

Title: “Changes in carboxylesterase activity, nuclear and mitochondrial lipid composition of human placenta associated to environmental exposure to pesticides”

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Abbreviations: CEs, carboxylesterases; RBC-AChE, red blood cell acetylcholinesterase; OP, organophosphate pesticides; PCh, plasma cholinesterase; PP, pulverization period; RP, recess period;

ABSTRACT

Carboxylesterases (CEs) are sensitive indicators of environmental organophosphate pesticides (OP) exposure and catalyze several lipid metabolic reactions. The aim of this pilot study was to assess the impact of OP exposure on placental CE activity and lipid composition. We performed a study of forty healthy women who live in a cultivation area. Samples were collected during the pulverization period (PP) and recess period (RP). The plasma cholinesterase and placental CE activity decreased in PP, suggesting that women were exposed to OPs and that these pesticides reached the placenta. The cardiolipin content increased and the phosphatidylethanolamine content decreased in the light mitochondrial fraction while total cholesterol and sphingomyelin increased in the nuclear fraction. These changes in lipid profiles suggest repair by hyperplasia of the cytotrophoblast. Decreased CE activity may have clinical and toxicological implications and thus entail potential risks to damage the developing fetus.

Keywords: organophosphate pesticides, carboxylesterase, lipid composition, placenta

1. Introduction

Epidemiological studies have linked exposure to pesticides during pregnancy with impairment of fetal growth and development [1,2,3,4,5,6].

One of the most important classes of chemicals actively applied to the environment is the cholinesterase-inhibiting organophosphate (OP). Almost every person is, or has been, exposed to OP insecticides in their home or work environment or as trace dietary contaminants [7,8]. Residing near pesticide-treated areas or in agricultural regions contributes to exposure, as do house and yard pesticide treatments. Studies of the toxicokinetics and placental transfer of OPs in exposed pregnant rats showed that the placenta was a poor barrier against the pesticide and this organ functioned as a temporary depot [9,10]. Although the health of the placenta is a prerequisite for the health of fetus [11], not many studies have focused on pesticide placental toxicity. Levario Carrillo et al. [12] investigated the placental morphology of women living in an agricultural area exposed to parathion. These authors observed microcalcifications, microinfarctions and atypical characteristics of tertiary villi. Alterations in the homogeneity of the maturity within placental tissue from women exposed to pesticides have also been reported [13]. We previously showed significant changes in the activity of acetylcholinesterase and catalase associated with periods of OP pulverization in placenta samples of women residing in a rural area and a correlation of catalase activity with newborn head circumference [14].

Lipid pathways and transport are very active in the placenta [15] and contribute to progesterone synthesis, membrane-dependent events and supply of fatty acids to the fetus [16]. As pesticides are lipophilic compounds, they may interfere with the lipid metabolic pathways that occur in membranes. In fact, *in vitro* studies from our laboratory have shown that in cell-free homogenates of placental tissue, OPs affected

the activity of phosphatidylinositol 4-kinase as well as the metabolism of phosphoinositides, minor lipids associated to signaling [14]. In addition, lipid peroxidation was found to be the most common molecular mechanism of action of OPs in experimental models both *in vitro* [17] and *in vivo* [18]. However, there is a lack of information about the possible effect on placental lipid composition associated to environmental human exposure.

Among the enzymes involved in lipid metabolism, carboxylesterases (CEs), which catalyze several cholesterol and fatty acid metabolic reactions [19], participate in the maintenance of the membrane structure [20] and in the hydrolysis and transesterification of xenobiotics [19]. CEs are considered sensitive indicators of environmental OP exposure [21, 22]. CEs form part of the B-class of esterases, enzymes that are inhibited by OPs and carbamates [23], and showed decreased catalytic efficiency in preeclampsia [24].

This pilot study was conducted to assess the potential impact of environmental pesticide exposure on CE activity and the lipid profile of the placentas of women residing in an agricultural area. As specific phospholipids are required by mitochondrial membrane-bound enzymes [25] and as they have functional significance in nuclear events [26], the lipid composition of both placental organelles, mitochondria and nuclei, were studied.

