Concentration of hepatic vitamins A and E in rats exposed to chlorpyrifos and/or enrofloxacin

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Abstract

The aim of the study was to determine the level of antioxidant vitamins A and E in the liver of rats exposed to chlorpyrifos and/or enrofloxacin. Chlorpyrifos (Group I) was administered at a dose of 0.04 LD₅₀ (6 mg/kg b.w.) for 28 days, and enrofloxacin (Group II) at a dose of 5 mg/kg b.w. for 5 consecutive days. The animals of group III were given both of the mentioned above compounds at the same manner as groups I and II, but enrofloxacin was applied to rats for the last 5 days of chlorpyrifos exposure (i.e. on day 24, 25, 26, 27 and 28). Chlorpyrifos and enrofloxacin were administered to rats intragastrically via a gastric tube. The quantitative determination of vitamins was made by the HPLC method. The results of this study indicated a reduction in the hepatic concentrations of vitamins A and E, compared to the control, which sustained for the entire period of the experiment. The four-week administration of chlorpyrifos to rats resulted in a significant decrease of vitamins in the initial period of the experiment, i.e. up to 24 hours after exposure. For vitamin A the maximum drop was observed after 24 hours (19.24%) and for vitamin E after 6 hours (23.19%). Enrofloxacin caused a slight (3-9%) reduction in the level of the analysed vitamins. In the chlorpyrifos-enrofloxacin co-exposure group reduced vitamins A and E levels were also noted, but changes in this group were less pronounced in comparison to the animals intoxicated with chlorpyrifos only. The decrease in the antioxidant vitamin levels, particularly noticeable in the chlorpyrifos- and the chlorpyrifos combined with enrofloxacin-treated groups, may result not only from the increase in the concentration of free radicals, but also from the intensification of the secondary stages of lipid peroxidation.

Key words: chlorpyrifos, enrofloxacin, vitamin A, vitamin E, rats

Introduction

Environmental pollution plays a crucial role in the occurrence of health problems affecting animals and human beings. Investigations which have been conducted in recent years indicate the need for studies estimating the potential harmful effects of substances of high biological activity, such as pesticides and medicines. A great deal of emphasis has been placed on evaluating the potential harmfulness of co-intoxication with these compounds (Liu et al. 2006, Wielgomas and Krechniak 2007).
Environmental pollution by pesticides is a major environmental concern due to their extensive use in agriculture and in public health programmes (Casida and Quistad 2004,Aktar et al. 2009). Organophosphorus (OP) compounds constitute the largest and the most diverse group of insecticides. Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is a widely used OP insecticide for agricultural and domestic applications in the whole world (Lemus and Abdelghani 2000). Similar to other phosphorothioate pesticides, chlorpyrifos acts primarily by inhibiting neuronal acetylcholinesterase (AChE) activity, thereby interfering with normal cholinergic nerve transmissions (Nigg and Knaak 2000). Recent studies have indicated that chlorpyrifos affects several biochemical pathways that are independent of the modulation of the AChE enzyme. One such mechanism associated with both acute and chronic poisoning is oxidative stress. Several studies point to the generation of ROS as a secondary molecular mechanism of the toxicity of some pesticides (Akhgari et al. 2003, Bebe and Panemanogalore 2003).

Another group of compounds, which are more and more often used in medicine, comprise fluoroquinolones. They are currently one of the main classes of antimicrobial agents used worldwide. The clinical use of these drugs is not restricted to human medicine, but is also widely applied in the treatment and prevention of veterinary diseases (Ihrke et al. 1999, Gottfried and Grossman 2010). Enrofloxacin belongs to the group of synthetic 6-fluoroquinolones and it is marketed specifically for use in veterinary medicine (Martinez et al. 2006). It is effective against some Gram-positive and Gram-negative bacteria, mycoplasmas and some rickettsial organisms (Vancutsem et al. 1990). The mechanism of action of enrofloxacin consists of the inhibition of bacterial DNA gyrase, which plays a basic role in the process of DNA replication, leading to the inhibition of the synthesis of bacterial proteins (Wang et al. 2010). Even though during therapy temporary gastrointestinal as well as biochemical and hematological disorders are observed, enrofloxacin is classed as a relatively safe drug, similarly to the whole group of fluoroquinolones (Tras et al. 2001, Stahlmann 2002). Investigations conducted in recent years suggest that one negative effect of fluoroquinolones administration could be the generation of reactive oxygen species (ROS) (Ibrahim and Yarsan 2011). Side effects such as phototoxicity and cartilage damage may be related to the production of ROS (Pouzaud et al. 2006, Rampal et al. 2008).

