THE EFFECT OF SUBLETHAL SHORT-DURATION EXPOSURE OF PARAOXON ON PREGNANCY AND FETUSES IN MICE

Syed M. Nurulain¹,*, Mohamed Shafiullah¹, Charu Sharma², Mahmoud A. Ali¹ and Shreesh Ojha¹

¹ Department of Pharmacology and Therapeutics, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, Abu Dhabi, UAE
² Department of Internal Medicine, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, Abu Dhabi, UAE

*Corresponding author: nurulain@uaeu.ac.ae

Abstract: A convincing number of epidemiological studies have reported on the exposure to and consequences of organophosphorus compounds (OPCs) in pregnant women. However, there is still a knowledge gap and paucity of systematic literature from animal studies. This study was undertaken with the hypothesis that short-duration sublethal exposure to OPCs can produce maternal and fetal lethal effects as chronic exposure. This study examines the teratogenicity and embryotoxicity of paraoxon (POX) in mice at a dose that is non-lethal to non-pregnant mothers. Pregnant mice were injected intraperitoneally (i.p.) with paraoxon (50 nmol/mouse) on the 4th and 5th days of gestation, and the effect of the treatment was assessed on day 18 of gestation. This dose was fatal to pregnant mice in 21% of instances as compared to non-pregnant animals in which 0% mortality was detected, even after daily injection of a similar dose for five days. Significant inhibition of red blood cell acetylcholinesterase (RBC-AChE) was observed in pregnant mice as compared to non-pregnant ones; however, no apparent neuronal effect was detected. Of note were fetal weight decrement, pregnancy termination, intrauterine growth retardation and maternal death. We concluded that exposure to even a non-toxic dose might be critical for pregnant mothers, the pregnancy as well as fetuses. In addition, even exposure of short duration can be detrimental and capable of producing profound and fatal effects.

Key words: Intrauterine growth restriction (IUGR); gestation day (GD); RBC-AChE; organophosphorous compound (OPC); paraoxon (POX).

Received November 14, 2014; Revised November 29, 2014; Accepted December 1, 2014

INTRODUCTION

Over the last 100 years, the use of organophosphorus compounds (OPCs) has dramatically increased with new applications still being developed. In agriculture, the environmental exposure of OPCs poses a risk of inadvertent toxicity through contamination of food or water. Studies have shown an increased exposure to pesticides by women and children and suggest an association between environmental exposure to certain agricultural pesticides such as OP, and adverse reproductive outcomes in peoples living near farms (Peiris-Johna and Wickremasinghe, 2008). Elevated risks of neural tube defects (NTDs) and anencephaly or spina bifida were also associated with exposures to different chemicals groups including OP pesticides. (Rull et al., 2006; Stemp-Morlock, 2007). The exposure of rodent dams during pregnancy to certain OP pesticides such as chlorpyrifos (Chanda et al., 1995; Muto et al., 1992) and dimethoate (Srivastava and Raizada, 1996) has been associated with a reduction in fetal weight in some studies. However, some studies (Institoris et al., 1995, Clemens et al., 1990; Spyker and Avery, 1977) have shown no association with fetal growth. There have been conflicting results in the literature regarding fetal and embryo toxicity of OPCs (Nurulain and Shafiullah, 2012). For example, OPs such as diazinon, malathion and dichlorvos induce maternal toxicity
but did not show teratogenicity (Vogin et al., 1971; Talens and Wooley, 1973). Teratogenicity produced by parathion in quail was reported by Meiniel et al. (1975, 1976) in successive studies. No similar literature could be retrieved on parathion and this demonstrates that there is a big gap on evidence based scientific knowledge. Similarly, dipterex has been shown to cause teratogenic effect at high concentration (Staple et al., 1976) and acephate was found to cause developmental toxicity at maternal toxic dose to mice (Farag et al., 2000). Chung et al., 2002 reported that fluapyrazofos causes fetal growth retardation at maternal toxic doses in rats. Ambali et al. (2010) reported that chlorpyrifos altered the conception pattern and caused pre-implantation losses in a dose-dependent manner in mice. Other likely functions associated to AChE (a biomarker enzyme of toxicity for OP poisoning), particularly with development, is the stimulation of neurite outgrowth and neural development (Sperling et al., 2012), adhesion (Paraoanu and Layer, 2008), regulation of cell differentiation (Zhang et al., 2002) and apoptosis (Landgraf et al., 2010).

