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Environmental pesticide exposure modulates cytokines, arginase and ornithine decarboxylase expression in human placenta

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ABSTRACT

To evaluate the cytokine balance and enzymatic alterations induced by environmental pesticide exposure during pregnancy, this transversal study explored placentas derived from non-exposed women (control group-CG), and from women living in a rural area (rural group-RG), collected during intensive organophosphate (OP) pesticide spraying season (RG-SS) and during non-spraying season (RG-NSS). The exposure biomarkers blood cholinesterase and placental carboxylesterase (CaE) were significantly decreased in RG-SS. Among the cytokines studied IL-8, IL-6, TNFα, IL-10, TGFβ and IL-13, the expression frequency of IL-13 increased in RG-SS. Arginase and ornithine decarboxylase (ODC) enzymes were induced in syncytiotrophoblast and endothelial cells. Interestingly, the decrease in CaE activity was associated with arginase and ODC activity induction. These findings suggest that environmental pesticide exposure impacts the placenta by increasing the expression frequency of the anti-inflammatory cytokine IL-13, which may be related to the up-regulation of enzymes implicated in tissue repair.

1. Introduction

One of the chemicals most widely used worldwide are the cholinesterase-inhibiting organophosphates (OPs). Humans may be exposed to OP pesticides by working in field crops [1], by living next to application areas [2], or by ingesting contaminated food [3]. In addition, several pesticides are also found in indoor dust [4] and as contaminants in watercourses [5]. Indeed, Bulgaroni et al. have reported that women and children living in close proximity to an agricultural area of the Río Negro River (Patagonia, Argentina) are exposed to anti-cholinesterase pesticides [6]. Recently, it has been postulated that pregnant women may be exposed to OP and carbamates as a consequence of poor industrial hygiene in the woman's workplace and re-exposures from pesticides introduced into their homes during work time [7].

In the last decade, the knowledge that many environmental chemicals are able to cross the placenta and reach the fetus has become a general concern. Exposure to toxic compounds during fetal development can increase the risk of adverse health consequences, including preterm birth, birth defects, childhood morbidity (*e.g.* neurodevelopmental effects and childhood cancer), and adult disease and mortality (*e.g.* cancer and cardiovascular effects) [8–10]. Animal studies on OP toxicokinetics and placental transfer, have demonstrated that this organ is a poor barrier which functions as a temporary depot [11,12]. The placenta has a finite life span and significant pathological processes can affect the placenta before involving the fetus, yet its examination is often neglected [13]. Consequently, few studies have focused on placental OP toxicity. Changes in placental morphology as atypical characteristics of tertiary villi [14] and alterations in the maturity homogeneity within placental tissue [15] have been observed in samples derived from OP exposed women. We have previously demonstrated changes in the activity of placental acetylcholinesterase and catalase [16] as well as changes in mitochondrial and nuclear lipid profiles associated with environmental OP exposure [17], in the North Patagonia agricultural area.

It has been recognized that placental health is a pre-requisite for fetal health [18]. In addition to providing the fetus with oxygen and nutrients, the placenta synthesizes and secretes hormones, growth factors, and cytokines [13]. Within the placenta,

Abbreviations: α-NA, alpha-naphthyl acetate; Ab, antibody; CaE, carboxylesterase; IL, interleukin; NO, nitric oxide; NSS, non-spraying season; OP, organophosphate pesticide; PCh, plasma cholinesterase; RBC-AChE, red blood cell acetylcholinesterase; TGFβ, transforming growth factor beta; TNFα, tumoral necrosis factor alpha; SS, spraying season.

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cytokines are involved in modulating fundamental processes such as -maternal–placental immune dialog, -trophoblast invasion and differentiation, -placental growth, proliferation and apoptosis, -placental metabolic and endocrine homeostasis, and -placental angiogenesis [19]. Also, the abnormal activation of immune components may be associated with pregnancy complications [20] such as preeclampsia and intrauterine growth restriction [19].

Immunotoxicological investigations demonstrated that OP exposure may modulate the major cytokines involved in the regulation of the immune response in animal models [21] as well as in *in vitro* experiments [22]. We have recently demonstrated that *in vitro* incubation with the OP phosmet or chlorpyrifos affected the cytokine pattern produced by trophoblast JEG-3 cells. OP exposure was able to increase TNFα levels in culture supernatants and to modulate IL-17, IL-6 and IL-13 mRNA expression pattern [23]. However, to our knowledge there is no information available about whether the environmental exposure of pregnant women to OP pesticides alter the cytokine expression profile, in the placenta.

The aim of the present research was to study whether the environmental exposure of pregnant women to pesticides, particularly to acetylcholinesterase inhibitors, alters the cytokine expression pattern (IL-6, TNFα, IL-8, IL-10, IL-13, and TGFβ) in the placenta. Considering that arginase and ornithine decarboxylase enzymes, relevant for trophoblast proliferation and placental growth, may be modulated by these cytokines, we also wished to determine whether their activity and expression can be altered by pesticide exposure. In addition, OP exposure was assessed by the determination of blood cholinesterase, and placental carboxylesterase activities.

2. Material and methods

2.1. Participants and recruitment

The study included 82 healthy pregnant women (15–35 years old) who were enrolled through 2008–2011. Forty six women, who attended to the Public Hospital in Centenario and in General Roca Cities, were considered rural group. They belonged to a population living in small towns near farms, where pears and apples are cultivated. In this area, North Patagonia-Argentina, pesticides are applied by ground-based spraying equipment during the dry season (September–December). Irrigation is performed by periodic flooding, providing a significant volume of water to the cultivation area. Associated with the irrigation technique, Loewy et al. have reported that pesticide residues, mainly chlorpyrifos, azinphos-methyl and carbaryl, are found both in water (surface and subsurface) and soil, indicating that off-site migration takes place [24]. Pregnant women who attended to the Castro Rendón Hospital in Neuquén City ($n = 36$), with no history of pesticide exposure were considered control group.

We restricted the study to women who had resided in urban (control group) or in the rural areas (rural group) for one or more years before pregnancy. Health status was checked by the medical staff, and women were excluded if they used illicit drugs, consumed medication (except those included in Group A according to U. S. Food and Drug Administration), had gestational diabetes, hypertension, history of preeclampsia, recurrent pregnancy loss or known infectious disease. A questionnaire was administered to document basic demographic information of participants, potential confounding factors such as smoking habit and daily contact with smokers, as well as risk behaviors for environmental pesticide exposure.