The toxicity of many xenobiotics is associated with the production of free radicals, which are not only toxic themselves, but are also implicated in the pathophysiology of many diseases. They may produce oxidative stress by generating free radicals and inducing or altering enzymatic and non-enzymatic antioxidant systems (Hybertson et al. 2011). To counteract the toxicity of active oxygen species the cells are equipped with a highly efficient antioxidative defence system, including both enzymatic constituents (SOD, CAT, GPx, GR) as well as low-molecular compounds (e.g. glutathione, ascorbic acid, carotenoids and tocopherols) (Sies 1997, Irshad and Chaudhri 2002). Each of these antioxidants has specific activities, and they often work synergistically to enhance the overall antioxidant capacity of the body.

Although it is known that both organophosphorus insecticides and fluoroquinolones may induce oxidative stress, the data regarding their influence on the level of antioxidant vitamins are very limited. It is also worth emphasizing that fluoroquinolones are widely and increasingly used in veterinary medicine, with enrofloxacin being the most common, and the use of chlorpyrifos is still increasing. Recently attention has been drawn not only to the adverse effects of individual xenobiotics, but also to their interactions with one another, so studies of such interactions are one of the important areas of toxicology research. Therefore, looking at the widespread applications of both enrofloxacin and chlorpyrifos, the aim of this study was to determine the concentrations of vitamins A and E in the liver of rats exposed to chlorpyrifos and enrofloxacin (given alone and in combination), and to assess the time interval for the persistence of changes following the discontinuation of the administration of the studied compounds. To the best of our knowledge no similar studies have been conducted before.

**Materials and Methods**

Chlorpyrifos (purity min. 99.9%) was obtained from the Institute of Industrial Organic Chemistry (Warszawa, Poland). Commercial formulation of enrofloxacin (ENFLOCYNA® SOL) was purchased from Biowet Puławy Ltd. (Puławy, Poland), and contained 50 mg/mL of the active substance. All the other chemical and reagents used were of analytical grade or better and commercially available.

Adult male rats of Wistar strain (n=120), weighing 170±10 g, were used for the study. The animals were kept under standard experimental conditions with ad libitum food and water.

The rats were randomly divided into three experimental groups and one control group of 30 animals each. Group I was administered chlorpyrifos in a sunflower oil solution at a dose of 6 mg/kg b.w. (0.04 LD₅₀) daily for 28 days. Group II was given with en-
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rofloxacin (ENFLOCYNA® SOL), in an aqueous solu-
tion, at a therapeutic dose of 5 mg/kg b.w.per day,
for five subsequent days. The animals of group III
were given both of the mentioned above compounds
at the same manner as groups I and II, but enro-
foxacin was applied to rats for the last 5 days of chlor-
pyrifos exposure (i.e. on day 24, 25, 26, 27 and 28).
The remaining group was untreated and used as the
control.

The experimental design and procedures has been
approved by the Local Ethics Committee for Animal
Experiments at the University of Warmia and Mazury
in Olsztyn, Poland.

Experimental material from 6 rats randomly se-
lected from each group was obtained after 3, 6, and 24
hours, and 3 and 7 days following the last applied dose
of the compounds under study. The livers were im-
mediately washed in ice-cold 0.9% NaCl, weighed and
frozen at -80°C for a further analysis of vitamins
A and E.