Exposure to chemicals during different stages of development such as pre- and periconception, fetal development has a different impact on the health of mother and offspring. Numerous animal studies have shown that in utero or early exposure to OP pesticides affects neurodevelopment (Eskenazi et al., 1999). In the present study, we investigated the effect of a non-toxic dose of paraoxon (POX) on non-pregnant mice and pregnant mice during the peri-implantation period. Chronic exposure is considered to be a cause of teratogenicity, but we hypothesized that a small dose and short duration exposure is sufficient to produce deleterious effects very similar to those of chronic exposure.

**MATERIALS AND METHODS**

**Experimental animals**

The TO (Theiler outbred) mice used in this study were initially obtained from Harlan Olac (England) and maintained in an outbred colony in our local

---

*Fig. 1. Acetylcholinesterase activity in saline control and paraoxon-treated pregnant and non-pregnant mice. Blood was collected before treatment on GD 4 and 24 h after last treatment, i.e. GD 6. * statistically significant.*
animal facility. The mice were housed in standard animal facility conditions at 21±2°C, about 55% relative humidity and a 12 h light:dark cycle. Adult female mice weighing 25±4 g and about 6 weeks of age were mated with males in the evening. On the following morning, a vaginal plug served to identify successful mating. Plug-positive day was regarded as Day 0 of gestation. Throughout the study, animals of all groups had free access to a commercial chow diet and water ad libitum. The experiments on animals were performed in compliance with the relevant regulations and institutional guidelines, and the protocol was approved from the Animal Ethics Committee of CMHS, UAE University.

**RBC-AChE activity**

Blood samples were taken from the tail vein for RBC-AChE measurements on GD3 (pretreatment) and on the next day following the last treatment (GD6 post-treatment). A similar procedure was applied to a saline control group. The number of samples measured for enzyme activity was not equal to the number of animals treated for intrauterine growth restriction (IUGR) because in some cases sufficient blood could not be drawn. For comparison, blood from non-pregnant POX-treated mice was also collected at pretreatment and 24 h after the 2nd injection (day 3). The RBC-AChE activity was measured in diluted whole blood samples in the presence of the selective butyrylcholinesterase inhibitor ethoproprazine as described previously by Worek et al. (1999). The AChE activity was calculated using an absorption coefficient of TNB at 436 nm (ε = 10.6 mM cm⁻¹).

**Drug administration and fetus collection**

Paraoxon (POX) was purchased from Sigma-Aldrich Chemie (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). POX stock solution (10mM) was prepared in dry acetone. The working solution was prepared extempore by diluting stock solution with normal saline. Groups of mice were administered i.p. a single dose of 50 nmol POX per mouse daily from GD4 to GD5 (two injections). Dose was selected in a pilot study on virgin mice i.p. injected daily for five days with 50 nmol POX. Non-lethality of the dose was further substantiated by the RBC-AChE. The controls were injected with a proportionate volume of saline (i.p.). All pregnant mice were killed by cervical dislocation on GD18 and the embryos were collected. Implantation and resorption sites were noted. The fetuses were blotted dry, weighed, fixed in 95% ethanol and examined for gross and visceral malformations. Fetuses weighing 2 x less than the mean of the corresponding control group were regarded as IUGR. Fetus collection and storage was carried out following a previously described method (Padmanabhan et al., 2008).

