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Prenatal exposure to pesticides: analysis of human placental acetylcholinesterase, glutathione S-transferase and catalase as biomarkers of effect

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Abstract

Pre- and perinatal exposure to pesticides is deleterious on foetal and neonatal development, but information regarding possible effects on environmental low-dose exposure to pesticides is scarce. Most epidemiological studies of the health effect of pesticides have been based on self-reported information. However, detailed information on past pesticide use is difficult to reconstruct. This is a current study conducted among pregnant mothers attending a delivery care and perinatal programme at a public hospital. The study investigates biomarkers of early effects in placentas from women living in an area with an intensive use of pesticides in the northern part of Patagonia, province of Río Negro, Argentina, and it assesses the consistency of the information provided by self-reports. The study confirms that placental acetylcholinesterase and catalase activities are significantly associated with periods of organophosphorus pesticides application, while glutathione S-transferase is not affected. We found a positive correlation between environmental exposure to organophosphorus pesticides and carbamate insecticides and newborn head circumference. The findings provide a further indication of a link between placenta acetylcholinesterase and catalase activity and prenatal exposure to pesticides in population studies. Both placenta enzymes may be used as biomarkers in health surveillance programmes for early diagnosis of exposure related alterations produced by organophosphorus pesticides and carbamate pesticides.

Keywords: *Placenta biomarkers, organophosphorous pesticides, acetylcholinesterase, glutathione S-transferase, catalase, intrauterine exposure*

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Introduction

Chemical contaminants, including metals, organic compounds and radionuclides, occur in the environment as the result of human activities, and they represent a threat to the health and well-being of ecosystems (Mansour 2004). Pesticides are a vast and important class of these chemicals, which are intentionally released in the

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environment due to their application in agriculture (Pope 1999). Recently, several studies have demonstrated widespread pesticide exposures in different populations (Hura et al. 1999, Berkowitz et al. 2004, Mansour 2004) with particular emphasis on farm residents (Fenske et al. 2000, Cooper et al. 2004, Quandt et al. 2004).

Some classes of pesticides, the chlorinated (OC) ones, belong to a family of persistent lipophilic compounds detected all over the world in water, sediments, soil and air, and accumulate in the ecosystem through the food web (Iwata et al. 1993, Lagueux et al. 1999). Other classes of pesticides producing concern are the organophosphorus (OP) and carbamates pesticides. These compounds are rapidly hydrolysed in the environment, but their potentiality to produce neurotoxicity has been recently reviewed (Costa 1998, Eskenazi et al. 1999, Gil & Pla 2001, Kamel & Hoppin 2004). However, information regarding the effects on pregnant women produced by chronic low-dose environmental exposure is scarce. The studies combining epidemiological dates with molecular biomarkers are even fewer (Perera et al. 2003, Eskenazi et al. 2004).

There is an increasing consensus that young children and foetus are probably more sensitive than adults to the toxic effects of certain pesticides (Perera et al. 2004). There is accumulating evidence indicating that the carryover of pesticides to foetus through the human placenta takes place (Benjaminov et al. 1992, Abu-Qare & Abou-Donia 2001, Gladen et al. 2003, Falcón et al. 2004, Whyatt et al. 2004). OC compounds and metabolites have been detected in fat and body fluids including newborn, umbilical cord blood, placenta, foetal subcutaneous fat tissues and organs (Martínez Montero et al. 1993, Waliszewski et al. 2000). In addition, morphological changes of term placenta tertiary villi have been reported in women living in agricultural area and exposed to parathion, an OP (Levario Carrillo et al. 2001).

