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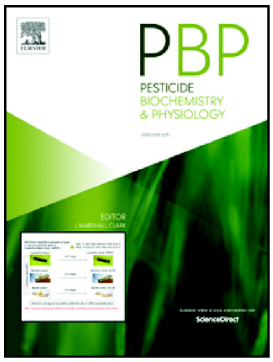

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The insecticides chlorpyrifos and acetamiprid induce redox imbalance in umbilical cord blood erythrocytes in vitro.

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Abbreviations: UCBE umbilical cord blood erythrocytes¸ ROS reactive oxygen species; CAT catalase; SOD superoxide dismutase; OPs organophosphates; HCT hematocrit, Hb hemoglobin; HNE 4-hydroxy-2-nonenal; NBT nitroblue tetrazolium, PMA phorbol 12-myristate 13-acetate

ABSTRACT

Organophosphate and neonicotinoid compounds belong to different pesticide families, and are used worldwide for agricultural purposes. The potential deleterious health effects associated with pesticide exposure during pregnancy has become a major public health concern. The present study analyzed the effects of the organophosphate chlorpyrifos and the neonicotinoid acetamiprid on oxidative stress biomarkers in human umbilical cord blood erythrocytes (UCBE). The reactive oxygen species (ROS) levels, the catalase (CAT) and superoxide dismutase (SOD) activities, the levels of 4-hidroxynonenal (HNE) and UCBE osmotic fragility were determined.

Both pesticides modified the oxidative status of UCBE. Chlorpyrifos increased ROS levels at 40 and 400 nM, while only decreased CAT activity at the higher concentration assayed (400 nM), with no modification in SOD activity. The insecticide acetamiprid increased ROS levels at all concentrations assayed, and decreased CAT and SOD activity at 40 and 400 nM, Chlorpyrifos and acetamiprid (40 and 400 nM) modified HNE content. Non-significant changes in UCBE osmotic fragility were induced by chlorpyrifos or acetamiprid treatments.

In conclusion, both pesticides assayed increased ROS production and decreased anti-oxidant enzyme activity in UCBE, even though changes were of different extent and depended on the insecticide analyzed. Interestingly no changes in erythrocyte osmotic fragility were registered; suggesting that the oxidative stress triggered under these experimental conditions was not sufficient to induce a functional damage to the UCBE membrane.

Keywords: UMBILICAL CORD BLOOD, PESTICIDES, CHLORPYRIFOS, ACETAMIPRID, OXIDATIVE STRESS.

1 Introduction

Following the introduction of organophosphates (OPs) as insecticides in the early 1940s, this insecticide family became the most used worldwide, mainly for agricultural purposes [1,2]. The neonicotinoids, another insecticide family of growing importance, are the most important chemical class of insecticides introduced to the global market since the synthetic pyrethroids [3]. Both insecticide families, organophosphates and neonicotinoids, are widely used to control insect pest in different crop types. The primary mechanism of OP toxicity is acetylcholinesterase inhibition, which increases acetylcholine. Nevertheless, other toxic effects have been observed at OP concentrations that do not inhibit acetylcholinesterase activity [4,5]. Neonicotinoids also impact central nervous system acting as selective agonists for the nicotinic acetylcholine receptors in insects, however *in vitro* studies have demonstrated that neonicotinoids may be also toxic to mammalian cells [6-8].

Chlorpyrifos, an OP insecticide, is a broad spectrum insecticide currently used worldwide to control insect pests (such as cutworms, corn root worms, grubs, flea beetles, flies, etc) on a variety of crops. Prior to June 2000, chlorpyrifos was also used for indoor pest control in USA, but most house-hold uses were phased out by 2001 [9]. Acetamiprid is a neonicotinoid insecticide commonly used against Hemipteran, Thysanopteran and Lepidopteran insect pests. It is frequently detected in agricultural products owing to its widespread and extensive use [10].

Once pesticides are released to the environment they follow a dynamic fate which includes gaining access not only to pest-specific target but also to non-target organisms, such as human beings. Potential health effects associated with pesticide exposure during pregnancy have become a major public health concern due to maternal and fetal high sensitivity to xenobiotics. Reports indicate that toxic manifestations induced by

pesticides may be associated with an enhanced production of reactive oxygen species (ROS) [11,12]. This later was demonstrated in human OPs exposure [13], and in carbamate and neonicotinoid animal exposure models [14,15].

Among ROS, superoxide anions, hydroxyl radicals and hydrogen peroxide enhance the oxidative process and induce lipid peroxidative damage in cell membranes [16,17] altering globular resistance in red blood cells [18,19]. Cells display several mechanisms to alleviate oxidative stress and to repair damaged macromolecules. Defense is offered by enzymatic and non-enzymatic antioxidants which scavenge ROS, such as superoxide dismutase (SOD), catalase (CAT) or the glutathione peroxidase system, among others.

To further delineate the role of oxidative stress in the toxicity of the OP chlorpyrifos and the neonicotinoid acetamiprid, the effect of doses representative of human exposure was studied on umbilical cord blood erythrocytes *in vitro*. Reactive oxygen species production, anti-oxidant enzyme activity, lipid peroxidation and osmotic fragility in umbilical cord blood erythrocytes were determined.

