Histopathological lesions, oxidative stress and genotoxic effects in liver and kidneys following long term exposure of rabbits to diazinon and propoxur

Christina Tsitsimpikou a, Manolis Tsatzarakis b, Persefoni Fragkiadaki b, Leda Kovatsi c, Polychronis Stivaktakis b, Alexandra Kalogeraki d, Demetrios Kouretas e, Aristidis M. Tsatsakis b,*

a Section of Hazardous Substances, Mixtures and Articles, Directorate of Environment, General Chemical State Laboratory of Greece, Athens, Greece
b Laboratory of Toxicology, School of Medicine, University of Crete, Voutes 71003, Heraklion, Greece
c Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki, University Campus, Thessaloniki 54124, Greece
d Department of Pathology, Medical School Voutes–Stavrakia, Heraklion, 71110 Crete, Greece
e Department of Biochemistry and Biotechnology, University of Thessaly, Larissa 41221, Greece

1. Introduction

Diazinon (O,O-diethyl O-[4-methyl-6-(propan–2-yl)pyrimidin-2-yl] phosphorothioate), is an organophosphate insecticide which acts by inhibiting acetylcholinesterase. Propoxur (Baygon) (2-isopropoxyphenyl N-methylcarbamate) is a carbamate insecticide with a similar mechanism of action through acetylcholinesterase inactivation. Both insecticides have been extensively used in Greece with proven acute and chronic adverse effects on health (Tsatsakis et al., 1996a, 1996b, 2008). They both accumulate in various tissues, such as hair, and analysis of these tissues enables the determination and establishment of long-term exposure (Maravgakis et al., 2012; Tsatsakis and Tutudaki, 2004; Tutudaki et al., 2003; Tutudaki and Tsatsakis, 2005).

Diazinon exposure has been linked to the development of serious histopathological lesions in the liver, the kidneys and the brain (Kalender et al., 2005; Shah and Iqbal, 2010; Yehia et al., 2007). On the other hand, propoxur exposure has only been linked to the decrease in weight of several organs (Institoris et al., 2002, 2004).

Pesticide exposure has been linked to oxidative stress (Merhi et al., 2010). Indeed, it has been established that both propoxur and diazinon induce oxidative stress (Abdou and ElMazoudy, 2010; Banerjee et al., 1999; Gupta et al., 2009; Shah and Iqbal, 2010; Suke et al., 2006) and studies have shown that oxidative stress contributes to diazinon-induced toxicity (Yilmaz et al., 2012).

Telomerase is an enzyme, which adds DNA sequence repeats to the ends of eukaryotic chromosomes, called telomere regions. This addition prevents the constant loss of important DNA from chromosome ends. Alterations in telomerase activity have many clinical implications, such as aging, cancer, diabetes mellitus and many more (Blackburn, 2005). Due to its central role in the maintenance of steady state telomere length, telomerase is a principal target for
regulatory mechanisms, while simultaneously being highly susceptible to oxidative stress (Rentoukas et al., 2012).

We have currently studied histopathological lesions induced in liver and kidneys, oxidative stress (through the determination of total antioxidant capacity – TAC, thiobarbituric acid reactive species – TBARS, catalase, reduced glutathione – GSH and protein carbonyl(s) and genotoxic effects (both the effect on telomerase activity, as well as oxidative DNA damage) induced in rabbits by the long term exposure to diazinon and propoxur.

2. Materials and methods

2.1. Animals and administration protocol

Ten healthy New Zealand white female rabbits (weighing between 3200 and 3500g each) were used in this study. The animals were housed in individual metal cages and kept in a 12-h dark/light cycle, at a temperature between 20 and 21 °C, in the laboratory animal house facilities of the School of Medicine, University of Crete, Heraklion. They were fed with commercial rabbit pellets ad libitum and provided with drinking (tap) water. As confirmed by daily inspection, animals were consuming consumable amounts (between 2500 mg/day per rabbit) (250 mg/kg BW) (200 ml water per day). The rabbits were acclimatized under laboratory conditions for 2 weeks, whereupon the treatment period began.