An optimal maternal-foetal exchange is necessary for a successful pregnancy. The placenta plays a major role in the embryo's nutrition and growth, in the regulation of the endocrine functions and in drug biotransformation (Rama Sastry 1999, Haggarty et al. 2002, Gude et al. 2004). Exchange involves not only physiological constituents, but also substances that represent a pathological risk for the foetus. The understanding of what xenobiotics do to the placenta and what the placenta does to the xenobiotics should provide the basis for the use of placenta as a tool to investigate and predict some aspects of developmental toxicity (Myllynen et al. 2005). Moreover, pathological conditions in the placenta are important causes of intrauterine or perinatal death, congenital anomalies, intrauterine growth retardation, maternal death, and a great deal of morbidity for both, mother and child (Guillette et al. 1998, Levario Carrillo et al. 2001).

The present study investigates biomarkers of pesticide exposure in placenta samples from pregnant women living in an area of agricultural exploitation with intensive pesticide application. Approximately 1.500 Tn were applied annually between 1995 and 1998 in the agricultural area known as the upper Valley of the Negro and Neuquén rivers (Loewy et al. 2003) where the community under study is located. We evaluated the association between prenatal exposure to these compounds and placental acetylcholinesterase (AChE), glutathione S-transferase (GST) and catalase (CAT) activities, as well as foetal growth.

Materials and methods

Participants and data collection

A study was conducted on a cohort of 200 women (their newborns and placentas) entering prenatal care at Cinco Saltos Public Hospital, Province of Río Negro, Argentina. This hospital, which is located in the region considered to be the major producer of pears and apples in the country, serves a low-income population, many of whom live in farms. Insecticides, mainly OP such as azinphos methyl, phosmet, chlorpyrifos and dimetoate, are applied aerially with farm tractors in the area, at least four to five times in October–February. They are usually finely dispersed as droplets or particles at the time of application and the aerial drift from the target area is frequent, increasing exposure to public and environment.

The most frequently used pesticide in the area was azinphos methyl and its presence was reported in 66% of the groundwater samples analysed (Loewy et al. 2003), suggesting that a large proportion of resident people, including pregnant women, may have been continuously exposed to low doses of these pesticides. This project was subject to review in accordance with institutional regulations.

All women entering the programme between June 2002 and June 2003 were asked to participate and consent was obtained from each participant before they were interviewed. Participants were selected as potentially exposed to pesticides because they were all living in the agricultural area. A questionnaire was administered to document physical characteristics, reproductive history, educational level, lifestyle habits (diet, smoking), and urban or farm residence.

Women with serious chronic diseases (arterial hypertension, gestational diabetes, thyroid disease), medication and those with serious pregnancy complications affecting foetal development were excluded. Information about pregnancy complications and the status of the newborn at birth was collected from medical records (weight, height, head circumference, gestational age and placental weight).

Biochemical determinations

At term, placenta tissue was used for determination of AChE, GST and CAT activities.

Placentas delivered vaginally were collected immediately after expulsion, weighted and frozen at -20°C until the time of analysis. Small pieces of the tissue were cut from the mother site's side. Biopsies were taken at the same sampling location in all placentas analysed. They were repeatedly washed with physiological solution and homogenized in ice-cold buffer according to each enzyme procedure. Then homogenates were filtered through a muslin cloth.

Total AChE activity was determined at 30°C following the method of Ellman et al. (1960), using acetylthiocholine as the substrate. AChE activity is expressed as μmoles product developed min^{-1} mg^{-1} proteins.

Total GST activity was measured at 22°C after the homogenate centrifugation at 20 000*g* during 40 min, according to Habig et al. (1974). 1-Chloro-2,4-dinitrobenzene (CDNB) was used as a substrate. GST activity was expressed in μmoles product developed min^{-1} mg^{-1} proteins.

CAT activity was determined on a subgroup of samples at 25°C as described by Beers and Sizer (1952). Specific activity was expressed in mmoles substrate hydrolysed min^{-1} mg^{-1} proteins.

Maternal blood samples were obtained during the second trimester by venipuncture. They were collected into heparin tubes and refrigerated at 4°C until analysis. Red blood cell (RBC) and plasma cholinesterase activities were measured at 30°C following the method of Voss and Schasse (1970). To increase reliability, a blank was added to every subject's own blood and each sample was determined in duplicate. RBC cholinesterase activity was normalized by RBC count and expressed as nmoles hydrolysed substrate $\times min^{-1} \times$ millions of RBC. Plasma cholinesterase activity was expressed as nmoles hydrolysed substrate $\times min^{-1} \times \mu l^{-1}$ whole blood.