2. Material and methods

2.1 Reagents

Reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA), unless otherwise stated. The OP chlorpyrifos and the neonicotinoid acetamiprid were of standard analytical grade according to the supplier (Chem Service, West Chester, PA, USA) with a 99.5% purity.

2.2 Sample Collection

Women in the third trimester of pregnancy were asked to participate in this study. They were included if they had medium income level and belonged to the same ethnic group –Hispanic. Women were excluded if they smoked, suffered from a serious chronic disease or were medicated (except those included in Group A according to U. S. Food and Drug Administration), or developed a pregnancy complication (i.e., gestational diabetes, hypertension, preeclampsia). The study included twelve pregnant women with no history of pesticide exposure, who attended to Castro Rendón Public Hospital in Neuquén City, Argentina, and underwent cesarean deliveries.

Written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki of the 59th WMA General Assembly, guidelines for the protection of human subjects. Study protocol was approved by the ethical committee of the local Advisory Committee of Biomedical Research in Humans. Samples were collected immediately after delivery by venipuncture after clamping and cutting the babies' end of the cord. Blood collection was done into sample sterile syringes containing heparin. Ten mL of umbilical cord blood were obtained and refrigerated at 4ºC until analysis.

2.3 Insecticide treatments

Samples were centrifuged at 3000 rpm for 5 min at 4 °C for red blood cells isolation. Plasma and buffy coat were removed. Red blood cells were washed three times with isotonic phosphate-buffered saline (PBS) at 4 °C and centrifuged at 1000 rpm. After the final wash, the red blood cells were suspended in the glucose buffer (2.1 g NaCl, 0.09 g KCl, 0.27 g Na_2 HPO_4 ($2H_2O$), 0.048 g, NaH_2 PO 4, 0.03 g $MgSO_4$ and 0.22 g glucose in 250 mL final volume, pH 7.4) until a hematocrit of 24% was reached.

With both pesticides separately, red blood cells suspensions were incubated at different concentrations 4, 40 and 400 nM for 3 h at 37°C in glucose buffer. Controls were incubated under the same conditions in presence of 0.02% DMSO. Cells were then washed with PBS, centrifuged and 50% hematocrit (HCT) restored by dilution with PBS. In all samples the hematological parameters of hemoglobin (Hb), total erythrocytes and HCT were analyzed by a Cell-Dyn 1400 hematological analyzer (data not shown).

The concentrations selected for the experimental design were in the concentration range reported after chlorpyrifos sub-chronic exposure, and below the concentrations reported after acetamiprid acute exposure. Chlorpyrifos concentration in serum after sub-chronic exposure was lower than 30 ng/mL (approximately, 85 nM) [20]. Others have reported, in umbilical cord blood serum, a chlorpyrifos concentration of 1.33 ng/mL (approximately, 3.8 nM) [21]. Acetamiprid concentration, in human serum, of a woman who ingested 100 mL solution containing 2% acetamiprid was reported as 268 µM [22]. Others have reported that following an acute poisoning blood acetamiprid level was 95 µM [23].

2.4 Reactive oxygen species analysis

After exposure of umbilical red blood cells to xenobiotics, red blood cells were washed three times with PBS, and 200 µl nitroblue tetrazolium (NBT) solution (0.01%), containing phorbol 12-myristate 13-acetate (PMA) 0.125 µM, was added and incubated at 37°C for 45 minutes. Red blood cells were washed twice with PBS and lysed with 250 µl of KOH (2N) and dimethyl sulfoxide (DMSO) solution. Formazan crystals were solubilized and absorbance was recorded at 630 nm in a final volume of 0.25 mL (Rayto TR 2100C microplate reader) and results were expressed as percentages. Assay was performed three times, and each condition was processed as triplicate.

2.5 Catalase and Mn-superoxide dismutase activities

Catalase (CAT) (E.C.1.11.1.6) activity was determined by recording the continuous decrease of absorbance at 240 nm. The reaction was performed in 1 mL PBS (50 mM, pH 7.0) containing 25 mM H_2O_2. Baseline absorbance remained constant; to initiate the reaction 16 µL of hemolyzed UCBE was added, at 37ºC, in a final volume of 1.016 mL. The absorbance of the reaction mixture was strictly controlled to be 1,000 absortion units. Specific activity was expressed as mmol /min x mg protein, using a molar extinction coefficient of 40 M^{-1} cm^{-1} [24]. Assay was performed three times, and each condition was processed at least as triplicate.

Mn-superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined in hemolyzed UCBE at 30ºC, by the adrenochrome spectrophotometric assay at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine (pH 10) [24], in a final volume of 1 mL. Enzyme activity was expressed as USOD/min x mg protein.

Linear conditions for all enzyme activities were previously adjusted. Assays were performed at least three times, and all measurements were performed in triplicate and a mean value was considered for the calculations. Protein content was quantified by the Lowry method [25]. Enzyme activity measurements were carried out in a UV/Vis 1603 Shimadzu Spectrophotometer.

2.6 Western blot analysis

4-hydroxy-2-nonenal (HNE), is one of the major lipid peroxidation products of n-6 polyunsaturated fatty acids. HNE is a trifunctional molecule, both the hydroxy group and the conjugate system consisting of a C=C double bond, and a carbonyl group contribute to the high reactivity of HNE, which make it a powerful biomarker of cellular oxidative stress [26,27].