<table>
<thead>
<tr>
<th></th>
<th>Untreated control</th>
<th>Saline control</th>
<th>POX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>7</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Maternal toxicity</td>
<td>0</td>
<td>0</td>
<td>4(21%)</td>
</tr>
<tr>
<td>Pregnancy termination</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>No. of Implantation</td>
<td>58</td>
<td>35</td>
<td>69</td>
</tr>
<tr>
<td>Resorption</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Alive</td>
<td>56</td>
<td>35</td>
<td>68</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.26±0.17</td>
<td>1.24±0.07</td>
<td>1.06±0.14</td>
</tr>
<tr>
<td>IUGR -2SD (%)</td>
<td>4%</td>
<td>3%</td>
<td>55%</td>
</tr>
<tr>
<td>IUGR -1SD (%)</td>
<td>9%</td>
<td>6%</td>
<td>25%</td>
</tr>
</tbody>
</table>
Statistical analysis

The litter was used as the statistical unit. The Mann-Whitney order rank test by SPSS software 21.0 was used to test the levels of significance between experimental and control groups. Significance was set at p ≤0.05.

RESULTS AND DISCUSSION

RBC-AChE activity

RBC-AChE activity in whole blood is presented in Fig. 1. The saline control group showed a 14.08% increase in enzyme activity (pretreatment 0.142±0.01; post-treatment 0.162±0.02 mU/µmol; n=5). However, this increase is not statistically significant. POX treatment of pregnant mice caused significant inhibition (59.23%; p ≤0.05) of RBC-AChE activity in comparison to the enzyme level of the same animals before treatment (pretreatment 0.157±0.01; post-treatment 0.064±0.01 mU/µmol Hb; n=9). Treated non-pregnant mice with the same two doses exhibited 14.41% inhibition (pretreatment 0.118±0.02; post-treatment 0.101±0.03 mU/µmol Hb; n=5). The data clearly demonstrates the enhanced effect of POX in pregnant mice.

Maternal effects

The data to evidence maternal effect are represented in Tables 1, 2, 3 and Fig. 2. The injected dose of 50 nmol/mouse was observed to be toxic to pregnant mice (21% mortality) after the second injection on GD 6. In nine out of the remaining 15 pregnant mice (60%), the pregnancy terminated. The total loss of pregnancy was found to be 4+9=13 out of 19 pregnant mice (58%). However, there was no pregnancy termination in the untreated control and saline control mice, and resorption was found to be 3.4% in the untreated control and 0% in the saline control.

Fetal effects

The data from the control and POX-treated pregnant mice are presented in Table 1. The average number of implantations and resorption was not significantly different between the control and POX-treated groups of mice. There were no dead fetuses in any experimental groups. The reduction in fetal weight was significant (P <0.05) in the POX-treated group in comparison to the controls. About 55% of the treatment group fetuses were found to be growth-restricted by -2sd, and 25% of the treatment group fetuses were found to be growth-restricted by -1sd. The descriptive analysis is presented in Tables 2 and 3.

