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Effect of the subchronic and early-life permethrin exposures on rat liver: oxidative stress and endogenous antioxidant responses

DIPLOMA THESIS

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RNDr. Lucie Škarydová, Ph.D.

Camerino and Hradec Králové 2012   Matyáš Palan

„Hereby I declare that this thesis is my original author's work. All literature and other sources, which I used for the elaboration of this thesis, are stated in the references and properly cited in the text. The thesis has not been used to obtain different or the same degree."

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ABSTRACT

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Title of diploma thesis: Effect of the subchronic and early-life permethrin exposures on rat liver: oxidative stress and endogenous antioxidant responses

Permethrin is a widely used insecticide with a low acute toxicity to mammals; however, there are concerns about consequences of a long term and an early life exposure to this compound. Two different animal models were chosen in order to study effects of permethrin on liver. In the *model of subchronic treatment*, adolescent rats treated for 60 days with permethrin (150 mg/kg body weight/day) were used and sacrificed immediately after the treatment. As far as the *model of early life* is concerned, the treatment with permethrin (34.05 mg/kg body weight/day) lasted for 15 consecutive days in early life and sacrifice took place after the interval of 300 days since the beginning of the treatment.

To evaluate the impact of permethrin treatment on liver tissue, markers of oxidative stress (lipid peroxidation, content of carbonyl groups), amount of superoxide dismutase, catalase, glutathione peroxidase, activity of glutathione transferase, amount of glutathione and fluidity of membranes were measured.

This work demonstrated that permethrin treatment caused an oxidative stress and alterations in antioxidant enzymes within both models. The changes were more pronounced in the *model of subchronic treatment* as the liver in the *model of early life treatment* showed a high ability to renew a damaged tissue before the sacrifice was made. An increased oxidative stress is one of the factors leading to several serious diseases, thus an exposition to permethrin might be associated with an outbreak of these diseases. Further studies to evaluate effects of permethrin on molecular level, especially on gene expression, are necessary to be done.

ABSTRAKT

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Název diplomové práce: Účinek podání permetrinu po narození a ve formě subchronických dávek na játra potkanů: vliv na oxidační stres a antioxidační odpověď

Permetrin je široce používaný a pro savce málo toxický insekticid. Nicméně dlouhodobá expozice nebo expozice v počáteční fázi života může způsobovat problémy. Pro studium vlivu permethrinu na játra byly vybrány dva různé zvířecí modely. V *modelu subchronické expozice* byli použiti adolescentní potkani, kterým byl podáván permethrin (150 mg/kg tělesné váhy/den) po dobu 60 dní. Byli usmrceni ihned po ukončení expozice. Pro *model expozice v počáteční fázi života* byla použita mláďata potkanů, kterým byl podáván permethrin (34.05 mg/kg tělesné váhy/den) po dobu 15 po sobě následujících dní. Potkani byli usmrceni po 300 dnech od začátku léčby.

Za účelem vyhodnocení dopadu expozice byly měřeny markery oxidačního stresu (peroxidace lipidů, obsah karbonylových skupin), množství superoxid dismutázy, katalázy, glutathion peroxidázy, aktivita glutathion transferázy, množství glutathionu a fluidita membrán.

Měření ukázala, že permethrin způsobil oxidační stres a změny v obsahu antioxidantů u obou modelů. Výrazněji se změny projevily u *modelu subchronické expozice*. Játra u *modelu expozice v počáteční fázi života* totiž před tím, než bylo provedeno usmrcení, prokázala vysokou schopnost regenerovat poškozenou tkáň. Vzhledem k tomu, že zvýšený oxidační stres je jedním z faktorů vedoucích k různým závažným chorobám, může mít expozice permethrinem vliv na propuknutí těchto chorob. Další studie vyhodnocující vlivy permethrinu na molekulární úrovni, zejména na genovou expresi, jsou nezbytné.

CONTENTS

1   INTRODUCTION

Permethrin, a member of the family of pyrethroids, is a widely used, relatively cheap, and potent insecticide with low acute toxicity to mammals. It has been proved recently, that despite the low acute toxicity an exposition to permethrin increases oxidative stress within the cells of mammals. For that reason, permethrin might be one of the possible factors leading to various diseases connected with an increased occurrence of reactive oxygen species. Thus, a research on the connection between permethrin and oxidative damages within a liver cell is the main subject of the thesis. Also, changes in susceptibility of an organism during different life stages represent one of the issues that are dealt with in the present research.

This work makes part of a broad research of effects of pyrethroid insecticides on mammals carried out by Dr. Rosita Gabbianelli and her team at the Department of Molecular Biology of University of Camerino. Dr. Rosita Gabbianelli's team has published various papers dealing with the effects of pyrethroids on different tissues at the biochemical and the molecular level.

The present research deals with effects of permethrin on liver as a vital organ responsible for the major part of metabolism, and one of the most exposed organs if permethrin is administered by oral route. Observing changes in liver that were induced by permethrin might be essential for understanding the mechanism of how the organism deals with an exposition to this compound. The present thesis might help to better evaluate the potential risks of pyrethroids.

2   GENERAL PART

In the present part of the thesis information about uses, classification, metabolism and mechanism of action of pyrethroids with the main focus on permethrin is provided. Also, selected antioxidant enzymes and molecules, involved in dealing with stress induced by administration of pyrethroids, are described in this chapter.

2.1   PYRETHROIDS

Pyrethroids (PYR) are widely used insecticides derived from pyrethrins, natural organic compounds found in genus Chrysanthemum. It is a very potent group next to organochlorides, organophosphates, and carbamates containing more than 3500 registered formulations. Their advantages are high lipophilicity, short half-life in the environment, low toxicity to terrestrial vertebrates, and no accumulation in food chains. Their importance has grown since 1970s after the ban on organochloride DDT (Schleier and Peterson 2011). PYR are used to control the insect in agriculture, households, textile industry, as anti-woodworm agent, and also in human and veterinary medicine (Bradberry S. M. et al. 2005; Aldridge W. N. 1990).

The risks for the environment result from their widespread use which is not strictly controlled. PYR are not selective and they kill both beneficial insect and pest insects. Well known is the toxicity to aquatic animals despite the low half-life time of PYR in water (Schleier and Peterson 2011).

The mechanism of action is based on the blockage of different types of sodium channels and therefore there emerge undesirable effects. Regardless of their low acute toxicity profile, there are concerns about negative effects to immune, nervous, and hormonal system after a long lasting exposition. PYR might also influence the development of foetus. According to Berkowitz et al. (2003), their metabolites can be found in the urine of pregnant women from the New York City urban area. Moreover due to their widely use the presence of pyrethroid metabolites in urines of all population have been detected (Barr D. B. et al. 2010; Saieva C. et al. 2004). On the other hand, PYR have an irreplaceable role in the control of malaria. For example, PYR are the only insecticides recommended for the treatment of mosquito nets (Internet 1).

2.1.1 Classification

PYR are divided into two groups according to the chemical structure and the presence of a typical intoxication syndrome. Type I PYR are molecules without the cyano group (Fig. 1) and intoxication is manifested by tremor. Type II has the cyano group (Fig. 1), and a typical intoxication syndrome is characterised by choreoathetosis with salivation (Soderlund et al. 2002). Type I examples are: permethrin (PERM), tetramethrin, allethrin, bifenthrin. Type II includes among others: cypermethrin, deltamethrin and cyfluthrin. Differences between these two types can be found in action on motor nerve terminals, and in modification of sodium channels. Type I cause presynaptic repetitive discharges, while type II causes a tonic release of transmitter from the motor nerve terminal. Type I modifies channels in closed state, whilst type II in their open state (Schleier and Peterson 2011). Another target of type II are the GABA receptors on chloride channels, but the difference of intoxication syndrome cannot by fully explained by this fact (Burr et al. 2003).
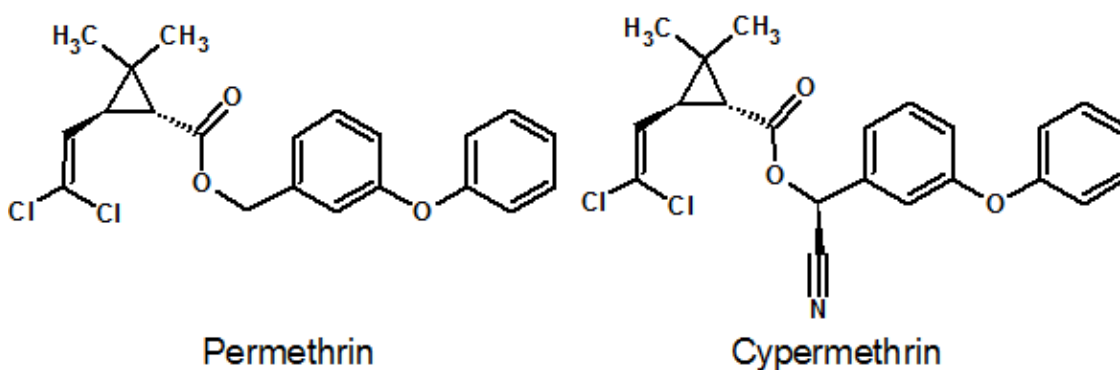
Figure 1 Type I pyrethroid permethrin and type II pyrethroid cypermethrin.

2.1.2 Structure

PYR are esters derived from natural structure of Pyrethrin I and Pyrethrin II (Fig. 2). Changes in alcohol and acid moiety lead to more stable structures with improved insecticidal potency. Replacing the alkenyl side chain and the cyclopentenolone by an aromatic or heterocyclic substituent within the alcohol moiety created more potent compounds with increased photostability and less mammalian toxicity. Finally, replacements of the acid moiety increased the photostability (e.g. chlorines for methyl groups in PERM (Fig. 2)) to a sufficient level allowing agricultural use.

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Deltamethrin (Fig. 2) is an example of a photostabile compound with added α-cyano group that leads to a greater insecticidal potency. Other pyrethroid-like compounds can lack the ester bond on condition that the overall configuration remains preserved (Soderlund 2002).
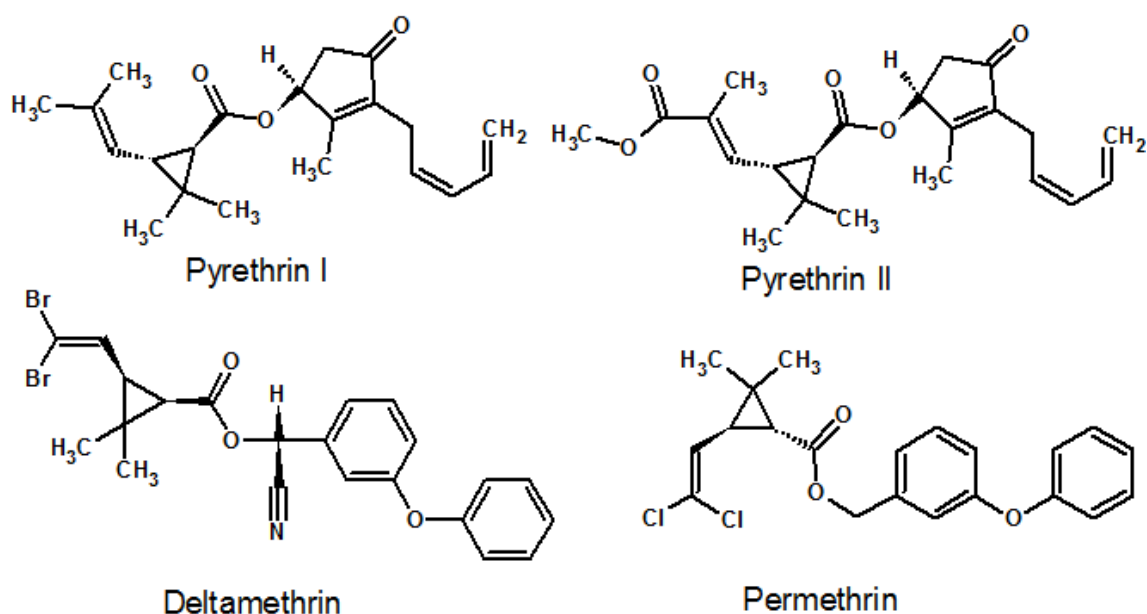
Figure 2 Natural pyrethrins and artificial pyrethroids.

2.1.3   Uses

PYR are estimated to have a 23% share in the insecticides market (Schleier and Peterson al. 2011). According to the United States Environmental Protection Agency, they were number six in 2007 in home and garden US market sector of conventional pesticides. Only carbaryl belonging to carbamates was more commonly used insecticide. The overall trend in the total insecticide use is: a decrease of percentage of organophosphates and an increase of all other insecticides, PYR included (Internet 2).

2.1.3.1 Agriculture

In agriculture, the PYR are most commonly used for a crop protection (e.g. fields of cotton, cereals and vegetable). Also fruits, bulbs of plants or wood can be preserved by PYR. Despite their high toxicity for water animals, they are used in small doses against sea-lice on salmonid fish (Lawrence et al. 2010). The advantage of PYR in agriculture is a short half-life. For that reason, the residue levels after application decline into acceptable values within days, and the crop production can be used without limitations after relatively short pre-harvest interval (Ripley et al. 2001).