The current study was carried out with full ethical permission of the local Ethical Assessor Committee of Biomedical Research in Humans, and at the hospital's Teaching and Investigation advisor committees that participated in the study. Written informed consent was obtained at the time of third trimester routine blood testing. However, for the aims of this study, data were processed anonymously. Additionally, the authors declare that this investigation has been conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Sample collection

Third trimester maternal blood samples were obtained by venipuncture. Heparinized blood samples were analyzed for cholinesterases activity and EDTA-treated samples were analyzed for red cell count in a hematological counter Cell-Dyn 1400.

The placenta was obtained after normal vaginal delivery and immediately sampled. Each placenta was laid with the fetal surface up facing the observer. Two 2×10 cm pieces were taken from the area around the umbilical cord attachment and snap frozen in liquid nitrogen. Then, all placental samples were stored at −80 °C until analysis. In addition, a 2×2 cm piece of the placenta was cut and immersed in PBS (pH 7.4) 4% formalin and kept at room temperature until processed. Blood and placenta samples of rural residents collected from September to December, were considered samples of spraying season (SS, $n = 23$) and those collected from April to August were considered samples of non-spraying season (NSS, $n = 23$).

2.3. Determination of acetylcholinesterase and carboxylesterase activities

Red blood cell acetylcholinesterase activity (RBC-AChE) and plasma cholinesterase (PCh) activities were measured using heparinized samples at 30 °C following the Voss and Sachsse method [25]. A blank was added to every subject's blood. RBC-AChE activity was normalized by red blood cell count and expressed as nmol of hydrolyzed substrate × min^{-1} × $million^{-1}$ of erythrocytes. PCh activity was expressed as nmol of hydrolyzed substrate × min^{-1} × μl^{-1} of whole blood. Measurements were carried out at 412 nm using the substrate acetylthiocholine iodide and a molar absorption coefficient of 13.6 mM^{-1} cm^{-1}.

Carboxylesterase (CaE) activity was measured using α-naphthyl acetate (α-NA), as described previously [17]. Briefly, placenta 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. Then, samples were centrifuged at 20,000 × *g* for 10 min at 4 °C and the supernatant was collected for determination of CaE enzymatic activity. The hydrolysis rate of α-NA was monitored at 550 nm.

Total protein content was quantified by the Lowry method [26]. All measurements were performed in triplicate and a mean value was considered for the calculations. The measurements were carried out with a UV/vis 1603 Shimadzu Spectrophotometer.

2.4. Cytokine reverse transcription-PCR analysis

A standard reverse transcription-PCR (RT-PCR) assay was used in this study. Briefly, the total RNA was isolated from the placenta, using TriZol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Reverse transcription reactions were carried out using 2 to 3 μg of total mRNA in a 25 μl mixture, with the total RNA being first incubated with 0.5 μg of oligo (dT) primer (Promega, Madison, WI, USA) for 10 min at 65 °C and allowed to stand at room temperature for 2 min. Samples were then incubated with 1.25 mmol/L deoxynucleotide triphosphates (Promega, Madison, WI, USA), 10 units of RNAse inhibitor (Invitrogen, Carlsbad, CA, USA), and 16 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1 h at 42 °C in reverse transcriptase buffer. The cDNA obtained was subjected to PCR amplification using the following primers; for TNFα forward: 5′ TCT CTA ATC AGC CCT CTG GCC CAG G 3′, reverse: 5′ TAC AAC ATG GGC TAC AGG CTT GTC AC 3′, for IL-6 forward: 5′ GGA TGC TTC CAA TCT GGA TTC AAT GAG 3′, reverse: 5′ CGC AGA ATG AGA TGA GTT GTC ATG TCC 3′, for IL-8 forward: 5′ CGTGGCTCTCTTGGCAGCCTTCCTGAT 3′, reverse: 5′ TCAAAAACTTCTCCA-CAACCCTCTGCA 3′, for IL-13 forward: 5′ TGC AGT GCC ATC GAG AAG AC 3′, reverse: 5′ AGC ACA GGC TGA GGT CTA AGC 3′, for TGF-β forward: 5′ GTT GAG CCG TGG AGG GGA AA 3′, reverse: 5′ TGC CGC ACG CAG CAG TTC TT 3′, for IL-10 forward: 5′ GCA CCC ACT TCC CAG GCA ACC 3′, reverse: 5′ AAG GCA TTC TTC ACC TGC TCC ACG 3′ and cyclophilin A forward: 5′ GTC AAC CCC ACC GTG TTC TT 3′, reverse: 5′ CTG CTG TCT TTG GGA CCT TGT 3′. The amplification protocol included: 1 cycle at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 1 cycle at 72 °C for 10 min and 1 cycle at 4 °C for 10 min [23]. Experiments were conducted to confirm that all the PCR amplifications were in the linear range. The PCR products were visualized in 2% agarose gels with ethidium bromide staining. Results are expressed as band presence or absence.

2.5. Arginase activity

Arginase activity was measured by determining the amount of urea [27]. Briefly, placenta samples (500 mg) were homogenized, using a glass homogenizer in 1 mL of 0.1% Triton X-100 containing protease inhibitors. 40 μl of the lysate was added to Tris–HCl (25 mM, pH 7.5) containing $MnCl_2$ (5 mM) and incubated at 56 °C for 10 min to activate the enzyme. Arginine hydrolysis was initiated by the addition of 25 μl of 0.5 M L-arginine, pH 9.7, at 37 °C for 60 min. The reaction was stopped by adding a mixture of acids (1 H_2SO_4:3 H_3PO_4:7 H_2O), and the urea concentration was measured at 540 nm after the addition of 25 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95 °C for 45 min. The mixture was further cooled at room temperature in the dark for 10 min. The results are expressed as μg of urea × h^{-1} mg $protein^{-1}$. The measurements were carried out with a UV/vis 1603 Shimadzu Spectrophotometer.

