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RESEARCH ARTICLE

Neonatal, placental, and umbilical cord blood parameters in pregnant women residing in areas with intensive pesticide application

María Martha Quintana[1,2] · **Berta Vera**[1,2] · **Gladis Magnarelli**[1] · **Natalia Guiñazú**[2,3] ·
María Gabriela Rovedatti[4,5]

Abstract In rural populations, the proximity to areas with intensive pesticide application represents a risk factor of xenobiotic exposure. Here, we investigated whether newborns born to mothers residing in an area with intensive pesticide application show alterations in placental and neonatal morphometric standards, umbilical cord blood (UCB) biochemical parameters, and/or biomarkers related to oxidative stress and oxidative damage. Samples were collected from 151 healthy pregnant women residing in a rural area (rural group; RG) during the pesticide spraying (SS) and nonspraying (NSS) seasons, as well as from women from an urban population (control group; CG), and grouped according to the delivery type (vaginal or cesarean). In the vaginal delivery group, the placental weight and placental index were higher in the RG groups than in the CG ($p = 0.01$), whereas in the cesarean delivery group, newborn weight was lower in the RG-SS group than in the CG. In the RG-SS group, UCB erythrocyte osmotic fragility and the DNA damage index (DI) were higher, and superoxide dismutase (SOD) activity was lower than in the RG-NSS group. Acetylcholinesterase and SOD activities were found to be inversely correlated with the DI.

Keywords Residential exposure · Neonate parameters · Umbilical cord blood · Osmotic fragility · Superoxide dismutase · DNA damage

Introduction

The Alto Valle of Río Negro and Neuquén provinces is the Argentine region with the largest agricultural fruit production. In 2012, 80% of the 50,000 ha with artificially added irrigation produced 1,400,000 tons of pears and apples, with 650,000 tons being exported. The average cropland size is between 5.1 and 20 ha, and 900 tons of pesticides are applied

✉ María Gabriela Rovedatti
rovedattimg@gmail.com

María Martha Quintana
quintana_maru@hotmail.com

Berta Vera
vera.berta02@gmail.com

Gladis Magnarelli
ggmag23@gmail.com

Natalia Guiñazú
natanien@hotmail.com

[1] Facultad de Ciencias Médicas, Universidad Nacional del Comahue, Av. Luis Toschi y Los Arrayanes, 8324 Cipolletti, Río Negro, Argentina

[2] Centro de Investigaciones en Toxicología Ambiental y Agrobiotecnología del Comahue (CITAAC), CONICET, Universidad Nacional del Comahue, Buenos Aires 1400, 8300 Neuquén, Argentina

[3] Departamento de Ciencias del Ambiente, Facultad de Ciencias del Ambiente y la Salud, Universidad Nacional del Comahue, Buenos Aires 1400, 8300 Neuquén, Argentina

[4] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, C1428EGA Buenos Aires, Argentina

[5] Laboratorio de Toxicología de Mezclas Químicas (LATOMEQ), Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 4to piso, Intendente Güiraldes 2160, C1428EGA Buenos Aires, Argentina

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every year for fruit pest control (Huerga and San Juan 2009). The insecticide families mostly used in this region are organophosphates (OP) (such as chlorpyrifos and azinphos-methyl), carbamates (such as carbofuran and pirimicarb), and neonicotinoids (such as tiacloprid and acetamiprid). OP and carbamates share at least one mode of action, the inhibition of acetylcholinesterase (AchE) activity, and there is in vitro evidence that mixtures of these pesticides may have additive inhibitory effects (Mwila et al. 2013). In contrast, the toxicity of neonicotinoids in insects is known to be conferred by target site specificity for nicotinic acetylcholine receptors, but little is known about the mode of action of these compounds in humans (Ding and Peng 2015).

In rural populations, the proximity to areas of intensive pesticide application is a risk factor that may favor xenobiotic exposure (Rowe et al. 2016). Several studies have demonstrated that pregnant and nonpregnant women living close to crops in North Patagonia are exposed to pesticides and that these cause alterations in the placenta and the fetus (Bulgaroni et al. 2013; Cecchi et al. 2012; Vera et al. 2012). Other studies have also reported associations of prenatal exposure to OP and carbamate exposure with pregnancy complications and adverse fetus and child development (Eskenazi et al. 2004; Naksen et al. 2015). Remarkably, few studies have examined possible associations of insecticide exposure with changes in umbilical cord blood and neonatal and placental parameters.

Biomonitoring is critical to evaluate the health of populations exposed to environmental pollutants (Angerer et al. 2007). In this sense, the intrauterine effects of pesticide exposure may be evaluated by studies on alterations in biomarkers, as these alterations may reflect underlying toxicological processes. Noninvasive matrices, such as the placenta and umbilical cord blood (UCB), are recommended for these studies. The study of UCB at delivery allows checking the transfer of contaminants from the mother to the fetus, which, if present, represents the prenatal exposure of newborns to contaminants (Yusa et al. 2015). Also, the evaluation of biochemical parameters allows assessing alterations in the last trimester of pregnancy (Camkurt et al. 2016). Additionally, since environmental contaminants may also affect placental function (Acosta-Maldonado et al. 2009), a parameter such as the placental weight to fetal weight ratio has been correlated with pregnancy alterations due to environmental exposure to toxicants and may be considered as a marker of placenta plasticity (Londero et al. 2013).