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2.1.3.2 Human medicine

PYR are used in human medicine as pediculicide, especially against head lice and scabies. Their use is very extensive. According to Turner et al. (2010), three million people of the British population need anti-head lice treatment each year, and pyrethroid PERM was one of the two most commonly used active ingredients for scabies treatment.

A great importance of PYR lies in controlling the malaria disease. According to WHO, group of PYR is the only one recommended group of long lasting insecticides for treatment of mosquito nets (Internet 2).There has been evidence that using bed nets treated by PERM in order to protect children younger than five is highly cost effective, and this kind of treatment also has a positive effect on non-protected population living nearby (Wiseman et al. 2003). However, there has been a rising concern about growing resistance of mosquitos in areas with extensive use of photostabile and highly effective PYR. In Western Kenya, there were even found mosquitos with a cross resistance to PYR and DDT (Kawada et al. 2011; N'Guessan et al. 2010).

2.1.3.3 Veterinary medicine

Veterinary pharmaceuticals are another source of PYR, the population can easily get into contact with them. These are very popular over-the-counter drugs massively used to control pet's fleas (Turner et al. 2010). Other dermatological animal diseases e.g. scabies can be treated by drugs with content of PYR as well.

2.1.3.4 Household uses

PYR are relatively safe contact insecticides used against crawling and flying insect which are available for domestic use. Volatilisation is necessary to provide a direct contact with flying insect. There have been several studies concerned with exposition to PYR. For example in a questionnaire study from New York City 70 % of pregnant women told that they were exposed to indoor pesticides and in 55 % of their urine samples indeed detectable amounts of metabolites of PYR were found (Berkowitz et al. 2003).

Bedbugs represent an internationally increasing problem often solved by PYR, and misuse of insecticides against bedbugs can lead to acute intoxications. However, the most serious cases of intoxication reported from seven states of the USA from 2003 to 2010 were caused by totally unrespect for directions written on the label or even by use of insecticide designed for other purposes (Internet 3).

Exposition to PYR is increased by a slower degradation process in indoor conditions. Any organic matter or dust can serve as a reservoir (e.g. carpets infused by PYR). Due to lack of extreme temperatures, sunshine, and rain residues can persist for a longer time than outside (Quiros-Alcalá 2011).

Another possible contact with PYR is connected with the previously mentioned administration of insecticides to domestic animals and in certain regions with application on bed nets.

2.2   PERMETHRIN

PERM, type II pyrethroid, was the first pyrethroid ever with sufficient photostability for agricultural use synthesised in 1972. Since this breaking point, the whole pyrethroid group became one of the most spread groups of insecticides with PERM as a leader type II structure.

2.2.1   Stereochemistry

PERM (3-phenoxybenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) consists of two possible enantiomeric pairs (Fig. 3) of which 1-RS diastereomers (cis, trans) are used. [1-R,cis]-PERM possesses both insecticidal activity and acute toxicity for mammals whereas [1-R,trans]-PERM lacks measurable acute toxicity but preserves its insecticidal potency. The low toxicity of trans isomer was ascribed to its faster degradation by esterase, however, even a direct intracerebral administration did not cause the toxicity syndrome (Soderlund et al. 2002; Schleier and Peterson 2011). It also emerged from toxicokinetic studies that cis isomer has a much lower clearance than trans isomer and persists longer within the body (Soderlund et al. 2002). On the other hand, trans isomers show higher potency to influence estrogenic receptors with a stimulation effect on gene transcription (Jin et al. 2008; Nillos et al. 2010). We can conclude that each of the PERM isomers differs in its toxicity but only mixtures of both isomers are broadly available.
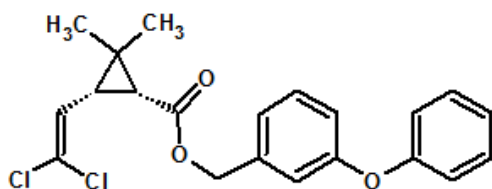
1RS,3RS - permethrin
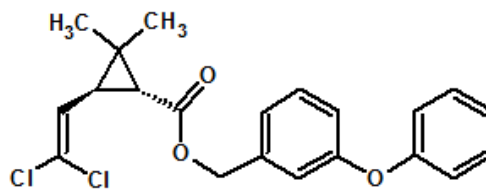
1RS,3SR - permehtrin
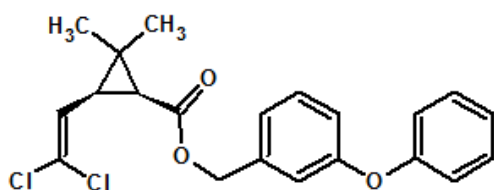
1SR,3SR - permethrin
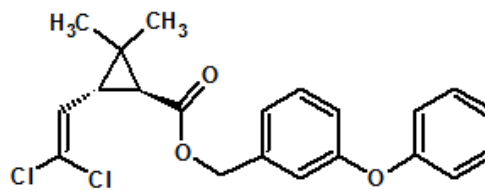
1SR,3RS - permethrin

A. cis enantiomers

B. trans enantiomers

Figure 3 Stereoisomers of permethrin.

2.2.2 Mechanism of action

The primary target of PERM is a voltage-gated sodium channel abundant in nerve cells (Cao et al. 2011). The essence of these channels consists in transferring the action potential throughout the nerve fibre or to muscles. The stimulation results in opening of the channel and Na^+ ions can influx into the cell. The higher amount of Na^+ ions inside of the cell causes depolarisation and changes in Na^+ and K^+ permeability. The inside of the nerve cell becomes positively charged in respect of the outside. An absolute refractory period when the cell cannot be stimulated is followed by a partially refractory period in which the cell can be forced to action by a stimulus of a high density (Schleier et al. 2011).

PERM binds with higher affinity to sodium channels occurring in an opened action state and causes prolongation of this state. This results in a multiple long-lasting action potential and spike discharges. An overall disruption of nerve impulses can follow.

Ca-ATPase and (Ca-Mg)-ATPase are among the other targets of PYR, but together with GABA receptors they represent sites with a minor influence to the overall effect, contrary to the main mechanism through sodium channels (Schleier et al. 2011).

2.2.3 Metabolism in human

After an oral administration, PYR are rapidly absorbed and within hours they can be found in every tissue (Miyamoto 1976). The high lipophilicity of PYR enables also other ways of absorption, e.g. penetration through an epidermis or diffusion along cells. Another source of intoxication is the inhalation of vapours (Schleier et al. 2011).

PERM is rapidly metabolised in blood and liver. The first phase of the main detoxification pathway involves cleavage of the ester bond by esterase resulting in cis- and trans-(2,2-dichlorovinyl)-3,3-dimethylcy-clopropane-l-carboxylic acid (cis- and trans-DCAA) and 3-phenoxybenzyl alcohol (Fig. 4). The alcohol moiety is then oxidised by cytochrome P450 up to 3-phenoxybenzoic acid (3PBA). All metabolites are subsequently excreted by urine in non-conjugated or conjugated form with more polar compounds (Rose et al. 2005).

As previously mentioned, the liver metabolism of cis PERM is generally slower than the metabolism of trans PERM (Miyamoto 1976).
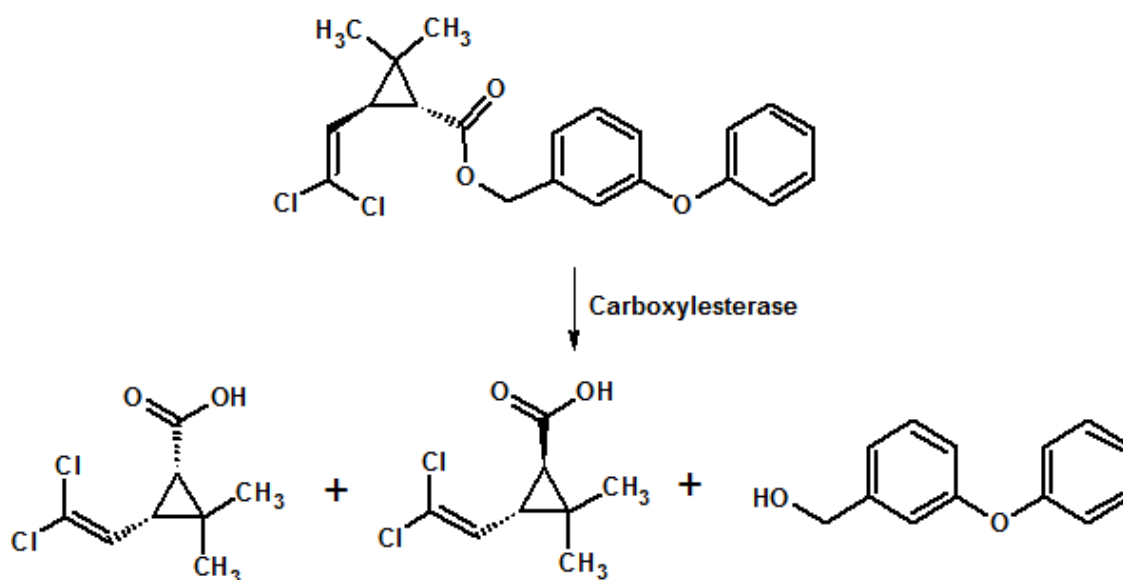

Figure 4 First step of permethrin detoxification.

2.2.4 Effects of permethrin

PERM and its metabolites have various effects on the organism. Because of its actual mechanism of action, PERM can also alternate human voltage-gated sodium channels, and the result is dose dependent acute toxicity. Unlike insects, mammals possess multiple different isoforms of these channels, thus mammals are much less susceptible to intoxication (Soderlund et al. 2001; Parkin and Quesne 1982). However, a long-lasting exposition can lead to neurodegeneration, heart and liver damage, and to alteration of immune responses. All these effects are probably based on the oxidative stress.

PERM is an inducer of oxidative stress (decribed in 2.3) that causes cellular damage (Giray et al. 2000; Gupta et al. 1999), rats treated with PERM in daily oral doses of 150mg/kg showed DNA damages, decrease of GSH levels, changes in a plasma membrane fluidity and alternation of a locomotor activity (Nasuti et al. 2007; Falcioni et al. 2010). Any compound that increases oxidative stress can be considered as potentially harmful (Miqliore and Coppedé 2009). It is important to note that the aspects of possible adverse effects have not been completely recognized until now.

2.2.4.1 Histopathological changes in liver

The liver is the main organ responsible for the metabolism of compounds naturally occurring in a body and of xenobiotics as well. PERM is also a substance which is rapidly metabolised mostly by the liver; no residues accumulate in body tissues of exposed organisms (Santos et al. 2011). Due its detoxifying function, the liver is very often the organ most exposed to toxic substances. Despite their high ability to renew a damaged tissue if the liver is overloaded by toxic substances, lesions may occur (Roma et al. 2011).

The PERM treatment causes several histopathological and histochemical changes in the liver tissue. In general, alterations are most visible in peripheral regions because they get into contact with xenobiotics first. A reduced size of nuclei, increased amount of Kupffer cells, altered amount of proteins, polysaccharides, lipids and extended vacuoled regions were observed in comparison to the control group. Also, hepatic capillaries got increased in a diameter probably due to infiltration of defence cells trying to perform phagocytosis (Roma et al. 2011).

2.2.4.2 Immune system

Previous studies with monocytes, lymphocytes and polymorphonuclear neutrophils showed that PERM induced changes in these cells of the immune system. PERM treatment reduced the capacity of monocytes to produce ROS during the respiratory burst, thus the reaction to possible pathogen was weakened (Gabbianelli et al. 2009a). Also, it was proved that PERM can interact with DNA of lymphocytes and cause significant damages (Gabbianelli et al. 2004). In the population of neutrophils an increased production of ROS during respiratory burst was found, suggesting that PERM might affect neutrophils as a priming agent (Gabbianelli et al. 2009a). In both cases changes in fluidity of plasma membrane bilayer were found. Since parts of NADPH complex, necessary for producing ROS during respiratory burst, are located on a plasma membrane, PERM can influence the complex through increased oxidation of lipids and proteins near to this complex (Gabbianelli et al. 2009a; Gabbianelli et al. 2009b).

2.2.4.3 Nervous system

PERM influences both peripheral and central nervous system, and causes repetitive discharges with similar effects to DDT. The mechanism of action of PERM, as mentioned above, could be described by slowing the kinetics of opening and closing of Na channels. One of the studies demonstrated that a daily dermal dose of 0.13mg/kg of PERM within 30 days proved no evident neurotoxicity but behaviour impairment, neurodegeneration, and alteration of regulatory mechanisms of CNS were observed on animal models (Abdel-Rahman et al. 2004).

Findings that foetuses and children are exposed to PYR raise concerns over possible developmental neurotoxicity (Berkowitz et al. 2003). One of the PYR, bioallethrin, was reported to cause permanent changes in behaviour of adult rats if administered during a neonatal period (Talts et al. 1998). Also, an experimental model of Zebrafish embryos showed a potential risk of developmental neurotoxicity after an exposition to several PYR, PERM included (DeMicco et al. 2009). In one study, the prenatal exposition of rats to PYR evoked changes in a locomotor activity and dopaminergic system measured at 60 and 140 days of age (Ray and Fry 2006).

