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Does diazinon-sprayed market melon alter cholinesterase activity in healthy consumers? A randomized control trial

M. NEMATY[1], A. TASHAKORI-BEHESTI[1], B. MEGARBANE[2], M. BAKAIYAN[3], M. HABIBI[3], R. AFSHARI[4]

[1]Nutrition Research Centre, Mashhad University of Medical Sciences, Mashhad, Iran
[2]Department of Toxicological and Medical Intensive Care Unit, Lariboisière Hospital, INSERM U1144, Paris-Diderot University, Paris, France
[3]Medical Toxicology Research Centre, Medical Toxicology Centre, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Addiction Research Centre, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract. – OBJECTIVE: Food contributes in measurable body burden of the widely used organophosphate pesticides. We designed a randomized controlled open label trial in Mashhad University Hospital in Iran, to study the possible alterations in cholinesterase activity resulting from consuming market melon known to be exposed to diazinon.

PATIENTS AND METHODS: Fifty-three young healthy volunteers were recruited. Participants were randomized to consume 250 g per day of organic (N = 22) vs. market melon (N = 31) during fifteen days. The primary outcome was the variation of red blood-cell (RBC) cholinesterase activity between day 15 (after) and day 0 (prior the intervention). The secondary outcome was a variation of the plasma cholinesterase activity between both dates.

RESULTS: Baseline RBC [5.21 ± 0.93 vs. 5.53 ± 0.99 IU/mL, mean ± SD] and plasma cholinesterase activities [54.0 ± 8.1 vs. 57.4 ± 8.6%] did not significantly differ between organic and market melon-exposed participants, respectively. RBC [5.86 ± 1.27 vs. 5.11 ± 1.2 IU/mL] and plasma cholinesterase activities [58.7 ± 10.0 vs. 50.5 ± 13.0%] significantly increased in organic melon-exposed vs. market melon-exposed participants ($p = 0.002$ and $p = 0.001$, respectively).

CONCLUSIONS: RBC and plasma cholinesterase activities significantly improved after eating organic instead of market melon during fifteen days. However, the consequences on the health of the observed cholinesterase alterations attributed to diazinon dietary intake remain to be determined.

Key Words:
Cholinesterase, Contamination, Diazinon, Food, Organophosphate, Randomized controlled trial.

Introduction

Air, dust, water, soil, as well as food contribute in measurable body burden of organophosphate (OP) pesticides that are widely used in agriculture[1]. Although debated, dietary exposure from the ingestion of contaminated food is considered to represent one of the major sources of pesticide exposure in humans, especially in children, despite likely additional environmental exposure[2-8]. Interestingly, changing conventional fruits and vegetables in food to organic items has been shown to significantly reduce urinary malathion and chlorpyrifos as well as their metabolites to close to non-detectable levels[9].

Exposure to well-characterized high-doses of OP compounds is responsible for the inhibition of esterase enzymes, especially acetylcholinesterase in synapses and on red blood-cell (RBC) membranes, and butyrylcholinesterase in plasma[10]. Rising acetylcholine concentration at the muscarinic and nicotinic cholinergic receptors in synapses results in the well-known acute cholinergic crisis including miosis, sweating, rhinorrhea, lachrymation, salivation, involuntary defecation urination, confusion, ataxia, seizures, acute respiratory insufficiency, muscle fasciculation and paralysis[10,11].

Exposure to OP at doses too low to produce cholinergic signs has been also shown to produce significant clinical effects. A variety of features ranging from enhanced maze learning to slowed nerve conduction as well as alterations in immune cell functions have been assessed in animal studies[12-14]. In humans, chronic low level exposure to

Corresponding Author: Reza Afshari, MD; e-mail: AfshariR@mums.ac.ir

OP in non-poisoned subjects has been associated with impaired neurobehavioral and immune functions in some cohort studies. Increased risk of asthma, attention-deficit/hyperactivity disorder, delayed polyneuropathy, and neurobehavioral deficits in school-age children have been attributed to direct beverage and food contamination or even prenatal exposure to OP[15-21].