Protein concentration was determined according to Lowry et al. (1951).

Data analysis

Results are expressed as mean ± standard deviation. Statistical significance between means was determined by analysis of variance (ANOVA), followed by a *t*-test.

Pearson correlation models were used to test the association between placental biomarkers and newborn weight, head circumference and body mass index, and placenta weight.

Results

The demographic characteristics of the group under study are presented in Table I. The women averaged 25 years of age; 53.5% had primary education, 41.2% had a high school degree and 4.9% had attended tertiary level. A total of 64.9% were residing in the urban area. Almost all the women had a normal body index; only 10% reported alcohol consumption (less than two alcoholic beverages/day); and 20% had smoked during pregnancy. No one had record drug consumption. Approximately 14%

Table I. Mean demographic characteristic of the study group ($n=200$).

Mean maternal age (years)	24.78
Maternal education (%):	
Less than a high school degree	53.5
High school degree	41.2
Greater than a high school degree	4.9
Maternal residence(%):	
Urban	64.9
Rural	35.1
Maternal body mass index (%):	
Normal	94
Abnormal	6
Environmental tobacco smoke (%):	
Reporting a smoker	20.5
Passive smoke exposure	60.5
Alcohol consumption (%)	10.3
Ground water consumption (%)	14.5
Self-reported indoors pesticides use (%)	47.5

of the women consumed ground water and about 50% of the group reported the domestic use of pesticides. The number of previous pregnancies varied from zero to nine with a mean of 1.79.

The alterations registered in the normal course of pregnancy were 19 threats of premature birth (9.5%), ten threats of spontaneous abortion (5%) and five premature births (2.5%). Ten newborns with low weight birth (<2.5 kg) were reported, four of them presented IUGR (2%) and only one presented general anomaly (0.5%). Pregnancy complications observed in this study are within standard values (Willis et al. 1993).

The morphometric characteristic of the neonates and placentas are presented in Table II. The mean neonate's characteristics were length 48.66 cm, weight 3.373 g, head circumference 34.85 cm and body mass index 1.42 g cm^{-2}. The mean placental weight was 613.14 g.

When the neonates' parameters were analysed considering the women's place of residence and indoor use of pesticides, statistical differences were observed. Rural newborn head circumference was larger (35.16 ± 1.55 cm, $n=75$) than urban neonates' (34.65 ± 1.67 cm, $n=109$; ANOVA: $p<0.05$). A similar effect was observed in association with indoor pesticides use (yes: 35.08 ± 1.68 cm, $n=75$ versus no: 34.51 ± 1.55 cm, $n=79$; ANOVA: $p<0.03$).

Additionally, head circumference was associated with periods of pesticides applications. When the first trimester of pregnancy coincided with the pesticides application period, an increase in the head circumference was also detected (35.15 ± 1.51 cm, $n=102$).

Another parameter affected by maternal residence was placental weight. Rural mothers' placentas were heavier (656.34 ± 141.85 g, $n=52$) than urban mothers' placentas (587.07 ± 125.14 g, $n=99$; ANOVA: $p<0.002$).

Newborn length and weight were not associated with pesticide use or exposure.

Placenta AChE activity was not associated with women's demographic characteristics (rural or urban residence, habits or ground water consumption), newborns' parameters (weight, height, head circumference, gestational age, body index), placental weight and difficulties in pregnancy. However, a clear temporal trend was detected. Comparing the average AChE activity obtained in placentas collected during the non-application period of pesticides (March–October) with placentas collected during the application season (November–February), an increased AChE activity was observed in the last period (Figure 1). During this last period, maternal plasma cholinesterase was 18% reduced ($p<0.01$) indicating that at least a minimum amount of pesticides was absorbed by the mother. No significant changes were detected in RBC cholinesterase (Table III).