2. Material and methods

2.1. Chemicals

The chemicals used were of reagent grade and were obtained from Sigma Chemical Co. (Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). Organic solvents were from Carlo Erba (Milan, Italy).

2.2 . Participant recruitment and collection of samples

We performed a study of 40 healthy women between the ages of 15 and 36, entering prenatal care at the Cinco Saltos Public Hospital, province of Río Negro, Argentina, between December 2006 and August 2008. They were asked to participate by a physician in their third trimester of pregnancy and consent was obtained from each participant before they were interviewed. This study was carried out with the full ethical approval of the local Advisory Committee of Biomedical Research in Humans, which approved the study protocol.

The patients included in this study were residents of farms or communities surrounding fruit cultivation areas where pesticides, mainly OPs, such as azinphos methyl, phosmet, chlorpyrifos and dimethoate, as well as carbamates are applied six months a year, during the dry seasons: spring and summer (September to February). Pesticides are usually finely dispersed as droplets or particles at the time of pulverization and aerial drift from the target area is frequent, increasing the potential environmental exposure of the population. Samples collected from September to December were considered samples of the PP, and those collected from April to August were considered samples of the RP. A questionnaire was administered to document physical characteristics, educational level and lifestyle habits. Women with chronic diseases, on long-term medication (except those included in Group A according to the FDA), and those with serious pregnancy complications were excluded. Groups were matched for reported smoking habit and alcohol consumption.

Maternal blood samples (n=40) of the third trimester of pregnancy were obtained by venipuncture. Heparinized blood samples were analyzed for cholinesterases activity.

EDTA-treated samples were analyzed for red cell count in a Cell-Dyn 1400 hematology analyzer. Villous placental samples were collected within 20 min of vaginal delivery. Suitable amounts from the central area of the maternal side of the placenta were obtained as the expression of various components may vary according to the location [27]. For CE activity determinations (n=40), samples were frozen at -20°C until use. For subcellular fractions and lipid extracts only samples from births occurring in the morning were considered because they require 6 hours of laboratory work. These samples (n=19) were collected in ice-cold Hepes buffer with NaCl 0.85 %, pH 7.0, containing butylated hydroxytoluene as an antioxidant and were processed immediately.

2.3. Cholinesterase assay

Red blood cell acetylcholinesterase activity (RBC AChE) and plasma cholinesterase (PCh) activity were measured using heparinized samples at 30°C following the method by Voss and Shasse [28]. A blank was added to every subject's blood. RBC AChE activity was normalized by red blood cell count and expressed as nmoles of hydrolyzed substrate $\times \text{min}^{-1} \times \text{millions}^{-1}$ of erythrocytes. ChP activity was expressed as nmoles of hydrolyzed substrate $\times \text{min}^{-1} \times \mu\text{l}^{-1}$ of whole blood.

2.4. Carboxylesterase assay

The enzymatic activity toward α -naphthyl acetate or α -naphthyl butyrate arbitrarily assigned as CE-1 and CE-2, respectively, was determined, essentially as described previously [29] in 20,000 \times g homogenate supernatant. All enzymatic determinations were conducted in triplicate and expressed as means \pm SD. Proper linear conditions for

enzymatic activity were previously adjusted for each enzyme determination. The activity was normalized according to the sample protein content [30].

2.4. Mitochondrial and nuclear fraction isolation

Isolation of both organelles was essentially performed according to the procedures described by Corso and Thomson [31]. All the steps were carried out with cold solutions and centrifugation was performed at 4°C. The nuclear fraction, the heavy mitochondrial (HM) fraction, corresponding to the cytotrophoblast and the light mitochondrial (LM) fraction, corresponding to the syncytiotrophoblast, were obtained.

2.5. Lipid extraction and analysis

The lipid extraction of each fraction obtained was conducted using the method by Bligh and Dyer [32]. Aliquots were used for the determination of phospholipid phosphorus [33] and total cholesterol, using a commercial kit (Colestat Enzyme Kit, Wiener Laboratory, Rosario, Argentina). Individual phospholipids were separated by two-dimensional thin layer chromatography (TLC). Spots were visualized using iodine vapor, scraped off and used for individual phospholipid quantification [33]. The results were corrected by the percentage of each plate recovery. Results were adjusted according to the protein content [30] of each subcellular fraction.