Oxidative stress has been reported as a main toxicity mechanism triggered by pesticides. Oxidative stress has been demonstrated in human OP exposure (López et al. 2007; Lukaszewicz-Hussain 2010) as well as in animal models of carbamate and neonicotinoid exposure (El-Bini Dhouib et al. 2015; Bal et al. 2012). Organisms display different mechanisms to alleviate oxidative stress and to repair damaged macromolecules. Defense is offered by enzymatic and nonenzymatic antioxidants, by scavenging free radicals and reactive oxygen species, such as superoxide dismutase (SOD), catalase (CAT), and the glutathione peroxidase system. When defense is overpassed, oxidative stress may damage different cell structures such as cell plasma membrane (Ben Amara et al. 2013) and DNA (Argentin et al. 2015).

The aim of this study was to investigate whether newborns born to mothers residing in an area of intensive pesticide application show alterations in placental or neonatal morphometric standards, as well as in UCB biochemical parameters or biomarkers related to OP exposure, oxidative stress, and oxidative damage.

Materials and methods

Participants and recruitment

The study included 151 healthy pregnant women (16–35 years old), enrolled between 2009 and 2015, who delivered single healthy babies at term, either by vaginal ($n = 74$) or cesarean ($n = 77$) delivery. One hundred and thirteen of these women attended the public hospitals of the localities of Cinco Saltos and Allen in Río Negro Province, Argentina, and were considered as the rural group (RG). These localities, which have ≅23,000 inhabitants, include other rural areas and small towns surrounded by farms, with pear and apple crops. In this area of North Patagonia (between 37° 35′ and 42° S and 62° 47′ and 71° 55′ W), OP insecticides are applied for three consecutive months during the year (October to December) and OP residues have been found in the soil, surface water, and shallow groundwater (Loewy et al. 2011). Pesticides are finely dispersed as droplets or particles at the time of spraying, and aerial drift from the target area is frequent, increasing the potential environmental exposure of nearby communities. Samples collected from October to December were considered as samples from the spraying season period (RG-SS), whereas those collected from April to August were considered as samples from the nonspraying season period (RG-NSS).

The other 38 pregnant women included in the study had no history of pesticide exposure, attended the Castro Rendón Public Hospital in Neuquén City, had vaginal ($n = 19$) or cesarean deliveries ($n = 19$), and were considered as the control group (CG). Neuquén City has a population of ≅231,000 inhabitants and is located 22–24 km far from Cinco Saltos and Allen.

Women who were asked to participate in this study were in the third trimester of pregnancy. They were included if they had medium income level and belonged to the same ethnic group (Hispanic), and excluded if they smoked, suffered from a serious chronic disease or were medicated (except with medications included in group A according to the U.S. Food and Drug Administration), or developed some pregnancy

complication (such as gestational diabetes, hypertension, or preeclampsia). At the time of recruitment, women were asked to complete a guided questionnaire including place of residence, physical characteristics, level of education, and lifestyle.

Placentas were weighed immediately after delivery (in both modes of delivery). Information about pregnancy complications, the newborn morphometric parameters at birth (weight, height, head circumference), and gestational age were collected from medical records. Values of weight, length, and head circumference were adjusted according to the gestational age and gender by using the standardized Z-score table of the Argentine Society of Pediatrics.

Written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki of the 59th WMA General Assembly, guidelines for the protection of human subjects. The study protocol was approved by the ethical committee of the local Advisory Committee of Biomedical Research in Humans.

Sample collection and hematological parameters

UCB (10 mL) was obtained from the CG ($n = 19$ vaginal and $n = 19$ cesarean deliveries), RG-SS ($n = 22$ vaginal and $n = 23$ cesarean deliveries), and RG-NSS ($n = 33$ vaginal and $n = 35$ cesarean deliveries) groups. Samples were collected immediately after delivery by venipuncture with sterile syringes containing heparin, after clamping and cutting the babies' end of the cord. UCB samples were then refrigerated at 4 °C until analysis. Hematological parameters (white cell number, red cell number, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were obtained by an automatic counter Cell-Dyn®1400 hematology analyzer.

Biochemical determinations

AchE (E.C.3.1.1.7) and butyrylcholinesterase (BchE) (E.C.3.1.1.8) activities were determined following the method of Voss and Sachsse (1970). A blank was added to every subject's blood. AchE activity was normalized by red blood cell count and expressed as nanomoles per minute red cells (1×10^6). BchE activity was expressed as nanomoles of hydrolyzed substrate per minute per microliter of whole blood. Reactions were performed at room temperature in 10 mL of 0.066 mM phosphate buffer (pH 8.0) containing 0.225 mM 5,5-dithio-2-bis-nitrobenzoate (DTNB), 0.9% NaCl, and 10 μL of sample. Measurements were carried out at 412 nm using the substrate acetylthiocholine iodide and a molar absorption coefficient of 13.6 mM^{-1} cm^{-1} (Vera et al. 2012).