Placental GST activity was not statistically associated with rural or urban residence, ground water consumption, difficulties in pregnancy or pesticides application periods (Figure 2).

Table II. Morphometric information (mean $\pm$ standard error) for newborns ($n=194$) and placentas ($n=156$).

Newborns, height (cm)	48.66 ± 23
Newborns, weight (g)	3373.73 ± 533
Head circumference (cm)	34.85 ± 1.62
Body mass index (g cm^{-2})	1.42 ± 0.15
Placenta weight (g)	613.14 ± 134

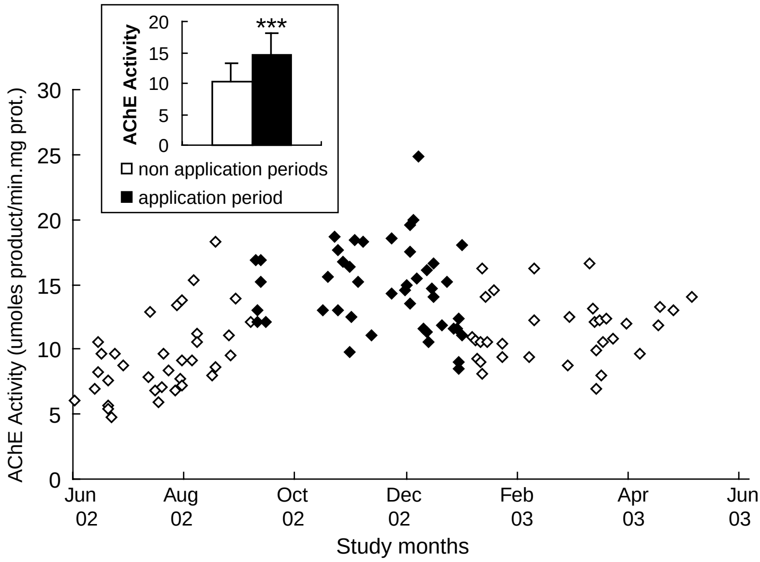

Figure 1. Seasonal variation of acetylcholinesterase activity in human placenta. Mean activity during non-pesticides applications period (white diamond) was 10.39 ± 2.93 (valid $n=68$), while mean activity during pesticides applications period (black diamond) was 14.66 ± 3.31 (valid $n=45$). Activity is expressed as µmoles product developed min^{-1} mg^{-1} proteins. ANOVA: $^{***}p<0.0001$.

CAT, like AChE, showed temporal activity patterns. Its average activity was lower in placentas collected during the non-application period (Figure 3, insert). No statistical association of CAT related to women's residence or lifestyle habits was observed. A correlation analysis revealed a statistically significant relationship between AChE and CAT activity (Figure 3).

In general, no significant correlation was obtained between the enzymes measured and the newborn body mass index and placenta weight except for GST. Figure 4A presents a moderate statistically significant inverse relationship between GST activity and newborn body mass index. Figure 4B shows an inverse relationship between GST activity and placental weight. A positive correlation was obtained between CAT activity and head circumference (Figure 5).

Table III. Plasma and red blood cell cholinesterase activities in pregnant women in relation to pesticide spraying.

	Plasma cholinesterase, mean ± SD	Red blood cell cholinesterase, mean ± SD	*n*
Non-spraying season	2.74 ± 0.84	1.14 ± 0.33	32
Spraying season	2.25 ± 0.55*	1.27 ± 0.36	33

Red cell cholinesterase activity was normalized by red blood cell count and expressed as nmoles hydrolysed substrate $min^{-1} \times$ millions red blood cells. Plasma cholinesterase activity was expressed as nmoles hydrolysed substrate $\times min^{-1}$ μl^{-1} whole blood.
*Significant differences at $p<0.01$.