2.6. Ornithine decarboxylase activity

Ornithine decarboxylase (ODC) activity was measured using L-[^{14}C]-ornithine. Briefly, placenta samples (500 mg) were homogenized, using a glass homogenizer in 1 mL of 50 mM Hepes buffer pH 7.4 containing 0.1 mM EDTA, 0.04% Triton X-100, 1 mM dithiothreitol, 0.5 mM pyridoxal-5′-phosphate and protease inhibitors (0.05 mM phenylmethanesulfonyl fluoride and 0.001 μg/μL leupeptin, 0.001 μg/μL aprotinin and 0.001 μg/μL NaF). Then, samples were centrifuged at 13,000 × *g* for 40 min at 4 °C and the supernatant was collected for determination of ODC enzymatic activity. ODC activity was assayed by measuring the release of $^{14}CO_2$ from

Table 1
Social and demographic characteristics of the study participants.

Variable	Control (*n*:36)	Rural spraying season (*n*:23)	Rural non-spraying season (*n*:23)
Educational level			
Greater	28.5	4.5	0
Secondary complete	51.4	22.7	22.7
Secondary incomplete	5.7	27.3	13.6
Primary complete	5.7	45.5	45.4
Illiterate/primary incomplete	8.6	0	18.2
Age (years)	24.69 ± 5.8	24.69 ± 5.8	23.68 ±4.9
Parity	0.97 ±1.08 (range 0–4)	1.32 ± 1.52 (range 0–5)	1.59 ±1.84 (range 0–6)
Body-mass index			
Normal	85.3	95.2	95.5
Smoking status			
Active	8.3	9.1	0
Pasive			
	11.1	27.3	36.4
Indoor pesticide use	0	17.3[*a]	17.3[*a]
Groundwater consumption	0	21.7[*b]	17.3[*b]
Neonate sex			
Female	42.9	63.6	52.4
Male	57.1	36.4	47.6
Gestational age (weeks)	38.95 ± 1.09	39.03 ± 1.16	39.65 ± 1.08
Neonate weight (g)	3269 ± 505.1	3453 ± 537.0	3438 ± 374.5
Placenta weight (g)	534.4 ± 89.3	532.6 ± 139.3	538.6 ± 111.9
Placenta weight/neonate weight ratio	0.167 ± 0.02	0.152 ± 0.02	0.157 ± 0.03

Data are presented as mean ± SD, or as percentages when correspond.
[*a] $p = 0.034$ by χ^2 test.
[*b] $p = 0.019$ by χ^2 test.

L-[^{14}C]-ornithine according to [28,29], with slight modifications. Standard reaction mixture consisted of homogenization buffer plus L-ornithine and L-[^{14}C]-ornithine (1 mM, 0.1–0.2 μCi) in a final volume of 50 μL. Enzyme reaction was initiated by the addition of 15 μL of samples. Blank solvent and sample controls were run in parallel, replacing sample with homogenization buffer and difluoromethylornithine (DFMO) in inactivated samples, respectively. The reaction was performed for 1 h at 37 °C in agitation and $^{14}CO_2$ was trapped on a 2 × 2 cm piece of filter paper soaked with 2 N KOH. The reaction was stopped by the addition of 50 μL of 0.25 N $HClO_4$ and maintained under the same conditions for 1 h. Filter papers were then transferred to scintillation vials and 0.5 mL 1% Triton X-100 were added along with 5 mL of scintillation liquid Optiphase "Hisafe". Radioactive CO_2 was then measured in a liquid scintillation counter (WallacWinspectral 1414).

2.7. Western blot of ODC, arginase I and arginase II

Placenta samples (500 mg) were homogenized on ice, using a glass homogenizer in 1 mL lysis buffer (1% Triton X-100, 0.5% sodium deoxicolate, 9% SDS, 5% DTT, 1 mM sodium ortovanadate, 10 μg PMSF, 30 μg aprotinin). Equal amounts of protein (80 μg) were diluted in SDS sample buffer, boiled at 100 °C for 3 min, and applied to precast 10% or 12% acrylamide Tris–glycine gels and run at 150 V for 1 h. Pre-stained protein molecular mass standards (Bio-Rad Laboratories) were run in parallel. Samples were transferred to nitrocellulose membrane (Bio-Rad Laboratories) at 100 V for 1 h. These membranes were probed using the rabbit polyclonal Abs: anti-ODC, anti-arginase I and anti-arginase II from human origin (Santa Cruz Biotechnology) at 1/400, 1/300 and 1/300 dilution, respectively. Incubations with specific Abs were followed by an anti-rabbit peroxidase conjugated at 1/10,000 (Sigma). The bands were detected by enhanced chemiluminescence. β-Actin protein was used as an internal standard.

2.8. Immunohistochemistry

Paraffin-embedded sections (4 μm thick) of placenta samples were mounted onto silane-coated slides, and then deparaffinized. Antigen retrieval was carried out by microwave oven (at 10% energy level for 15 min), and then endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in absolute methanol for 10 min. After washing with PBS (pH 7.4), the sections were incubated for 30 min at room temperature with 2% bovine serum albumin, in order to prevent non-specific binding. The sections were then incubated with anti-ODC (1/100), anti-arginase I (1/200) or anti-arginase II (1/200) polyclonal Abs (Santa Cruz Biotechnology, USA) for 1 h at room temperature. Slides were washed with PBS three times after incubation with primary antibody, and finally incubated for 30 min at room temperature with mouse/rabbit Polyscan™ HRP/DAB detection system (CELL MARQUE, Rocklin CA, USA). The immunostained sections were counterstained with Mayer's hematoxylin. Negative controls were prepared by replacing the primary antibody with appropriately diluted normal mouse or rabbit serum.

2.9. Statistical analysis

Categorical variables were compared using the Pearson's chi-squared test (χ^2). Comparison between groups was performed using one-way ANOVA followed by the *post hoc* Tukey's multiple comparison test. A value of $p < 0.05$ was considered to be significant. Correlation studies were performed on various parameters (Pearson's rank test and Spearman test for normally and abnormally distributed data, as appropriate).