CAT (E.C.1.11.1.6) activity was determined by recording the continuous decrease in absorbance at 240 nm. The reaction was performed in 1 mL of phosphate buffered saline (PBS) (50 mM, pH 7.0) containing 25 mM H$_2$O$_2$. Baseline absorbance remained constant; to initiate the reaction, 16 μL of hemolyzed UCB was added, at 37 °C. The absorbance of the reaction mixture was strictly controlled to be 1000 absorption units. Specific activity was expressed as millimoles per minute × milligrams of protein, using a molar extinction coefficient of 40 M^{-1} cm^{-1} (Rivero Osimani et al. 2016).

SOD (E.C.1.15.1.1) activity was determined in hemolyzed UCB at 30 °C, by the adrenochrome spectrophotometric assay at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine (pH 10) (Rivero Osimani et al. 2016). Enzyme activity was expressed as units of SOD (USOD) per minute × milligrams of protein. Linear conditions for all enzyme activities were previously adjusted. All measurements were performed in triplicate and a mean value was considered for the calculations. Protein content was quantified by the Lowry et al. (1951) method.

Methemoglobin levels

Methemoglobin was measured by a spectrophotometric method. Briefly, 0.1 mL erythrocytes were lysed with 8 mL PBS 0.05 M pH 6.6, and absorbance was recorded at 630 nm (measurement A1). Afterward, 0.1 mL sodium azide (70 mM) was added, left for 5 min, and absorbance measured again at 630 nm (measurement A2). In parallel, 0.1 mL of a potassium ferricyanide solution (50 g/L) was added to the lysed erythrocytes and absorbance measured at 630 nm (measurement A3). Sodium azide (70 mM) was then added to the mixture, and after 5 min, absorbance was measured again at 630 nm (measurement A4).

The percentage of methemoglobin was determined according to the following formula (Davidsohn and Bernard 1978): methemoglobin (%) = [(A1 − A2)/(A3 − A4)] × 100.

Red blood cell osmotic fragility

UCB red blood cell osmotic fragility was determined by adding a decreasing concentration of NaCl solutions, and total hemolysis was achieved with incubation with distilled water (Bautista et al. 2003). Soluble hemoglobin derived from lysed red blood cells was measured spectrophotometrically. Osmotic fragility was determined by plotting the hemolysis percentages versus the buffer sodium chloride concentrations, yielding a logistic function which allowed determining the buffer concentration at which specific percentages of hemolysis were achieved. Results are expressed as NaCl concentration (g/L) at 50 and 45% hemolysis for each group analyzed.

A stock solution of buffered sodium chloride (osmotic equivalent to 10% NaCl) was prepared by adding 90 g NaCl, 14.4 g Na$_2$HPO$_4$, and 2.7 g NaH$_2$PO$_4$ H$_2$O, diluted to 1 L with distilled water. Different dilutions of the buffered sodium chloride solutions were prepared to provide the

equivalent of 0.80, 0.50, 0.45, 0.40, 0.30, 0.10, and 0.00 g/L of NaCl. Then, 5 mL of each solution was placed in triplicate test tubes, and 20 μL of heparinized cord blood was added. All the samples were mixed and allowed to stand at room temperature for 30 min, and then centrifuged at $600 \times g$ for 5 min. The absorbance of supernatants was determined at 540 nm.

All spectrophotometric measurements were carried out with an UV/vis 1603 Shimadzu spectrophotometer (UV-16030).

Single cell electrophoresis (comet assay)

Cell viability was determined by the trypan blue exclusion technique. Samples were included in the comet assay when they presented a cell viability of 92% or greater. The alkaline comet assay was performed as previously described (Simoniello et al. 2010). Blood samples (80 μL) were embedded in 1% low melting point agarose (200 μL). Slides were placed in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris; pH 10.0–10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulfoxide. Then, the slides were incubated in alkaline buffer solution (10 N NaOH and 200 mM Na_2EDTA; pH > 10) in darkness for 20 min. DNA electrophoresis was performed for 20 min at 25 V (0.90 V/cm) and 300 mA. The alkaline solution was neutralized with 0.4 M Tris (pH 7.5) at room temperature, and the slides were fixed with 100% ethanol and stained with 40 μL ethidium bromide (20 μg/mL). Positive and negative internal controls consisted of UCB lymphocytes treated with 100 μM of H_2O_2 or 0.1 M PBS (pH 7.4), respectively, at 37 °C for 2 h. For microscopic analysis, slides were randomized and coded to blind the scorer. Then, 100 cell images of two replicate slides were randomly selected and observed at ×20, using an epifluorescence Nikon® Eclipse 80i microscope coupled with a digital camera with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Comet assays were analyzed visually on a scale of 0–4 (categories depending on the DNA damage level). The four categories were established according to the total comet length: I (<20 μm), II (20–40 μm), III (40–80 μm), and IV (>80 μm). The genotoxic damage index (DI) was calculated by means of the following formula: DI: cell number cat. I + (2 × cell number cat. II) + (3 × cell number cat. III) + (4 × cell number cat. IV).

Statistical analysis

Power calculations to determine the sample size required were based on available data of the analytical variables. Results are expressed as means ± SD. Statistical analyses of categorical variables were compared using the Pearson's chi-squared test. Groups were compared using one-way ANOVA followed by the post hoc Tukey's multiple comparison test, and Kruskal-Wallis and Dunn's tests were performed to evaluate data with nonparametric distribution. The associations between analytical variables and morphometric parameters were estimated by calculating the Pearson correlation coefficient. Infostat® software was used and statistical significance was assumed as $p < 0.05$.