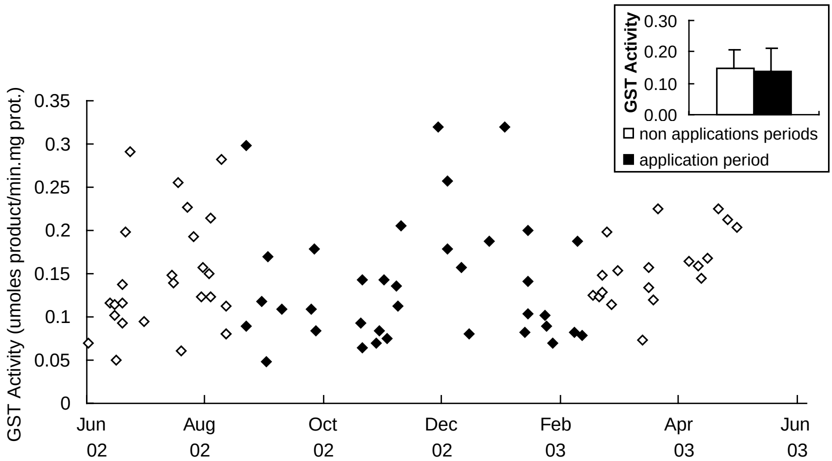

Figure 2. Seasonal variation of glutathione S-transferase activity in human placenta. Mean activity during non-pesticides applications period (white diamond) was 0.149 ± 0.05 (valid $n = 45$) while mean activity during pesticides applications period (black diamond) was 0.138 ± 0.07 (valid $n = 37$). Activity is expressed as µmoles product developed min^{-1} mg^{-1} of proteins. ANOVA: n.s.

Discussion

Pre- and perinatal exposure to pesticides is considered to be deleterious for foetal and neonatal development. Windows of vulnerability exist during foetal development when small exposures to xenobiotics may produce profound effects in adults (Jacobson & Jacobson 1996). Preconception, intrauterine and postnatal periods have been associated with childhood carcinogenesis related to pesticides exposure (Flower et al. 2004).

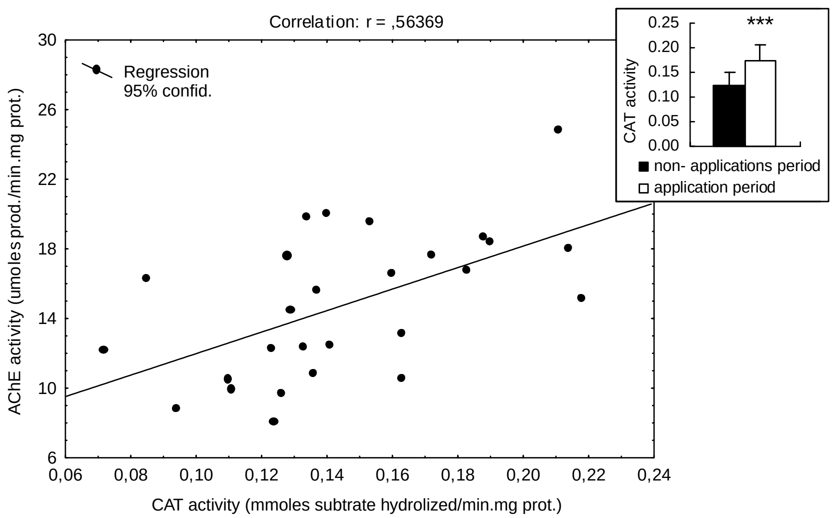

Figure 3. Relationship between acetylcholinesterase and catalase activities in human placenta. A positive correlation between acetylcholinesterase and catalase activity was shown ($p < 0.05$) (A). An increased CAT activity was observed on placentas collected during pesticides application period (B). ANOVA: $^{***}p < 0.0001$. Acetylcholinesterase activity is expressed as µmoles product developed min^{-1} mg^{-1} proteins; CAT activity is expressed on mmoles substrate hydrolysed min^{-1} mg^{-1} proteins.