2.6. Morphometric parameters

The placenta was weighed immediately after childbirth. Information about the status of the newborn at birth (weight, length, head circumference, gender and gestational age) was collected from medical records. Weight, length and head circumference were

adjusted according to gestational age and gender using the standardized Z-scores table of the Argentine Society of Pediatrics [34].

2.7. Statistical Analysis

Categorical variables were compared using the Pearson's chi-squared test (χ^2).

Statistical significance between means was determined by the *t*-test. The associations between analytical parameters, as well as between placental weight and analytical parameters, were estimated by calculating the Pearson's correlation coefficient. All statistical analyses were performed using R software version 2.6.0. Statistical significance was assumed as $p < 0.05$.

3. Results

3.1. Demographic characteristics

The demographic characteristics of the groups under study are presented in Table 1. The women who participated in this study were a relative homogenous group, since, they were similar in terms of demographical characteristics and habits.

3.2. Morphometric characteristics of the newborns and placentas

Table 2 shows the morphometric characteristics of the newborns and placentas collected. No significant differences were observed between RP and PP.

3.3 Enzymes activity

Comparing the average blood cholinesterase activity of RP vs. PP, PCh decreased significantly (20%, $p < 0.01$), suggesting maternal anticholinesterase pesticide exposure in PP (Table 3).

Figure 1 shows the average CEs activity of placental villi. CE-1 activity decreased significantly 40% ($p < 0.01$) in PP while there were no changes in CE-2 activity.

Neither PCh nor placental CE-1 activity were statistically associated with maternal lifestyle habits.

3.4 Lipid composition

Studies of lipid composition (Table 4), expressed as total cholesterol and total phospholipid phosphorus content, revealed that LM and HM composition showed no changes associated to the pesticide pulverization period. However, the nuclear cholesterol content and cholesterol/phospholipid ratio significantly increased in PP (61% and 82%, respectively). In addition, the correlation analysis revealed a statistically significant relationship between nuclear cholesterol content and placental weight ($\beta=10.10$, $p=0.03$, $\text{adj } r^2 = 0.33$).

Regarding mitochondrial phospholipid composition, comparing PP vs. RP, an increase in cardiolipin content (8%, $p < 0.05$) and a decrease in phosphatidylethanolamine (16%, $p < 0.05$) was observed in LM (Table 5) as well as in the percentage distribution (Figure 2B). No changes were observed in HM (Table 5 and Figure 2A). Sphingomyelin content (Table 5) and percentage distribution (Figure 2C) significantly increased in the nuclear fraction (34 % , $p < 0.05$); 29%, $p < 0.05$ respectively) in PP. Statistical

analysis showed no association between CE-1 activity and the lipid content of the organelles studied.

4. Discussion

RBC-AChE and PCh activity are usually used as sensitive biomarkers to evaluate exposure to OPs and carbamate pesticides [35,36]. In this study we determined the activity of both cholinesterases in the blood of pregnant women living in an area of intensive pesticide use in two periods: PP and RP. The mean values for the activity of both cholinesterases for samples collected in RP were similar to those reported by Peyster et al. [37], who studied blood cholinesterases during pregnancy in a Hispanic population. As seen in our previous study in pregnant women living in this area [14], during PP, the average PCh activity decreased significantly (Table 3) with respect to the samples collected during RP. These results corroborate that this enzyme may be a more sensitive indicator of exposure than RBC-AChE [38,39]. The degree of decrease (20%) observed in our study is of great concern as it is not so different than the decrease reported by Remor et al. and Bhalli et al. [40,41] in exposed workers relative to control groups (24% and 30%, respectively).

CEs are widely used to evaluate tissue exposure to OPs and carbamate pesticides. It has been reported that there is a differential tissue distribution and microlocalization of CEs family members in mammals [19]. In fact, the placenta expresses three isoforms of CEs [42]. As placental CEs activity has been found in microsomal and cytosolic fractions [42,23], we determined the hydrolytic activity in 20,000 x g supernatant of homogenate microvilli. The activity range determined in RP (74 - 178 nmol/mg prot x min), using α -naphthyl acetate as substrate, was similar to the range reported by Yang et al. (1996)

[42]. for human placenta using the same substrate (85- 170 nmol/mg prot x min).