Results

Social and demographic characteristics of study participants

The sociodemographic characteristics of the women recruited for the study are presented in Table 1. The three groups studied showed similar age in both modes of delivery, and none of the participants reported alcohol or tobacco consumption during pregnancy.

In the vaginal delivery group, the level of education in RG-SS was significantly different from the others ($p = 0.003$), and no differences were recorded in the pesticide exposure risk behavior such as groundwater consumption. In the cesarean delivery group, the education level of the three groups was similar, but the maternal nutritional status in CG was significantly different from the others ($p = 0.015$). Similar to that observed in the vaginal delivery group, no differences were recorded in the groundwater consumption.

Morphometric characteristics of the newborns and placentas

In the vaginal delivery group, the newborns' weight, height, head circumference, and gestational age were similar between groups (Table 2). In this group, both placental weight and the placental weight to newborn weight ratio (pw/nw), an indicator of placental functional efficiency, were higher in RG-SS and RG-NSS than in CG ($p = 0.001$ and $p = 0.01$, respectively).

In the cesarean delivery group, the newborns' height, head circumference, and gestational age were similar between groups, whereas the newborns' weight was significantly lower in RG-SS (difference of 14%) than in CG ($p = 0.04$). No differences were found in the placental weight and placental weight to newborn weight ratio.

Umbilical cord blood parameters

No significant differences were found in the UCB hemogram parameters (white and red blood cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) of all groups analyzed (Table 3). In addition, no significant differences were found in the methemoglobin levels of all the groups studied.

Table 1 Demographic characteristics of the study groups

Variable	CG		RG-NSS		RG-SS	
	Type of delivery					
	Vaginal ($n = 19$)	Cesarean ($n = 19$)	Vaginal ($n = 33$)	Cesarean ($n = 35$)	Vaginal ($n = 22$)	Cesarean ($n = 23$)
Age (years)	23.95 ± 6.10	27.69 ± 5.79	23.10 ± 5.54	24.64 ± 5.16	23.06 ± 6.83	28.47 ± 5.75
Education level (%)						
Greater	13	9	6	17	0[a]	50
Secondary complete	20	75	55	58	27	50
Primary complete	67	16	39	25	73	0
Nutritional status						
Malnutrition	0	0	5	0	0	0
Normal	89	73	95	100	87	92
Overweight	11	26[b]	0	0	13	8
Passive smoker (%)	0	6	11	0	13	0
Groundwater consumption (%)	6	17	0	7	6	20
Alcohol consumption (%)	0	0	9	0	0	0

The results were expressed as mean ± SD or as percentage when indicated

[a] For the vaginal group by chi-square test ($p = 0.003$)

[b] For the cesarean group by chi-square test ($p = 0.014$)

Umbilical cord blood cholinesterase activity

It is known that the primary targets of anticholinergic pesticides are the B-esterases AchE and BchE, which are early biomarkers of environmental exposure to carbamate and OP compounds (Vera et al. 2012). Since this study included women living in a rural area where pesticides are intensively applied and since exposure to these xenobiotics has been demonstrated before in pregnant women of this area (Souza et al. 2005; Cecchi et al. 2012; Bulgaroni et al. 2013; Rivero Osimani et al. 2016), AchE and BchE activities were evaluated to determine the impact of anticholinesterase pesticides on the activities of these enzymes in UCB. No significant differences were found in AchE (Fig. 1a, b) or BchE (Fig. 1c, d) activities between the three groups studied, in both delivery modes.

Table 2 Morphometric parameters of newborns and placenta

Variable	CG		RG-NSS		RG-SS	
	Type of delivery					
	Vaginal ($n = 19$)	Cesarean ($n = 19$)	Vaginal ($n = 33$)	Cesarean ($n = 35$)	Vaginal ($n = 22$)	Cesarean ($n = 23$)
Neonate weight (kg)[a]	3.38 ± 0.63	3.66 ± 0.59	3.38 ± 0.39	3.39 ± 0.39	3.37 ± 0.40	3.27 ± 0.23[b]
Neonate height (cm)[a]	47.85 ± 2.82	46.83 ± 4.44	45.62 ± 3.32	47.96 ± 3.07	47.33 ± 3.13	46.15 ± 2.92
Head circumference (cm)[a]	32.42 ± 2.42	34.41 ± 1.83	30.95 ± 2.67	34.14 ± 1.79	31.84 ± 3.44	34.43 ± 2.33
Placental weight (g)	518.7 ± 92.72	658.7 ± 146.1	601.60 ± 117.8[c]	691.0 ± 123.9	610.0 ± 76.24[c]	664.8 ± 87.71
Placental weight/neonate weight ratio	0.15 ± 0.03	0.18 ± 0.02	0.18 ± 0.02[d]	0.20 ± 0.03	0.18 ± 0.01[d]	0.20 ± 0.02
Gestational age (weeks)	38.89 ± 1.66	38.17 ± 1.14	38.50 ± 1.58	38.52 ± 0.88	39.44 ± 0.82	38.55 ± 0.88