Chromatographic quantitation of retinol and
α-tocopherol levels in the liver was performed in a re-
versed-phase liquid chromatography (Shimadzu
HPLC system: pumps LC-20AD, degasser
DGU-20A3, column oven CTO-10AS). The analytical
column was a Nucleosil C18 (250 × 4.6 mm) with
a particle size of 5 μm, supplied by Mecherey-Nagel.
The mobile phase was methanol:H2O (95:5 v/v), flow
rate 1 mL/min, loop 20 μL. The liver concentrations of
retinol were determined using detector UV-vis – 326
nm and α-tocopherol using detector RF-20A FLU-
ORESCENCE: E<sub>ex</sub> 293 nm and E<sub>em</sub> 326 nm. Concen-
trations of vitamin A and E were calculated by com-
parison to external standards: α-Tocopherol
(DL-all-rac α-tocopherol) and Retinol Vitamin
A – alcohol (Sigma-Aldrich). The data was expressed
as μg/g of liver tissue.

All data were expressed as a mean ± SD (standard
deviation) and analyzed using one-way analysis of
variance (ANOVA) followed by Newman-Keuls t-test
as a post hoc test for a comparison between the
groups. The differences were considered significant
at p<0.05. Statistical calculations were performed using
Statistica 10 PL software (StatSoft, Poland).

Results

The results of hepatic vitamins A and E concen-
trations in particular groups and time intervals are
presented in Tables 1-2.

Exposure of rats to chlorpyrifos and enrofloxacin,
administered either alone or in combination, had no
effect on the animals’ behaviour or body weight.

The concentration of vitamins A and E in the liver
was decreased in comparison with the control in all
experimental groups throughout the experiment and
did not reach the control values until the completion
of the study, i.e. up to the 7th day.

In the group of rats exposed to enrofloxacin only
a slight decrease (3-9%) in the content of vitamin
A and E was observed compared to the control group.
However, the level of these vitamins was higher than
that found in the chlorpyrifos- and the chlorpyrifos
combined with enrofloxacin-treated groups.

The content of vitamins A and E in subacute in-
toxications with chlorpyrifos was significantly (p<0.05)
reduced in the initial period of the experiment. For
vitamin A the maximum drop was observed after 24
hours (19.2%), and for vitamin E after 6 hours
(23.2%), compared to the control group. The de-
crease in the level of vitamin E in this group was
greater than that of vitamin A.

Combined application of chlorpyrifos and enro-
foxacin to rats resulted in a significant decrease in
vitamin A content, compared to controls, after 24
hours (17.1%). Vitamin E levels decreased gradually
up to 24 hours, and were, respectively 17.3%, 15.2%
and 14.8% after 3, 6 and 24 hours. In this group
a slightly reduced level of these vitamins was observed
in comparison with the chlorpyrifos group, but this
decrease was greater compared to the enrofloxacin
group.

Discussion

Among numerous chemical compounds posing
a health hazard to animals and humans, substances
with high biological activity, such as pesticides and
drugs, are particularly important. However, no special
attention has been paid to the simultaneous adminis-
tration of agrochemicals and medicaments, such as
those investigated in this work.

Cells have several ways to alleviate the effects of
oxidative stress, either by repairing the damage or by
directly reducing the prooxidative state via enzymatic
and non-enzymatic antioxidants which have been
shown to scavenge free radical and ROS (Kulikowska-
-Karpińska and Moniuszko-Jakoniuk 2004, Bando et
al. 2005). Most studies involving antioxidants and
their role in combating cellular oxidative stress in ani-
mals have focused on the alteration in various en-
dogenous enzymatic and non-enzymatic components
of the antioxidant defence system (such as SOD,
CAT, GPx, GSH). In contrast, only a few studies have
examined exogenous antioxidants such as retinoids
and vitamin E (Rodriguez-Estival et al. 2011, Defo et
al. 2014).

Antioxidant vitamins can prevent the uncontrolled
Table 1. Concentration of vitamin A in the liver of rats exposed to chlorpyrifos, enrofloxacin and their combination (μg/g of fresh tissue).