Regarding the sampling period, differential effects were observed for CEs activity (Table 3). In accordance with previous reports showing that mammalian CEs occur with different inhibition sensitivities [43], CE-1 but not CE-2 was inhibited during PP. The inhibition of CEs can be important in cumulative toxicity with exposure to multiple anticholinesterase pesticides, as is the case of the study population. They are assumed to provide protection against OP and carbamate pesticide poisoning through the hydrolysis of ester bonds and also by stoichiometric binding for active pesticides, which reduces the amount of OPs/carbamates available for AChE inhibition. However, the esterase detoxification system also protects the fetus from drugs prescribed to pregnant women and detoxifies narcotics, such as heroin and cocaine [44]. In addition, CEs are involved in pyrethroid detoxification [45,46]. Therefore, the inhibition of CEs during pregnancy would have toxicological consequences in fetal development.

The uptake, biosynthesis and metabolism of cholesterol and other lipids are exquisitely regulated by feedback and feed-forward pathways in humans [47]. In fact, phospholipid percentual distribution of rat liver mitochondria was not sensitive to diets enriched in carbohydrates [48] or unsaturated fatty acids [49]. Also, the cholesterol/phospholipid ratio of liver mitochondria was unchanged in rats feeding on diets containing trans fatty acid. However, in our study, environmental exposure to pesticides modified the lipid content (Table 4), the phospholipid content (Table 5) and the percentage phospholipid composition of the nuclear fraction (Figure 2C) as well as the phospholipid content (Table 5) and the percentage phospholipid composition of the LM fractions (Figure 2B). In line with these findings, another environmental contaminant, the peroxisome proliferator, di-(2-ethylhexyl)-phthalate, and its metabolites, changed the lipid metabolome in a rat HRP-1 trophoblast model[50].

Mitochondria is a target organelle of pesticide toxicity, as was demonstrated by Binukumar et al. [51]. These authors observed alterations in the mitochondrial electron transfer enzyme activities and an increase in reactive oxygen species (ROS) levels in liver chronically exposed to dichlorvos (OP). Astiz et al. [52] showed a loss of cardiolipin and glutathione content and increased lipid peroxidation in the mitochondria of substantia nigra associated to the activation of apoptosis in rats treated with low doses of OPs. Owing to its high unsaturated fatty acids content and its location near the site of ROS production, cardiolipin is found almost exclusively in the inner mitochondrial membrane and is particularly prone to peroxidative attack by ROS [53]. Cardiolipin oxidation is a critical step in apoptosis. During apoptosis, cardiolipin may form stable complexes with specific mitochondrial glycoproteins which are translocated to the plasma membrane [54]. However, our results do not concur with the above findings. Unexpectedly, the cardiolipin content increased while the phosphatidylethanolamine content decreased LM in samples collected in PP with respect to samples collected in RP Although further study is required to understand the mechanisms involved in the changes observed in mitochondrial composition, it may be assumed that changes in the LM lipid profile may affect LM function. In fact, we recently studied the progesterone content in placentas collected in the same area under study and found significantly lower progesterone levels in PP than in RP (data not shown). Interestingly, progesterone synthesis occurs in the inner mitochondrial membrane of the syncytiotrophoblast [55].

Lipids *in toto* comprise approximately 5% by weight of the nucleus, resulting in high-buoyant density that facilitates isolation of these organelles in high purity. Lipids of the membrane as well as those of the endonuclear loci are now recognized as the source of numerous signaling reactions [56]. Therefore, the study of nuclear lipid composition is a

topic of interest. In our study, total cholesterol increased and the cholesterol/phospholipid ratio significantly increased in PP. In addition, the nuclear cholesterol content was positively associated to placental weight. Also, the sphingomyelin content decreased in PP. These changes in lipid content resemble the results of Cascianelli et al. [57], who showed an increased sphingomyelin and cholesterol content in nuclear microdomains of regenerated hepatocytes. Interestingly, increased hepatic cardiolipin synthesis, which is presumed to be under the control of transcription factors that regulate mitochondrial biogenesis [58], as well as the pool size of cardiolipin, increased during liver regeneration [59]. Although this study cannot explore the precise mechanisms of the effects observed, our results suggest that the changes in lipid content in the LM and nuclear fractions may be associated with an increase in the trophoblast proliferation rate. In support of this, it has been reported that the OP malathion was mitogenic at lower levels of exposure in human liver carcinoma cells [60]. It is accepted that moderate levels of ROS may function as signals to promote cell proliferation and survival [61].