The single non-toxic dose of POX given on GD4 and GD5 to pregnant mice caused significant maternal toxicity, pregnancy termination and intrauterine growth restriction in mice. A moderate inhibition of RBC-AChE was also observed. There are conflicting reports on the association of pesticide exposure, including OP, on developmental processes. Short-duration exposure studies have been rarely done and we could not retrieve any literature with the same treatment regime. However, the available animal data suggests that OP poisoning may cause severe teratogenicity and consequently termination of pregnancy following exposure in the first trimester (Kamanyire and Karalliedde, 2004). Weitman et al. (1983, 1986) found higher concentrations of paraoxon in pregnant mice than in virgin mice though a similar dose was
used in both groups. It is obvious that several physiological, endocrine and other biochemical changes occur during pregnancy that causes an altered maternal metabolism and physiology. This altered physiology may influence the lethality of poison as observed in our study. It has been reported that cholinergic syndromes appears at about 50% AChE inhibition, and death is believed to occur at more than 90% AChE inhibition (Morita et al., 1987). Ambali et al. (2010) injected 25% of LD₅₀ of chlorpyrifos from GD2 to GD6 and reported findings similar to those observed in the present investigation. However, Ambali et al. used a higher dose and five injections in comparison to our two sublethal doses of POX. Many other factors may play a role in the termination of pregnancy, such as altered endocrine physiology during pregnancy. Chung et al. (2002) investigated the developmental toxicity of an OP, flupyrazofos, in rats and reported maternal toxicity as well as fetal growth restrictions at high dose. When evaluating the effect of anticholinesterases such as paraoxon on fetuses and embryos, it should be kept in mind that an active cholinergic system also exists in the placenta. Moreover, acetylcholine (ACh), a natural substrate for AChE, plays a vital role in the maturation of the placenta in addition to many physiological roles other than non-neuronal (Gupta, 2007). This shows that the placenta is susceptible to OPCs and a physiologically normal placenta is a prerequisite for the development and maturation of a healthy fetus. The lipophilic nature of OPCs makes possible their easy penetration through biological barriers. Moreover, fetuses already having 50-70% less cholinesterase activity than the mother (Jones and McCance, 1949) may be at more risk from OPC poisoning. Moreover, substantial evidence now suggests that the canonical (cholinesterase-based) mechanism of OP toxicity cannot alone account for the wide-variety of adverse consequences of OP exposure that have been described in literature. The expression of AChE is not restricted only to cholinergically innervated tissues during growth and development. Moreover, cholinergic mechanisms are involved greatly during embryonic growth, not only of the neural tube but also of other organ systems (Layer et al., 2013). An increased concentration of ACh at the muscarinic and nicotinic receptors stimulates contraction of the uterus as well as autonomic imbalance, which may cause uteroplacental complications (Arbuckle et al., 2001). The increased rate of abortion in our study might be explained by this phenomenon. The present investigation shows that a profound lethal effect in pregnancy may be anticipated even with a short-duration exposure of a non-lethal dose. Further detailed studies with different sublethal doses and exposure regimes need to be undertaken.

Table 2. Descriptive statistics of the fetal weight/growth restriction in the saline control group. Seventeen pups were normal in comparison to two 2sd pups (this is not statistically significant).

<table>
<thead>
<tr>
<th>Number of pups</th>
<th>Mean fetal weight +SD</th>
<th>95% confidence interval</th>
<th>Minimum fetal weight</th>
<th>Maximum fetal weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17</td>
<td>1.26±0.04</td>
<td>1.24-1.28</td>
<td>1.19</td>
</tr>
<tr>
<td>1SD</td>
<td>1</td>
<td>1.17±0.01</td>
<td>---</td>
<td>1.17</td>
</tr>
<tr>
<td>2SD</td>
<td>2</td>
<td>1.10±0.04</td>
<td>0.76-1.44</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Table 3. Descriptive statistics of fetal weight/growth restriction in the POX-treated group. Only six pups were normal in comparison to twenty-three 2sd pups (this is highly significant; p ≤0.001).

<table>
<thead>
<tr>
<th>Number of pups</th>
<th>Mean fetal weight +SD</th>
<th>95% confidence interval</th>
<th>Minimum fetal weight</th>
<th>Maximum fetal weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>1.21±0.03</td>
<td>1.18-1.25</td>
<td>1.18</td>
</tr>
<tr>
<td>1SD</td>
<td>5</td>
<td>1.16±0.01</td>
<td>1.15-1.17</td>
<td>1.15</td>
</tr>
<tr>
<td>2SD</td>
<td>23</td>
<td>1.00±0.13</td>
<td>0.95-1.06</td>
<td>0.69</td>
</tr>
</tbody>
</table>
Authors’ contribution: SMN and SO was responsible for the study concept, design and overall supervision. SMN and MS performed the experiments and collected samples. CS performed the data analysis and MAHA helped in the experimental conduct. SMN and SO drafted the manuscript. All the authors provided critical revision of the manuscript for important intellectual content, reviewed content, and approved the final version for publication.

Conflict of interest: The authors declare that they have no conflict of interest.

REFERENCES