2.2.4.4 Carcinogenicity

The U.S. EPA classifies PERM as likely to be carcinogenic (Internet 4). Some studies on animal models showed a potentially increased risk of carcinogenicity (Gabbianelli et al. 2004; Ishmael and Lithfield 1988; Price et al. 2007), but the prospective Agricultural Health study with more than 49 thousands of pesticide

applicators showed no association between PERM and any type of cancer. The only fact that could raise concerns is an elevated risk for a multiple myeloma. However, in this particular case number of workers with multiple myeloma was low and future studies are necessary to have conclusive evidence (Rusiecki et al. 2009).

Suggestions that PERM can be carcinogenic were based on a hypothesis that PERM is involved in a breakdown of tryptophan leading to carcinogenic products (el-Toukhy et al. 1989), and also on its involvement in the inhibition of intercellular communication through gap junctions (Tateno et al. 1993). But carcinogenic effects are highly dose dependent and a real life exposure is much lower than exposure used in studies on animal models (Deguchi et al. 2009; Rusiecki et al. 2009).

2.2.4.5 Hormonal system

PERM is considered, along with the other PYR, to be an endocrine disruptor (Garey et al. 1998; Jin et al. 2011; Go et al. 1999). Several studies revealed that PERM has a potential to induce histological changes in testis and alternate levels of testosterone (Issam et al. 2011; Zhang et al. 2008). It was also shown that different enantiomers have a different reproductive toxicity. According to Jin et al. (2011), (+)-cis-PERM has the greatest disruption activity and (+)-trans-PERM the lowest one. These results are in accordance with Zhang et al. (2008) suggesting that only cis isomers have adverse effects on the reproductive system. PERM has, according to Garey and Wolf (1998) and Go et al. (1999), no estrogenic or progestagenic activity; other researchers propose that the metabolites of PERM possess this activity (Nillos et al. 2010; Jin et al. 2008). Although the results are unequivocal, there are remaining concerns about possible effects of PERM and its metabolites to the endocrine system.

2.3   OXIDATIVE STRESS

The organism has several defence mechanisms whose function is to cope with reactive oxygen species (ROS). The ROS, e.g. hydroxyl radical or superoxide radical, are created by free radicals containing unpaired electrons and causing oxidation of other molecules. ROS may be generated by an exposure to UV, heat, ionizing radiation or chemical able to produce free radicals. However, they can also be produced endogenously (e.g. respiratory burst or by leakage from respiratory chain). Defence mechanisms include antioxidant compounds (e.g. glutathione, arginine, citrulline, vitamins A, E, C) and antioxidant enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase). Under physiological conditions these mechanisms are able to deal with the oxidative stress. However, an excess of the ROS of any origin

can damage the organism and result in neurodegeneration or DNA damage (Migliore and Coppedè 2008).

To reveal the oxidative stress in human tissues measureable biomarkers are employed, e.g. lipid peroxides are markers of lipid oxidation, the amount of protein carbonyls serves as a marker of protein oxidation, and level of 8-hydroxy-guanin is a marker of DNA damages (Takahashi et al. 2001; Kale et al. 1999; Fagan et al. 1999).

2.3.1 Selected antioxidant enzymes and molecules

As stated in 2.2.4., PERM increases the oxidative stress. Changes in the amount or activity of several antioxidant enzymes and molecules are directly connected with oxidative processes within cells. In the following paragraphs a more detailed description of important enzymes and molecules, to which the attention is paid in the experimental part, will be provided.

2.3.1.1 Catalase

The catalase (CAT) is a ubiquitous tetrameric metalloprotein abundant in blood and liver peroxisomes that catalyses decomposition of a hydrogen peroxide to water and dioxygen (Fig. 5). The CAT acts not only as a protecting enzyme against the ROS, but it has also an impact on detoxification, e.g. oxidation of short chain alcohols to aldehydes. The CAT is a part of homeostatic mechanisms of cell, and together with other antioxidant enzymes and molecules helps to keep a balance between creation and decomposition of ROS (Voet 2011). An overexpression of CAT and SOD can provide protection against harmful effects of ROS (Day 2009). On the other hand, a lack of CAT is not lethal but manifests itself as an Acatalasia. A decrease of CAT activity can be compensated by an increase of another antioxidant and vice versa (Kasapoglu and Özben 2000). After an acute bout of exercise, CAT levels showed mostly no alterations (Ji 1999). An exposition to PYR results in different effects to the CAT activity. An important distinguishing feature here is the type of the tissue, duration of exposition and the dose (Eraslan et al. 2008; Gabbianelli et al. 2004; Kale et al. 1999; Manna et al. 2004; Nasuti et al. 2003).
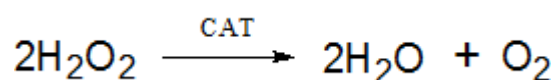
$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Figure 5 Catalase reaction.

2.3.1.2 Superoxide dismutase

Superoxide anion (O_2^-) is mostly created by an occasional leakage of electron from cytochromes. It has been estimated that 0.1 to 0.4 % of an electron flow in mitochondria forms O_2^- which is under normal circumstances removed by superoxide dismutase (SOD). The O_2^- can convert other molecules to radicals with even higher reactivity, and the excess of these ROS is a factor of numerous diseases (Golden and Patel 2009). SOD catalyses dismutation of O_2^- yielding to hydrogen peroxide and dioxygen (Fig. 6), and thus creating less reactive forms of oxygen. The SOD did not show any beneficial effects if it was administered to animal models without other antioxidant enzymes (Day 2009). This fact supports the idea of a connection among protective antioxidant enzymes. A complete lack of mitochondrial SOD is lethal; deficiency in the cytosolic and extracellular SOD leads to the oxidative damage of DNA, increased cancer rates and higher susceptibility to stress (Golden and Patel 2009). Variable effects on SOD levels were observed after an exposition of animal models to PERM or to other PYR (Gabbianelli et al. 2002; Gabbianelli et al. 2004; Manna et al. 2004; Nasuti et al. 2003).

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} 2H_2O + O_2$$
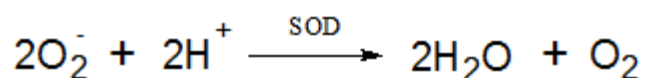
Figure 6 Superoxide anion dismutation.

2.3.1.3 Glutathione peroxidase

Glutathione peroxidase (GPx) is selenium containing tetrameric enzyme that catalyses a decomposition of hydrogen peroxides yielding to water and dioxygen with concomitant oxidation of GSH (Fig. 7). The oxidised glutathione is subsequently reduced by glutathione reductase (GSR) using NADPH (Day 2009). The GPx is more efficient enzyme than CAT (Kasapoglu and Ösben 2001), and is also responsible for a breakdown of lipid peroxides, thus GPx is linked up with hydroperoxides status of cell membrane. Subchronic exposition of adolescent rats to PERM as well as the early life treatment led to a decrease of GPx in different tissues (Gabbianelli et al. 2002; Gabbianelli et al. 2004; Nasuti et al. 2003; Nasuti et al. 2007). The decrease of GPx was observed also after an exposition to several other PYR (Chargui et al. 2010; Fetoui et al. 2010; Raina et al. 2009).
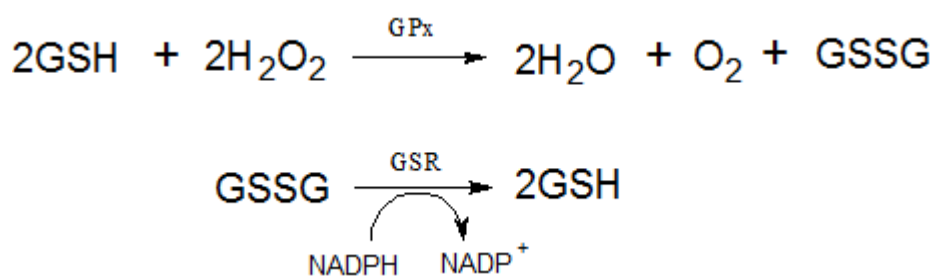
$$2GSH + 2H_2O_2 \xrightarrow{\text{GPx}} 2H_2O + O_2 + GSSG$$

$$GSSG \xrightarrow{\text{GSR}} 2GSH$$
$$\overset{\curvearrowright}{NADPH \quad NADP^+}$$

Figure 7 Reaction catalysed by the GPx and regeneration of GSH.

2.3.1.4 Glutathione transferase

Glutathione transferases (GST) are enzymes widely distributed in organisms with three distinguished families. Mitochondrial and cytosolic GST are soluble proteins; the third one, microsomal GST, is a membrane associated protein. The GST catalyses reaction of glutathione (GSH) with compounds containing electrophilic carbon. GST is also responsible for detoxification of broad spectrum of xenobiotics (e.g. arene oxides, substituted benzenes, quinones), for inactivation of endogenous molecules (e.g. aldehydes, epoxides, hydroperoxides), and it is equally involved in biosynthesis of hormones and local signalling molecules. Disruption of genes regulating GST leads to higher susceptibility to xenobiotics, oxidative stress, increased risk of cancerogenesis and cardiovascular diseases, changes in inflammatory response and to alteration of lung function (Hayes et al. 2005). After the subchronic or chronic exposition of animal models to PYR, an increase of the level of GST was observed (Raina et al. 2009). Short term exposition demonstrated various effects on the levels of GST (Giray et al. 2001; Singh et al. 2009; Kale et al. 1999).

2.3.1.5 Glutathione

GSH, the most abundant non-protein thiol source in a cell, keeps intracellular environment in a reduced state. The ratio of reduced GSH and its oxidized form determines the oxidation status of sulfhydryl group of cell proteins (Hazelton and Lang 1979). GSH is not solely an antioxidant molecule; it is equally involved in detoxification of broad spectrum of compounds, regulation and synthesis of signalling molecules, transport of molecules, metabolism of iron and DNA repair (Ji 1999; Hazes et al. 2005). In the majority of cases, animal models treated with PERM showed a decrease of the GSH levels measured in striatum, heart and liver (Girai et al. 2001; Nasuti et al. 2007; Nasuti et al. 2008; Vadhana et al. 2011a).

2.3.2 Fluidity of membranes

The oxidation process at protein and lipid levels can modify the physical-chemical state of plasma membrane, thus measurement of the plasma membrane fluidity is useful to quantify the impact of the oxidative stress on membranes. Changes in fluidity are associated with lipid peroxidation, carbonyl group formation and with changes in composition of the plasma membrane bilayer. Probes with diverse region of incorporation can be used to examine a fluid state of membranes.

One of the probes is 1,6-diphenyl-1,3,5-hexatriene (DPH) which localizes in hydrophobic region of the bilayer. Steady-state fluorescence anisotropy (r) of DPH is calculated using an excitation and emission wavelengths of 360 and 430 nm, respectively, according to the equation (Shinitzky and Barenholz, 1978):

$$r = (I\| - I\perp g) / (I\| + 2I\perp g)$$

where g is an instrumental correction factor, and $I\|$ and $I\perp$ are the intensities measured with the polarization plane parallel and perpendicular to that of the exciting beam.

Another often used probe is 2-dimethylamino-6-lauroylnaphthalene (laurdan) which localizes in hydrophilic-hydrophobic part of the bilayer. Generalized polarization of Laurdan is calculated according to Parasassi equation (Parasassi et al. 1990):

$$GP_{340} = (I_B - I_R) / (I_B + I_R)$$

where I_B and I_R are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum and correspond to the fluorescence emission maximum in he gel and liquid-crystalline phases of the bilayer (Parasassi et al., 1991).

3   AIM OF THE WORK

The aim of the present thesis was as follows:

- to measure markers of oxidative stress and determine the levels of antioxidant enzymes and GSH in liver of rats from two different animal models prepared in advance (models of early life and subchronic treatment with PERM)

- to determine changes in fluidity of plasma membrane in liver cells of both models caused by exposition to PERM

- to evaluate alterations of the antioxidant system of liver cells of both models

- to compare the impact of two different types of treatment with PERM on liver of animal models

- to compare changes in liver of both models with previous studies performed on different organs and tissues

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Chemicals

All reagents were of pure and analytical grade. Albumin from bovine serum (≥96%), Folin & Ciocalteu's phenol reagent, catalase from bovine liver (2000-5000 units/mg protein), 1-chloro-2,4-dinitrobenzene, copper sulphate, deoxycholic acid, ethylenediaminetetraacetic acid disodium salt, 5,5'-dithiobis-(2-nitrobenzoic acid), L-Glutathione reduced (≥98%), glutathione peroxidase from bovine erythrocytes (≥300 units/mg protein), glutathione reductase (≥12 units/mg protein, recombinant, expressed in Escherichia coli), glutathione-s-transferase from equine liver (≥25 units/mg protein), superoxide dismutase bovine (recombinant, expressed in Escherichia coli, ≥2500 units/mg protein), guanidine hydrochloride and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, USA). Calcium chloride, natrium hydroxide, sodium azide, sodium bicarbonate, sodium carbonate, sodium tartrate, potassium tartrate, sodium phosphate dibasic and phosphoric acid were purchased from J.T.Baker (Deventer, Holland). The natrium chloride was purchased from VWR International (Haasrode, Belgium). Hydrogen peroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate dibasic, hydrochloric acid, ethanol absolute and ethylacetate were purchased from Carlo Erba (Milano, Italy). Sodium citrate and trichloroacetic acid were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Adrenalin was purchased from Merck (Darmstadt, Germany). Nicotinamide adenine dinucleotide phosphate, tetrasodium salt (≥97%)(NADPH) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). 1,6-diphenyl-1,3,5-hexatriene (DPH), diphenyl-1-pyrenylphosphine (DPPP) and 6-lauroyl-2-dimethylaminonaphtalene (laurdan) were purchased from Molecular Probes (Eugene, OR, USA). Technical grade (75:25, *trans:cis*; 94% purity) 3-phenoxybenzyl-(1R,S)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, PERM (NRDC 143) was generously donated by Dr. A. Stefanini of ACTIVA (Milan, Italy).