Therefore, we hypothesize that pesticide environmental exposure during pregnancy modifies placental ROS levels affecting the redox-sensitive signals involved in cell proliferation. However, considering that cardiolipin and oxidized cardiolipin species co-migrate in the TLC used to separate phospholipids [62], oxidative injury of the LM organelle characteristic of the syncytiotrophoblast cannot be discarded. As the transfer of pesticides from maternal circulation occurs across the endothelial-syncytial membrane of the placenta it may contribute to the injury of the syncytiotrophoblast, which would lead to repair by hyperplasia of the cytotrophoblast.

In summary, our study makes the novel observation that placental CE activity is sensitive to OP exposure during pregnancy. As mixing OPs and pyrethroid pesticides becomes a more common practice in the insecticide markets in developing countries [63], maternal exposure to pesticide mixtures may have important implications in fetal health. In addition, this information has an important clinical implication in the administration of medications designed to treat the mother but which are also able to cross the placenta and thus entail potential risks to damage the developing fetus. The data also suggest that changes in lipid profiles may be associated with an increase in the trophoblast proliferation rate. Further research is needed to corroborate this hypothesis and to assess their impact in the physiology of the placenta.

Conflict of interest

None

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Table 1

Mean demographic characteristic of the studied groups.

Characteristic		RP(n=18)	PP(n=22)
Age ^a		22.7 ± 4.8	23.5 ± 5.2
Level of instruction ^b	primary	6.5	18
	secondary	75	73
	tertiary	17	9
Parity ^a		1.1 ± 1,3	1.2 ± 1.6
Nutritional condition ^{b,c}	normal	100	98
Smoking status ^b	smoker	5	6.2
	Passive smoker	11	12
Occasional alcohol consumption ^b		1.2	0
Ground water consumption ^b		7.6	8.1
Self-reported indoor pesticide use ^b		6.5	5

^a The results were expressed as mean ± SD. Student *t*-test: NS.

^b The results are expressed as average. Test chi-square: NS.

^c On the basis of BMI .

Table 2

Morphometrical information of the neonates and the placentas.

	RP(n=18)	PP(n=22)
Newborns' weight (kg) ^a	3.35 ± 0.44	3.299 ± 0.32
Newborns' height (cm) ^a	47.09 ± 2.41	47.09 ± 5.11
Head circumference (cm) ^a	34.47 ± 1.25	34.47 ± 0.83
Placental weight (g)	629.04 ± 18.14	621.93 ± 17.76
Placental index ^b	0.17 ± 0.03	0.18 ± 0.04

The results were expressed as mean ± SD. Student *t*-test: NS.

^a Data corrected by gestational age and sex.

^b Placenta weight (kg) / newborns' weight (kg).

Table 3

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

	Plasma cholinesterase	Red blood cell cholinesterase
PR (n=18)	3.59 ± 1.00	1.00 ± 0.22
PP (n=22)	2.87 ± 0.79 **	0.94 ± 0.29

The results were expressed as mean ± SD . Red cell cholinesterase activity was normalized by red blood cell count and expressed as nmoles hydrolyzed substrate min⁻¹ x millions red blood cells. Plasma cholinesterase activity was expressed as nmoles hydrolyzed substrate x min⁻¹ μ⁻¹ whole blood . Student *t*-test: ** p < 0.01.

Table 4

Lipid content of placental heavy mitochondria, light mitochondria and nucleus in relation to pesticide spraying.

