72]. Placental and fetal growth retardation following partial PG withdrawal over the final third of rat pregnancy, the period of maximal fetal growth, has also been reported [73]. Here, we found a positive correlation between placental PG levels with placental and neonate weight in the CG, but no correlation in the RG. A wide range of factors have been studied as possible contributors to fetal anthropometric development, including parental anthropometric variables, socioeconomic status, prenatal smoking, and diet. In this study, ethnicity, prenatal smoking and nutritional state were similar among groups. The lack of correlation in RG, thus requires further investigation focusing on endocrine toxicity of pesticides such as PG receptor expression and PG receptor activity assays.

PG affects eNOS function by both genomic and non-genomic mechanisms [74]. eNOS catalyzes the cellular conversion of arginine to NO, which plays an important role in maternal cardiovascular adaptations, vasodilation of the systemic circulation, blood flow through the uterine and/or feto-placental circulations and in placental vasculogenesis and angiogenesis [52]. Amaral et al. (2014) [75] recently proposed a significant contribution of PG supplementation to decrease mean arterial pressure in pregnancy, which was also associated with positive modulation of the placental eNOS pathway. In the current work, similarly to placental PG levels, eNOS expression was significantly lower in the rural population. Kusinski (2012) showed that $eNOS^{-/-}$ mice develop reduced placental nutrient transport capacity and fetal growth. Mean weight of $eNOS^{-/-}$ fetuses was 10% lower than that of wild type fetuses, while placental weight was not altered [76]. Remarkably, neonate weight was significantly lower (9.6%) in RG compared to CG ($p = 0.0069$), without significant changes in the placenta weight.

This study has some limitations. The samples collected during NSS belonged to women who lived in the same agricultural area and long-term hazards may arise. In fact, we reported individual out-of-range PG and cortisol levels during the first and second trimester of pregnancy not only in SS but also in NSS [67]. More recently we showed that the cytokines TNFα and TGFβ, increased in pla-

cental samples of the rural group compared to the control group, but no differences were observed between SS and NSS [17]. Moreover, could have been interactions among toxicants that influenced results. The study population was probably at risk of exposure to other toxicants due to indoor pesticide use, passive smoking, well-water consumption and also by residing in an intensive agricultural area. The most frequently applied indoor pesticides by professional pest control services is the pyrethroid pesticide family. Additive or synergistic effects between OP and household pesticides could not be discarded. Taking in mind that pyrethroids are detoxified by the hydrolysis of the ester linkage by CE [77,78], the inhibition of CE observed in RG-SS may enhance pyrethroids toxicity. In summary, we consider that OP exposure is a crucial factor modifying complex IV activity. A variety of persistent organic pollutants which may accumulate in the placenta represent additional confounding factors that have not been addressed.

In summary, our study suggests the impact of pesticide exposure in the placenta, with the SCT mitochondria bioenergetics and steroidogenic function being a toxicity target. Furthermore, reduction in placental PG and eNOS expression may account for diminished newborn weight, and pregnancy alterations observed in the rural population.

Conflict of interest

All the authors declare that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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