3. Results

3.1. Socio-demographic characteristics

Table 1 provides the socio-demographic characteristics of pregnant women that participated in the study. Compared with pregnant women living in an urban area with no history of pesticide exposure (control group), the rural women were less educated (27.2% and 22.7% for spraying season (SS) and non-spraying season (NSS) groups, *vs.* 79.9% control group attained to a 12th grade or higher education level). Also, no women in the control group reported consuming ground well water, whereas a 17.3% (NSS) and 21.7% (SS) of the women living in rural areas did so. In addition, 17% of women living in rural areas reported using indoor pest control. Well water consumption and indoor pest control may indicate additional chemical exposure sources in the rural group. There was no significant difference in smoking status among groups. In addition, no significant changes in gestational age, newborn and placenta weight were observed among groups. The placenta weight to neonate weight (pw/nw) ratio, an indicator of placenta functional efficiency [30], also showed no significant changes among groups. The pw/nw ratio of all groups studied, were within the 5th and 95th centiles of published values at 37–42 weeks [31].

3.2. Blood cholinesterases and placenta carboxylesterase activities

The activity of OP pesticide exposure reference biomarkers, namely red blood cell acetylcholinesterase (RBC-AChE) and plasma

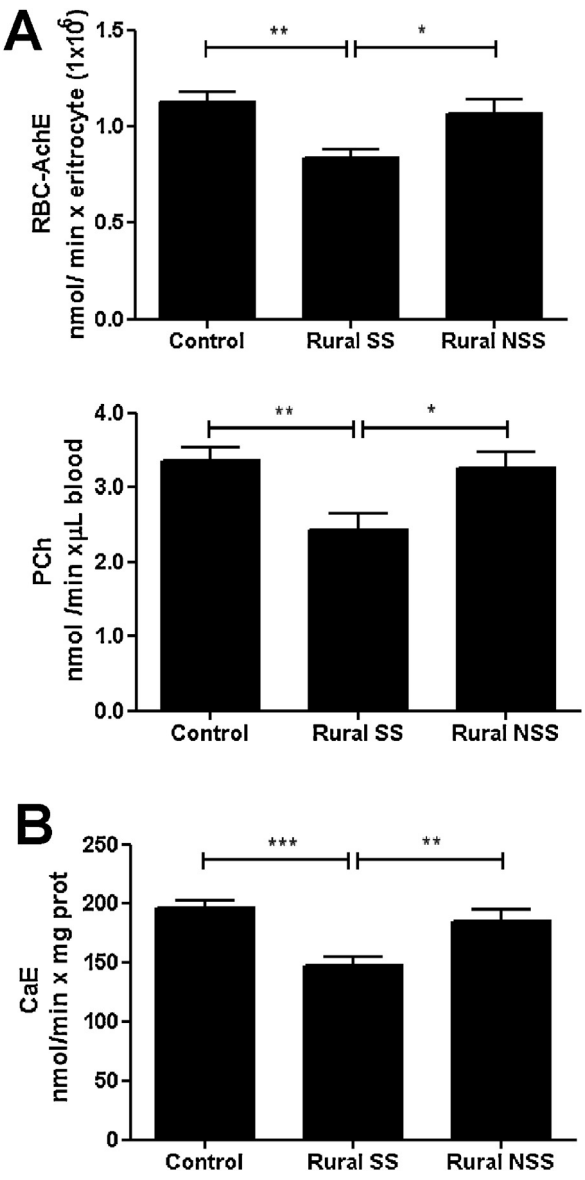

Fig. 1. Activity of the pesticide exposure biomarkers: red blood cell–acetylcholinesterase, plasma–cholinesterase and carboxylesterase. (A) Red blood cell acetylcholinesterase (RBC-AChE) activity, was assessed in the control, rural spraying season (SS) and rural non-spraying season (NSS) groups. RBC-AChE activity was normalized by red blood cell count and expressed as nmoles hydrolyzed substrate min^{-1} per million erythrocytes. Plasma cholinesterase (PCh) activity was assessed in the control, rural spraying season (SS) and rural non-spraying season (NSS) groups. PCh activity was expressed as nmoles hydrolyzed substrate min^{-1} per μL of whole blood. (B) Carboylesterase (CaE) activity, was assessed in the placentas from the control, rural spraying season (SS) and rural non-spraying season (NSS) groups. Results are expressed as nmol hydrolyzed substrate (α-naphthyl acetate)/min per mg protein. The graph shows the mean activity ± SEM, from three different experiments, with each sample processed as triplicates. $^{*}p \leq 0.05$, $^{**}p = 0.002$, $^{***}p = 0.0003$ one way ANOVA followed by Tukey's multiple comparison test.

cholinesterase (PCh) was analyzed. In addition, placental carboxylesterase (CaE) activity was also studied. Fig. 1A and B show the impact of environmental pesticide exposure in enzyme activity. Significant activity reductions were observed in RBC-AChE (14%), PCh (12.8%) and in placental CaE (12.5%) during SS relative to NSS. Also, significant decreases in all the enzyme activities studied were observed between SS and control group.

3.3. Detection of cytokine expression in the placentas

The mRNA expression of the pro-inflammatory cytokines TNFα, IL-6 and IL-8, as well as the anti-inflammatory cytokine IL-13 and the immunomodulatory cytokines IL-10 and TGFβ were analyzed by conventional RT-PCR in the placenta samples. Table 2 shows the cytokine mRNA expression frequency (expressed as %). The χ^2 test indicated that TNFα, TGFβ and IL-13 expressions, significantly changed between groups. Interestingly, IL-13 significantly increased only during SS in the rural group. In addition, the expression frequency of TNFα and TGFβ, increased in the rural group, but showed no differences between SS and NSS, corresponding to 87% and 78% for TNFα, 61% and 83% for TGFβ, respectively. Taking this result into consideration, the association between the expression of both cytokines was analyzed, χ^2 test indicated that TNFα and TGFβ co-expression is statistically significant ($p \leq 0.001$) (Table 3). In addition, cytokine seasonal variation was studied. Thus, mRNA expression frequency was analyzed in the control placentas collected during September–December ($n = 17$) and compared with the ones collected from April-August ($n = 19$), no significant changes were observed (data not shown).