Diazinon (o,o-diethyl-o-[2-isopropyl-6-methyl-4-pyrimidinyl]phosphorothioate) is an OP widely used for controlling insects in different kinds of agriculture products including crops, ornamentals, lawn, fruits, and vegetables[22]. It is used in melon production in Iran for protection against *Myiopardalis pardalin*, also known as melon fly. However, the growing use of diazinon has been found to contribute to an increasing number of OP poisoning[23]. Due to recent national concerns about the possible involvement of dietary exposure in the elevated number of mild non-occupational OP poisonings in Iran, we designed a study to assess the biological consequences in healthy humans of consuming market melon, known to be sprayed in Mashhad region with diazinon[24].

Patients and Methods

Study design

We conducted a randomized controlled open label trial in Mashhad University Hospital in Iran, from 2010-06-01 to 2010-12-01.

Eligibility Criteria

Young healthy volunteers were recruited among the medical students who were living at the university campus during summer holidays. Participants were rather fresh fruit-deprived due to limited market accessibility and familial care. Prior to recruitment, all participants were examined for potential fruit hypersensitivity and chronic diseases. A written informed consent was obtained from all of them.

Intervention

Participants were randomly divided into two groups, instructed to eat 250 g per day during two weeks of either organic melon or market melon. During the study period, food was provided by the University. All participants were asked to take no other fresh fruits and vegetables. They were allowed to wash their fruits as they usually did. Melon obtained from local markets had a background of diazinon exposure, previously estimated to correspond to a mean rest concentration of 107.64 ± 38.50 ng/kg[24]. By contrast, organic melon was ordered to be cultivated without exposure to any pesticide. The quantity of 250 g melon was calculated to be per serving each day[25]. Following participation in the study, nutrition advises were given to participants to adequately improve their fruit intake. Additional recommendations to wash fruits and vegetables before eating were provided to reduce pesticide exposure.

Randomization and Sample Size Determination

The method used to generate the random allocation (2:3) was based on a random-number table. Allocation sequence was generated by following the numbers from the random-number table. Choosing a type-1 error equal to 5% and a power equal to 80%, the sample size was calculated to detect a difference of one point between both study groups, assuming that the standard deviation was 1.25. Considering a multiple correlations close to 0.25 we needed to recruit a total of 50 subjects.

Outcome and Measurements

Traditional chromatographic methods are effective for the analysis of OP pesticides in the environment; however, they have significant limitations and prevent adequate monitoring[26]. Enzymatic methods including acetylcholinesterase and butyrylcholinesterase, have been promoted as possible alternatives to detect OP pesticides. The enzymatic methods are based on the activation or inhibition of the enzyme by the OP pesticide proportionally to its concentration. The primary outcome was the variation of RBC cholinesterase activity between day 15 (after) and day 0 (prior the intervention). Secondary outcomes included the variation of plasma cholinesterase activity between day 15 and day 0 as well as occurrence of alimentary intolerance and toxic symptoms that could be attributed to OP.

Blood samples were obtained in heparinized tubes in both groups at recruitment (day 0) and at the end of the study (day 15). Samples were immediately transferred on ice to the laboratory for measurements. RBC cholinesterase activity (normal range $\geq$4.2 IU/mL of erythrocyte) was measured by Ellman's method[27,28], using spectrophotometry (Perkin-Elmer Inc., Waltham, MA, USA). The quality assurance/control of cholinesterase analyzes was performed using acetyl cholinesterase Merck standard solutions on weekly intervals. Plasma cholinesterase activity (normal range $\geq$

Table I. Baseline demographics, red blood cell (RBC) and plasma cholinesterase activities in the two study groups.