The levels of the HNE lipid peroxidation products were determined in the erythrocyte membrane fractions by the western blot technique according to the protocol as follows. Equal amounts of protein (50 µg) were diluted in sodium lauryl sulfate (SDS) sample buffer, boiled at 100°C for 5 min and loaded onto a 10% SDS-PAGE gel and run at 150 V for 1 h. After migration, proteins were electrotransferred to nitrocellulose (Bio-Rad Laboratories) at 100 V for 1 h. The membrane was blocked in Tris buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.2% Tween 20 and 5% non-fat dry milk, washed and incubated overnight with the primary antibody anti-HNE (1:2000) with shaking. After washing the blots were incubated with secondary antibody anti-rabbit peroxidase conjugated (1:5000) at room temperature for 1 h. Protein antibody complexes were visualized by an enhanced chemiluminescence detection system [24].

2.7 Red blood cell osmotic fragility

Red blood cell osmotic fragility was carried out by adding a decreasing concentration of NaCl solutions, and total hemolysis was achieved with distilled water incubation [28]. Soluble hemoglobin derived from lysed UCBE was measured spectrophotometrically. Osmotic fragility was performed by plotting the hemolysis percentages versus the buffer sodium chloride concentrations, yielding a logistic function which allowed the determination of the buffer concentration at which specific percentages of hemolysis were achieved. The results were expressed as NaCl concentration (g/L) at 50% and 45% hemolysis for each group analyzed.

Buffered sodium chloride stock solution (osmotic equivalent to 10% NaCl) was prepared by adding 90g NaCl, 14.4g Na_2HPO_4 and 2.7g NaH_2PO_4 H_2O, diluted to 1 L with distilled water. Different dilutions of the buffered sodium chloride solutions were prepared to provide the equivalent of 0.80, 0.50, 0.450, 0.40, 0.30, 0.10 and 0.0 g/L of NaCl. Each solution was placed in triplicate test tubes, and 20 µL of heparinized cord blood were added, with a final volume of 5 mL. All the samples were mixed and allowed to stand at room temperature for 30 min., then centrifuged at 600xg for 5 min. Supernatants absorbance were determined at 540 nm. Assay was performed three times, and each condition was processed as triplicate.

2.8 Statistical analysis

Results were expressed as means ± SD. Comparison between groups was performed using one-way ANOVA followed by multiple comparison post hoc test, Dunnett's test, with a confidence level of 5%, using the Infostat® and Graphpad Prism 5® programs.

3 Results

3.1 Pesticide incubation increases reactive oxygen species production

ROS production was determined by NBT reduction assay. UCBE were incubated for 3 h with chlorpyrifos or acetamiprid 4, 40 and 400 nM. Figure 1A shows that chlorpyrifos 40 and 400 nM significantly increases ROS production by UCBE. Figure 1B shows that acetamiprid significantly increases ROS levels in UCBE at all concentration tested (4, 40 and 400 nm).

3.2 Pesticide incubation modulates antioxidant enzyme activity

Anti-oxidant enzyme activity was analyzed in UCBE after 3 h incubation with insecticides. Chlorpyrifos *in vitro* exposure decreased catalase activity at the highest concentration assayed (400 nM), and did not modify superoxide dismutase activity at all the concentrations assayed (Figure 2A and 2B). Acetamiprid *in vitro* exposure decreased both catalase and superoxide dismutase activities at 40 and 400 nM (Figure 2C and 2D).

3.3 Pesticide treatment modifies HNE content

HNE positive protein bands in umbilical UCBE were analyzed by western blot after 3 h *in vitro* exposure to chlorpyrifos or acetamiprid. Positive band density comparison, showed significant differences between control and chlorpyrifos or acetamiprid treatments (Figure 3A and 3B). Chlorpyrifos treatment increased HNE band intensity at 40 and 400 nM (Figure 3A), similarly acetamiprid treatment increased HNE band intensity at 40 and 400 nM (Figure 3B), compared with control treated UCBE.

3.4 Pesticide incubation not modifies UCBE osmotic fragility

Red blood cell osmotic fragility was then evaluated. NaCl concentrations that produced 45% and 50% hemolysis are presented in Figure 4. Non-significant changes were observed after chlorpyrifos or acetamiprid incubation for 3 h at all concentration tested (4, 40 and 400 nM).

4 Discussion

An adverse health impact of concern to human reproduction and development is the exposure to chemicals, including pesticides [29]. The fetus and infant have long been recognized as especially vulnerable to the effects of environmental agents that disrupt developmental processes, with possible lifelong consequences [30]. During pregnancy, these environmental pollutants can reach the fetus crossing the placenta and triggering oxidative stress. In this sense, pregnant women residing close to intensive application pesticide fields have altered indicators of umbilical cord blood oxidative stress and oxidative damage [31]. Moreover, insecticide residue levels in cord blood correlated with alterations in birth outcomes [32]. The role of oxidative stress in pesticide toxicity has been well established [13], and has also emerged as a likely promoter of several pregnancy-related disorders, such as spontaneous abortions, embryopathies, preeclampsia, fetal growth restriction, preterm labor and low birth weight [33].