4.1.2 Instruments and software

- Fluorescence methods were performed on a Hitachi 4500 spectrofluorometer, Hitachi Ltd. (Tokyo, Japan).

- Spectrophotometry was carried out on a Varian Cary 219 spectrophotometer, Varian, Inc. (USA).

- Centrifugation was carried out on the eppendorf centrifuge MPW Centrifuge MPW-65 R, MPW Med. instruments, Co-Operative (Warsaw, Poland), and on the Centrifuge 4225, Apparecchi per Laboratori Chimici (Milan, Italy).

- For the statistical analyses GraphPad Prism 4 was used. Graphs were created by Microsoft Excel 2010, chemical structures and equations were made by ChemSketch 12.01, and Microsoft Word 2010 was used as a text editor.

4.1.3 Solutions and buffers

- Phosphate buffer saline (PBS): sodium chloride 8 g/l, potassium chloride 0.2 g/l, disodium hydrogen phosphate dibasic 1.15 g/l, potassium dihydrogen phosphate monobasic 0.2 g/l, distilled water, pH 7.4.

- Precipitation solution: phosphoric acid 16.7 g/l, ethylenediaminetetraacetic acid disodium salt (EDTA) 2 g/l, sodium chloride 300 g/l, distilled water.

- DNTB solution: sodium citrate 10g/l, 5,5'-dithiobis-(2-nitrobenzoic acid)(DNTB) 0.4 g/l, distilled water.

4.2   MODEL OF SUBCHRONIC TREATMENT WITH PERM

4.2.1 Animals

Male Wistar rats from Charles River (Calco, LC, Italy), weighing 120–130 g and about 5 weeks old were used. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature-controlled room (21 ± 5°C) and maintained on a laboratory diet (pellet 4RF from Mucedula, Settimo Milanese, Italy) with tap water ad libitum. The light/dark cycle was from 7 a.m. to 7 p.m. Animal use in this study complied with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

4.2.2 Treatment

PERM was dissolved in corn oil and administered orally (5 ml/kg) (n = 12) for 60 days, at a dose of 150 mg/kg body weight per day (1/10 of LD50) by intragastric tube. Rats serving as controls (n = 8) received 5 ml/kg body weight of corn oil orally for 60 days by intragastric tube. The substances were administered daily to the rats and the volume administered was based on body weight. The animals were observed on a daily bases and weighed at regular intervals. After 60 days of treatment the rats were killed by CO_2 asphyxia. Liver samples were immediately frozen and stored in a freezer under -80°C until use.

4.2.3 Sample preparation

Pieces of liver of a proximate weight of 0.3 g were cut into smaller pieces by surgical blade, 1ml of ice cold PBS was added, and the liver tissue was homogenised in batches by pestle. Then, the samples were centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and diluted by 5 ml of PBS. The samples were put on the ice until Lowry assay, and they were subsequently stored at freezer under -80°C until use.

For the GST assay the same routine was performed but using a different buffer. Except of PBS, ice cold 0.1 M sodium phosphate buffer, pH 7.5 containing 0.3 % Triton X-100 was used.

Pools containing the same amount of protein were prepared from the samples taken from individual rats. Therefore, pools derived from the samples taken from three different individuals in a treated group, and pools derived from the samples taken from two different individuals in a control group were prepared. Eventually, the total number of samples was eight, each group counting four samples.

4.3 MODEL OF EARLY LIFE TREATMENT WITH PERM

4.3.1 Animals

Male and female Wistar rats from Charles River (Calco, LC, Italy) of the weight of 250–270 g and the age of approximately 90 days were used. The animals were housed in plastic (Makrolon) cages (five rats per cage) in a temperature controlled room (21 ± 5 °C) and maintained on a laboratory diet with water ad libitum. The light/dark cycle lasted from 7 p.m. to 7 a.m. Animal use in this study complied with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Rat pups born in our laboratory from primiparous dams were used in the study. The parturition day was set as Post Natal Day 0 (PND0). On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed, and the female pups were discarded. Two male pups were assigned to each dam until weaning (PND21). No cross-fostering was employed. At 2 days of age, litters were randomly assigned to two experimental groups (n = 6 rats for each).

4.3.2 Treatment

PERM was dissolved in corn oil and administered orally (2 ml/kg) at a dose of 1/50 of LD_{50} by intragastric tube. The LD_{50} was determined in adult rats (Cantalamessa, 1993), thus 34.05 mg/kg was the dose of PERM. The compounds were administered once a day in the morning from PND6 to PND21. Control rats were treated with vehicle (corn oil 2 ml/kg) according to similar schedule. The volume of the compound administered was adjusted daily, based on body weight measured during the dosing period. On PND21, the offspring were weaned and the littermates were housed together. In adult age (PND300), six animals from each group (PERM treated and control groups) were sacrificed by exposure to CO_2, and liver were collected and stored at freezer under -80°C until analyses. For the purpose of experiments, the groups of animals were formed by drawing animals from different litters so that no group contained siblings.

4.3.3 Sample preparation

Sample preparation was the same as described in 4.2.3.

4.4   METHODS

4.4.1   Determination of protein concentration

The Lowry method (Lowry et al. 1951) was employed to evaluate protein concentration of samples

The liver homogenate was used for the assay. Each sample consisted of 10 µL of the protein solution, 0.1 ml of 8 % deoxycholate, 890 µL of distilled water and of 2 ml of reagent (3); a mixture of reagent (1) (2% sodium carbonate in 0.1 M sodium hydroxide) and reagent (2) (0.5 % copper sulphate in 1% sodium and potassium tartrate) mixed in the ratio 50:1. Every sample was vortexed and allowed to stand at room temperature. After 10 minutes the 0.2 ml of Folin-Ciocalteu reagent (previously mixed in a ratio of 1:1 with distilled water) was added and immediately vortexed. After 30 minutes of incubation in dark, every sample was read at 750 nm using the spectrophotometer. A standard curve using bovine albumin was made to calculate a protein concentration.

4.4.2   Method for determination of lipid peroxidation

Lipid peroxides were detected using a diphenyl-1-pyrenylphosphine (DPPP) probe according to Takahashi et al. (2001).

0.4 mg of protein taken from every sample of liver homogenate was adjusted up to 1 ml with polyphosphate buffer (PBS; pH 7.4). Subsequently, 1 µL of 1 mM DPPP was added and samples were incubated in dark at 37°C for 5 minutes. After the incubation, fluorescence intensities were read by spectrofluorometer using 351 and 380 nm as excitation and emission wavelengths, respectively.

4.4.3   Determination of membrane fluidity

Two diverse probes (DPH and laurdan) with different localization within plasma membrane were employed to measure membrane fluidity.

Fluorescence anisotropy was measured using liver homogenate containing 0.4 mg of protein. Volume was adjusted to 1 ml with PBS and DPH was added to a final concentration of 1 µM. After 1 hour of incubation in dark at room temperature, samples were read by Hitachi 4500 spectrofluorometer.

The same concentration of protein was used to measure generalized polarization of Laurdan. Also, the final concentration of the probe laurdan was 1 µM. All samples were incubated for 5 minutes in dark at 20°C and then read by spectrofluorometer using excitation wavelength of 340 nm, and measuring emission

wavelengths of 440 and 490 nm. Results were calculated according to Parasassi equation (Parasassi et al. 1990).

4.4.4 Method for detection of protein carbonyl groups

Content of protein carbonyl groups was measured using modified method of Lentz (1989).

The liver homogenate was used for the assay. Amount of 0.6 mg of protein was incubated with 0.5 ml of 10 mM DNPH in 2.5 M HCl for one hour at room temperature with continuous shaking. A blank lacking DNPH was prepared for each sample. Then the protein was precipitated using 0.5 ml of 20% trichloroacetic acid and centrifuged at 4000 rpm for 5 minutes followed by washing the protein pellet by ethanol/ethylacetate (1:1). The last step was repeated three times, every time followed by centrifugation. As the last step, the protein was dissolved in 0.6 ml 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer, pH 6.5, vortexed, incubate at 37°C for 15 minutes, and then read at 370 nm. Results were calculated using a molar absorption coefficient of 22 000 M^{-1} cm^{-1}.

4.4.5 Superoxide dismutase assay

For determination of SOD, method based on the inhibition of autooxidation of epinephrine was used (Misra and Fridovich 1971).

Total cuvette volume was 2 ml and consisted of 50 µL of 0.05 M epinephrine dissolved in 0.06 M HCl, 0.05 M sodium carbonate buffer with 0.1 mM EDTA, pH 10.2 and of sample solution of liver homogenate. Suitable volume of the sample solution was determined with respect to the standard curve. Kinetics of the reaction was measured on spectrophotometer with set up wavelength at 480 nm, temperature of 37°C and the buffer as a reference. The most linear part of sigmoidal curves was taken into account, and the percentage of inhibition was calculated. The solution made only of buffer and epinephrine was considered as a solution with 0% inhibition. A standard curve was utilized to calculate the amount of SOD. Results were reported as µg of SOD in 1 mg of protein.

4.4.6 Method for determination of catalase

The method is based on a detection of decrease of UV absorption of H_2O_2 caused by decomposition of H_2O_2 catalysed by CAT (Luck 1974).

Solution containing 66 mM sodium phosphate buffer, pH 7.0 and pure 9 M H_2O_2 mixed in the ratio 1:625, was prepared for the assay. Total volume of 3 ml

consisted of 100 µl of liver homogenate and 2.90 ml of solution with H_2O_2. Time dependent absorption changes were measured on spectrophotometer using wavelength of 240 nm at 20°C. Results were calculated using a standard curve and they were reported as µg of CAT in 1 mg of protein.

4.4.7 Colorimetric assay of glutathione

To estimate the level of glutathione in the liver tissue, method according to Butler et al. (1994), was employed.

The liver homogenate was used for the determination of the level of glutathione. Assay consisted of 2 mg of protein topped up to 1 ml by 2.7 mM ethylenediaminetertaacetic acid disodium salt. Subsequently, 1.5 ml of precipitation solution as described in 4.1.3 was added, vortexed and centrifuged for 10 minutes at 4000 rpm. One millilitre of the supernatant was collected and 2 ml of 0.37 M disodium hydrogen phosphate dibasic were added. As the last step 0.5 ml of DNTB solution was added, immediately vortexed and read at 412 nm at room temperature. Samples were measured against the blank and results were calculated using a standard curve made of different concentrations of GSH.

4.4.8 Method for determination of glutathione peroxidase

Glutathione peroxidase (GPx) was determined according to the method of Paglia and Valentine (1967).

Assay consisted of: 2.755 ml of 0.05 M sodium phosphate buffer containing 5mM EDTA pH 7.0, 50 µl of 8.4 mM NADPH, 5 µl of GSR (205 units/ml), 10 µl of 0.125 M NaN_3, 50 µl of GSH 0.15 M, 100 µl of 2.2 mM H_2O_2 and 30 µl of liver homogenate. Hydrogen peroxide was added directly into cuvette as the last one, and the decrease of absorption was measured at 340 nm at 20°C with phosphate buffer as a reference. Results were calculated using a standard curve and they were reported as µg of GPx in 1 mg of protein.

4.4.9 Colorimetric assay of glutathione transferase

Glutathione transferase (GST) assay was performed according to the Sigma-Aldrich protocol available online (Internet 4).

Total cuvette volume of 3 ml consisted of: 2.75 ml of 100 mM potassium phosphate buffer with 1.0 mM EDTA, pH 6.5, 100 µL of 75 mM GSH (dissolved in buffer), 100 µL of 30 mM CDNB (dissolved in absolute ethanol), and 50 µL of liver homogenate (protein concentration 0.1 mg/ml). Enzyme solution was added as the last

one directly into cuvette already placed in the spectrophotometer termostatted at 25°C. Changes in absorption during first 5 minutes were recorded against the blank consisting of buffer, GSH and CDNB. Results were calculated using a standard curve and reported as units of GTS in 1 mg of protein.