<table>
<thead>
<tr>
<th>Time after intoxication</th>
<th>Control (n = 6)</th>
<th>Chlorpyrifos (n = 6)</th>
<th>Enrofloxacin (n = 6)</th>
<th>Chlorpyrifos and Enrofloxacin (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>109.92 ± 2.06a</td>
<td>94.99 ± 5.68b</td>
<td>100.78 ± 0.78ab</td>
<td>97.30 ± 4.84ab</td>
</tr>
<tr>
<td>6 h</td>
<td>114.19 ± 6.71a</td>
<td>96.58 ± 5.69b</td>
<td>105.23 ± 4.48ab</td>
<td>101.65 ± 5.89ab</td>
</tr>
<tr>
<td>24 h</td>
<td>117.85 ± 3.18a</td>
<td>95.21 ± 6.19a</td>
<td>108.01 ± 6.92b</td>
<td>107.66 ± 2.17b</td>
</tr>
<tr>
<td>3 d</td>
<td>110.42 ± 3.72ab</td>
<td>96.56 ± 8.72a</td>
<td>104.66 ± 4.59ab</td>
<td>108.34 ± 4.29b</td>
</tr>
<tr>
<td>7 d</td>
<td>114.28 ± 2.26a</td>
<td>108.60 ± 6.23a</td>
<td>110.69 ± 6.26a</td>
<td>114.82 ± 4.62a</td>
</tr>
</tbody>
</table>

Values expressed as means ± SD; means marked by the same letter within the rows are not significantly different.

Table 2. Concentration of vitamin E in the liver of rats exposed to chlorpyrifos, enrofloxacin and their combination (μg/g of fresh tissue).

<table>
<thead>
<tr>
<th>Time after intoxication</th>
<th>Control (n = 6)</th>
<th>Chlorpyrifos (n = 6)</th>
<th>Enrofloxacin (n = 6)</th>
<th>Chlorpyrifos and Enrofloxacin (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>17.61 ± 2.06a</td>
<td>14.11 ± 2.85b</td>
<td>16.78 ± 0.78a</td>
<td>14.57 ± 1.84a</td>
</tr>
<tr>
<td>6 h</td>
<td>16.19 ± 0.82a</td>
<td>12.43 ± 1.54a</td>
<td>14.75 ± 1.45a</td>
<td>13.74 ± 2.53a</td>
</tr>
<tr>
<td>24 h</td>
<td>15.60 ± 0.9a</td>
<td>12.47 ± 1.36a</td>
<td>14.32 ± 1.05a</td>
<td>13.30 ± 1.12a</td>
</tr>
<tr>
<td>3 d</td>
<td>16.15 ± 2.12a</td>
<td>13.84 ± 1.97a</td>
<td>14.88 ± 1.84a</td>
<td>14.68 ± 1.88a</td>
</tr>
<tr>
<td>7 d</td>
<td>15.40 ± 2.69a</td>
<td>13.66 ± 1.36a</td>
<td>14.72 ± 2.32a</td>
<td>14.01 ± 1.47a</td>
</tr>
</tbody>
</table>

Values expressed as means ± SD; means marked by the same letter within the rows are not significantly different.

formation of free radicals, and inactivate oxygen species or inhibit their reaction with biological structures. Vitamin A (retinol) and vitamin E (α-tocopherol) are low molecular compounds that exhibit high biological activity against oxidants and free radicals (Sies and Stahl 1995). They are absorbed by blood via the lymphatic system, and reach the liver and fatty acid tissues where they are stored. Despite low concentrations, these vitamins prevent the accumulation of free radicals through either neutralising them or increasing the defensive capabilities of other endogenous antioxidants. Lipophilic vitamin A plays an important role in the functioning of the cell and the entire organism. It can react with peroxide radicals, and so interrupts the chain reaction of lipid peroxidation forming hydroperoxides. Vitamin A is also capable of reacting directly with ROS (Edge et al. 1997).

Vitamin E is a major lipid-soluble antioxidant present in the membranes of cells and cellular organelles, where it plays an important role in the suppression of lipid oxidation (Traber and Atkinson 2007). It reacts more rapidly than polyunsaturated fatty acids with peroxyl radicals, and hence breaks the chain reaction of lipid peroxidation. In addition to its antioxidant role, vitamin E might also have a structural role in stabilizing membranes (Kamal-Eldin and Appelqvist 1996).

