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EFFECT OF LAMBDA-CYHALOTHRIN - AN INSECTICIDE FROM THE GROUP OF SYNTHETIC PYRETHROIDS - ON THE CONCENTRATIONS OF NF-κB AND VEGFR2 IN THE LIVER OF ALBINO SWISS MICE AS MARKERS OF ITS DAMAGE

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ABSTRACT

Background: Lambda-cyhalothrin (LCH) is a one of the type II synthetic pyrethroids which is widely used in veterinary medicine and in agriculture to protect crops from pest insects. In previous studies, there are few reports about the influence of pyrethroids on the liver and its damage. Analyzing numerous publications, nuclear factor-κB (NF-κB) and vascular endothelial growth factor 2 (VEGFR2) seem to be sensitive indicators of microdamages occurring at the cellular level in the liver. The aim of the study was to investigate the effect of subacute poisoning with LCH on the concentration of NFκB and VEGFR2 in the livers.
Methods: The experiment was carried on 32 Albino Swiss mice (16 females and 16 males). The animals were divided into 4 groups. Controls received canola oil, the rest received LCH orally in oil at a dose of 2 mg/kg bw for 7 days. The NF-κB and VEGFR2 were mesuredin mice livers with ELISA kits.
Results: The mean NF-κB concektration in control femals' livers was 3.27ng/mL and after LCH it was 6.12ng/mL ($p<0.05$). In control males it was 5.49ng/mL and it did not significantly differ after LCH when it was 5.27ng/mL. The mean VEGFR2 in control females was 84.28ng/mL and after LCH it was 173.81ng/mL ($p<0.05$). In control males it was 170.61ng/mL and after LCH 170.06ng/mL.
Conclusion:The NF-κB and VEGFR2 can be used as markers of liver damage after subacute poisoning with LCH on female mice. Females are more sensitive to LCH than males.
Keywords: Lambda-cyhalothrin; nuclear factor-κB; vascular endothelial growth factor2; liver damage.

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INTRODUCTION

Pyrethrum is one of the oldest natural insecticides of plant origin known to man. The insecticidal properties of pyrethrum became more widely known in the mid-nineteenth century, when it was noticed that many Caucasian tribes used plants rich in this substance to combat human lice [1]. Pyrethrins are prepared from the dried flower heads of *Chrysanthemum cinerariaefolium* and / or *Chrysanthemum cineum* [2,3]. The flower extract is a heterogeneous mixture of pyrethrins, with a significant difference in proportions consisting of pyrethrin I and II, cinerin I and II, and jasmoline I and II, which are collectively known as pyrethrins [4]. Pyrethrin I and II differ in their insecticidal properties - pyrethrin I shows a greater lethal effect, and pyrethrin II causes a stronger knock-down effect [5].

Due to the fact that the cultivation of plants is subject to significant fluctuations, the search for synthetic pyrethrin derivatives began. The result of the scientists' work was the synthesis of pyrethroids [6]. Most pyrethroids were obtained by modification of the pyrethrin I chrysanthemum acid moiety and esterification of alcohols. Their activity is enhanced by the addition of a synergist such as piperonyl butoxide which inhibits the metabolic degradation of the active ingredient. Their widespread use began in the 1970s after the development of photostable forms such as permethrin and fenvalerate. At the same time, synthetic pyrethroids maintain a low toxicity to terrestrial vertebrates [7]. They are approximately 2,250 times more toxic to insects than to mammals [8].

Currently, it is estimated that 23% of the insecticides available on the world market are pyrethrins and pyrethroids. More than 3,500 registered formulations of these compounds are widely used in public health, agriculture, food processing and insect control in housing estates and in urban areas. In medicine, they are used to treat scabies and head lice [9].

Pyrethrins and pyrethroids belong to the group of neurotoxic compounds and have a similar mode of action that distinguishes them from other insecticides. There are several ways for pyrethrins and pyrethroids to enter the body. The first is rapid penetration through the epidermis, followed by uptake by blood and haemolymph carrier proteins, and then distribution in the body. Pyrethroid diffusion along the epidermal cells is the major route of distribution into the central nervous system (CNS) after penetration [10].