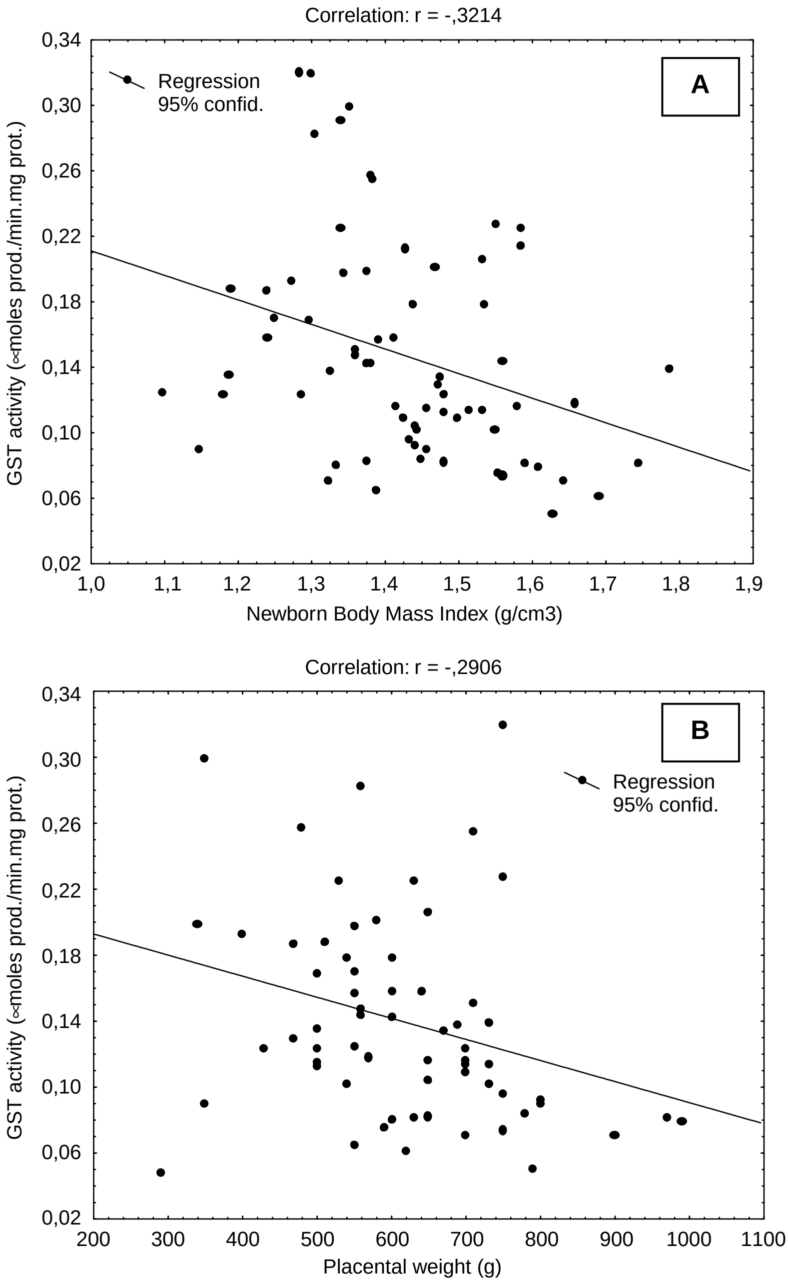

Figure 4. Relationship among glutathione S-transferase activity in human placenta and newborn parameters. A statistically significant inverse relationship between glutathione S-transferase activity and newborns body mass index (A) was observed ($p < 0.05$). Similarly, a significant inverse relationship was observed between glutathione S-transferase activity and placental weight (B) ($p < 0.05$). Enzymatic activity is expressed as μmoles product developed min^{-1} mg^{-1} proteins.