3.4. Placental arginase activity and expression

It has been demonstrated that arginase expression may be modulated by cytokines, in particular type 2 cytokines as IL-13, as well as immunomodulatory cytokines such as IL-10 and TGFβ [32,33]. Taking into consideration that IL-13 and TGFβ expression frequency significantly augmented in the rural group, arginase activity was also explored in these placentas. Fig. 2A shows that arginase activity, significantly augmented (2.6-fold) in the placentas collected during SS compared to the ones collected during NSS. Enzyme activity was also up-regulated (3.2-fold) in the rural group during SS compared to the control group. The association between CaE and arginase activities was also analyzed. Table 4 shows a weak inverse association, which suggest that a decrease in CaE activity may be linked to an increase in arginase activity.

The Western blot analysis of arginase I/II expression demonstrated that arginase II protein content is augmented in rural placentas during SS compared to the ones collected during NSS and controls (Fig. 2B). Arginase I/II expanded western blots are available as supplemental file (Supp. Fig. 1A and B).

3.5. Placental ornithine decarboxilase activity and expression

The extrahepatic isoform of arginase, arginase II, degrades L-arginine into L-ornithine and urea. Ornithine is then, converted to putrescine by ornithine decarboxilase (ODC). Similar to arginase, it has been reported that the expression of ODC, may be regulated by IL-13 [34]. Therefore, ODC activity and expression were analyzed in the placentas from rural and control groups. Similarly to arginase, ODC activity increased in the rural placentas collected during SS compared to the ones collected during NSS (7.8-fold) and to the control group (6.4-fold) (Fig. 3A). The associations among CaE, ODC and arginase activities were also analyzed. Table 4 shows a weak inverse association between CaE and ODC activities, while a positive stronger association was observed between ODC and arginase.

The western blot analysis showed that ODC protein expression is induced in the placentas collected during SS compared to NSS and controls (Fig. 3B). ODC expanded western blots are available as supplemental file (Supp. Fig. 2).

3.6. Tissue distribution of arginase and ornithine decarboxilase

Immunostaining for arginase I/II and ODC were performed in order to evaluate tissue localization. No changes in arginase I tissue

Table 2
Cytokine expression frequency in term placentas.

	IL-6	TNFα	IL-8	IL-10	IL-13	TGFβ
Control group	61% 22 positive 14 negative	58% 21 positive 15 negative	22% 8 positive 28 negative	44% 16 positive 20 negative	0% 0 positive 36 negative	36% 13 positive 23 negative
Rural SS	52% 12 positive 11 negative	87%* 20 positive 3 negative	13% 3 positive 20 negative	35% 8 positive 15 negative	32%*** 7 positive 15 negative	61%** 14 positive 9 negative
Rural NSS	57% 13 positive 10 negative	78%* 18 positive 5 negative	4% 1 positive 22 negative	65% 15 positive 8 negative	4% 1 positive 22 negative	83%** 19 positive 4 negative

* $p = 0.04$ by χ^2 test.
** $p = 0.001$ by χ^2 test.
*** $p = 0.0002$ by χ^2 test.

distribution was observed among control, rural SS and NSS groups (Fig. 4). In contrast, arginase II staining was found in the syncytiotrophoblast in control, and rural NSS groups, whereas a localized intense staining was observed in the syncytiotrophoblast and in endothelial cells in rural SS group (Fig. 4). Referred to ODC, a homogeneous staining was found in the syncytiotrophoblast in the control and rural NSS groups, resembling the tissue distribution found for arginase II (Fig. 4). No signal was found in negative controls (data not shown). These results were consistent with western blot analysis of placenta homogenates, that showed an increased expression of arginase II and ODC in rural SS group (Figs. 2 and 3).

4. Discussion

Organophosphate and carbamate pesticides are designed to inhibit AChE. Many enzymes used for the detection of pesticides are inhibited by the pesticide and the extent of inhibition correlates with the concentration of the analyte [35]. Through the exposure biomarkers studies, RBC-AChE and PCh, our results emphasize that pregnant women, living in close proximity to crop fields, are actually exposed to OP pesticides in the North Patagonia rural area, as previously reported [17,36]. In addition to the systemic impact of OP pesticides in pregnant women, we have also demonstrated the local impact, since CaE activity is decreased in the placentas of rural women during SS, compared to NSS and to the control group. CaEs are considered sensitive indicators of environmental exposure to OP and carbamate pesticides in several systems [17,37].

In order to determine whether environmental pesticide exposure may impair cytokine balance at the placenta, we analyzed the pro-inflammatory cytokines IL-8, IL-6, TNFα, and the anti-inflammatory IL-10, TGFβ and IL-13 mRNA expression. These cytokines were chosen because they have been previously found to be induced in trophoblast or placental explant cultures by *in vitro* xenobiotic exposure [23,38,39] or because they are known to be involved in the control of trophoblast life/death balance [40]. Among the anti-inflammatory cytokines studied the most remarkable result is the significant increase in the expression frequency of IL-13 in the rural population during SS. It has been reported that IL-13 mRNA can be detected in trophoblast of first trimester human placentas [41], while in 28 weeks' gestation and term placentas no expression is immunohistochemically detected [42]. As shown in Table 2, IL-13 mRNA was expressed in term placentas in the rural population during SS (32%) and in only one placenta during NSS (4%), while no expression was detected in the control group. It has been proposed that cytokine imbalance at the materno-fetal interface, particularly of Th2 cytokines, would possible promote a skewed atopy-predisposing immune response in the fetus [42]. Moreover, in a prospective birth cohort study in California, having a mother working in agriculture was found to be associated with increased levels of Th2 cytokines in children at 2 years age [43]. Nevertheless, since in this work cytokine protein content was not determined, further studies are needed in order to confirm cytokine mRNA translation, and to evaluate whether IL-13 increase in placentas from OP pesticide exposed women may be related to asthma or atopy-predisposing immune response in the children.

Our results also demonstrate that placentas from the rural population show increased expression frequency of TNFα, during both NSS and SS. Despite the well-known limitations of *in vitro* models, it is worth noting the similarity of chlorpyrifos induced effects in JAR [38] and JEG-3 trophoblast cell lines [23]. Indeed, chlorpyrifos and phosmet favored TNFα and IL-13 mRNA expression [23]. Interestingly, both OP are widely used in North Patagonian crops [24,44]. Regarding placental cytokine production, different cell types are able to produce TNFα, such as trophoblasts, Hofbauer, epithelial and decidual cells [45]. TNFα has been widely studied in reference to many pregnancy complications [46], since it provokes a variety of biological effects on placental and endometrial cell types such as cell migration, hormone production and apoptosis [40]. Nevertheless, the small sample size and the number of missing data concerning pregnancy alterations, limit the present study's potential for detecting significant associations between TNFα expression and pregnancy complications.