Among currently used pesticides the families mainly studied are organophosphates and pyrethroids. Toxic effects of the neonicotinoids insecticide family, although regarded as a substitute to the organophosphate insecticides [3], have been studied to a lesser extent. It has been postulated that pesticide toxicity may be mediated by aims of redox imbalance. However, few studies analyze umbilical cord blood erythrocytes sensitivity to pesticide toxicity. This study reports the effects of two insecticides, currently used worldwide, on ROS induction, anti-oxidant enzyme activity modulation, lipid

peroxidation, and membrane integrity in UCBE *in vitro*. The results obtained show that both pesticides increased ROS production by UCBE. It has been postulated that the increase in ROS triggered by pesticides may be a result of xenobiotic metabolization (i.e. cytochrome P450s), were ROS are generated as a byproduct [34,35]. In addition, ROS increase may result in antioxidant homeostasis changes leading to antioxidant depletion [13]. In this sense, SOD and CAT activity inhibition, can also be related to the presence of free radicals such as singlet oxygen, superoxide and peroxyl radicals [17,36]. In line with this, ROS increase in UCBE incubated with the insecticides was accompanied by a decrease in antioxidant enzyme activities. Other authors have reported redox imbalance and decreased anti-oxidant enzyme activity after human erythrocytes *in vitro* incubation with several OP insecticides, such as chlorpyrifos-ethyl [11], phosalone [17] and methidathion [37]. Recently Deeba et al. (2017) have reported that exposure to chlorpyrifos at concentrations indicative of field use, decrease CAT and SOD activities in human adult erythrocytes [34]. Results shown here demonstrate that chlorpyrifos incubation only impacted CAT activity at the highest concentration assayed (0.14 ppm). Differences may be due to erythrocyte origin, umbilical cord blood vs adult, and the concentrations utilized 0.0014 to 0.14 ppm vs 100-2000 ppm.

On the other hand, acetamiprid decreased CAT and SOD activities. In this sense, it has been reported that acetamiprid triggers oxidative stress in different cell types. On bacteria this compound modulates SOD and CAT activities, although oxidative stress lasted for a relatively short time and do not caused a long-term damage [36]. Deleterious effects of acetamiprid on male mice reproductive system were also associated to a reduced activity of the antioxidant enzymes CAT, glutathione peroxidase and SOD [38].

Erythrocyte plasma membrane is sensitive to lipid peroxidation and to oxidative damage, due to polyunsaturated fatty acids presence among other characteristics [39]. Results indicate that both pesticides increased HNE content in UCBE. Others have also reported an increase in the end product of lipid peroxidation malonaldehyde concentration, after erythrocyte pesticide treatments [17,34]. Erythrocyte osmotic fragility can be influenced by several factors that include lipid membrane peroxidation, and any factor inhibiting or attenuating glycolysis, among others [39]. The results shown here demonstrate that pesticides increased ROS levels, decreased anti-oxidant enzyme activities, and augmented HNE adducts. Nevertheless, the concentrations and time analyzed were not sufficient to alter UCBE osmotic fragility. Others have reported that OP, at highest concentrations that the ones assayed here, impact redox balance in adult erythrocytes with changes in osmotic fragility [19,40]. There have been conflicting reports about the relative osmotic fragility of red blood cell in newborns and adults. Some reports contend that fetal red blood cells are more resistant to hemolysis than adult red blood cells, emphasizing the presence of a subpopulation of osmotically resistant cells in cord blood [41,28]. The current work does not ment to compare umbilical cord blood vs adult erythrocytes, however under an oxidative stress condition induced by both insecticides, non-significant changes in red cell hemolysis were observed.

Our results demonstrate that chlorpyrifos induced ROS at 40 and 400 nM and decreased CAT activity at 400 nM, changes in HNE content were observed at 40 and 400 nM. In contrast, acetamiprid exposure at 4, 40 and 400 nM increased ROS, and decreased CAT and SOD activities at 40 and 400 nM, while increased HNE content at the higher acetamiprid concentrations. Nevertheless, neither chlorpyrifos nor acetamiprid were able to alter UCBE osmotic fragility in the tested conditions. Other antioxidant defense

mechanisms present in red cells such as glutathione content and glutathione peroxidase enzyme, which were not evaluated in this work should be also implicated in the effects induced by the insecticides.

5 Conclusion

The present study demonstrates for the first time the ability of chlorpyrifos and acetamiprid to increase ROS production and to decrease anti-oxidant enzyme activity in UCBE. Interestingly no changes in erythrocyte osmotic fragility were registered; suggesting that the oxidative stress triggered under this experimental condition was not sufficient to induce a functional damage to the erythrocyte membranes.

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References

[1]. M. Aktar, D. Sengupta, and A. Chowdhury, Impact of pesticides use in agriculture: their benefits and hazards, Interdisciplinary Toxicology, 2 (2009) 1–12.

[2]. A. Debost-Legrand, C. Warembourg, C. Massart, C. Chevrier, N. Bonvallot, C. Monfort, and S. Cordier, Prenatal exposure to persistent organic pollutants and organophosphate pesticides, and markers of glucose metabolism at birth, Environmental Research, 146 (2016) 207–217.

[3]. P. Jeschke, R. Nauen, and M. Schindler. Overview of the Status and Global Strategy for Neonicotinoids, Journal of Agricultural and Food Chemistry, 59 (2011) 2897–2908.