4.4.10 Statistical analyses

SOD and GPx assays were performed in duplicate, all other experiments in triplicate. Non-panoramatic t-test was used for a statistical analysis. Experimental data were expressed as mean value ± SEM, and P value < 0.05 was considered significant.

5 RESULTS

5.1 MODEL OF SUBCHRONIC TREATMENT WITH PERM

5.1.1 General findings

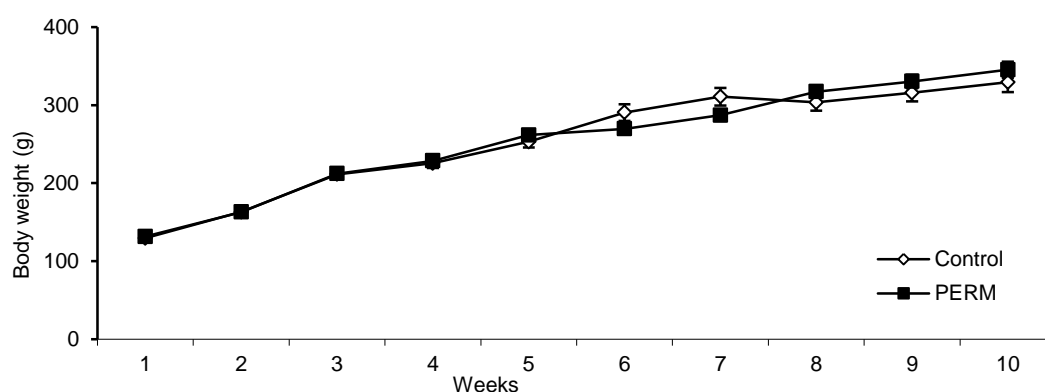No signs of poisoning, gross abnormalities or significant differences in body weight (Fig. 8) were observed.

Figure 8 Body weight of PERM-treated and control rats during 60 days of PERM exposure (150 mg/kg). Data are presented as mean ± S.D.

5.1.2 Lipid peroxidation

The results show that there was a significant difference ($P < 0.01$) between control and treated group. As shown in Fig. 9, within the group of adolescent rats treated for 60 days with PERM (adolescent rats), the treated ones had a higher (24 %) rate of lipid peroxidation (1760 ±75.1 a.u.) with respect to control (1336 ±84.9 a.u.).
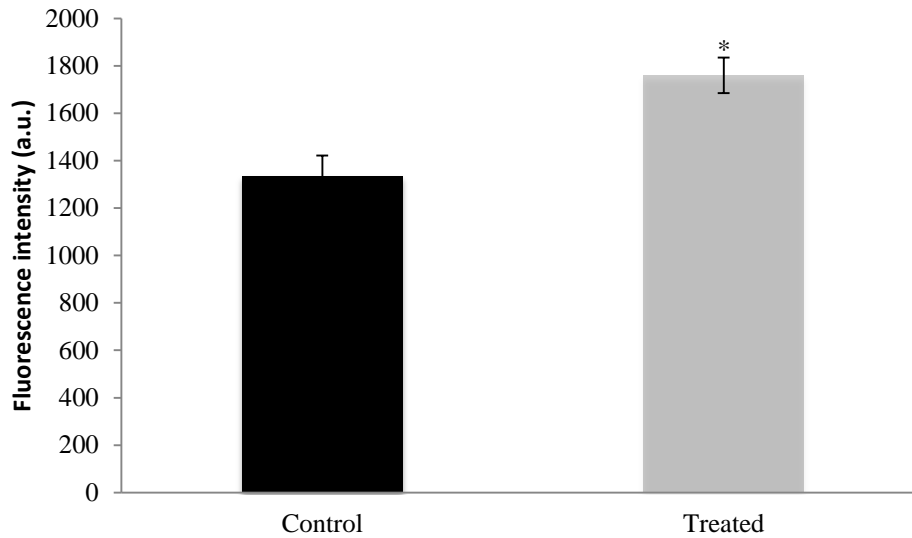
Figure 9 Lipid peroxidation in liver of adolescent rats treated for 60 days * P < 0.01.

5.1.3 Anisotropy, probe DPH

No difference in anisotropy was found (P > 0.05) between control (r = 0.141 ±0.006) and treated group (r = 0.141 ±0.003) in experiments performed on adolescent rats (Fig. 10).
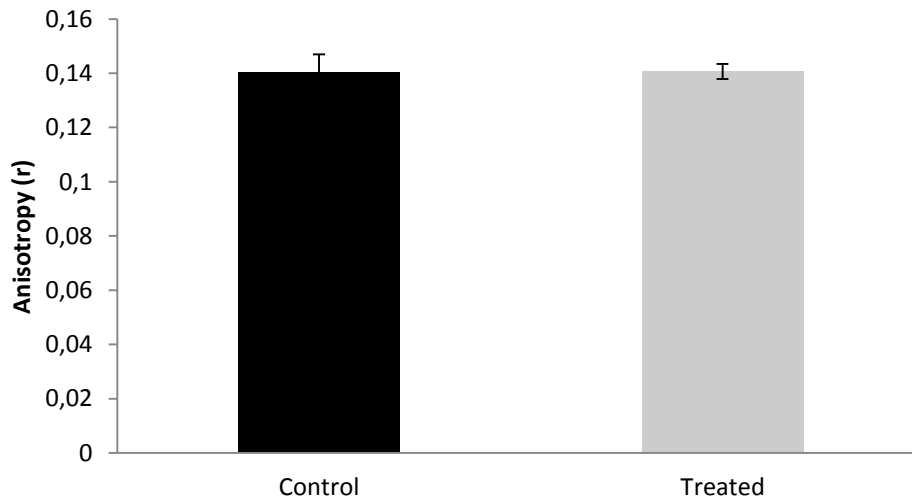

Figure 10 DPH florescence anisotropy measured in liver of adolescent rats treated for 60 days.

5.1.4 Generalized polarization of Laurdan

A significant increase (P < 0.001) of Laurdan generalized polarization was observed in the treated group (GP_{340} = 0.003 ±0.012) of adolescent rats compared to the control one (GP_{340} = −0.058 ±0.006) (Fig. 11). Laurdan probe localizes in hydrophilic-hydrophobic region of the plasma membrane bilayer. Accordingly, results acquired from treated adolescent rats showed a reduction of membrane fluidity in this region in comparison to the control group.
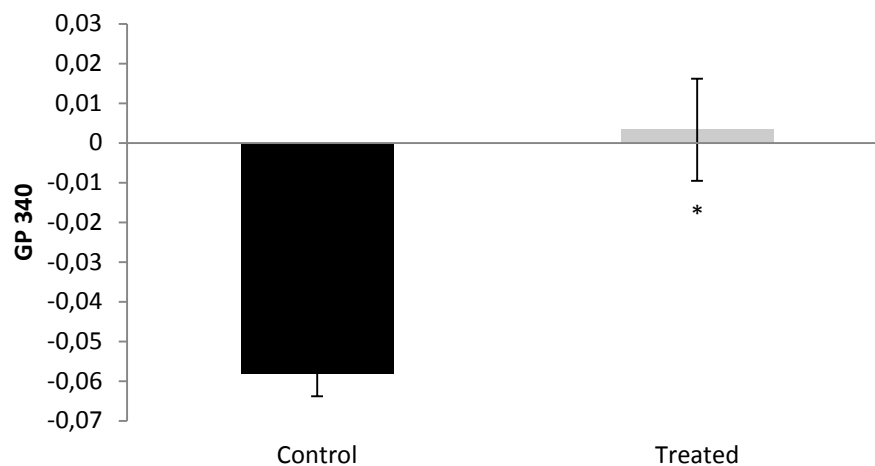

Figure 11 Laurdan generalized polarization in liver of adolescent rats treated for 60 days days * P < 0.001.

5.1.5 Carbonyl groups

Carbonyl group formation, the marker of protein oxidation, significantly (P < 0.001) increased in adolescent rats treated with PERM (6.15 ±0.16 nmol/mg prot.) compared to control group (4.99 ±0.15 nmol/mg prot.) (Fig. 12).
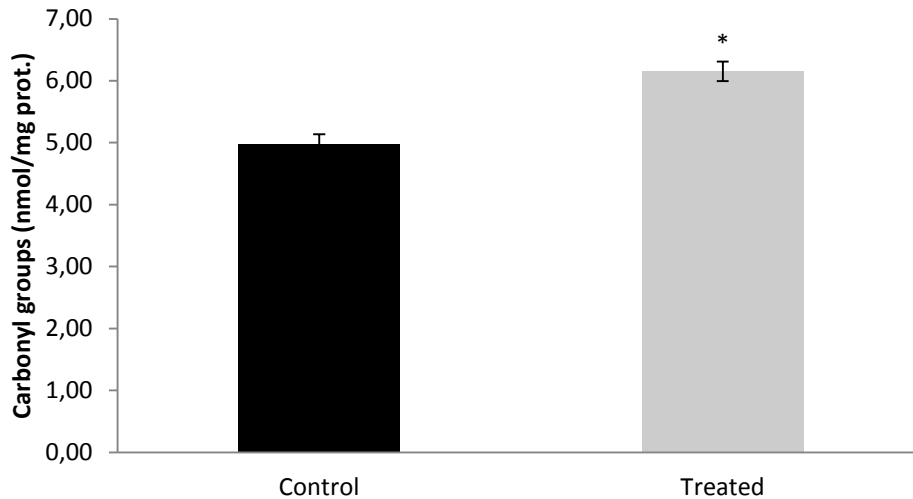
Figure 12 Content of Carbonyl groups measured in liver of adolescent rats treated for 60 days * P < 0.001.

5.1.6 Superoxide dismutase

No differences were observed in levels of SOD (Fig. 13), the antioxidant enzyme responsible for elimination of superoxide anion in experiments with adolescent rats (Treated: 3.34 ±0.22 μg/mg prot.; Control: 3.31 ±0.27 μg/mg prot.).
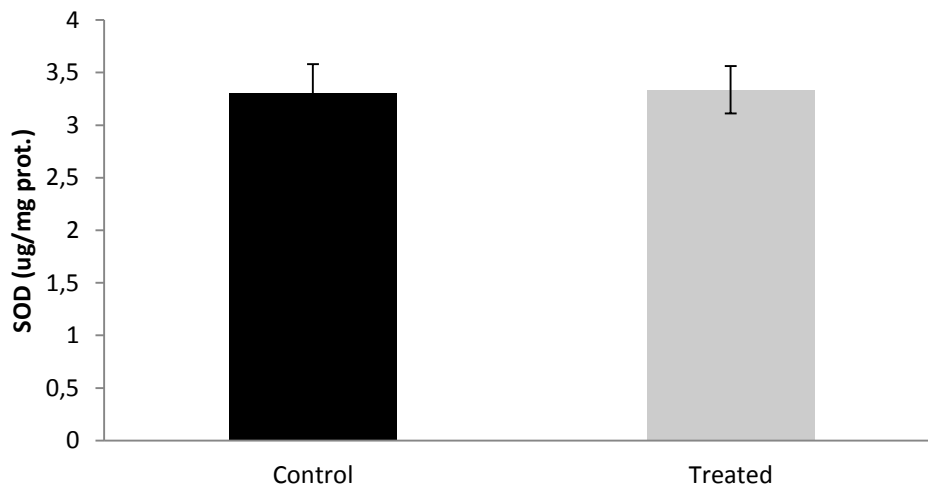

Figure 13 Amount of SOD in liver of adolescent rats treated for 60 days.

5.1.7 Catalase

The liver of adolescent rats treated with PERM had a significantly (P < 0.001) higher content of CAT in comparison with the control group, 21.93 ±0.53 μg/mg prot. and 17.76 ±0.19 μg/mg prot., respectively (Fig. 14).
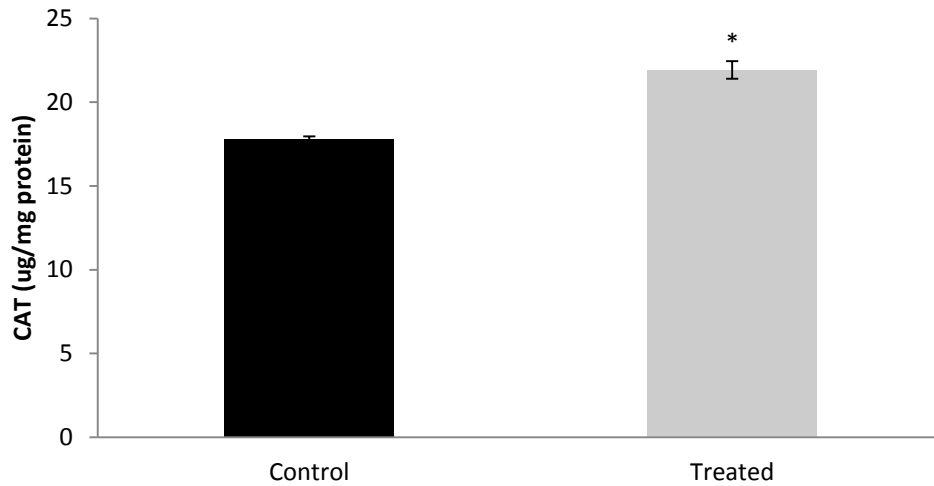
Figure 14 CAT content in liver of adolescent rats treated for 60 days * P < 0.001.