Data are expressed as mean ± SD or as percentages when indicated

[a] Data were corrected by gestational age and sex

[b] RG-SS versus CG for the cesarean group. Kruskal-Wallis test followed by Dunn's multiple comparison test ($p = 0.04$)

[c] RG-SS and RG-NSS versus CG for the vaginal group. One-way ANOVA followed by Tukey's multiple comparison test ($p = 0.010$)

[d] RG-SS and RG-NSS versus CG for the vaginal group. One-way ANOVA followed by Tukey's multiple comparison test ($p = 0.023$)

Table 3 Umbilical cord blood parameters

Variable	CG		RG-NSS		RG-SS	
	Type of delivery					
	Vaginal ($n = 19$)	Cesarean ($n = 19$)	Vaginal ($n = 33$)	Cesarean ($n = 35$)	Vaginal ($n = 22$)	Cesarean ($n = 23$)
White blood cells ($\times 10^3$/mm^3)	11.56 ± 1.9	10.30 ± 2.0	11.02 ± 3.1	10.64 ± 2.6	11.97 ± 2.6	9.50 ± 2.8
Red blood cells ($\times 10^6$/mm^3)	4.49 ± 0.58	4.24 ± 0.63	4.73 ± 0.49	4.36 ± 0.43	4.94 ± 0.42	4.26 ± 0.39
Hemoglobin (g/dL)	16.30 ± 1.68	15.16 ± 2.65	15.60 ± 1.77	15.38 ± 1.55	16.22 ± 1.53	14.86 ± 1.50
Hematocrit (%)	48.38 ± 5.63	45.14 ± 7.98	45.09 ± 4.97	46.44 ± 4.88	48.87 ± 4.70	45.02 ± 4.39
Mean corpuscular volume (μm^3)	107.7 ± 2.27	106.1 ± 4.07	103.8 ± 6.12	107.2 ± 3.38	105.5 ± 4.66	105.9 ± 3.59
Mean corpuscular hemoglobin (pg)	35.85 ± 1.2	34.63 ± 1.52	35.43 ± 2.40	35.41 ± 1.36	34.99 ± 1.36	35.1 ± 1.01
Mean corpuscular hemoglobin concentration (g/dL)	33.28 ± 0.98	32.66 ± 0.82	34.43 ± 2.58	33.11 ± 0.89	33.19 ± 1.23	33.17 ± 0.89
Methemoglobin (%)	6.16 ± 3.62	5.86 ± 4.42	4.03 ± 3.25	7.09 ± 3.90	6.91 ± 5.39	6.32 ± 4.11

Data are expressed as mean ± SD or as percentages when indicated

Umbilical cord blood biomarkers of oxidative stress and DNA damage

During vaginal delivery, both the mothers and their newborns show signs of oxidative stress (Raijmakers et al. 2003, Watanabe et al. 2013). In addition, in this delivery mode, differences in labor duration (Cindrova-Davies et al. 2007) may contribute to dispersion of results. In contrast, programmed cesarean deliveries represent a more homogeneous labor background than vaginal deliveries. Thus, and taking into consideration that
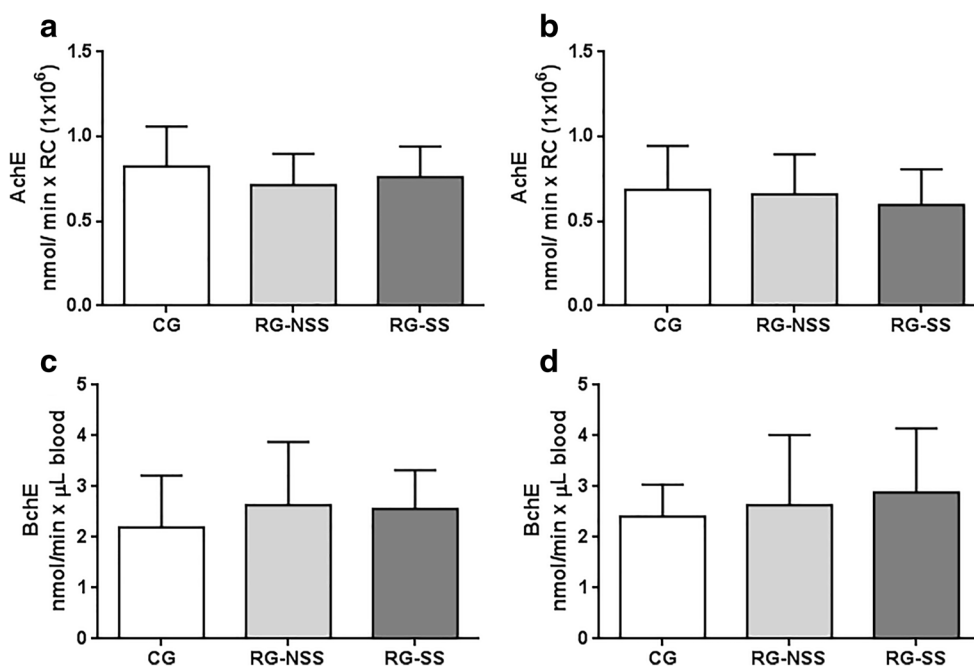
Fig. 1 Activity of biomarkers of OP exposure. Acetylcholinesterase activity was assessed in red cells (*RC*) from the control group (*CG*) and the rural group (*RG*) during nonspraying (*NSS*) and spraying (*SS*) seasons **a** in vaginal and **b** in cesarean deliveries. Results are expressed as nanomoles per minute × RC (1×10^6). The graph shows the mean activity ± SD. Differences between groups were evaluated with one-way ANOVA, followed by Tukey's multiple comparison test. No significant differences were found. Butyrylcholinesterase activity was assessed in red cells (*RC*) from the control group (*CG*) and the rural group (*RG*) during nonspraying (*NSS*) and spraying (*SS*) seasons **c** in vaginal and **d** in cesarean deliveries. Results are expressed as nanomoles per minute × microliters of blood. The graph shows the mean activity ± SD. Differences between groups were evaluated with one-way ANOVA, followed by Tukey's multiple comparison test. No significant differences were found
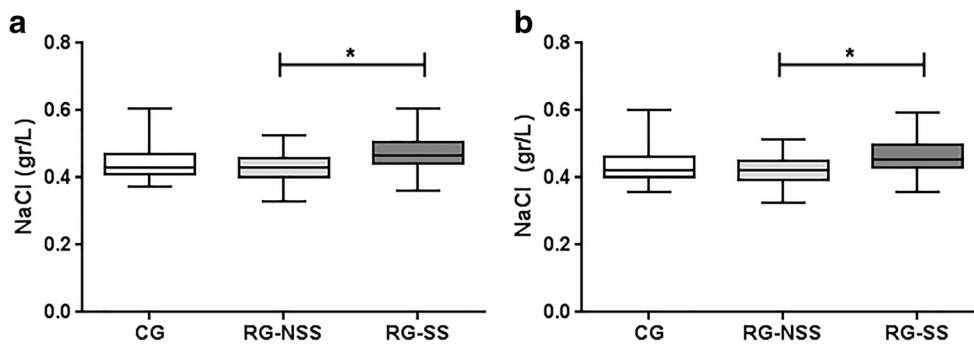
Fig. 2 Distribution of NaCl concentrations at which **a** 45% hemolysis and **b** 50% hemolysis were observed in cesarean delivery. The box plots show the median and the 25–75 percentiles. The *error bars* represent the 10 and 90 percentiles. Differences between groups were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. Data represent mean ± SD. *$p = 0.04$ and *$p = 0.034$, respectively