[4]. V. Rauh, R. Garfinkel, F. Perera, H. Andrews, L. Hoepner, D. Barr, and R.W. Whyatt, Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children, Pediatrics, 118 (2006) e1845–e1859.

[5]. T. Slotkin, and F. Seidler, Comparative Developmental Neurotoxicity of Organophosphates In Vivo: Transcriptional Responses of Pathways for Brain Cell Development, Cell Signaling, Cytotoxicity and Neurotransmitter Systems, Brain Research Bulletin, 72 (2007) 232–274.

[6]. A. Kocaman, and M. Topaktas, In vitro evaluation of the genotoxicity of acetamiprid in human peripheral blood lymphocytes, Environmental and Molecular Mutagenesis, 48 (2007) 483–490.

[7]. M. Calderón-Segura, S. Gómez-Arroyo, R. Villalobos-Pietrini, C. Martínez-Valenzuela, Y. Carbajal-López, M. Calderón-Ezquerro, J. Cortés-Eslava, R. García-Martínez, D. Flores-Ramírez, M. Rodríguez-Romero, P. Méndez-Pérez, and E. Bañuelos-Ruíz, Evaluation of genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed in vitro to neonicotinoid insecticides news, Journal of Toxicology, 2012 (2012) 612647.

[8]. T. Cavas, N. Cinkilic, O. Vatan, D. Yılmaz, and M. Coskun, In vitro genotoxicity evaluation of acetamiprid in CaCo-2 cells using the micronucleus, comet and cH2AX foci assays, Pesticide Biochemistry and Physiology, 104 (2012) 212–217.

[9]. U.S. Environmental Protection Agency. 2002. Interim reregistration eligibility decision: Chlorpyrifos. Washington, DC: U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances.

[10]. Y. Akiyama, N. Yoshioka, and M.Tsuji, Pesticide residues in agricultural products monitored in Hyogo prefecture, Japan, FYs 1995-1999. Journal - Association of Official Analytical Chemists, 85 (2002) 692–703.

[11]. F. Gultekin, M. Ozturk, and M. Akdogan, The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant enzymes (in vitro), Archives of Toxicology 74 (2000) 533-538.

[12]. V. Duzguner, and S. Erdogan, Acute oxidant and inflammatory effects of imidacloprid on the mammalian central nervous system and liver in rats, Pesticide Biochemistry and Physiology, 97 (2010) 13–18.

[13]. A. Lukaszewicz-Hussain, Role of oxidative stress in organophosphate insecticide toxicity – Short review, Pesticide Biochemistry and Physiology, 98 (2010) 145–150.

[14]. I. El-Bini Dhouib, M. Lasram, A. Annabi, N. Gharbi, and S. El-Fazaa, A comparative study on toxicity induced by carbosulfan and malathion in Wistar rat liver and spleen, Pesticide Biochemistry and Physiology, 124 (2015) 21-28.

[15]. R. Bal, G. Türk, Ö. Yilmaz, E. Etem, T. Kuloğlu, G. Baydaş, and M. Naziroğlu, Effects of clothianidin exposure on sperm quality, testicular apoptosis and fatty acid composition in developing male rats, Cell Biology and Toxicology, 28 (2012)187–200.

[16]. I. Ben Amara, A. Karray, A. Hakim, Y. Ben Ali, A. Troudi, N. Soudani, and N. Zeghal, Dimethoate Induces Kidney Dysfunction, Disrupts Membrane-Bound ATPases and Confers Cytotoxicity Through DNA Damage. Protective Effects of Vitamin E and Selenium, Biological Trace Element Research, 156 (2013) 230–242.

[17]. I. Altuntas, N. Delibas, D. Doguc, S. Ozmen, and F. Gultekin, Role of reactive oxygen species in organophosphate insecticide phosalone toxicity in erythrocytes in vitro, Toxicology in Vitro, 17 (2003)153–157.

[18]. I. Grattagliano, S. Russmann, V. Palmieri, P. Portincasa, G. Palasciano, and B. Lauterburg, Glutathione peroxidase, thioredoxin, and membrane protein changes in erythrocytes predict ribavirin-induced anemia, Clinical Pharmacology and Therapeutics, 78 (2005) 422–432.

[19]. C. Uchendu, S. Ambali, J. Ayo, K. Esievo, and A. Umosen, Erythrocyte osmotic fragility and lipid peroxidation following chronic co-exposure of rats to chlorpyrifos and deltamethrin, and the beneficial effect of alpha-lipoic acid, Toxicology Reports, 1 (2014) 373–378.

[20]. R. Nolan, D. Rick, N. Freshour, and J.H. Saunders, Chlorpyrifos: pharmacokinetics in human volunteers, Toxicology and Applied Pharmacology, 73 (1984) 8-15.

[21]. K. Huen, A. Bradman, K. Harley, P. Yousefi, D. Boyd Barr, B. Eskenazi, and N. Holland, Organophosphate pesticide levels in blood and urine of women and newborns living in an agricultural community, Environmental Research, 117 (2012) 8-16.

[22]. T. Imamura, Y. Yanagawa, K. Nishikawa, N. Matsumoto, and T. Sakamoto, Two cases of acute poisoning with acetamiprid in humans, Journal of the American Academy of Clinical Toxicology and European Association of Poisons Centres and Clinical Toxicologists, 48 (2010) 851–853.