It is also interesting to note that TGFβ and TNFα expression followed the same behavior, since both expression frequencies increased in rural SS and NSS groups. It has been reported that TGFβ1/2 levels in the chorionic villi of normal placenta were variable depending on the pregnancy trimester. Specifically, the expression is very low or absent in third trimester normal placenta [47,48]. Since TGFβ and TNFα are positively modulated in the rural group regardless of whether pesticides are being applied or not, these prompt us to consider that a common factor present in SS and NSS may affect interleukin expression. In this respect,

Table 3
Co-expression of TNFα and TGFβ in all samples.

	TNFα (+)	TNFα (−)
TGFβ (+)	40**	6
TGFβ (−)	19	17

** $p \leq 0.001$, by Fisher's exact test.

Table 4
Correlation coefficients between carboxylesterase, arginase and ornithine decarboxylase activities.

	CaE	Arginase
CaE	–	0.16 (β −0.3172)*
ODC	0.13 (β −0.0009)*	0.45 (β 0.002)***

* $p \leq 0.05$, by linear regression test.
*** $p \leq 0.0001$, by linear regression test.

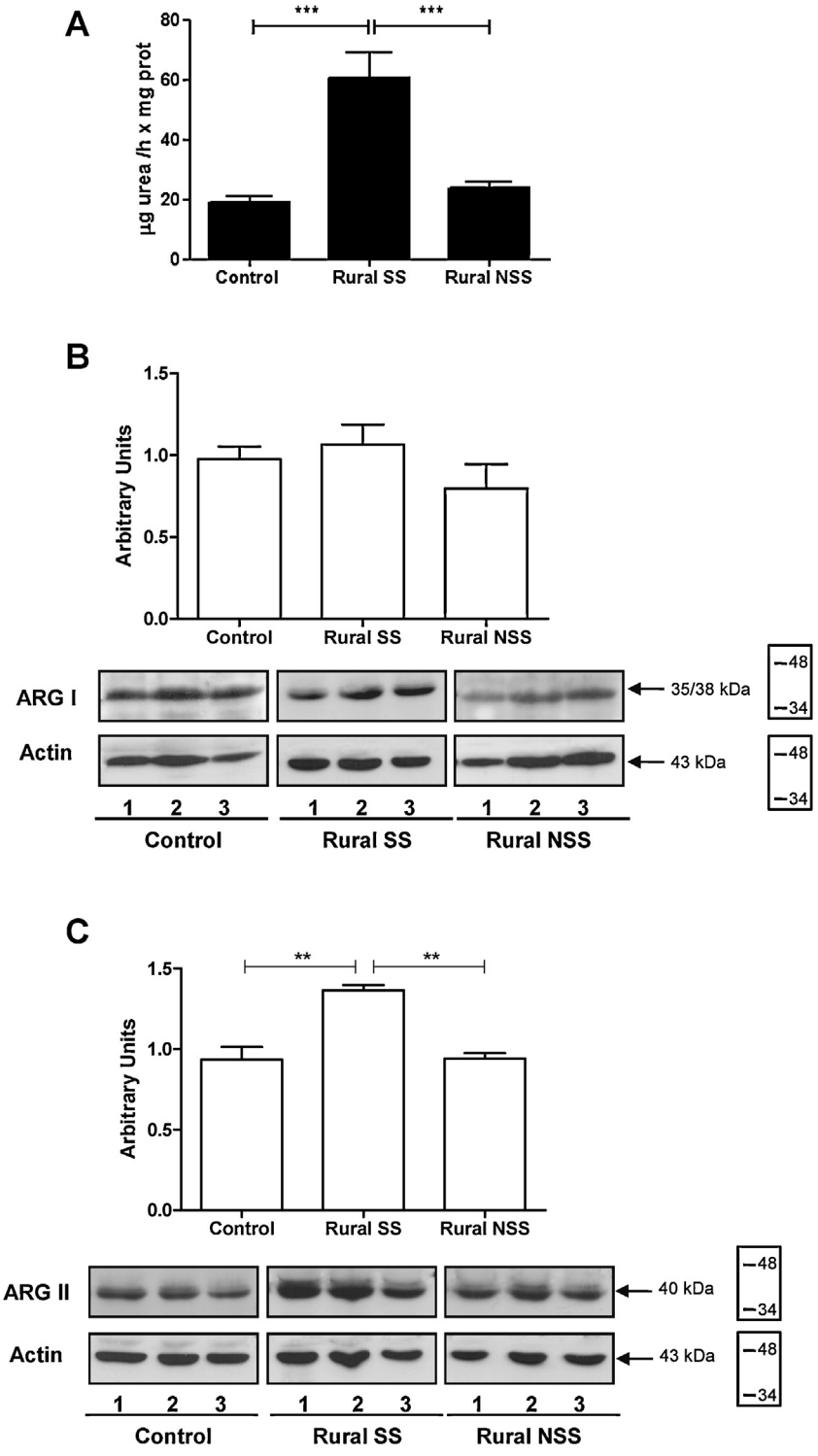

Fig. 2. Activity and expression of arginase isoforms. (A) Arginase activity was assayed in placenta lysates obtained from control, rural spraying season (SS) and rural non-spraying season (NSS) groups. Lysates were incubated with L-arginine, and urea production was measured. Results are expressed as µg of urea produced in 1 h considering the total protein content in the lysate (mg). The graph shows the mean activity ± SEM from 3 independent experiments, with each sample processed as triplicate. $^{***}p = 0.0001$, one way ANOVA followed by tukey's multiple comparison test. 2B, 2C, the expression of arginase I and II isoforms in placenta lysates was evaluated by western blot. Equal amounts (80 µg) of protein obtained from control, rural spraying season (SS) and rural non-spraying season (NSS) groups were tested. Bands are representative of 2 independent experiments. Band relative intensity was estimated by standardization with densitometry analysis (Gel Pro Analyzer 3.2 program). β-Actin was used as a loading control. Right panels show the molecular weight markers (kDa). $^{*}p \leq 0.05$, $^{**}p = 0.008$, one way ANOVA followed by Tukey's multiple comparison test.