		RP (n=9)	PP (n=10)
heavy mitochondria	μg Pi/mg protein	14.85 ± 10.41	14.46 ± 5.65
	μg Chol/mg protein	26.12 ± 10.29	35.61 ± 15.54
	Chol/PL	0.18 ± 0.09	0.22 ± 0.08
light mitochondria	μg Pi/mg protein	19.55 ± 12.15	21.77 ± 11.07
	μg Chol/mg protein	38.32 ± 19.58	32.63 ± 24.06
	Chol/PL	0.21 ± 0.02	0.22 ± 0.02
nucleus	μg Pi/mg protein	4.62 ± 2.51	5.78 ± 1.87
	μg Col/mg protein	7.54 ± 2.46	12.12 ± 4.5*
	Chol/PL	0.11 ± 0.07	0.20 ± 0.11*

The results were expressed as mean ± SD. Pi : phospholipid phosphorus, Chol: total cholesterol, Chol/PL: molar ratio cholesterol/phospholipids. Student *t*-test: * p < 0.05.

Table 5

Selected phospholipid content of placental heavy mitochondria, light mitochondria and nucleus in relation to pesticide spraying.

		RP (n=9)	PP (n=10)
light mitochondria	PC	0.506 ± 0.34	0.314 ± 0.39
	PE	0.37 ± 0.29	0.31 ± 0.44*
	CL	0.048 ± 0.044	0.052 ± 0.041*
nucleus	PC	5.17 ± 0.93	3.99± 0.55
	PE	4.04± 1.01	2.95± 0.87
	SM	0.50 ± 0.18	0.67 ± 0.25 *

The results are expressed as $\mu\text{g Pi/mg protein}$ (mean \pm SD). Pi : phospholipid phosphorus, PC: phosphatidylcholine. PE: phosphatidylethanolamine, SM: sphingomyelin, CL: cardiolipin + oxidized cardiolipin. Student *t*-test: * $p < 0.05$.