[23]. M. Todani, T. Kaneko, H. Hayashida, K. Kaneda, R. Tsuruta, S. Kasaoka, and T. Maekawa, Acute poisoning with neonicotinoid insecticide acetamiprid, Chudoku Kenkyu, 21 (2008) 387-390.

[24]. V. Rivero Osimani, S. Valdez, N. Guiñazú, and G. Magnarelli, Alteration of syncytiotrophoblast mitochondria function and endothelial nitric oxide synthase expression in the placenta of rural residents, Reproductive Toxicology, 61 (2016) 47–57.

[25]. O. Lowry, N. Rosebrough, A. Farr, and R. Randall, Protein measurement with the Folin phenol reagent, Journal of Biological Chemistry, 193 (1951) 265–275.

[26]. J. Zhou, B. Cai, Y.P. Jang, S. Pachydaki, A. Marie, and J.R. Sparrow, Mechanisms for the induction of HNE- MDA- and AGE-adducts, RAGE and VEGF in retinal pigment epithelial cells, Experimental Eye Research, 80 (2005) 567–580.

[27]. W. Lee, H. Wong, Y. Chai, C. Shi, N. Amino, S. Kikuchi, and S. Huang, Lipid peroxidation dysregulation in ischemic stroke: Plasma 4-HNE as a potential biomarker?, Biochemical and Biophysical Research Communications, 425 (2012) 842–847.

[28]. M. Bautista, W. Altaf, R. Lall, and R. Wapnir, Cord blood red cell osmotic fragility: A comparison between preterm and full-term newborn infants, Early Human Development, 72 (2003) 37–46.

[29] A. Wang, A. Padula, M. Sirota and T.J. Woodruff, Environmental influences on reproductive health: the importance of chemical exposures. Fertility and Sterility 106 (2016) 905-929.

[30]. M. Vrijheid, M. Casas, M. Gascon, D. Valvi and M. Nieuwenhuijsen, Environmental pollutants and child health—A review of recent concerns, International Journal of Hygiene and Environmental Health, 219 (2016) 331–342.

[31]. M. Quintana, B. Vera, G. Magnarelli, N. Guiñazú, and G. Rovedatti, Neonatal, placental, and umbilical cord blood parameters in pregnant women residing in areas with intensive pesticide application, Environmental Science and Pollution Research, 24 (2017) 20736-20746.

[32]. D.B. Barr, C.V. Ananth, X. Yan, S. Lashley, J.C. Smulian, T.A. Ledoux, P. Hore and M.G. Robson. Pesticide concentrations in maternal and umbilical cord sera and their relation to birth outcomes in a population of pregnant women and newborns in New Jersey. Science of the Total Environment 408 (2010) 790–795.

[33]. K.H. Al-Gubory, P.A. Fowler and C. Garrel. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. The International Journal of Biochemistry & Cell Biology 42 (2010) 1634–1650

[34]. F. Deeba, I. Raza, N. Muhammad, H. Rahman, Z. Rehman, A. Azizullah, and M. Daud, Chlorpyrifos and lambda cyhalothrin-induced oxidative stress in human erythrocytes: In vitro studies, Toxicology and Industrial Health, 33 (2016) 297-307.

[35]. F. Demir, F. Gökce, D. Durak, and Y. Kalender, Subacute chlorpyrifos-induced oxidative stress in rat erythrocytes and the protective effects of catechin and quercetin, Pesticides. Biochemistry. Physiology, 99 (2011) 77–81.

[36]. X. Yao, H. Min, and Z. Mei, Response of Superoxide Dismutase, Catalase, and ATPase Activity in Bacteria Exposed to Acetamiprid, Biomedical and Environmental Sciences, 19 (2006) 309-314.

[37]. I. Altuntas, N. Delibas, and R. Sutcu, The effects of organophosphate insecticide methidathion on lipid peroxidation and anti-oxidant enzymes in rat erythrocytes: role of vitamins E and C, Human Experimental Toxicology, 21(2002) 681-685.

[38]. Z. Jiao-jiao, W. Yi, X. Hai-yang, L. Meng-xue, L. Wen-hao, M. Kai-ge, and W. Xian-zhong, Oxidative Stress: Role in Acetamiprid-Induced Impairment of the Male Mice Reproductive System, Agricultural Sciences in China, 10 (2011) 786–796.

[39]. M Clemens, and H. Waller, Lipid peroxidation in erythrocytes., Chemistry and Physics of Lipids, 45(1987), 251–268.

[40]. S.F. Ambali, J.O. Ayo, S.A. Ojo, and K.A.N. Esievo, Vitamin E protects Wistar rats from chlorpyrifos-induced increase in erythrocyte osmotic fragility, Food Chemistry. Toxicology, 48 (2010) 3477–3480.

[41]. R.E. Serrani, D. Alonso, and J.L. Corch, States of stability/lysis in human fetal and adults red blood cells. Arch Int Physiol Biochim, 97 (1989) 309-316.

Figure Legends

Figure 1: Reduction of NBT (%) in umbilical red blood cells treated with Chlorpyrifos (A) and Acetamiprid (B) for 3 h. Red blood cells were treated with 4, 40 and 400 nM of pesticide or diluent DMSO 0.02% (CONTROL). Data are expressed as percent reduction of NBT relative to the control group. The mean ± SD of three independent experiments performed in triplicate is shown. ANOVA-Tukey. ** p <0.01; *** p <0.001, **** p <0.0001.