clearly the feature shared by the rural population is the place they are living (next to farms), also some habits, such as consuming well water and using domestic pest control. Both features may increase the risk of pesticide exposure. Related to this, in this geographic area it has been demonstrated that pesticide residues (mainly OP) are detected in the water during NSS, and that the leaching process represents a continuous and slow supply of pesticide residues to the groundwater [24]. Then, location and habits may be involved in maintaining continue low-level pesticide exposure during NSS, which increases during SS, as demonstrated by

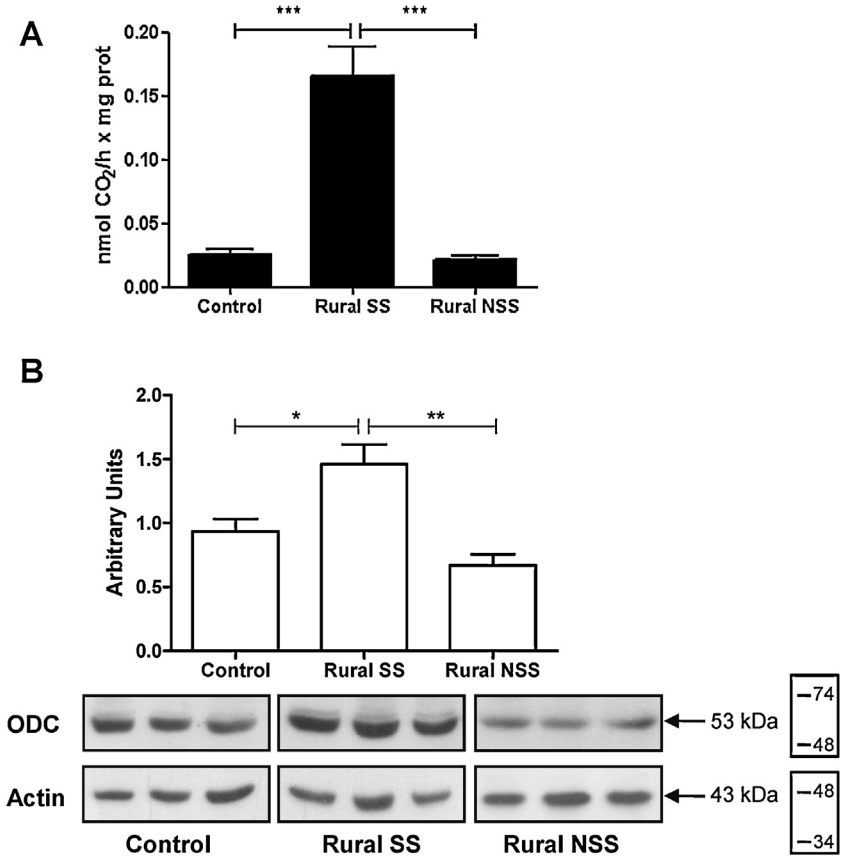

Fig. 3. Activity and expression of ornithine decarboxylase. 3A, ornithine decarboxylase (ODC) activity was assayed in placenta lysates obtained from control, rural spraying season (SS) and rural non-spraying season (NSS) groups. Values are expressed as nmol of CO_2 released per hour considering the total protein content in the lysate (mg). The graph shows the mean activity ± SEM from 3 independent experiments, with each sample processed as triplicate. $^{***}p = 0.0001$, one way ANOVA followed by Tukey's multiple comparison test. 3B, the expression of ODC in placenta lysates was evaluated by western blot. Equal amounts (80 μg) of protein obtained from control, rural spraying season (SS) and rural non-spraying season (NSS) groups were tested. Bands are representative of 2 independent experiments. Band relative intensity was estimated by standardization with densitometry analysis (Gel Pro Analyzer 3.2 program). β-Actin was used as a loading control. Right panels show the molecular weight markers (kDa). $^{*}p \leq 0.05$, $^{**}p = 0.008$, one way ANOVA followed by Tukey's multiple comparison test.

the reference biomarkers. Our results also demonstrated that independent of the groups studied when the placenta expressed TNFα mRNA, also expressed TGFβ mRNA (Table 3). In particular, there is evidence demonstrating that TNFα influences the expression of TGFβ-1 [49,50]. In this regard, it has been recognized that the anti-inflammatory cytokine TGFβ is involved in the homeostatic regulation of pro-inflammatory cytokines such as TNFα [51].

It has been suggested that an exquisite cytokine regulatory feedback mechanism between the fetal immune cells and the placental unit exists. This may indicate an early role for the placenta in mounting and maintaining the correct cytokine environment in which the developing conceptus can mature [42]. Moreover, enzyme expression may be influenced by cytokine balance [33]. Particularly Th2 (IL-4, IL-13) and immunoregulatory (IL-10, TGFβ) cytokines have been linked to arginase and ODC induction [34,52]. Mammalian arginases catalyze the hydrolysis of arginine to ornithine and urea and are composed of two distinct isozymes: arginase I and arginase II. Ishikawa et al. [58] have reported that human placenta expresses both isozymes, and that enzyme activity follows the expression grade of arginase II. We found a significant increment in arginase activity and arginase II expression, in the placentas of women exposed to OP pesticides. It has been reported that arginase II expression increases in preeclampitc placenta, however the mechanism responsible for this up-regulation remains to be elucidated [53]. Recently it has been demonstrated that arginase activity and arginase II expression are induced by hypoxia in human umbilical vein endothelial cells [54]. In this regard, it has been suggested that an increased arginase II activity can deplete L-arginine as a substrate, which can result in the decreased synthesis of nitric oxide (NO) [55] and consequently prevent the damage associated with excessive NO production [56]. Moreover, arginases may have a role in placental vessels counteracting the nitric oxide synthase-dependent relaxation, which is differentially regulated in placental artery and vein endothelial cells [57]. Interestingly, by immunostaining we demonstrate an increased expression of arginase II not only in syncytiotrophoblast but also in the endothelial cells.