the birth weight in RG-SS was lower than that in CG only in the cesarean delivery group, the results described below were analyzed only in the cesarean delivery group.

Red blood cell osmotic fragility was evaluated in UCB. The PBS concentration that induced 50 and 45% hemolysis of UCB red blood cells was significantly higher in the RG-SS than in the RG-NSS group ($p = 0.039$ and $p = 0.038$, respectively) (Fig. 2a, b).

The activities of the antioxidant enzymes CAT and SOD were studied to determine the antioxidant enzyme status. SOD activity was significantly decreased in RG-SS compared with RG-NSS and CG ($p = 0.010$) (Fig. 3a). In contrast, CAT activity showed no significant changes between groups (Fig. 3b).

To investigate possible DNA damage, UBC samples were processed by the alkaline comet assay. The DI was calculated taking into account the comet's tail length (μm), as indicated in the "Materials and methods" section. This index was significantly higher in RG-SS than in CG ($p = 0.003$) (Fig. 3c), but not significantly different between RG-SS and RG-NSS.

Associations between variables

Possible correlations between enzyme activities (AchE, BchE, SOD, CAT), DI, placental parameters, and newborn morphometric parameters were analyzed (Table 4). Interestingly, AchE activity showed a weak but significant inverse correlation with the DI ($r = -0.37$, $p = 0.0065$, $n = 52$), whereas SOD activity showed a significant inverse association with the DI ($r = -0.314$, $p = 0.014$, $n = 54$). AchE and SOD activities showed a significant positive correlation ($r = 0.38$, $p = 0.032$, $n = 54$). Additionally, nonsignificant associations were found when the other parameters were analyzed.

Discussion

In this study, we analyzed newborn, placenta, and UCB parameters in babies born to mothers from the rural population of the Alto Valle of Río Negro and Neuquén provinces in
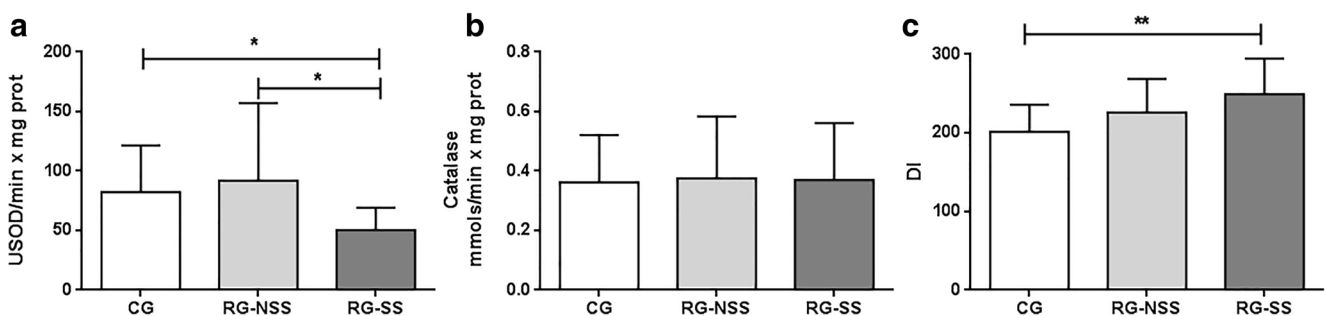
Fig. 3 Damage biomarkers and activity of the enzymatic antioxidant defense system in umbilical cord blood red cells, derived from the control group (*CG*) and the rural group (*RG*) during nonspraying (*NSS*) and spraying (*SS*) seasons. **a** Mean values (±standard deviation (SD)) of DNA damage in umbilical cord blood cells, damage index (*DI*). Differences between groups were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. Data represent mean ± SD. **$p = 0.0026$. **b** Mn-SOD activity is expressed as units of SOD (USOD) per minute × milligrams of protein. Differences between groups were evaluated by the Kruskal-Wallis test followed by Dunn's multiple comparison test, *$p = 0.0101$. **c** CAT activity is expressed as micromoles per minute × milligrams of protein. Differences between groups were evaluated with one-way ANOVA, followed by Tukey's multiple comparison test. No significant differences were found