Figure 2: Enzymatic antioxidant defense system in umbilical red blood cells treated with Chlorpyrifos (A, B) and Acetamiprid (C, D) for 3 h. Red blood cells were treated with 4, 40 and 400 nM of pesticide or diluent DMSO 0.02% (CONTROL). catalase (CAT) activity is expressed as percentage of activity (µmol/min x mg protein) , superoxide dismutase (SOD) activity, is expressed as percentage of activity (USOD/min x mg protein). Difference among groups was evaluated by Anova Test followed by Dunnet `s Multiple Comparison Test. *p = 0.01. The mean ± SD of three independent experiments performed in triplicate is shown.

Figure 3: 4-hydroxy-2-nonenal (HNE)-modified proteins in umbilical red blood cells treated with Chlorpyrifos (A) and Acetamiprid (B) for 3 h. Red blood cells were treated with 4, 40 and 400 nM of pesticide or diluent DMSO 0.02% (CONTROL). HNE modified proteins were determined by western blot. Equal amounts of protein (50 µg) were tested. Results shown are representative of 3 independent experiments. Band relative intensity was estimated by standardization with densitometry analysis (Gel Pro Analyzer 3.2 program). Difference between groups was evaluated with one-way ANOVA followed by Dunnet `s multiple comparisons test. *p = 0.015, **** p <0.0001.

Figure 4: Osmotic fragility of umbilical red blood cells treated with Chlorpyrifos (A, B) and Acetamiprid (C, D) for 3 h. Red blood cells were treated with pesticide (4, 40 and 400 nM) or diluent DMSO 0.02% (CONTROL). Distribution of NaCl (g/L) concentration for 45% (A and C) and 50% (B and D) Box diagrams show the median and 25-75 percentiles. The mean ± SD of three independent experiments performed in duplicate is shown. Error bars represent the 10 and 90 percentiles. Differences between groups were assessed by ANOVA followed by a Dunnet multiple comparison test.