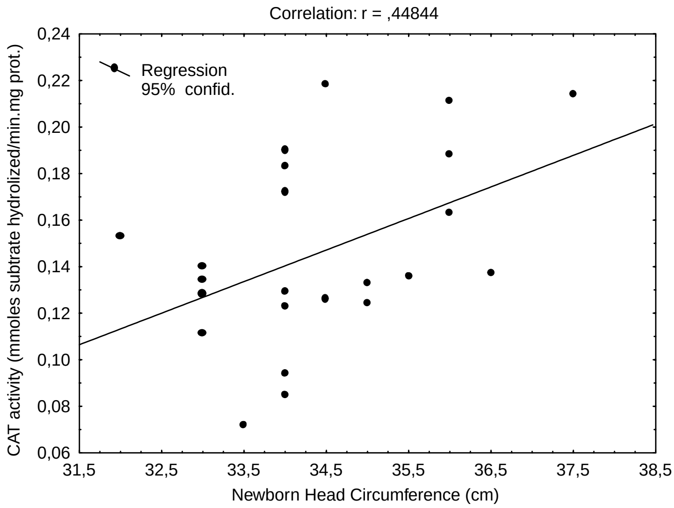

Figure 5. Relationship among catalase activity in human placenta and newborn head circumference. Catalase activity presented a positive correlation with newborn head circumference ($p < 0.05$). Enzymatic activity is expressed as mmoles substrate hydrolysed min^{-1} mg^{-1} proteins.

OP and carbamate insecticides are thought to elicit toxicity through a common mechanism via inhibition of cholinesterases.

The present work determined RBC and plasma cholinesterase in pregnant women in order to evaluate residential exposure in an area of intensive pesticide use during the application (November–February) and non-application (March–October) periods. Both enzyme activities have been widely used to assess OP exposure. In our data, plasma cholinesterase appears to be inhibited more effectively by the OPs applied than RBC cholinesterase (Table III), corroborating the fact that this enzyme might be a more sensitive indicator of exposure (Dyer et al. 2001, Walker & Nidiry 2002). We have also reported similar seasonal variations in plasma cholinesterase in pesticide fumigators working in this area (Rovedatti et al. 2004). However, to date there is no single biomarker that can state subchronic exposure in populations with a high risk of pesticide exposure and which can be questioned given the inter-individual variability.

Nevertheless, an increase in placental AChE level has been observed in placental tissue during the same period of pesticide application (Figure 1). The occurrence of AChE activity in human placenta, a tissue without innervations, has been known for more than 60 years (Rama Sastry 1997). The classical role of AChE is to terminate cholinergic neurotransmission by hydrolysis of acetylcholine (ACh), but nowadays it is known that elevated levels of AChE are present in non-neuronal tissues such as haematopoietic cells, osteoblasts, oocytes and early embryos (Patinkin et al. 1994, Grisaru et al. 1999, Inkson et al. 2004). Recently, AChE was implicated in stress-induced changes in brain neurons and it is also involved in the modulation of cell growth (Deutsch et al. 2002). Transient elevation of ACh levels produced by AChE inhibition stimulate the expression of genes located in the 'cholinergic locus' and produce long-term effects reminiscent of human post-traumatic stress disorders (Kaufer et al. 1998). The molecular events switched on by the inhibition of AChE include a cascade of c-Fos transcriptional responses involving a feedback mechanism that enhances the synthesis of AChE. An increase of AChE-R isoform was noted in those cases. The expressions of genes for synthesis and packaging of ACh are repressed at the same time. All these events lead to decreased ACh levels (Grisaru et al. 1999, Kaufer et al. 1999).

Considering that the reduction observed in the maternal plasma cholinesterase (ChE) level (Table III) also reflects similar changes occurring in other organs, we postulate that the increase in the activity of term placenta AChE observed during the months of pesticide application (Figure 1) may be the long-term effect produced by placenta AChE inhibition through a feedback mechanism that stimulates enzyme overproduction. We have not yet characterized the placenta AChE isoforms, but according to the literature there is accumulating evidence in experimental animals that AChE-R appeared in embryonic and tumour cells and is induced under chemical and physical stress (Chan et al. 1998, Grisaru et al. 1999).