5.1.8 Glutathione

The liver tissue of adolescent rats treated with PERM (1.09 ±0.05 µg/mg prot.) had a significantly (P < 0.001) decreased level of GSH with respect to the control (0.81 ±0.05 µg/mg prot.) (Fig. 15).
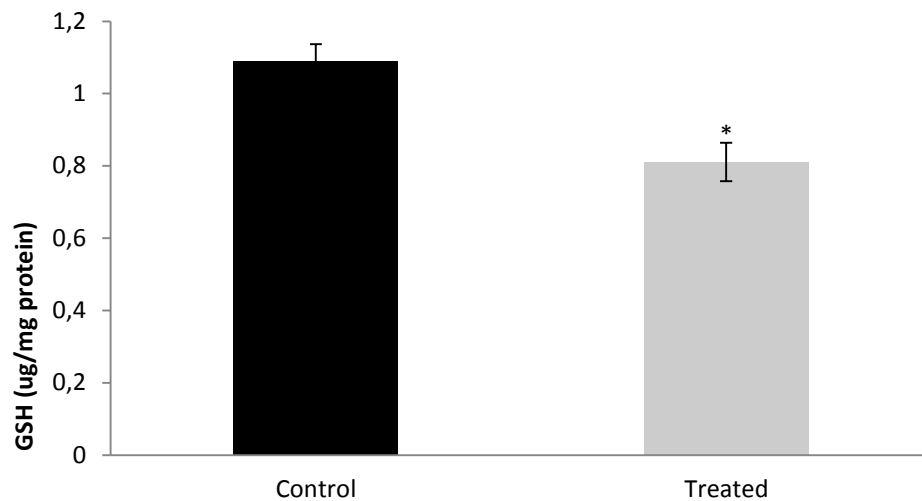

Figure 15 GSH level in liver of adolescent rats treated for 60 days * P < 0.001.

5.1.9 Glutathione peroxidase

The amount of GPx, an antioxidant enzyme that catalyses reaction between GSH and hydrogen peroxide, was significantly ($P < 0.05$) decreased in samples from PERM treated (5.56 ±0.17 µg/mg prot.) adolescent rats with respect to the control group (7.73 ±0.67 µg/mg prot.) (Fig. 16).
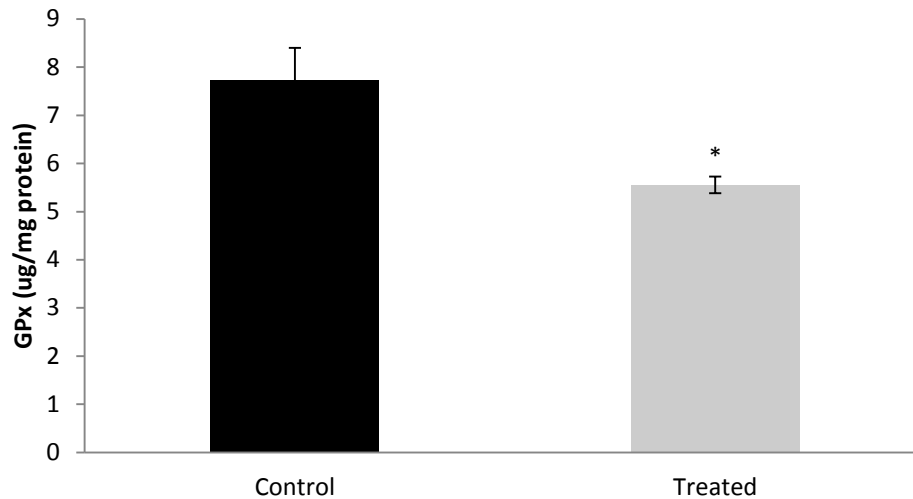
Figure 16 GPx levels in liver of adolescent rats treated for 60 days * $P < 0.05$.

5.1.10 Glutathione transferase

Adolescent rats treated with PERM (6.46 ±0.29 units/mg prot.) had a significantly ($P < 0.05$) increased activity of GST compared to the control group (5.47 ±0.18 units/mg prot.) (Fig. 17). One unit is defined as the amount of GST that will conjugate 1.0 µmole of CDNB with GSH per minute at pH 6.5 at 25°C.

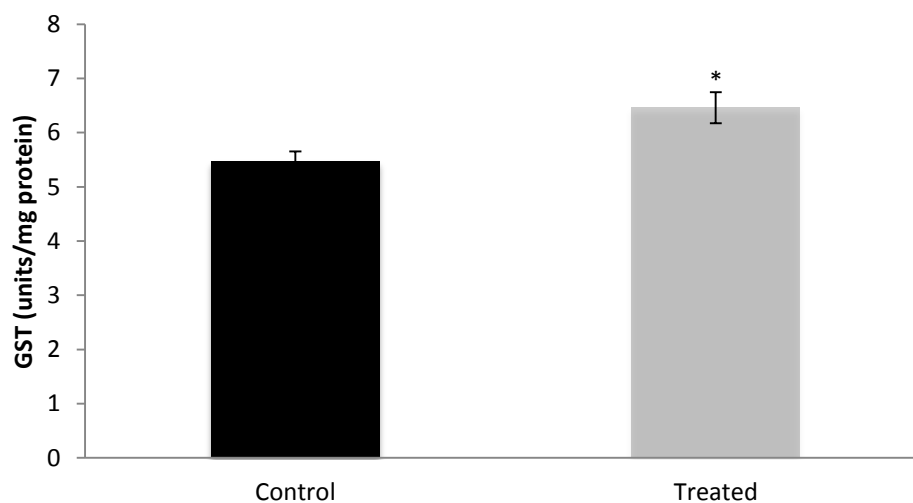**Figure 17** Activity of GST in liver tissue of adolescent rats treated for 60 days * $P < 0.05$.

5.1.11 Adolescent rats, summary of results

Following results were found within the group of adolescent rats treated for 60 days: increased lipid peroxidation, no changes in DPH fluorescence anisotropy, increase of Laurdan generalized polarization, increased amount of carbonyl groups, no difference in levels of SOD, increased content of CAT, decreased content of GSH, decreased amount of GPx and increased activity of GST.

5.2   MODEL OF EARLY LIFE TREATMENT WITH PERM

5.2.1   General findings

No signs of poisoning, gross abnormalities or significant differences in body weight (Fig. 18) were observed.
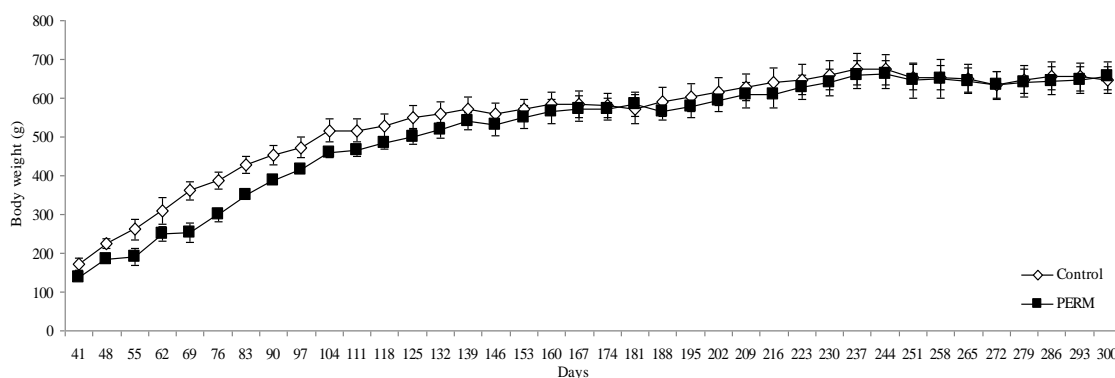
Figure 18 Body weights of control and PERM-treated rats during the life time after the early life exposure to PERM (34.05 mg/kg). Data are presented as mean ± S.D.

5.2.2   Lipid peroxidation

In experiments performed with liver tissue taken from old rats treated in early life with PERM for 15 days (old rats), an increase (10%) of lipid peroxides in treated group (2431 ±29.2 a.u.) was found in comparison to the control group (2193 ±20.7 a.u.) (Fig. 19).
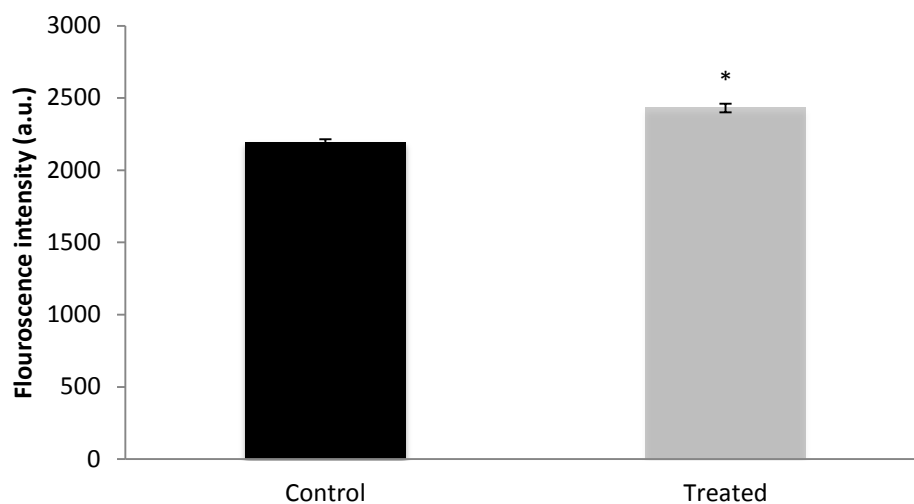
Figure 19 Lipid peroxidation in liver of old rats treated in early life for 15 days * P < 0.01.

5.2.3   Anisotropy, probe DPH

Differences in DPH florescence anisotropy were statistically significant in old rats. The P value was lower than 0.05. Samples from rats treated with PERM had lower anisotropy (r = 0.181 ±0.009) with respect to the control group (r = 0.220 ±0.008) (Fig. 20).
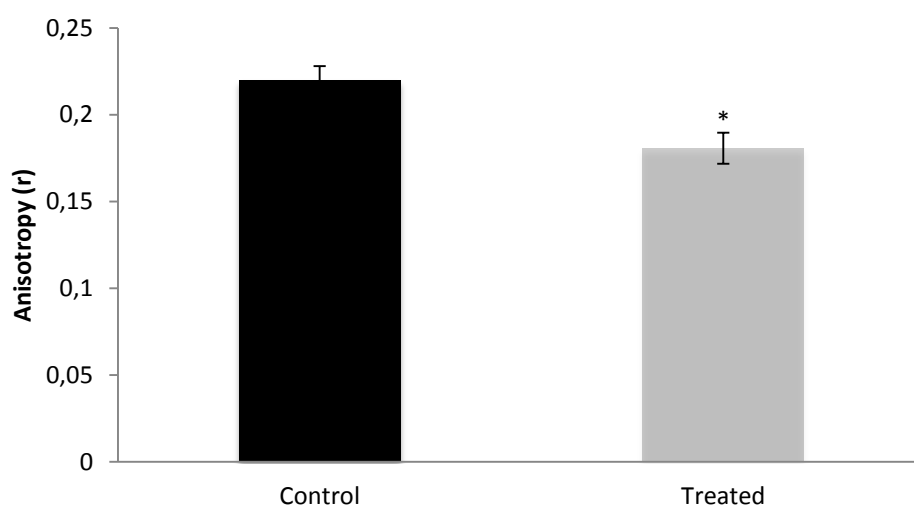
Figure 20 DPH florescence anisotropy measured in liver of old rats treated in early life for 15 days * P < 0.05.

5.2.4 Generalized polarization of Laurdan

Experiments conducted on old rats proved no significant difference between treated (GP_{340} = –0.176 ±0.013) and control group (GP_{340} = –0.169 ±0.06) (Fig. 21).
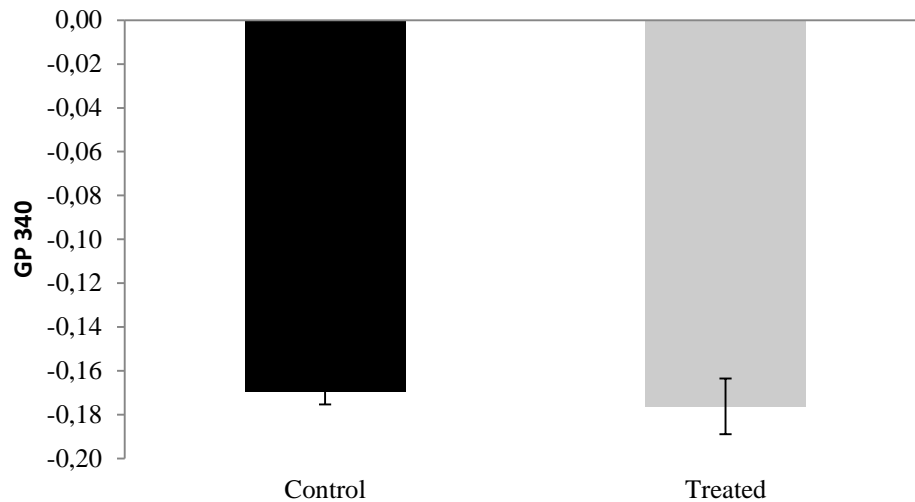
Figure 21 Laurdan generalized polarization in liver of old rats treated in early life for 15 days.