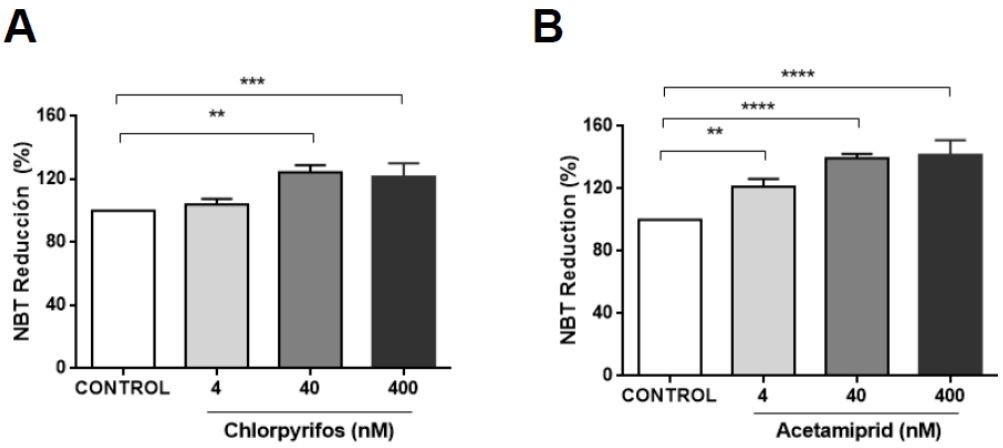


Figure 1

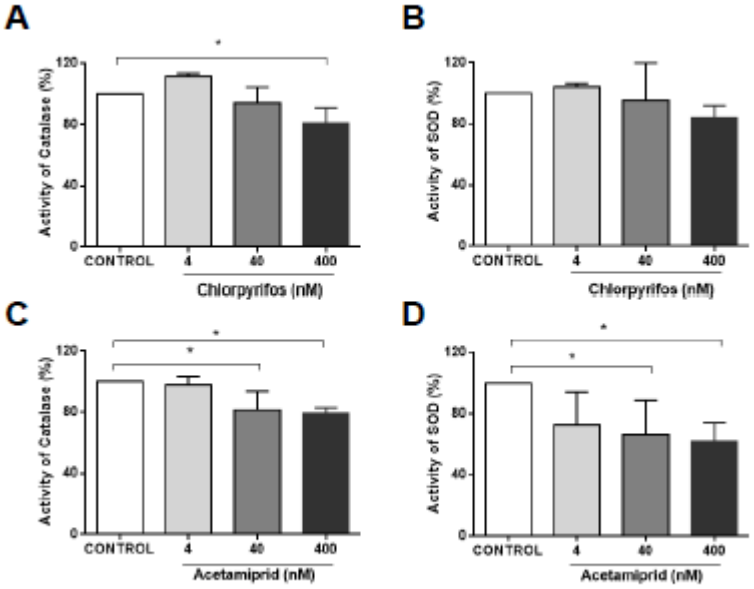


Figure 2

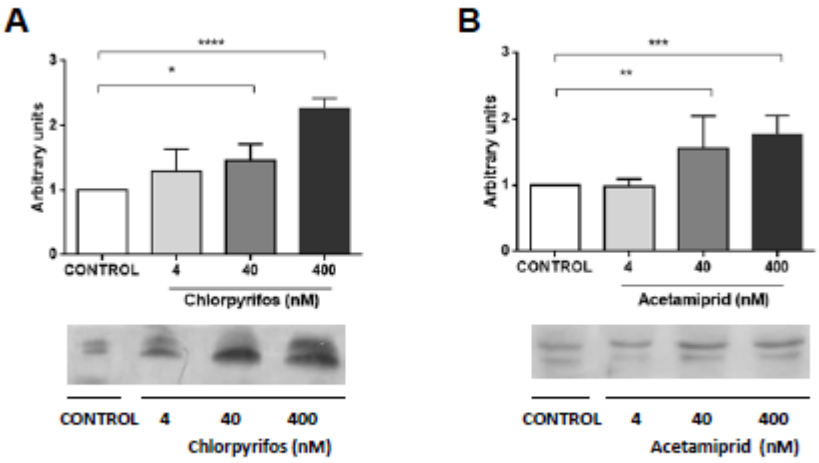


Figure 3

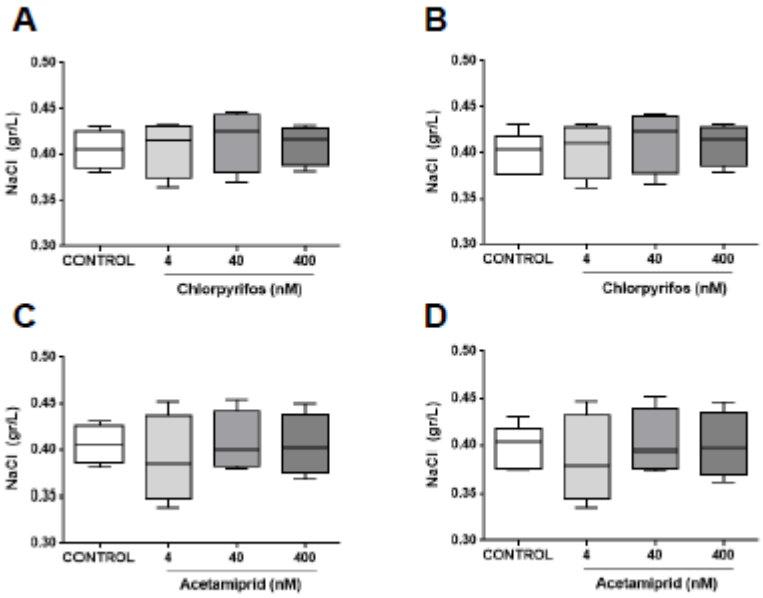
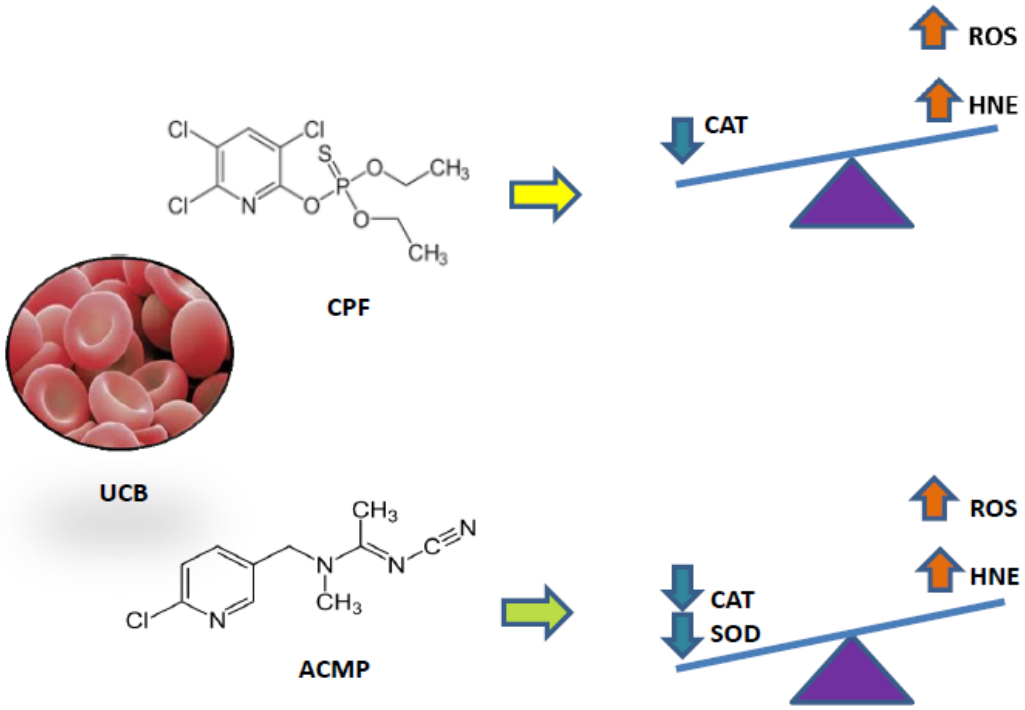


Figure 4



Graphical abstract

Highlights

- Insecticides increased ROS production by umbilical cord blood erythrocytes
- Insecticides altered anti-oxidant catalase and superoxide dismutase activities
- Insecticides augmented HNE lipid peroxidation products
- Insecticides did not alter erythrocyte osmotic fragility