These results are consistent with our previous reports indicating that OP pesticides are the compounds most intensively used in the valley of Río Negro and Neuquén for agricultural purposes. Additionally, several OP are frequently detected on surface and groundwater during the period indicated in this study (Loewy et al. 1999, 2003).

Median GST activity was similar in our study compared with those reported by Raijmarkers et al. (2002) in human placenta after an uncomplicated vaginal delivery. GSTs are a super family of xenobiotic-metabolizing enzymes that catalyse the conjugation of glutathione to this potentially hazardous reactive species in order to detoxify and facilitate their excretion. Consequently, the expression of these enzymes affects the level of cellular damage and, hence, modulates the disease risk. The absence of correlation between GST activity and the period of intensive pesticide application (Figure 2) may be explained by the large variation in the activity of the enzyme among individuals, suggesting that the system ACh/AChE would be a more sensitive and appropriate indicator of the OP environmental contamination.

Some OC and OP consume glutathione (GSH) through a GST-catalysed reaction as a major way of detoxifying these chemicals (Agrawal et al. 1991, Peña Llopis et al. 2003). The enzyme activity was negatively correlated with newborn body mass index and placental weight (Figure 4A, B). However, we still have no explanation for these associations.

Also, the chemical stress caused by pesticide exposure to the placenta is illustrated by the increased CAT activity observed during the pesticides applications period (Figure 3). This finding is consistent with previous observations (John et al. 2001). A positive correlation between the increase of AChE and CAT placental activity is shown in Figure 3. It allows the introduction of CAT as an excellent non-specific bioindicator of cell damage in this kind of study. The cell-damaging effects of highly reactive oxygen species (ROS), such as superoxide and hydrogen peroxide have been implicated in many diseases and in ageing (Qanungo et al. 1999). ROS are capable of damaging collagen in the chorioamnion and lead to preterm premature rupture of membranes (Woods 2001). In fact, Nguyen et al. (2004) reported that exposure to pesticides during pregnancy increased the risk of preterm delivery.

Previous studies examined the association of prenatal pesticide exposure and foetal growth. Our results are consistent with those of Eskenazi et al. (2004) who reported no association between maternal exposure to OP and birth weight and length, but a small increase in head circumference. However, the results are different from those reported by Whyatt et al. (2004), who described decreased birth weight and length in association with chlorpyrifos levels in umbilical cord plasma.

Head circumference has been shown to correlate with CAT (Figure 5) and is also indicative of brain weight. Both brain sizes and head circumferences are, in turn, predictive of IQ and cognitive ability (Lindley et al. 1999). Animal studies found that low-dose exposure *in utero* or in early postnatal life can produce neurochemical and neurobehavioural changes (Berkowitz et al. 2004). In fact, psychological and physiological differences in functional abilities were reported between children at 4 and 5 years of age living in agricultural areas (Guillette et al. 1998).

Finally, the increased placental weight observed in this study could be associated with atypical placental villi produced by a compensatory mechanism to low blood cholinesterase observed in women living in agricultural areas (Levario Carrillo et al. 2001) and/or alteration in the hormone signalling mechanism described by Souza et al. (2004).

In conclusion, the integrated use of placental AChE and the antioxidant enzyme CAT seems to be useful tools for environmental monitoring programmes for the detection of the *in utero* exposure/effect induced by anticholinesterase pesticides on women living in areas of intensive pesticide application. The stimulation in the activity of both enzymes was produced by the adaptive response to the xenobiotic and no statistical association of both enzymes was observed with lifestyle habits. The placenta is often the most accessible and readily available component of the triad mother, infant, placenta. It shows cumulative effects of pregnancy-related events, reflects the intrauterine environment and can be examined to a degree that the infant usually cannot (Stoll et al. 2003). We found a positive correlation relationship between environmental exposure to OP and carbamate and head circumference.

The long-term goal of this study is to develop valid biomarkers to be included in epidemiological monitoring programmes in order to clarify the relationship between prenatal pesticides exposure and adverse developmental effects.

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