Figure 1

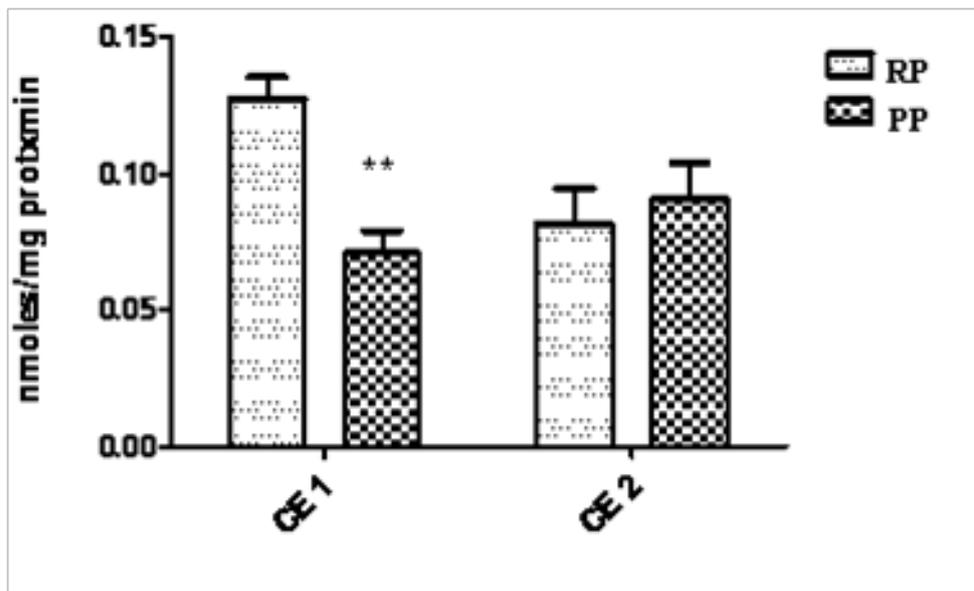
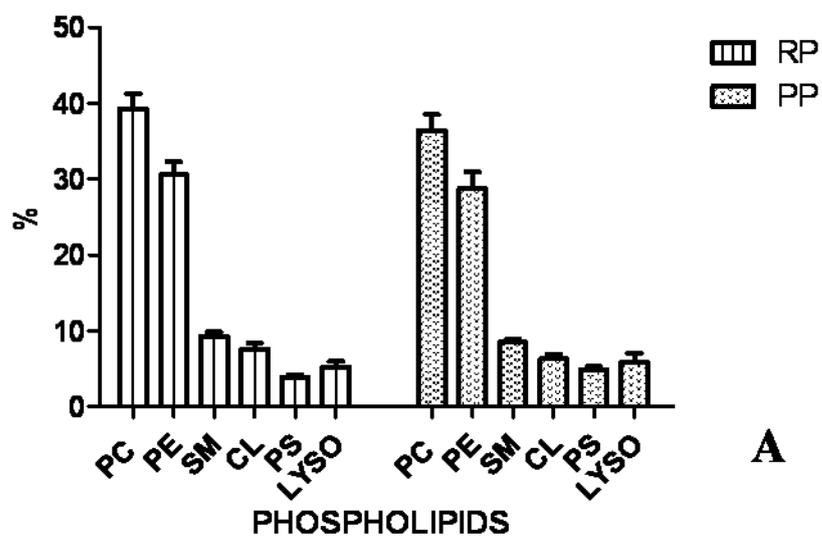
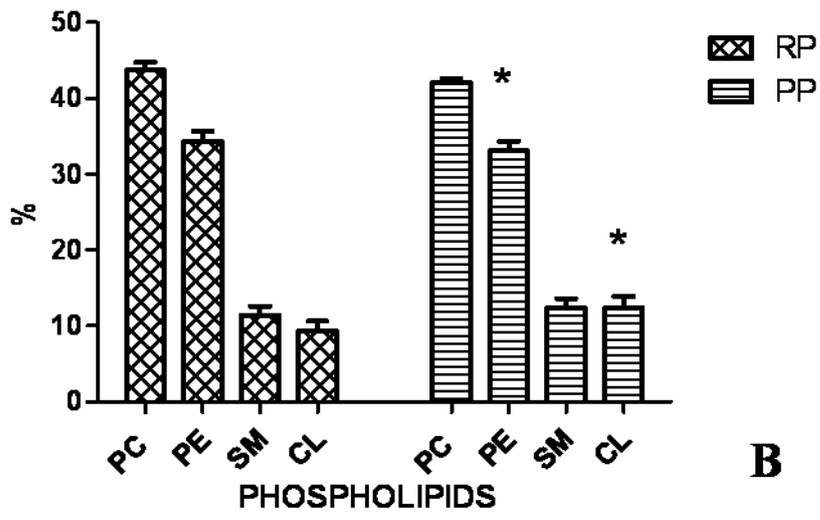


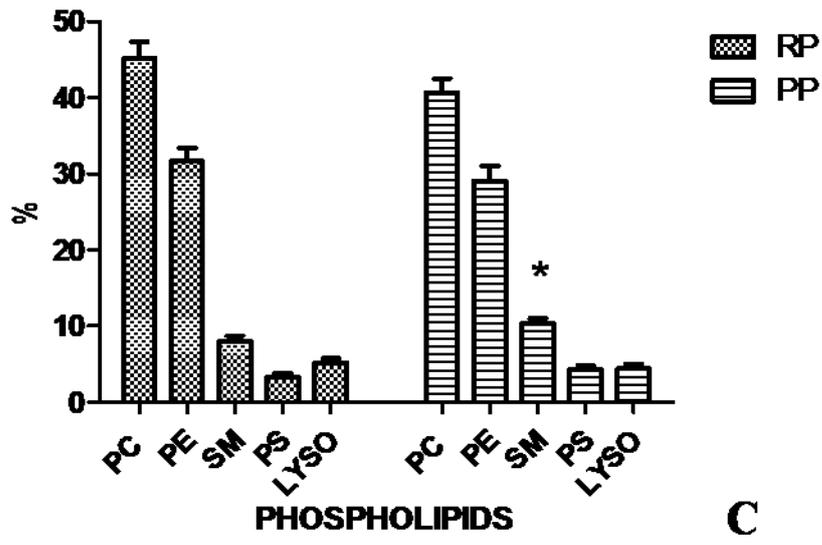
Figure 2



A



B



C