Table 4 Univariate analyses stratified by parameter analysis

	AchE	SOD	DI	Neonate weight	Placental weight	Placental weight/ neonate weight ratio
AchE		$r = -0.3716$ $p = 0.0057$ $n = 54$ *	$r = -0.37$ $p = 0.0065$ $n = 52$ *	$r = -0.184$ $p = 0.18$ $n = 53$	$r = -0.068$ $p = 0.18$ $n = 55$	$r = -0.192$ $p = 0.171$ $n = 52$
SOD	$r = -0.3716$ $p = 0.0057$ $n = 54$ *		$r = -0.314$ $p = 0.0205$ $n = 54$ *	$r = 0.072$ $p = 0.607$ $n = 52$	$r = 0.008$ $p = 0.95$ $n = 54$	$r = -0.033$ $p = 0.815$ $n = 52$
DI	$r = -0.37$ $p = 0.0065$ $n = 52$ *	$r = -0.314$ $p = 0.0205$ $n = 54$ *		$r = -0.108$ $p = 0.442$ $n = 52$	$r = 0.069$ $p = 0.618$ $n = 54$	$r = 0.125$ $p = 0.377$ $n = 52$
Neonate weight	$r = -0.184$ $p = 0.18$ $n = 53$	$r = 0.072$ $p = 0.607$ $n = 52$	$r = -0.108$ $p = 0.442$ $n = 52$		$r = 0.282$ $p = 0.026$ $n = 62$	$r = -0.403$ $p = 0.0012$ $n = 62$ ***
Placental weight	$r = -0.068$ $p = 0.18$ $n = 55$	$r = 0.008$ $p = 0.95$ $n = 54$	$r = 0.069$ $p = 0.618$ $n = 54$	$r = 0.282$ $p = 0.026$ $n = 62$		$r = -0.403$ $p = 0.0012$ $n = 62$ ***
Placental weight/neonate weight ratio	$r = -0.192$ $p = 0.171$ $n = 52$	$r = -0.033$ $p = 0.815$ $n = 52$	$r = 0.125$ $p = 0.377$ $n = 52$	$r = -0.403$ $p = 0.0012$ $n = 62$ ***	$r = -0.7575$ $p = 0.00012$ $n = 62$ ***	

Data are expressed as covariates with a significance level of $p < 0.05$ (*) and number of samples. Covariates with a significance level of $p < 0.010$ (***)

North Patagonia, Argentina. The samples were grouped into delivery type (vaginal or cesarean) and according to the pesticide spraying season and compared with the parameters derived from an urban population. Sociodemographic data from questionnaires took into account potential risk factors of pesticide exposure such as groundwater consumption and passive smoking, which gave nonsignificant differences between groups.

In the cesarean delivery group, we found a significant decrease in the newborns' weight (11%) during the spraying season. A similar finding was reported by Rivero Osimani et al. (2016) in rural residents from the same North Patagonian area. Others have described a reduced birth weight related to detection of pesticide mixtures, including chlorpyrifos (Whyatt et al. 2003; Naksen et al. 2015). Previous studies in pregnant women from this region have demonstrated decreased cholinesterase activity in maternal blood (Souza et al. 2005; Cecchi et al. 2012; Vera et al. 2012; Bulgaroni 2013), indicating maternal exposure to anticholinesterase pesticides. Results presented here showed no significant changes in the activities of cholinesterase enzymes (AchE and BchE) in UCB. These apparently contrasting results raised the concern of whether the impaired fetal development observed could be due to xenobiotic exposure rather than to AchE inhibition. In this sense, Flaskos (2012) concluded that the developmental toxicity of OP metabolites is not related to the inhibition of

AchE activity, but may be due to direct metabolite interference with the morphogenic activity of AchE, including interference with cellular targets as signaling molecules and cytoskeletal proteins. Alternatively, it is also possible that the placenta would act as a metabolic barrier, since it expresses not only B-esterases, such as acetylcholinesterase and carboxylesterases (Bhuiyan et al. 2006; Vera et al. 2012), but also a range of metabolic enzymes (Prouillac and Lecoeur 2010). Thus, the placenta may have the ability to dampen the toxic impact of pesticides on fetal components, including UCB. Concordantly, the UCB hematological parameter studied showed no differences between the SS and NSS groups.