The animals were divided into 5 groups, consisting of 2 animals each. The limited number of animals per group constitutes the main limitation of the study. Group 1 and group 2 received diazinon at 2 different doses, namely 2.64 (low dose diazinon, LDD) and 5.28 mg kg⁻¹ day⁻¹ (high dose diazinon, HDD), respectively. Group 3 and group 4 received propoxur at 2 different doses, namely 8.76 (low dose propoxur, LDP) and 17.52 mg kg⁻¹ day⁻¹ (high dose propoxur, HDP), respectively. Group 5 served as the control group (C) and its animals received only tap water. Doses were administered every 2 days. The administered doses of diazinon and propoxur corresponded to 1/30 and 1/15 of the respective LD₅₀ for LD and HD treatment groups (Fish et al., Encyclopedia, 2007) in order to avoid any overt toxicity. Originally, the appropriate amounts of pesticides were diluted in 500 ml tap water.

The yearly based experimental scheme of exposure, selected in continuation of previous studies conducted in order to mimic seasonal human exposure to pesticides (Marganti and Tatsakos, 2009), consisted of two oral administration periods, lasting 3 months and 1 month, respectively, interrupted by an 8-month wash-out period (total duration 12 months). The animals were sacrificed at the age of 12 months (weight 3.6–4.2 kg) by administering an injection of 5 ml thiopental (Thiopental sodium solution, 25 mg/ml), according to the bioethical rules of the University of Crete. During the study period, the animals were weighed every month and their food consumption was recorded. All rabbits were regularly observed and their condition was closely monitored. No clinical signs were observed at any point. At the end of both administration periods, urine and serum samples were collected. Following the animals’ sacrifice, tissue samples from kidneys and liver were also collected and stored at −20 °C.

2.2. Histopathological lesions

Tissue block samples, embedded in paraffin and sectioned at 4 μm, were stained with eosin–hematoxylin and were subsequently examined under light microscopy by a histopathologist without knowing the source of the tissues.

2.3. Oxidative stress

TAC, expressed in mmol dityphenyl-1-pircyhydrazyl (DPPH)/L reduced to DPPH·, was determined by the DPPH spectrophotometric assay using stable DPPH radical as reagent. The plasma was mixed with PBS and DPPH, it was then incubated and centrifuged and the absorbance was read at 520 nm.

TBARS, expressed in μmol/L, were measured in blood plasma using a previously described method (Paschalis et al., 2007). Briefly, plasma was mixed with TCA, Tris–HCl, Na₂SO₄, and thiobarbituric acid and incubated at 95 °C. TCA was added again, centrifuged and the absorbance was read at 530 nm. GSH and catalase, expressed in μmol/gHb and U/mgHb, respectively, were determined in red blood cell lysate.

Protein carbonyls, expressed in nmol/mg protein, were determined in plasma, as previously described (Paschalis et al., 2007).

2.4. OxSelect Oxidative DNA Damage Quantitation Kit (aparinic/apyrimidinic – AP or abasic sites)

Genomic DNA was isolated from tissue samples with the QuiaGen FlexiGene DNA kit. Oxidative DNA damage was determined in the isolated DNA using the OxSelect Oxidative DNA Damage Quantitation Kit (AP Sites), which uses an aldehyde reactive probe (ARP) to react specifically with an aldehyde group on the open ring form of AP sites. In this way, AP sites are tagged with biotin, which is later detected with streptavidin–enzyme conjugate. The quantity of AP sites in the DNA sample (expressed as the number of AP sites per 100.000 base pairs), is determined by comparing its absorbance with a standard curve generated from the provided DNA standard, containing predetermined AP sites.

2.5. Telomerase activity

Telomerase activity in tissue samples and peripheral blood mononuclear cells (PBMCs) was measured using a commercial telomerase PCR–ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA), based on the telomeric repeat amplification protocol (Kim et al., 1994). The method for the isolation of PBMCs is described elsewhere (Tsirpanlis et al., 2006). PBMCs isolation with density gravity centrifugation from pesticide-treated animals did not result in enough DNA to proceed with the PCR elongation/amplification step of the commercial telomerase activity kit due to technical errors.

2.6. Determination of propoxur and diazinon in urine and serum samples

The determination and quantification of propoxur and diazinon in serum and urine samples was performed by liquid–liquid extraction according to previously conducted studies (Jochen et al., 1999; Nagaraja et al., 1995; Tarabhi et al., 2001).