Also arginase activity has been postulated as a limiting or regulatory factor for polyamine production [58]. *Via* L-ornithine production arginase can promote the synthesis of polyamines, such as spermine, spermidine and putrescine which are involved in cell growth, proliferation, and wound healing [59,60]. The first enzyme in the polyamine biosynthetic pathway is ODC, which catalyzes the transformation of L-ornithine into putrescine. It is a highly inducible, cytosolic enzyme which responds to a range of trophic stimuli [61]. In this sense, ODC gene promoter region contains multiple sequences that allow response to hormones, growth factors, and tumor promoters [62]. Moreover, it has been reported that ODC expression may be regulated by IL-13 [34]. Results presented here, showed that ODC activity was significantly associated with arginase and CaE activities, suggesting that a repair process may be induced after OP exposure. Reinforcing this idea, Vera et al., by

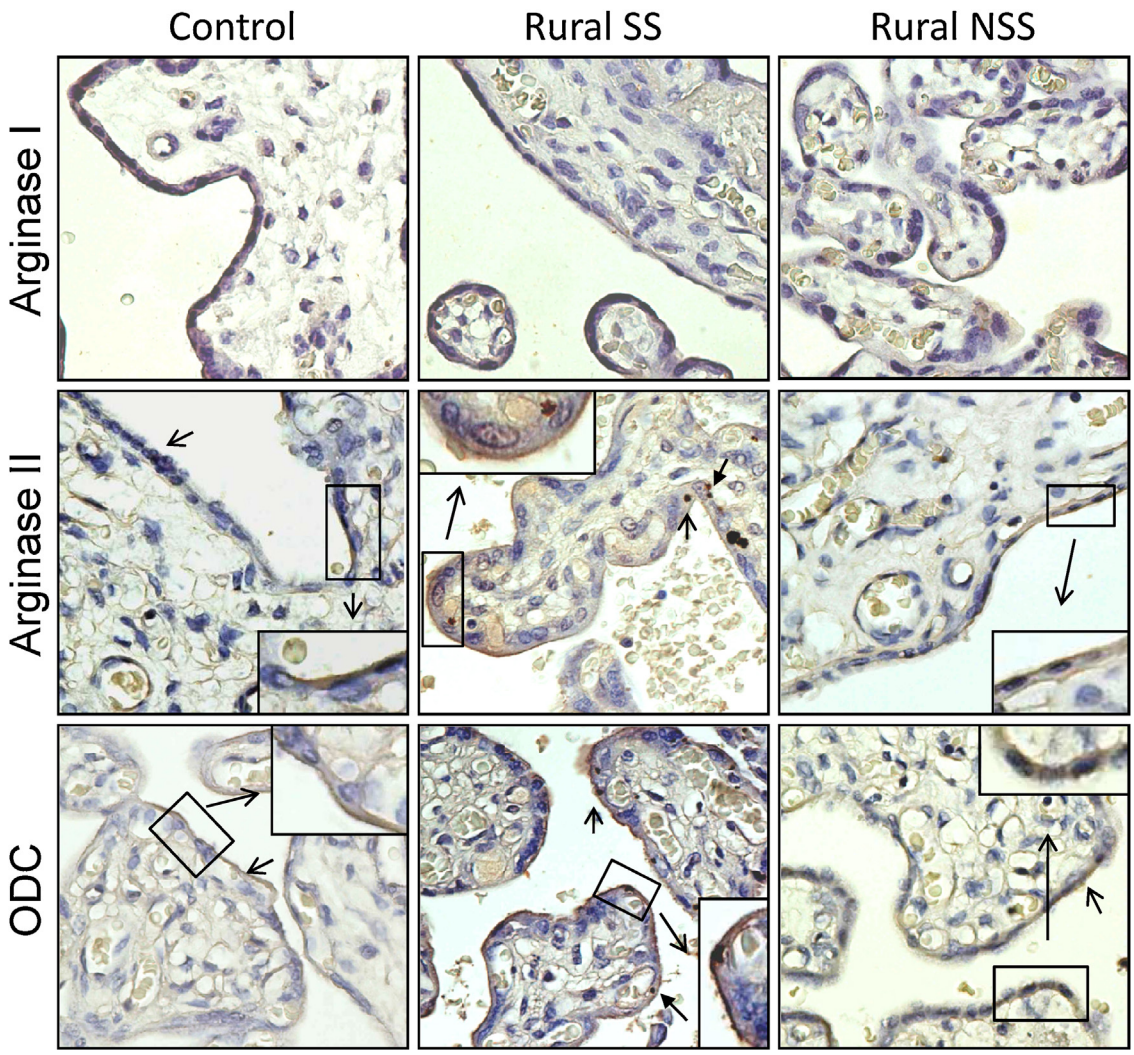

Fig. 4. Immunohistochemical staining of arginase-I, arginase-II and ornithine decarboxylase. Staining was performed on placenta sections from control, rural spraying season and rural non-spraying season groups. Stainings are representative of 2 independent experiments (magnification 600×). Panel, indicates positive staining in the syncytiotrophoblast layer; open arrow, indicates syncytiotrophoblast and closed arrow, endothelium.

studying placentas from pregnant women exposed to OP found that the cytotrophoblast nuclear lipid profile exhibited changes possibly associated to a regenerative process [17]. Moreover, it has been recently demonstrated that OP insecticides are capable of inducing ODC activity and altering polyamine metabolism during amphibian embryogenesis [29].

5. Conclusion

This study demonstrates the impact of OP pesticides in the placenta, concomitant with the alteration of both pro-inflammatory and anti-inflammatory cytokines, and the induction of enzymes relevant for cell proliferation and tissue repair. Furthermore the increase in IL-13 expression, arginase and ODC activities, may create an ideal environment to dampen excessive tissue damage and stimulate tissue repair. In this sense, arginase and ODC up-regulation may be associated with cytotrophoblast proliferation and placenta repair as a compensatory mechanism in response to OP induced injury. In line with this hypothesis, we found no significant changes in placenta efficiency among groups. Together, our data suggest that the replacement of injured cells by functional cells predominated over fibrosis. However, the initial tissue injury inflicted by OP exposure, which eventually triggers this reparative response in the placenta, remains to be established. Follow-up studies of this newborn cohort will determine the clinical significance of these findings in the immune response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2013.03.010.

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