Organophosphate insecticides have been extensively studied for their toxic potential (Sultatos 1994, Eddleston 2012). However, pesticide-induced oxidative stress has been the focus of toxicological research for over a decade and many investigations have been focused on the alteration of particular components of the antioxidant system (Giordano et al. 2007, Ben Amara et al. 2014). The number of studies concerned with the impact of insecticides on the level of antioxidant vitamins is limited.

Results of the present study show that after 28-days intoxication with chlorpyrifos, the concentration of vitamins A and E decreased in comparison to the control group for the entire period of the experiment. A significant (p<0.05) drop in the content of these vitamins was observed in the initial period of the experiment, i.e. up to 24 hours. Similar results were also obtained by other authors. The decrease in the vitamin A level was observed in the serum of rats following a single intoxication with fenthion (Buyukokuroglu et al. 2008, Cemek et al. 2010). Ben Amara et al. (2012) reported that subacute exposure to dimethoate was characterized by the depletion of...
vitamin E in rat erythrocytes. Other types of pesticides can also cause a reduction in vitamin A content. Fernie et al. (2005) recorded that hepatic retinol concentrations were nearly 50% reduced in PBDEs (polybrominated diphenyl ethers)-exposed American kestrels. Our results indicate that the reduction in the level of antioxidant vitamins may be attributed to the prooxidative properties of chlorpyrifos. Potentially, chlorpyrifos exposure began to exceed the capabilities of the antioxidant involving vitamins.

The majority of publications on fluoroquinolones mainly focus on the efficacy of its therapeutic activity (Suojala 2010, Zhang et al. 2012) and the data on its effects on antioxidant status are still limited (Albesa et al. 2004, Becerra et al. 2004). In our study, the exposure of rats to enrofloxacin caused an insignificant decrease in hepatic vitamins A and E levels. Because there is no available data investigating the effect of fluoroquinolones on these parameters, it is difficult to compare the results with the findings of other authors. However, the observed slight decline in the analysed vitamins may be the result of the prooxidative action of enrofloxacin, and may indicate impairment of antioxidant mechanisms.

In recent years there has been an increasing number of data concerning the interaction of organophosphate insecticides with different compounds and their impact on the oxidative processes. However, the majority of studies discuss the protective effect of vitamins or microelements with exposure to organophosphate insecticides (Verma et al. 2007, Abdallah et al. 2011) or the influence of mixtures of pesticides (El-Demerdash 2011, Ojha and Srivastava 2012). To the best of our knowledge a very limited number of investigations refer to the consequences caused by the co-administration of pesticides and medicaments (Gelal et al. 2001, Babu et al. 2006, Suarez et al. 2014), and only individual ones focus on oxidative stress (Barski and Spodniewska 2012, Spodniewska et al. 2014).

In the current study, in the group with combined intoxication with chlorpyrifos and enrofloxacin, the levels of vitamins A and E were reduced throughout the experiment. However, no synergistic effect of both compounds was found, as the observed changes of the vitamin levels were less pronounced as compared to the chlorpyrifos-treated rats. In the available literature, few papers concerning the content of lipophilic antioxidant vitamins in the tissues of animals exposed to pesticide mixtures were found. In rats and mice, exposure to PBDEs and PCBs mixtures have been shown to cause a reduction in hepatic α-tocopherol level (Hallgren et al. 2001). Twaroski et al. (2001) reported that various PCBs congeners treatments of rats led to a decrease in hepatic α-tocopherol and an increase in α-tocopheryl quinine (an oxidized form of α-tocopherol). In research on rats treated with pesticides (dimethoate, zineb and glyphosate), alone and in combination, Astiz et al. (2009) showed a decline of α-tocopherol in the liver and brain (30-60%), particularly noticeable in the case of mixed intoxication. On the other hand, the non-dioxin-like PCBs did not significantly alter the retinol content, indicating that in the case of exposure to these PCBs at environmental levels, no effects, or only marginal effects, can be expected.

In our study, the largest decrease in the content of vitamins A and E in the liver of rats was noted in the chlorpyrifos-treated group, as well as in the group with combined intoxication with chlorpyrifos and enrofloxacin. These changes were particularly noticeable in the initial period of the study, i.e. up to the 24th hour following exposure. This may indicate that during this period both increased generation of free radicals and intensified oxidative stress occurred.

