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Pesticide exposure and genetic variation in xenobiotic-metabolizing enzymes interact to induce biochemical liver damage

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ABSTRACT

Metabolic activation of pesticides in the liver may result in highly reactive intermediates capable of impairing various cellular functions. Nevertheless, the knowledge about the effect of pesticide exposure on liver function is still limited. This study assessed whether exposure to pesticides elicits early biochemical changes in biomarkers of liver function and looked for potential gene-environmental interactions between pesticide exposure and polymorphisms of pesticide-metabolizing genes. A longitudinal study was conducted in farm-workers from Andalusia (South Spain), during two periods of the same crop season with different degree of pesticide exposure. Blood samples were taken for the measurement of serum and erythrocyte cholinesterase activities as well as for determining clinical chemistry parameters as biomarkers of liver function. Serum lipid levels were also measured as they may help to monitor the progress of toxic liver damage. A reduction in serum cholinesterase was associated with decreased levels of all clinical chemistry parameters studied except HDL-cholesterol. Conversely, a decreased erythrocyte cholinesterase (indicating long-term pesticide exposure) was associated with increased levels of aspartate aminotransferase and alkaline phosphatase and increased levels of triglycerides, total cholesterol and LDL-cholesterol, but reduced levels of HDL-cholesterol. Changes in liver biomarkers were particularly associated with the $PON1_{55M/192R}$ haplotype. The obtained results therefore support the hypothesis that pesticide exposure results in subtle biochemical liver toxicity and highlight the role of genetic polymorphisms in pesticide-metabolizing enzymes as biomarkers of susceptibility for developing adverse health effects.

1. Introduction

Pesticides are widely used to enhance food production in agricultural practice and, to a lesser extent, to control unwanted pests and disease vectors in public health. *N*-methylcarbamates (NMC), pyrethroids (PYR), dithiocarbamates, neonicotinoids and organophosphates (OPs) are among the pesticides most commonly used in plastic-covered greenhouses widely spread in Andalusia (South Spain) for intensive vegetable production (Hernández et al., 2003). Furthermore, NMC, OPs, PYR and neonicotinoids are, in this order, the compounds more often involved in acute pesticide poisoning in the same area and remain an important source of occupational intoxications (Hernández et al., 2010). Most of these pesticides undergo phase I reactions, rendering highly reactive molecules that may further interact with relevant molecular targets such as enzymes, nucleic acids and membrane phospholipids leading to cytotoxic changes, genotoxicity and cell necrosis, respectively (Milatovic et al., 2006; Androutsopoulos et al., 2013). Given that these metabolic reactions occur primarily in the liver microsomes, early hepatotoxicity at a biochemical level may be expected as one of the earliest toxic effects of pesticides.

The liver plays a key role in the maintenance of the homeostasis of the organism. Conventional liver test provide information about the integrity of hepatocytes, such as serum transaminases (alanine –ALT– and aspartate –AST– aminotransferases), with ALT being considered as the gold standard clinical chemistry marker of liver injury. The integrity of the biliary system is commonly assessed by measuring gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) (Ozer et al., 2008). Chronic overproduction of

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reactive species induces a redox imbalance leading to increased GGT, which has also been considered as an early marker of oxidative stress in humans (Sedda et al., 2008). Given that *de novo* synthesis of triglycerides (TG) and their incorporation into lipoprotein particles takes place in the liver, TG might be useful for monitoring the progress of toxic damage (Provost et al., 2003). However, so far no thorough study has assessed the usefulness of TG as a marker of liver dysfunction in humans.

Significant increases in serum biochemical indices, including ALT, AST, ALP, total cholesterol, TG, low density lipoprotein cholesterol (LDL-c) and lipid peroxidation by-products have been observed experimentally after subchronic or chronic exposure to OPs (Binukumar et al., 2010; El-Gharieb et al., 2010; Kalender et al., 2005, 2010; Rezg et al., 2008). A dose-dependent increase in total serum lipids concentration has also been observed in rats treated with atrazine (Santa Maria et al., 1987).

The enzymes primarily involved in pesticide metabolism include cytochrome P450s (CYP450s), paraoxonase-1 (PON1) and glutathione S-transferases (GSTs), although the profile of the participating isoforms is different for each agent (Hernández et al., 2008). Serum cholinesterase (BChE) and carboxylesterases can also inactivate OPs and NMC acting as stoichiometric scavengers. The marked inter-individual variation in expression of these enzymes can largely influence pesticide toxicity by increasing or decreasing the sensitivity to certain compounds. Hence, particular genotypes (or phenotypes) can be used as biomarkers of susceptibility for the detection of subgroups of individuals at an increased risk of adverse health effects.

The aim of this study was to assess whether exposure to pesticides in an intensive agriculture setting may lead to changes in liver function parameters. Secondly, to ascertain whether pesticide exposure interacts with genetic polymorphisms of pesticide-metabolizing enzymes resulting in alterations of the clinical chemistry parameters studied, by using new and non-conventional statistical analysis integrating data from exposure at two different time-periods.