All samples were analyzed by liquid chromatography–mass spectrometry (LC/MS) using a Discovery 250 mm column for the separation. A gradient formic acid 10 mM in methanol (A) and water (B) of LC/MS grade were selected as mobile phases at 0.65 ml/min for diazinon and for ionization APC source in positive mode was used (m/z 305.1 for diazinon and 302.2 for methylidiazin as internal standard). A gradient water (A) and methanol (B) of LC/MS grade were selected as mobile phases at a flow of 0.5 ml/min for the detection of propoxur and for ionization APC source in negative mode was used (m/z 210.0, 211.0 for propoxur and 201.0 for carbaryl as internal standard). For the evaluation of the extraction procedure, blank tissue samples were spiked at concentration levels ranging from 0.1 to 8 μg/ml. Recoveries in serum and urine samples were calculated to be 78.1 and 81.2% for propoxur and 69.4 and 75.2% for diazinon, respectively. All standard and spiked solutions tested exhibited good linearity (r² > 0.99).

3. Results

3.1. Analysis of urine and serum for the determination of the two insecticides

The concentrations of the two insecticides in urine and serum after the end of the two administration periods, as described in Section 2, are shown in Table 1. As expected, the concentrations of the two insecticides in urine and serum were statistically lower in the samples collected after the second administration period, since the 8-month wash out period had intervened, with the exception of diazinon concentrations in urine, probably due to its higher fat accumulation and its lower extent of metabolism (Repetto et al., 1996).

According to a previous study, rats exposed orally to 23 mg/kg diazinon had detectable levels of diazinon in the blood, adipose tissue, muscle, liver and brain. All levels peaked at day 4 except muscle and liver concentrations, which peaked at 12 and 8 days, respectively. Detectable levels were no longer found in any samples after 30 days (DRAFT Toxicological Profile for Diazinon, 2006).

3.2. Histopathological lesions

For high dose diazinon and propoxur and for low dose propoxur-exposed animals, focal inflammation and fibrosis were observed in the liver and kidneys for all pesticide-exposed animals, except the LDD group (Figs. 1–4).

In control animals as well as in pesticide-exposed animals extramedullary hematopoiesis in the liver (Fig. 5) was observed as expected, since it has been established that hematopoiesis in rabbits takes place in secondary lymphoid organs (Pyhälä et al., 1978).

3.3. Oxidative stress

The results of the effect of diazinon and propoxur on markers of oxidative stress are summarized in Fig. 6.
Table 1
Concentrations of the two insecticides in urine and serum after the end of the two administration periods.

<table>
<thead>
<tr>
<th>Pesticide administered</th>
<th>Biological matrix</th>
<th>Average concentration detected ( \times 10^{-1} ) (µg/ml)</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st administration period</td>
<td>2nd administration period</td>
</tr>
<tr>
<td><strong>Diazinon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose group (2.64 mg kg(^{-1}) day(^{-1}))</td>
<td>Serum</td>
<td>3.05 ± 0.764</td>
<td>1.55 ± 0.211</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>1.45 ± 0.231</td>
<td>2.42 ± 0.521</td>
</tr>
<tr>
<td>High dose group (5.28 mg kg(^{-1}) day(^{-1}))</td>
<td>Serum</td>
<td>8.57 ± 0.723</td>
<td>6.83 ± 0.432</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>4.52 ± 0.621</td>
<td>6.21 ± 0.467</td>
</tr>
<tr>
<td><strong>Propoxur</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose group (8.76 mg kg(^{-1}) day(^{-1}))</td>
<td>Serum</td>
<td>10.0 ± 0.876</td>
<td>4.02 ± 0.423</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>4.64 ± 0.512</td>
<td>0.644 ± 0.123</td>
</tr>
<tr>
<td>High dose group (17.5 mg kg(^{-1}) day(^{-1}))</td>
<td>Serum</td>
<td>23.3 ± 1.87</td>
<td>9.29 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>11.0 ± 0.987</td>
<td>3.03 ± 0.432</td>
</tr>
</tbody>
</table>

Fig. 1. Inflammation and fibrosis of kidney tissue from diazinon and propoxur treated animals.