Variables	Organic Melon group (N = 22)	Market Melon group (N = 31)
Age (years)	22.8 ± 1.4 (21; 27)	23.5 ± 3.0 (18; 34)
Gender (M/F), N (%)	12 (54.5%) / 10 (45.5%)	15 (48.4%)/16 (51.6%)
RBC cholinesterase (IU/mL)	5.21 ± 0.93 (3.25; 7.76)	5.53 ± 0.99 (4.04; 7.38)
Plasma cholinesterase (%)	54.0 ± 8.1 (35.0; 67.0)	57.4 ± 8.6 (43.0; 76.0)

40%) was measured with titrimetry as follows: 10 mL of a pre-prepared acetylcholine chloride solution consisting of 1 g acetylcholine chloride powder (Sigma-Aldrich Co., Iran) diluted in 545 mL water and 5 mL 10% acetic acid was added to 0.5 mL of the subject's serum as well as 2 drops of Cresol Red indicator (Sigma-Aldrich Co., Iran). This solution was then titrated with 0.1 N NaOH until it becomes purple. The result was kept in bain-marie at 37°C. Color change to yellow was checked every 10 min. If the solution became yellow, titration was repeated with 0.01 N NaOH.

To document the occurrence of any alimentary intolerance or toxic symptom, a predesigned questionnaire was developed and completed prior and after the intervention.

Ethics

This trial was registered in Mashhad University of Medical Sciences and approved by our University ethics committee (No.87133). The procedures followed were in accordance with the Declaration of Helsinki 1975, revised Hong Kong 1989. The trial was registered on the Iranian clinical trial website (IRCT138902151187N4, http://www.irct.ir/searchen.php?keyword=diazinon&field=g&lang=en) as well as on the World Health Organization international clinical trials registry platform (http://apps.who.int/trialsearch/trial.aspx?trialid=IRCT138902151187N4).

Statistical Analysis

Data are presented as mean ± SD (minimum-maximum). Comparisons were performed using Chi-2 for qualitative variables and Student's *t*-test for quantitative variables. The primary and secondary endpoints were compared between groups using an ANCOVA analysis, adjusted for sex and age. The difference of Lease square means between groups and their corresponding 95%-confidence intervals were given. P-values less than 5% were considered as significant. All statistical analyses were performed using SAS version 9.3.

Results

Fifty-three healthy volunteers [51% male; age: 23.3 ± 2.3 years (18.0-34.0)] were recruited in this study, twenty-two in the organic melon and thirty-one in the market melon group. Baseline values of demographics, RBC and plasma cholinesterase activities are presented in Table I, showing no significant differences between the two study groups.

After the intervention and based on our ANCOVA analysis, both RBC ($p = 0.002$) and plasma cholinesterase activities ($p = 0.001$) were significantly more elevated in the organic vs. the market melon group (Table II). During the study, no participant in either group complained of alimentary intolerance or toxic symptoms.

Discussion

We demonstrated that eating organic melons for two weeks while reducing other sources of OP pesticide exposure, significantly increases RBC and plasma cholinesterase activity in comparison

Table II. Comparison of red blood cell (RBC) and plasma cholinesterase activities after the intervention between the two study groups.

Variables	Organic melon group (N = 22)	Market melon group (N = 31)	least square means difference [95%-confidence intervals]	*p*-value
RBC cholinesterase (IU/mL)	5.86 ± 1.27	5.11 ± 1.2	-1.00 [-1.63;-0.38]	0.002
Plasma cholinesterase (%)	58.7 ± 10.0	50.5 ± 13.0	-10.6 [-16.8;-4.5]	0.001

to market melons, known to be contaminated by diazinon, as previously demonstrated[24].

Cholinesterase activity is the usual biomarker of OP exposure and toxicity[10,11]. RBC acetylcholinesterase is a good marker of synaptic function and therefore of poisoning severity[10,29]. Patients with RBC acetylcholinesterease activity of at least 30% have normal muscular function and do not need any treatment including atropine. Patients with less than 10% activity have deranged muscle function, and require treatment. Patients with activity among both values present moderate muscular impairment and need for atropine. In contrast, plasma cholinesterase activity, which corresponds to the butyrylcholinesterase activity, is not related to poisoning severity; however, it serves as sensitive marker of exposure to OP[10] and may be more effectively inhibited by some OP pesticides in comparison to RBC cholinesterase[30]. After OP's elimination from the body, butyrylcholinesterases which are produced by the liver, recover by about 7% of normal each day, while once RBC acetylcholinesterases have aged, recovery only occurs via erythropoiesis[10]. The regeneration rate of RBC cholinesterase activity is, therefore, slower at about 1% per day than plasma cholinesterase one.