2. Materials and methods

2.1. Study population

A longitudinal study was conducted on greenhouse workers from Almeria (a Southeastern province of Spain) during two periods of the same crop season. In the high exposure period (October–November) large quantities of pesticides were sprayed and in the low exposure period (April–May) pesticides were less heavily sprayed. A comparative group of non-exposed subjects was selected and assessed at the same periods. Both groups of individuals were recruited from the same villages in order to eliminate differential biases related to socio-economic status and background exposure to pesticide residues. The non-exposed group was specifically interviewed to ascertaining the lack of potential exposure to either pesticides or any other industrial chemical that could modify any of the enzyme activities assessed.

A total of 190 individuals agreed to participate in the study, 135 of them being greenhouse workers (14–59 years-old) and 55 acted as non-exposed controls (aged 23–55). All individuals were identified through occupational physicians involved in their health surveillance. However, only 118 subjects provided two blood samples during the course of the crop season; of the subjects, 81 were pesticide applicators and 37 age-matched healthy controls. The primary reason of dropping from the follow-up was change of work. Individuals presenting any type of chronic disease (e.g., liver dysfunction, diabetes, renal failure, cancer) were excluded from the study to avoid any interference with the biochemical parameters measured. Subjects taking any medication on a long-term basis were also excluded. Exposed subjects performed different activities within greenhouses, including the application of pesticides. Pesticide categories more often used were NMC, PYR, dithiocarbamates, neonicotinoids and OPs.

2.2. Collection of information

The study population completed a structured questionnaire containing questions on sociodemographic characteristics (age, gender), anthropometric measures (weight and height), lifestyle (alcohol and smoking habits) and occupational features (lifetime exposure to pesticides, use of personal protective equipment and type of pesticide used). The application of questionnaires and collection of blood samples were done by nursing personnel specially trained and standardized for this purpose. They did not know the objectives of the study. All the individuals gave informed and written consent after explaining the procedures and main objectives of the study and they were also informed of their right to withdraw at any time throughout the study after which each subject participated voluntarily. The study was approved by the Ethics Committee of the University of Granada.

2.3. Samples collection

Blood samples were taken in fasting conditions by the nursing personnel using heparinized Vacutainer® tubes and Vacutainer® serum tubes. Samples were centrifuged at 2500 rpm for 20 min to separate serum and erythrocyte package. Erythrocytes were washed twice in NaCl 0.9% and diluted in an equal volume of saline. Serum and erythrocytes aliquots were stored frozen at −20 °C until analysis, not longer than 1 month thereafter.

2.4. DNA extraction and genotyping

Genomic DNA was extracted from the buffy coat layer of blood samples using a standard phenol–chloroform extraction protocol and purified DNAs were stored at −20 °C until analysis. PON1 and GST polymorphisms were determined by polymerase chain reaction amplification followed by polymorphism specific restriction digestion (PON1 genotypes) or allele specific oligonucleotide probe ligation (GST polymorphisms). The resulting fragments were separated by gel electrophoresis analysis and identified by visualization of the band pattern following the procedure described elsewhere (Hernández et al., 2003 and Hernández et al., 2005).

2.5. Enzyme activities and lipid profile in serum

BChE and AChE activities were determined by the method of Ellman et al. (1961) as previously described (Hernández et al., 2005). Exposure to pesticides was characterized by distinguishing short-term and long-term exposures based on depression of BChE and AChE, respectively. BCHE variants were determined by measuring the inhibition of benzoylcholine hydrolysis with dibucaine and fluoride at 240 nm (Whittaker, 1984). Serum enzymes reporters of liver function (AST, ALT, GGT and ALP), often referred to as liver function test, were determined by colorimetric assays using a Hitachi 747 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Lipid parameters, such as triglycerides (TG), total cholesterol, low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) were measured using the same instrument following routine and automatized procedures. All analyses were performed in certified clinical laboratories.

2.6. Statistical analysis

Because the distribution of some liver function test and serum lipid parameters was skewed to the right and did not fulfill normality criteria (Kolmogorov–Smirnov test) they were log-transformed for further statistical analysis. The significance of the differences in the mean values of raw clinical chemistry parameters was tested by the Student's *t*-test or the non-parametric Mann–Whitney test in the case of a non-normal distribution. Differences in lifestyle (tobacco and alcohol consumption) between the control and exposed groups were studied by the χ^2 test. Geometric means (GM) of paired data in the two crop season periods studied were compared by the paired Student's *t*-test and the Wilcoxon signed-rank test, depending on whether or not their distribution fulfilled normality criteria. Generalized estimating equation (GEE) models were developed to evaluate the association between indicators of pesticide exposure (serum and erythrocyte cholinesterases, crude exposure as a binary variable and accumulated lifetime exposure in years) and genetic polymorphisms of pesticide-metabolizing enzymes with serum biomarkers of hepatic function and serum lipid parameters These models extend generalized linear models for the situation of correlated data (as is the case of our study, where the population was assessed at two different time-points).

Interaction effects of BChE and AChE activities with genetic polymorphisms on liver function test and serum lipid parameters were estimated by means of GEE. Potential confounding variables, including age, body mass index (BMI), tobacco and alcohol consumption (with both being considered as dichotomous covariates) were included in the adjusted models based on their biological plausibility and on current scientific literature. Significant associations were considered when the *p* value was <0.10. All the statistical analyses were conducted with Stata version 8.2 (StataCorp LP. Texas) and SPSS version 15.0 (SPSS Inc. Chicago, IL).

3. Results

General characteristics of the study population are described in Table 1. A comparison of levels of cholinesterases, liver function test and serum lipid profile between the exposed population and