Pyrethroids can also enter the CNS directly through contact with receptors in the peripheral nervous system [11]. Pyrethroid in an aerosol can also enter the body through the respiratory tract, but penetration is only a small percentage due to the low vapor pressure of these compounds [12,13]. An important route of penetration of pyrethroids is through the gastrointestinal tract with food and water [14].

Based on the chemical structure, action on nerves pyrethroids are divided into: Type I (they produce syndrome T with tremor) and type II (producing syndrome CS -choreoathetosis with salivation) [15,16]. Aggressive ataxia, convulsions, tremors and extreme exhaustion are the hallmarks of the T syndrome. Moreover, as a consequence of muscle tremors, compounds classified as type I pyrethroids increase body temperature. In turn, the CS syndrome consists of profuse salivation, choreoathetosis, increased fear response and treatment-resistant seizures. Unlike type I pyrethroids, type 2 pyrethroids lower body temperature as a result of excessive salivation and wetting of the abdominal surface of the body [15,17,18]. The literature also describes combinations of the symptoms of T and CS syndromes, for example TS syndrome (tremor with saliviation) [16]. Detailed electrophysiological studies explained that the voltage-dependent sodium channels in the nerve membrane are the main target sites for pyrethroids in both insects and mammals, including humans [18,19].

Pyrethroids act very quickly, causing symptoms of loss of musculoskeletal coordination and paralysis known as the "knockdown" effect, often accompanied by cramps and tremors, inducing intense, repeated activation in the sensory organs and myelinated nerve fibers. Sometimes the contractions are so violent that in insects they may result in the loss of legs and wings [6].

Lambda-cyhalothrin (LCH) is a type II synthetic pyrethroid. It is one of the new type II pyrethroid insecticides with high efficacy and against a wide variety of arthropods, harmful both to human and animal health and to plant breeding. LCH is widely used in veterinary medicine for the control of lice, flies, and ticks in cattle, sheep and pigs, as well as in agricultural formulations for the control of numerous pests on fruit, vegetables in order to increase crops. It is used in soaking anti-mosquito nets used in malaria risk zones, as well as in many products used in skin spraying or sprayed in households to protect against unwanted insects. Regarding high efficacy, synthetic pyrethroids appear to be very good insecticides' as they are effective against a broad spectrum of pests. Due to their lipophilic nature, pyrethroid insecticides are well absorbed through the gastrointestinal tract and respiratory tract. Good lipid solubility promotes distribution to lipid-rich internal tissues, including adipose tissue, skin, liver, kidneys, ovaries, and the central and peripheral nervous system. The liver is one of the largest organs in the body. It has many important metabolic functions. It transforms the nutrients in our diet, stores them to supply the cells with the necessary substances when needed. Equally important, it performs the main detoxification function by transforming, neutralizing and eliminating toxins with the participation of hepatocyte enzyme systems [7,20,21].

Taking into account the wide and widespread use of pyrethories, it seems reasonable to analyze the impact of this group of pesticides on non-target organisms including humans. Pyrethroids are metabolized in the liver and their metabolites are passed with urine. The 3-phenoxybenzoic acid (3-PBA) is the most commonly detected urinary metabolite of several pyrethroids [22]. The pyrethroid metabolites are frequently detected in urine of children and adults from rural and urban areas, confirming widespread exposure of human population to these compounds. Non-occupational exposure occurs *via* ingestion with food and water, or contact with contaminated house dust after use of bednets, burning coils, pyrethroid-soaked mats, electrovaporizes, aerosols [23,24]. In previous studies, there are few reports of the influence of pyrethroids on the liver [25-27].