5.2.5 Carbonyl groups

Differences between treated (5.21 ±0.05 nmol/mg prot.) and control (4.84 ±0.16 nmol/mg prot.) group were not significant ($P > 0.05$) in experiments done with old rats (Fig. 22).
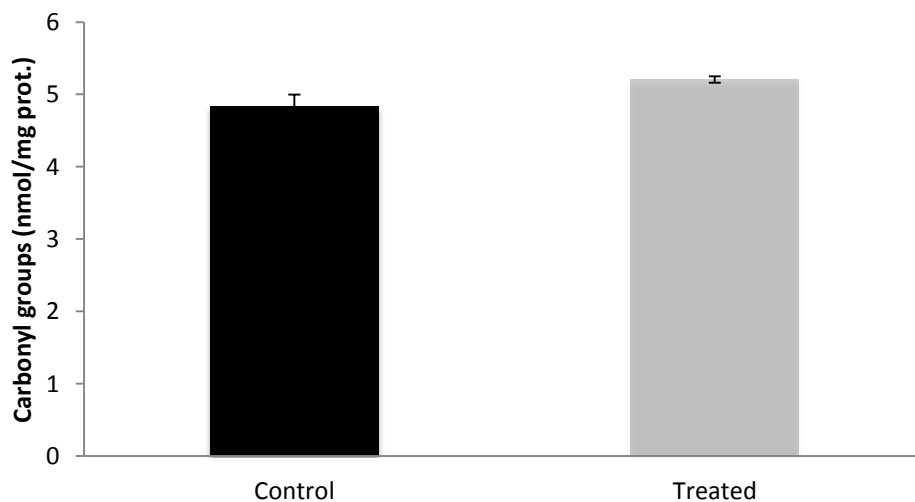
Figure 22 Content of carbonyl groups measured in liver of old rats treated in early life for 15 days.

5.2.6 Superoxide dismutase

No differences were observed in levels of SOD between control and treated group in experiments with old rats (Treated: 1.93 ±0.17 µg/mg prot.; Control: 2.08 ±0 µg/mg prot.) (Fig. 23).
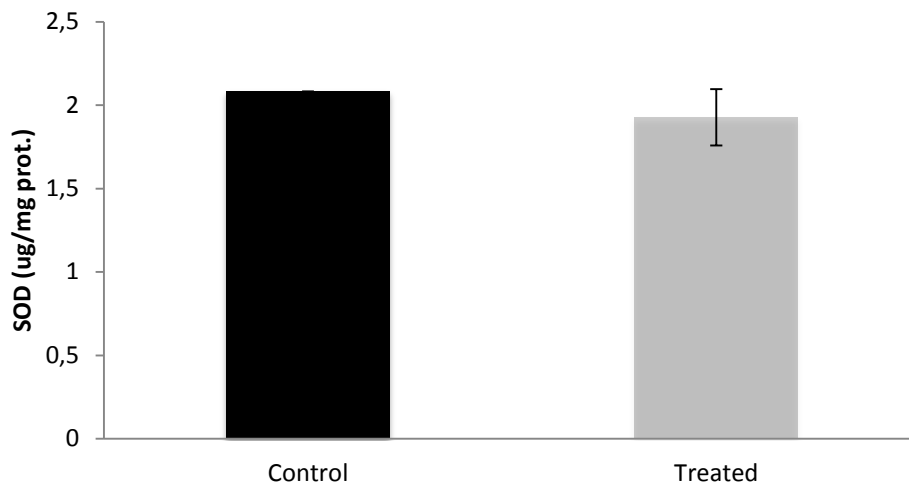
Figure 23 Amount of SOD in liver of old rats treated in early life for 15 days.

5.2.7 Catalase

The amount of CAT in liver of old rats was significantly ($P < 0.05$) decreased within the treated group (56.99 ±2.88 µg/mg prot.) with respect to the control group (45.39 ±3.52 µg/mg prot.) (Fig. 24).
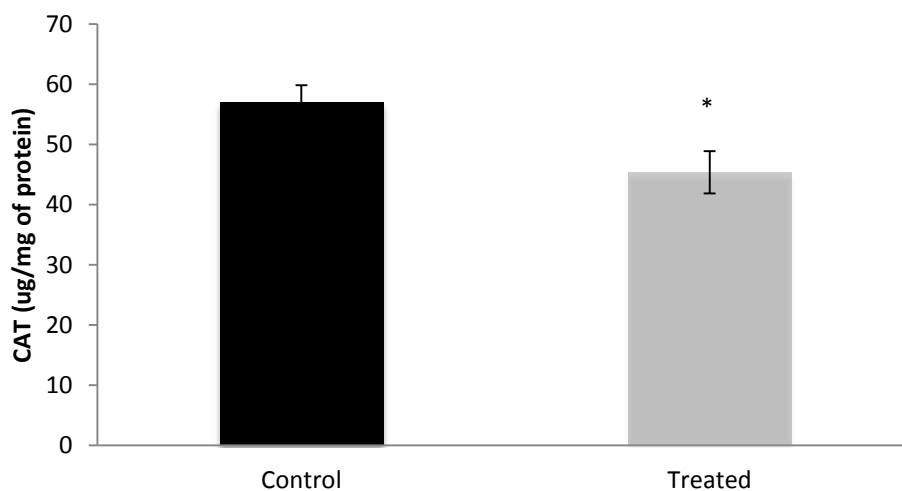
Figure 24 CAT content in liver of old rats treated for 15 days * $P < 0.05$.

5.2.8 Glutathione

The early life treatment had evidently no effect on the GSH level of old rats. No difference was observed between treated (1.11 ±0.06 µg/mg prot.) and control rats (1.15 ±0.06 µg/mg prot.) (Fig. 25).
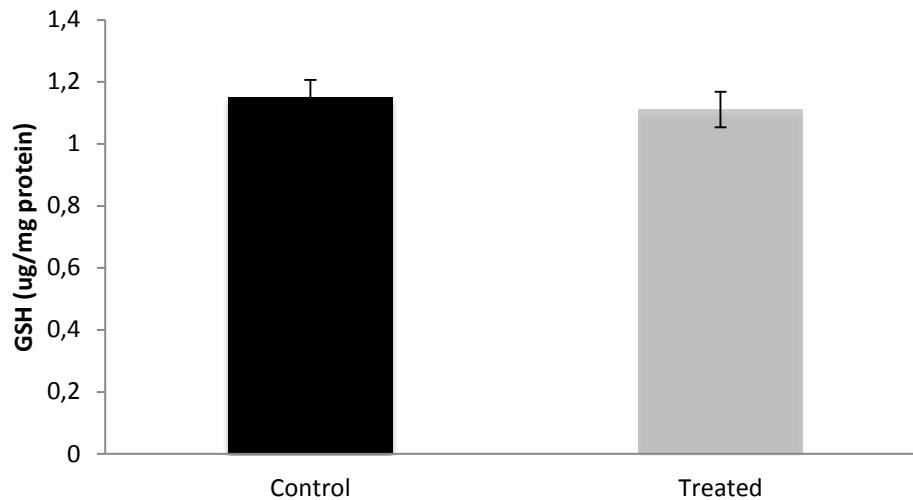
Figure 25 GSH level in liver of old rats treated in early life for 15 days.

5.2.9 Glutathione peroxidase

The measures performed on old rats showed no difference between treated (7.62 ±0.53 µg/mg prot.) and control group (7.89 ±0.21 µg/mg prot.) (Fig. 26).
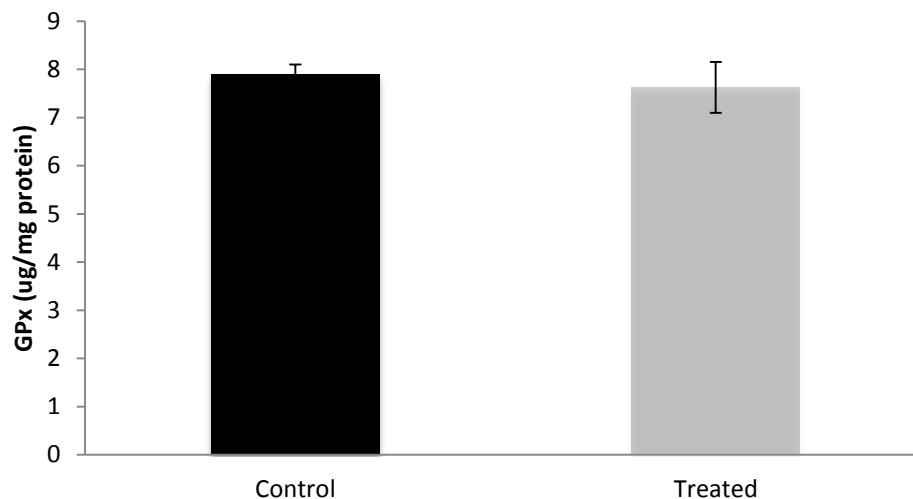
Figure 26 GPx levels in liver of old rats treated in early life for 15 days GPx levels in liver.

5.2.10 Glutathione transferase

No differences were found between treated (6.46 ±0.24 units/mg prot.) and control group (6.35 ±0.21 units/mg prot.) of old rats (Fig. 27).
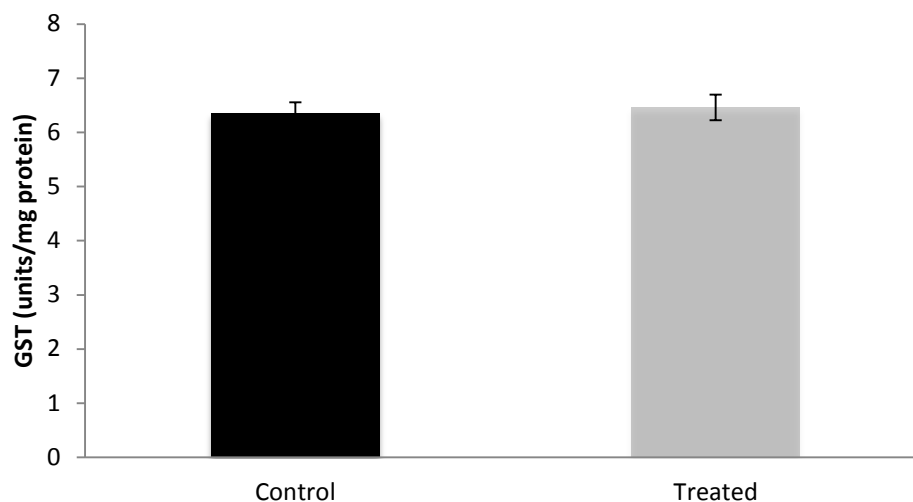
Figure 27 Activity of GST in liver tissue of old rats treated in early life for 15 days.

5.2.11 Early life treatment, summary of results

Following results were found within the group of old rats treated in early life: increased lipid peroxidation, decrease in DPH fluorescence anisotropy, no changes in generalized polarization of Laurdan, no differences in content of carbonyl groups, no differences in levels of SOD, decrease in content of CAT, no changes in amount of GSH, no changes in levels of GPx and no differences in activity of GST.

6   DISCUSSION

The oxidative stress and the inability of organism to deal with it are basic factors of several diseases (e.g. cardiovascular diseases, neurodegenerative diseases, and cancer), as well as of ageing (Migliore and Coppedè 2008; Dalle-Donne et al. 2003). Therefore, a higher exposition to compounds which cause the oxidative stress might increase the risk of these diseases. PERM as a widely used and a broadly available insecticide (Schleier et al. 2011) might be one of the compounds which can get easily into the organism and increase the oxidative stress. That is the reason why the attention should be focused on the research of PERM.

The involvement of oxidative stress in toxicity of PERM has been recently demonstrated on various tissues (Gabbianelli et al. 2004; Otitoju et al. 2008; Nasuti et al. 2008; Vadhana et al. 2011a). The survey was carried out on animal models as well as *in vitro*, and it has been proved that the exposition to PERM provokes various changes in the composition of antioxidant apparatus of cells. In the present research two different models were chosen. The first one, the model of subchronic treatment, simulated the worst case scenario – the administration of relatively high doses for a continuous period of 60 days. Limited data are available for the evaluation of impact of the subchronic treatment with PERM at biochemical level. Thus, the present research should provide the data for future surveys on molecular level. The second model was chosen in order to study the effects of PERM in adulthood if administered during first 15 days of life. This model served as a simulation of administration of PERM during the last months of pregnancy when enzymatic system is not fully developed and the organism is vulnerable. The effects still persisting in adulthood were recorded as the basis for future studies of changes in gene expression caused by PERM.