Upon exposure of the animals to diazinon, plasma TAC in the LDD group remained statistically unchanged \((p = 0.824)\) and GSH was significantly \((p < 0.001)\) increased compared to controls. On the other hand, the HDD group had significantly higher plasma TAC \((p = 0.011)\), with no significant differences in GSH levels \((p = 0.196)\). Furthermore, comparison of the TAC and GSH values between the LDD and the HDD groups, yielded a statistically significant difference \((p = 0.034\) and \(p < 0.001\), respectively), pointing to a concentration-dependent effect.

On the contrary, in the LDP group TAC was significantly higher \((p = 0.005)\), while GSH remained unchanged \((p = 0.939)\) compared to the control group. On the other hand, in the HDP group, plasma TAC was similar to that in the control group \((p = 0.609)\) and GSH values were significantly higher \((p = 0.002)\). The same concentration-dependent effect was substantiated (TAC in LDP vs HDP \(p = 0.034\), GSH in LDP vs HDP \(p = 0.002)\).

Propoxur and diazinon administration had no statistical or discrete effect on the other oxidative stress markers examined, such
3.4. Genotoxic effects

3.4.1. Oxidative DNA damage

Both diazinon and propoxur induced oxidative DNA damage in liver and kidneys, since both the low, as well as the high dose of the two insecticides increased the AP sites in DNA. The results are summarized in Fig. 7. In the liver, statistically significant differences \((p<0.001)\) in the AP sites were found when controls \((0.25 \pm 0.01)\) were compared with HDD animals \((2.49 \pm 0.34)\), as well as with LDD animals \((1.08 \pm 0.03)\). A statistically significant difference \((p<0.001)\) was also observed between the effect of the high and the low dose of diazinon. Similarly, statistically significant differences \((p<0.001)\) in the AP sites were found upon comparison of control animals \((0.25 \pm 0.01)\) with HDP animals \((5.32 \pm 0.25)\), as well as with LDP animals \((4.63 \pm 0.43)\). Nevertheless, there was no significant difference \((p=0.07)\) between the effect of the low and the high dose of propoxur, indicating that both doses had a similar effect.

In kidneys, statistically significant differences \((p<0.001)\) in the AP sites were found when control animals \((0.10 \pm 0.01)\) were compared with HDD animals \((0.52 \pm 0.05)\), as well as with LDD animals \((0.46 \pm 0.01)\). Nevertheless, there was no significant difference \((p=0.159)\) between the effect of the low and the high dose of diazinon. Similarly, a statistically significant difference \((p<0.001)\) in the AP sites was found upon comparison of control animals \((0.10 \pm 0.01)\) with HDP animals \((0.68 \pm 0.08)\), while there was a non-significant difference \((p=0.931)\) between control animals \((0.10 \pm 0.01)\) and LDP animals \((0.10 \pm 0.01)\). The difference between the effect of the low and the high dose of propoxur was found to be significant \((p<0.001)\).
3.4.2. Telomerase activity

Telomerase activity in PBMCs, following exposure to propoxur was significantly increased ($p = 0.012$), up to 229%, in the HDP group compared with the control group, while a non-significant increase ($p = 0.148$), of 134%, was observed in the LDP group. A statistically significant difference ($p = 0.024$) in telomerase activity between the LDP and the HDP groups was noted, suggesting that the effect of propoxur is concentration-dependent.

Telomerase activity in the liver and kidneys, following exposure to diazinon and propoxur is shown in Fig. 8. There was a statistically significant increase in telomerase activity both at low and high doses of both pesticides in liver (LDD and LDP $p = 0.017$, HDD and HDP $p = 0.027$). Nevertheless, when the two different dose groups were compared, a non-significant difference was observed. Similarly, a non-concentration dependent significant increase in telomerase activity in the kidneys of over 140% for diazinon and 180% for propoxur exposed animals was observed following exposure to the pesticides (LDD $p = 0.009$, HDD $p = 0.012$, LDP $p = 0.018$, HDP $p = 0.020$).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDD</td>
<td>187</td>
<td>154</td>
</tr>
<tr>
<td>LDP</td>
<td>178</td>
<td>141</td>
</tr>
<tr>
<td>HDD</td>
<td>155</td>
<td>145</td>
</tr>
<tr>
<td>HDP</td>
<td>166</td>
<td></td>
</tr>
</tbody>
</table>

4. Discussion

An unfavorable oxidative environment seems to be created in the cells due to exposure to both diazinon and propoxur. The results of the present study clearly show that the high doses of both pesticides, as well as the low dose of propoxur induced focal inflammation and fibrosis in the liver and kidneys. Nevertheless, no decrease in the organs’ weight was recorded, despite previously published findings (Institoris et al., 2002, 2004).