The mechanism of the action of the antioxidant compound follows more than one type to reduce the impact of the oxidative stress induced in living organisms. The reduction in the level of vitamins A and E may be explained by the intensified utilisation of these vitamins in protection against oxidative damage to tissues. Vitamin E effectively reacts with organic lipid radicals produced in the process of lipid peroxidation (Liebler 1993). Therefore, it can be assumed that the decrease in vitamin E concentration in the liver after chlorpyrifos and enrofloxacin exposure is due to the interaction of this vitamin with radicals generated during lipid peroxidation. A decrease in the antioxidant vitamin level may also result from the intensification of the secondary stages of lipid peroxidation. Chow et al. (1999) demonstrated that a decrease in the content of vitamin E leads to a marked increase in the production of H₂O₂ in both the mitochondria of skeletal muscles and the mitochondria of the liver.

It should also be remembered that the capacity of antioxidants in vivo is determined by many factors which should be taken into consideration in its assessment. One such factor is bioavailability. The antioxidants should be absorbed, transported, distributed and retained properly in the biological fluids, cells and tissues. Intestinal absorption of fat-soluble vitamins is dependent on the digestion volume and absorption rate of fat. Thus, any perturbation of lipid absorption would result in a similar perturbation in vitamin absorption. It has been suggested that the compounds used in our study might interfere with nutrient absorption and also with the levels of antioxidants present in feed. Lee et al. (2008) suggested that vitamin A might act as an antioxidant, and it is possible that it is not transformed and accumulated in the liver as
retinyl esters because it is rapidly consumed by scavenging free radicals. Another possibility is that the biosynthesis of retinyl esters is inhibited under conditions of oxidative stress, and this may have negative consequences for overall vitamin A homeostasis. The diminished content of vitamin A may also be caused by the loss or transformation of the storage stellate cells in the liver.

Another reason for the decrease in the level of the analysed vitamins could be the inflammatory processes resulting from the exposure to the compounds used in the experiment. As a result of the ROS generation during the inflammation, chemical changes occur within antioxidant molecules which lead to their inactivation. This is confirmed by a study concerning the level of vitamin E in inflamed intestinal mucosa, which demonstrated the level of α-tocopherol being two times lower than in the control group (Hengsternmann et al. 2008).

It seems interesting that in rats exposed simultaneously to chlorpyrifos and enrofloxacin the decrease in the levels of both vitamin A and E was smaller than in the group of rats intoxicated exclusively with chlorpyrifos. It is known that the hepatic metabolism of xenobiotics with the participation of cytochrome P450 may also be a source of disorders in the free radical metabolism.

In mammals, the cytochrome P450 isoforms (CYPs) involved in the metabolism of xenobiotics belong mostly to the families CYP1-CYP3. The same CYPs are often involved in the biotransformation of such compounds as pesticides and commonly used drugs (e.g. fluoroquinolones) (Anzenbacher and Anzenbacherova 2001, Mutch and Williams 2006). The most frequent consequence of that interaction is an increase in the toxicity of substances, the metabolism of which was inhibited. No such phenomenon was observed in our study. Similarly to our research, the study of Wiaderkiewicz et al. (2006) showed that the stimulatory effect of dimethoate on the content of cytochrome P450 was abolished in rats simultaneously administered with pyrantel.

The present study indicates that both chlorpyrifos and enrofloxacin and their combination reduce the concentrations of vitamins A and E in the liver of rats, indicating the occurrence of oxidative stress. The levels of these vitamins did not reach the control values until the completion of the study, i.e. up to the 7th day following the discontinuation of the administration of the compounds used in the experiment. The decrease in the antioxidant vitamin levels was particularly noticeable in the groups exposed to chlorpyrifos and the combination of chlorpyrifos and enrofloxacin, but changes to vitamin levels were less pronounced in the latter group. Even though enrofloxacin is considered to be a relatively safe medicament for animals, the conducted studies indicate that it may induce insignificant oxidative stress.

References


Cemek M, Buyukben A, Buyukkuroglu ME, Aymelek F, Tur L (2010) Protective roles of vitamin E (α-tocopherol), selenium and vitamin E plus selenium...