The placental index, calculated as placental weight divided by birth weight, reflects the balance between fetal and placental growth. The ratio decreases across gestation as the placenta matures, and correspondingly, fetal weight increases (Macdonald et al. 2014). Reports indicate that the placental weight and placental index may indicate maternal disease and thus predict obstetric outcome, perinatal morbidity and mortality, and child growth and development (Burkhardt et al. 2006; Macdonald et al. 2014). In the present study, we observed differences in placental weight and birth weight between vaginal and cesarean deliveries, as reported by others (Burkhardt et al. 2006). Nevertheless, results showed a significant increase in both placental weight (16%) and placental index (20%) in the vaginal delivery group during the spraying

season. In contrast, the mean placental weight in CG (vaginal delivery) was in accordance with previous reports (Burkhardt et al. 2006; Raghunath et al. 2011; Grandi et al. 2016), and values correspond to 50 percentiles as reported by Burkhardt et al. (2006). The placenta has a high functional reserve capacity and a remarkable potential to increase its growth as a required adaptation. A study of 18,386 pregnancies found a high placental index among pregnancies characterized by poor outcomes, such as hypertensive disorders and small for gestational age infants (Londero et al. 2013). Thus, placental hypermaturity is an adaptation mechanism to preserve fetal well-being in adverse environments. In addition, a higher placental maturity index has been associated with pesticide exposure in pregnant women in Mexico (Acosta-Maldonado et al. 2009), suggesting a link between pesticide exposure and changes in placental parameters.

The generation of oxidative stress related to the delivery mode is still controversial. Some authors have postulated that labor and vaginal delivery promote redox imbalance (Raijmakers et al. 2003; Cindrova-Davies et al. 2007; Watanabe et al. 2013), whereas others have postulated that the cesarean section provides a more pro-oxidant scenario than the vaginal delivery (Noh et al. 2014), and others have reported no differences between delivery modes (Saphier et al. 2013). To investigate possible changes in oxidative stress biomarkers in UBC, and considering that birth weight was lower in the RG-SS group from cesarean delivery, studies were performed only in the cesarean delivery group.

A common pesticide toxic response, independent of primary target effects, is oxidative stress. Several pesticides, including OP, have been linked to induction of oxidative stress in human and animal exposure models (Mostafalou and Abdollahi 2013). Alterations in the oxidant balance, such as an increase in reactive species, a decrease in the radical scavenging system, or both, may affect erythrocyte integrity. In this sense, the polyunsaturated fatty acids present in the plasma membrane as well as the redox active hemoglobin render erythrocytes vulnerable to oxidative insults. In the rural group, we found a significant increase in UCB red cell osmotic fragility during the spraying season. The osmotic fragility found in the CG (50% lysis) was similar to that reported by Bautista et al. (2003) in UCB erythrocytes from term babies. In other models, pesticide exposure has been shown to increase erythrocyte osmotic fragility (Uchendu et al. 2014) and lipoperoxidative changes in the plasma membrane (Ambali et al. 2010). In relation to osmotic fragility, in the present study, SOD activity showed a decrease in RG-SS. The antioxidant defense repertoire of erythrocytes, such as GSH, CAT, SOD, and GSH-peroxidase (Ertabak et al. 2004), is also the target of different pregnancy complications and can even be modified by environmental factors (Mohorovic et al. 2010). A decreased antioxidant defense capacity may contribute to the damage of cell components, including lipids, proteins, and DNA (Ben Amara et al. 2013; Argentin et al. 2015). The comet assay is the method of choice to measure DNA damage in human cells such as lymphocytes. This technique has been extensively used in population-based studies of environmental and occupational exposure to toxic agents, including radiation, pesticides, and oxidative stressors (Dusinska and Collins 2008; Simoniello et al. 2010). In the present study, maternal exposure to pesticides used in fruit production induced an increase in UCB DNA damage. Regarding genotoxic damage, we found a significant inverse correlation between the DI and AchE activity and between the DI and SOD activity, which indicates that the lower the enzyme activity, the higher the DNA damage. We also found a weak positive correlation between SOD and AchE activities, which would explain that the redox imbalance that affected SOD activity also impacted AchE activity. Similarly, Chiapella et al. (2013) demonstrated that antioxidants prevent AchE inhibition. Moreover, in workers from an intensive agricultural area in Spain, López et al. (2007) found a positive correlation between SOD and AchE activities during the OP exposure period.

In conclusion, these results demonstrate that residing in areas of intensive pesticide application during pregnancy is a risk factor for the induction of significant changes in UCB form elements. This was demonstrated in erythrocyte osmotic response and antioxidant defense, as well as in lymphocyte DNA damage. These parameters may be more sensitive indicators of xenobiotic exposure in UCB than classical biomarkers (i.e., cholinesterases). Taking into consideration the vulnerability of the embryo and the fetus to environmental chemicals, our results are of great concern and point to the necessity of larger field studies in these rural populations to find out possible epidemiological relationships between long-lasting maternal pesticide exposure and its impact on placental physiology and fetal development.

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