Telomeres are indicators of oxidative stress (Saretzki et al., 2003; Saretzki, 2009). Under increased oxidative stress, telomere shortening is accelerated (von Zglinicki et al., 1995). Furthermore, the shortening of telomeres in blood cells during aging is attributed to oxidative stress (von Zglinicki et al., 2000). Increased telomerase activity in PBMCs observed in the present study depicts systemic inflammation (Rentoukas et al., 2012; Roth et al., 2003), in agreement with the histopathological findings recorded. Increase in telomerase activity found in the liver and in the kidneys, following exposure to propoxur and diazinon, which corresponds to an extension of the cells’ lifespan, could possibly represent a counteracting survival mechanism (López-Diazguerrero et al., 2012; Serra et al., 2003). Such a protective function has already been shown for telomerase, which, is excluded from the nucleus under oxidative stress, and is localized in the mitochondria in order to protect them from stress (Ahmed et al., 2008).

Our results on oxidative stress markers suggest that diazinon and propoxur have discrete and concentration-dependent mechanisms of action. The observed TAC and GSH increase is a rescue mechanism to counteract diazinon and propoxur-induced oxidative stress. However, each pesticide acts through a different pathway and, at different doses, different rescue mechanisms are induced. TAC is affected not only by GSH, but also by other antioxidant molecules, such as uric acid, cytochrome C and ubiquinone. Thus, at LDD GSH is increased to counteract oxidative stress, but other antioxidant molecules may be reduced, leading to practically unchanged TAC levels. However, at HDD GSH is reduced to normal levels, but this reduction is counterbalanced by the increase of other antioxidant molecules resulting in increased TAC. Propoxur acts differently compared to diazinon. Low concentration of propoxur does not affect GSH, but induces other antioxidant molecules as a rescue mechanism resulting in increased TAC. However, at higher concentrations of propoxur, the levels of some antioxidant molecules are reduced resulting in decreased TAC, while GSH is increased as a counterbalance. Furthermore, the increased GSH scavenged free radicals, and subsequently prevented lipid peroxidation (i.e. the non-significant TBARS increase) (Tchantchou et al., 2004).

Free radicals, which are constantly generated in vivo, but can further be increased following exposure to various chemicals, cause oxidative damage to various biomolecules, to the extent that it is permitted by the existence and normal function of the various antioxidant and repair systems. One of the most significant targets of oxidative damage is DNA.

In the present study, the AP or abasic sites, which represent one of the prevalent lesions of oxidative DNA damage, were monitored after exposure to both diazinon and propoxur. To the best of our knowledge, there is no available data on the induction of such DNA damage by diazinon or propoxur. It was found that in liver both insecticides increased the number of abasic sites in DNA in a dose-dependent manner. Similarly, when kidneys were examined, diazinon induced oxidative DNA damage in a dose-dependent manner, while propoxur increased the number of abasic sites in DNA only in those animals that received the high dose. These results are in agreement with previous studies reporting that organophosphorous pesticides (OP), such as diazinon, have been found to alter sperm chromatin and DNA. Their toxicity can be attributed to their oxygen analogs (oxons), which are formed during the OP oxidative activation. Furthermore, it has been shown that oxons are more toxic than the parent compounds, since they are more toxic to sperm DNA (evaluated by the SCSA parameter) than their corresponding parent compounds (Salazar-Arredondo et al., 2008). In another study, where diazinon was shown to induce testicular damage, it was suggested that this damage could be attributed to the generation of free oxygen radicals by diazinon, which induce alterations in the DNA and promote local apoptosis (Sarabia et al., 2009).

5. Conclusions

Long term exposure of rabbits to propoxur and diazinon caused histopathological lesions (focal inflammation and fibrosis) in kidneys and liver. Oxidative stress and genotoxic effects were also induced, documented through changes in TAC, GSH and oxidative DNA damage. Systemic inflammation has also started to accumulate, whereas the observed increase in telomerase activity in liver and kidneys probably counteracts for local tissue damage.

Conflict of interest statement

The authors declare that there are no conflicts of interest.