Despite its low acute toxicity to mammals, a little is known about accurate mechanisms of subchronic or chronic toxicity. A number of studies have demonstrated the effects of PERM on brain, blood cells, immune system, and on heart. But as far as the liver is concerned, a considerably lower number of studies have been made. Nevertheless, the liver is the vital organ responsible for metabolism, and equally, after an oral administration it becomes one of the most exposed organs (Roma et al. 2011). The observed effects of PERM are as follows: alteration in levels of antioxidant enzymes, GSH, lipid peroxides, and protein carbonyls. The evaluation

of changes induced in a liver tissue might be helpful in deducing the risks resulting from the exposition to PERM.

Increased levels of ROS are in direct connection with oxidative metabolism of PERM. PERM detoxification consist of several phases including oxidation by microsomal fraction of the liver; during the oxidation a leakage of ROS can occur (Rose et al. 2005; Schleier et al. 2011). PERM and its metabolites are lipophilic molecules which can easily pass through the plasma membrane bilayer and cause lipid peroxidation, protein oxidation, or create bridges between molecules. By that mechanism, PERM may cause a decrease in the plasma membrane fluidity (Vadhana et al. 2011b).

A decrease in fluidity was observed in the subchronic type of treatment in the present research. Specifically, the generalized polarization of Laurdan was increased in comparison with the control rats. The decrease in fluidity of a hydrophilic-hydrophobic region of the bilayer was in accordance with the results of other markers of oxidative stress, concretely with the lipid peroxidation and the content of carbonyl groups. Similarly, the decrease in fluidity was observed after the treatment with PERM in heart cells (Vadhana et al. 2011a; Vadhana et al. 2011b), striatum (Nasuti et al. 2008) and polymorphonuclear neutrophils (Gabbianelli et al. 2009). Different results showing an increased fluidity were found in erythrocytes of rats treated with PERM (Nasuti et al. 2003). Lipid peroxides together with bridges among molecules of fatty acids could reduce the natural movement of molecules in plasma membrane. Consequently, an increased amount of lipid peroxides could lead to a more rigid state of the membrane bilayer, changes in permeability to different substances, and in general to an alteration of metabolic processes (Wong-Ekkabut et al. 2007). The protein oxidation might be responsible for an increase in generalized polarization as well because the laurdan probe is localized in the external region which also contains proteins. A higher content of carbonyl groups could contribute to the decrease in fluidity within this region. Only the hydrophilic-hydrophobic region of the bilayer was affected and no alterations were observed in the inner hydrophobic region. One of the factors that could contribute to the higher rate of lipid peroxides was a decrease of GPx, one of the most efficient enzymes responsible for decomposing H_2O_2 and lipid peroxides (Kasapoglu and Ösben 2001; Day 2009). Similarly, a decrease in GPx was observed in studies examining liver and brain (Eraslan et al. 2008), erythrocytes (Gabbianelli et al. 2002), lymphocytes (Gabbianelli et al. 2004) and whole blood (Nasuti et al. 2007) isolated from rats treated with PERM or cypermethrin. Lower levels of GPx within the treated group might be compensated by higher levels of GST. This enzyme can dispose of lipid peroxides and even though GST is not directly responsible

for a PERM detoxification, the higher levels of GST are often connected with an exposition to PYR. Facilitation of detoxification reactions, sequestrating PYR, and disposing of lipid peroxides might be the main objective of GST (Kostaropoulos et al. 2001). An increase in the CAT level could be a result of compensation mechanisms which were counteracting the decrease in GPx and GSH levels (Kasapoglu and Özben 2000). CAT is a cytosolic antioxidant enzyme which does not need GSH for its functioning (Fig. 5). Thus, CAT could partially overtake the activity of GPx. The decrease in GSH could be explained by its higher consumption in reactions catalysed by GPx and GST. GSH is an essential molecule involved in numerous biochemical reactions. Likewise, a decrease in GSH was observed in striatum of rats receiving the same type of treatment (Nasuti et al. 2008) as well as in striatum and hearts of rats treated in early life (Nasuti et al. 2007; Vadhana et al. 2011a). SOD was the only antioxidant enzyme which did not show any changes. An overexpression of SOD can protect cells against the increase of ROS (Day 2009), however, in this particular case other antioxidant enzymes might compensate the raised oxidative stress. Therefore, we can suggest that the content of SOD in liver is very stable and PERM was not able to induce any changes in the content of this enzyme.

The early life treatment with PERM increased the level of lipid peroxides as well as subchronic treatment did. However, no decrease in fluidity was observed following subchronic treatment, on the contrary, a decrease in anisotropy was found in early life treated rats. This means that the fluidity of the inner part of hydrophobic region of plasma membrane bilayer was increased. Mostly fatty acids can be found in this region. No alterations of antioxidant enzymes and of GSH were found except for decrease in CAT. Previous studies based on the same type of treatment but dealing with different organs displayed more changes in antioxidants and fluidity as well. In heart, there was observed a decrease in fluidity and GSH and an increase in lipid peroxides and GPx. A decrease in GSH and GPx, but no changes in lipid peroxides were measured in striatum; the level of carbonyls, on the other hand, was increased. SOD was not altered in any of the cases (Vadhana et al. 2011a; Nasuti et al. 2007). We can assume that liver has a higher ability to renew damaged tissue in comparison with heart and striatum, and thus the levels of antioxidant enzymes and of GSH have been soon (sacrification at PND300) restored back to normal values. Hazelton and Lang (1979) demonstrated that the total content of GSH in liver is 10 times higher than in heart. Therefore, the antioxidant capacity of liver can be higher as well. Consequently, this might result in lower susceptibility of liver in comparison with other organs. However, PERM could influence the processes of composition of plasma membranes during the early life treatment, and the higher fluidity of hydrophobic region

could be a possible result of it. Thus, the only markers demonstrating that the oxidative stress took place in early life were lipid peroxides and the decrease in CAT. The comparison between results obtained from liver and from other organs suggests that other organs suffered a more extensive damage than the liver. It is supposed that the damage was caused by an incomplete development of liver enzymes catalysing detoxification of PYR.

It should be considered that the dosage administered to pups was lower compared to that subchronic treatment, according to a higher susceptibility of baby animals to PYR (Cantalamessa 1993), and it was close to "no observed adverse effect level" (NOAEL) for permethrin of 25 mg/kg. Heart damages and striatum impairment in adulthood were observed in previous studies applying the early life treatment. However, the subchronic treatment evidently affected the liver more than the treatment in early life did. It is possible that the gap between the last administration of PERM on PND21 and scarification on PND300 was sufficient for the liver tissue to renew. In the subchronic type of treatment the rats were sacrificed immediately after the 60 days long period of regular daily administration of PERM. Therefore, the liver was exposed to PERM up to the very last day of the experiment and the antioxidant apparatus of cells could not be restored to normal values as compared to the control rats. CAT and changes in fluidity were the two markers in which different tendencies were observed. The increase in CAT level during the subchronic treatment was explained in terms of a compensative reaction to a higher occurrence of ROS and to a decrease in other antioxidant enzymes. However, the decrease in the same enzyme during early life treatment could mean that PERM influenced an expression of CAT but did not influence other antioxidant enzymes. The early life treatment obviously caused changes in fluidity in a different region of membrane bilayer than the subchronic treatment. The increase in fluidity in the hydrophobic part of bilayer was previously explained by the incorporation of hydrophobic molecule of PERM (Gabbianelli et al. 2003), but in this particular case no PERM molecules could be incorporated because of the short elimination half-life (Soderlund et al. 2002). Hence, on PND300 there were no residues of PERM which may have caused it. On the contrary, the changes in hydrophilic-hydrophobic region observed on rats under the subchronic treatment could be explained in accordance with an increased number of carbonyl groups and with decreased levels of antioxidant enzymes. Thus, the difference in fluidity could be clarified in terms of a different susceptibility of pup rats and adolescent rats. A modification of synthetic processes leading to a more fluid composition of plasma membrane might have occurred because of the exposition to PERM.

There are several possible routes of PERM absorption. Due to the high lipophilicity, PERM can be easily absorbed through the skin, by inhalation or orally. However, a diet represents together with an indirect exposition (e.g. remnants of PERM in houses) the major source of PERM for a population (Schleier et al. 2011). PERM is widely used in agriculture and despite its relatively short half-life PERM persists on crops. And thus, the crops can become a potential source of exposition (Ripley et al. 2001). If ingested, PERM is rapidly absorbed and can be found in various tissues within a short interval (Miyamoto 1976). The detoxification of PERM starts with the ester cleavage by esterases in intestines and blood, and subsequently, the metabolism continues in liver being the organ through which the majority of blood from digestive system has to go through (Schleier et al. 2011). Therefore, liver can lower the levels of PERM to which other tissues would be exposed if some other absorption route took place. After the cleavage of ester bond by esterases, PERM is metabolized mainly by oxidation on cytochromes, and as a side effect increased levels of ROS occur (Rose et al. 2005). It is possible that the undeveloped enzyme equipment in early life leads to a slower metabolism of PERM and thus, the exposition of other organs might become higher (Cantalamessa 1993). The achieved results support this theory. The early life treatment caused more alterations of antioxidant enzymes in other organs than in liver. On the contrary, the changes in liver induced in the model with subchronic treatment were comparable with changes found in other organs (Nasuti et al. 2003, Gabbianelli et al. 2009), consequently, liver was amongst the organs most exposed to PERM activity.

According to the US EPA (Internet 2) in the United States, there is a continuous decline in organophosphate insecticides during three last decades. In the group of all other insecticides PYR included, a slight increase was recorded. The majority of PERM (70%) is used in non-agricultural settings and 41% of the totality of PERM is applied by non-professionals in residential areas. Risk/benefit analyses conducted by the Agency demonstrated that benefits of PERM outweigh the risks and that PERM offers a substantial benefit to users. An application of 5% PERM pharmaceutical cream possesses a 2300 times greater exposition to PERM than a combined exposition to other possible sources. Thus, a short-term exposition to PERM is considered as safe. The Agency also expects that all recreational expositions will be short-term in duration (Internet 4). However, the early life model proved that also a short-term exposition can lead to oxidative damages in different organs. For that reason, one should be concerned about the possible exposition of susceptible individuals (e.g. children, pregnant women) to PERM. The subchronic treatment demonstrated an involvement of oxidative stress in the toxicity of PERM.

Normally, the doses which is population exposed are lower than the doses used in the study. However, according to Santos et al. (2011), even an undetectable amount of PERM in tissues led to damages. Therefore even in low doses, PERM could be one of the factors which contribute to the increased amount of ROS, proved as the origin of various potentially serious diseases.

The present research revealed that PERM in subchronic treatment led to a much higher liver damage than that caused by the early life treatment. However, it might be useful to compare data from the last day of treatment (PND21) with results gained from the present research (data from PND300) in order to demonstrate the ability of liver to cope with oxidative stress in early life, and its capacity to renew during the time. This thesis does not cover all aspects of PERM effects on the model organism. Especially deeper knowledge of precise mechanisms of interaction of PERM and its metabolites with genome, and detailed mechanism of ROS creation after exposition to PERM should be the concern of future studies.

7 CONCLUSION

As stated in the introduction, the primary intention of the present thesis was to demonstrate the effects of PERM on liver and compare two different types of PERM treatment. The effects on enzymatic level, specifically, were the main subject of study of this thesis. It was proved that subchronic treatment (for 60days) with PERM caused an increase of oxidative stress markers and alterations in antioxidant enzymes. Namely an increased lipid peroxidation, content of carbonyl groups, generalized polarization of laurdan, content of CAT, and activity of GST was recorded. The amounts of GSH and GPx were decreased, and SOD was the only enzyme the levels of which did not differ between groups. Even though every each tissue reacts in a slightly different way, after the exposition to PERM in general the results were in accordance with previous findings gained from the experiments made on different organs of rats treated with the same or similar type of treatment.

Only minor changes were found in the liver of rats with early life treatment (for 15 days) compared to findings in other organs of the same rats. It was proposed that this phenomenon was caused due to high ability of liver to renew damaged tissue. The following differences between treated and control group were found: increased lipid peroxidation, decreased anisotropy, and increased amount of CAT. All other markers did not differ from the control group.

Still, PERM should be considered as a relatively save insecticide which brings us benefits if used in a correct way. However, its high affordability and widespread use amongst households might lead to mishandling, and initially invisible oxidative effects on cells of exposed humans might later contribute to factors associated with setting of a number of serious diseases. The misuse in agriculture should be prevented as well to protect the population against the excess of exposition through ingestion of crops treated by PERM and of products made of it. A special attention should be given to individuals with a not completely developed liver enzyme system, especially to new born children.

8   LIST OF ABBREVIATION

CAT	catalase
DNPH	2,4-Dinitrophenylhydrazine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPP	diphenyl-1-pyrenylphosphine
GSH	glutathione
GPx	glutathione peroxidase
GST	glutathione transferase
laurdan	2-dimethylamino-6-lauroylnaphthalene
LD$_{50}$	lethal dose 50
PERM	permethrin
PND	post natal day
PYR	pyrethroids
ROS	reactive oxygen species
SEM	standard error of mean
S.D.	standard deviation
SOD	superoxide dismutase

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