In all our participants, the level of cholinesterase activity was within the normal range and alterations far from any possible acute clinical consequences. However, we observed deterioration in both RBC and plasma cholinesterase activities in the participants who had consumed market melon during two weeks. A retrospective questioning of participants, who were rather fresh fruit-deprived students in terms of market accessibility and familial care, revealed that 250 g melon was actually above their total daily fruits intake, thus possibly contributing to the decrease in post-intervention cholinesterase activities.

As suggested by our study, dietary intake of OP pesticides represents one major source of human exposure[3,5,6,8]. Although no immediate clinical consequences occurred with this 14-day exposure to market melon, our study does not rule out a risk of significant consequences with long-term OP dietary exposure in market fruit consumers. Only rare studies have attempted to evaluate dietary exposure to OP[2-8]. Despite their wide use[1], the exact impact of OP on long-term health remains unknown. Several pathologies were attributed to OP exposure including topical irritant reactions, asthma, neurodevelopment impairments, reproductive dysfunction, and even death[15-21].

Our findings assessing that intake of market fruits even for a short period may sub-clinically affect biological markers challenge the currently accepted daily intake and maximum residue levels[31]. In the European Union, 3.0-5.5% of food samples contain levels of pesticide residues above the maximum residue levels, while 32-42% contain detectable residues below the limit, and no residues are detectable in 53-64%[32]. When assessing cumulative risk, managers have to consider what level of risk would be considered as "acceptable"[33]. Moreover, setting guidelines is more complicated when potential risks of combined exposures to multiple residues from pesticides in the diet are considered[33,34]. In the developing countries, this is challenging as food contamination with OP is even more important since law enforcement mechanisms are weaker, application rates exceeding manufacturers' recommendations and disregard of recommended pre-harvest intervals[35]. Consistently, more marked dietary OP exposures have been reported with imported products from the developing countries[36]. In contrast, in the US, a large number of "older pesticides" have been banned from the market and safer pesticide products registered as "reduced risk" or "biologicals" developed[34]. Since the implementation of the Food Quality Protection Act signed into law in 1996, regulatory efforts to reduce OP exposure have been effective, as assessed by the decrease in urinary concentrations of dialkyl-phosphate metabolites of OP pesticides in a sample representative of the US population[37].

Adopting an organic diet appears an obvious solution for reducing dietary OP exposure, as supported by biomonitoring studies[22,38-40]. There is a widespread belief that organic agriculture products are safer and healthier than conventional foods. Organic fruits and vegetables can be expected to contain fewer agrochemical residues than conventionally grown alternatives. However, it is difficult to come to conclusions. What should be made clear to the consumers is that "organic" does not automatically equal "safe". Additional research with adequate comparative data is still required.

Our study has several limitations. Our study was conducted among young fresh fruit-deprived volunteers. However, we do not believe that the study subjects would have been different from the general population, in terms of their susceptibility to OP effect on RBC and plasma cholinesterase. We did not measure the residual level of diazinon in the market melons. However, previous measurements performed on market melon in Mashhad

clearly demonstrated its contamination by diazinon[24], while no governmental intervention restricted OP use in melon culture in Iran. We did not measure the dialkyl-phosphate metabolites of diazinon in the urine of our study participants. However, biological monitoring of occupational exposure to diazinon was shown to be possible by either the determination of RBC cholinesterase activity or the measurement of diazinon metabolites in urine[22], despite the scarce published data on relationships between both measurements.

Conclusions

RBC and plasma cholinesterase activity significantly improves in healthy consumers after eating organic instead of market melon during two weeks. Our data support the necessity of reinforcement of public health controls of OP-contaminated market fruits and vegetables to limit possible short and long-term consequences on health. Investigations are still required to better characterize dietary-related sources of OP contamination in Mashhad region.

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Conflicts of interest

The authors declare no conflicts of interest.

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