Nuclear transcription factor NF- κB (NF-κB) is a pleiotropic transcription factor regulating the expression of more than 200 genes involved in the regulation of various cell functions [28]. In normal cells without the action of an activating factor, NF-κB dimers

are inactive, sequestered in the cytoplasm by inhibitory proteins: κB inhibitor (IκBα, β or λ) and inactive precursors: p100 and p105. In response to numerous stimuli including cytokines, viruses, bacteria and other stress factors at the cellular level, NF-κB is rapidly activated by phosphorylation [29,30]. A number of NF-κB-regulated target genes have been identified, including cytokinins, chemokinins, growth factors, acute phase proteins, immunomodulators, cell adhesion factors, cell stress response factors, apopototic response proteins, and enzymes [31]. A well-recognized function of NF-κB is the regulation of inflammatory responses [32]. NF-κB activation is a key transcription factor of macrophages M1 and is required for the induction of a large number of inflammatory genes, including those encoding TNF-α, IL-1β, IL-6, IL-12p40 and cyclooxygenase-2 [33]. NF-κB are present in cells in an inactive state and do not require new protein synthesis for activation. This allows NF-κB to be the first response to noxious cellular stimulation [34]. NF-κB are able to inhibit apoptosis and induce the expression of protooncogenes, as well as regulate the expression of various molecules favoring tumor cell invasion and angiogenesis [35,36].

Vascular endothelial growth factor (VEGF) plays an important role in the development of blood vessels. They have a decisive influence on both the formation of capillaries from progenitor cells during embryogenesis - vasculogenesis, and the formation of blood vessels in adults - angiogenesis [37-39]. The stages of angiogenesis are initiated by stimulation of endothelial cells (EC) by angiogenic growth factors [40].

Currently, the following factors can be included in the VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, placental growth factor (PlGF) and recently qualified to this group endocrine gland-derived vascular endothelial growth factor (EG-VEGF) [41–43]. VEGF is secreted not only by endothelial cells, but also, in response to oxygen deficiency, by: tumor cells, macrophages, platelets, keratinocytes, mesangial kidney cells, activated T cells, leukocytes, dendritic cells, retinal pigment epithelial cells, retinal cells, astrocytes, osteoblasts, bronchial and alveolar epithelial cells, pericytes, vascular smooth muscle cells [41,44].

Analyzing the above publications, NF-κB and VEGFR seem to be candidates for good and sensitive indicators of liver microdamages occurring at the cellular level due to subacute poisoning with xenobiotics.

AIM

The aim of the study was to investigate the effect of subacute poisoning with LCH on the concentration of NF-κB and VEGFR2 in the livers.

MATERIALS AND METHODS

The study project was accepted by The Local Ethical Committee in Lublin, Poland (permission Nr 69/2015 dated 11.12.2015). The uthors had certificates confirming training for conduction experiments on animals. The experiment was conducted according to European law regulation at the Center for Experimental Medicine (CEM) at The Medical University of Lublin. There were standard laboratory conditions.

A total of 32 (16 non-gravid females and 16 males) Albino Swiss mice bred at the Center for Experimental Medicine at The Medical University of Lublin 6 weeks of age at the beginning of the experiment were randomly divided into 4 groups of 8 animals:

- females-controls-received 0.9%NaCl daily by gavage for 7 consecutive days,
- males-controls- received 0.9%NaCl daily by gavage for 7 consecutive days,
- females-receiving 2mg/kg LCH daily by gavage for 7 consecutive days,
- males-receiving 2mg/kg LCH daily by gavage for 7 consecutive days.

Lambdacyhalothrin was purchased form Organic Chemistry Institute (Annopol 6, 03-236 Warsaw, Poland). It was dissolved in canola oil and administered daily by gavage. Canola oil "Kujawski", produced by ZT "Kruszwica" S.A. was used to make the suspension. Oral LD_{50} in mice 19.9 mg/kg [45]. We administered oral $0.1LD_{50}$ to the animals for 7 consecutive days. 0.9% saline B.Braun (Melsungen AG, Hessen, Germany) was used. Animals had free access to sterile water (sterilized with UV) and feed for rodents purchased from Altromin International (Lage, Germany). The animals were bred at CEM and the original source of the herd was Charles River Laboratories (Cologne, Germany).

On the eighth day of the experiment, the mice were weighed and then decapitated. No anesthetics were used as we wanted to eliminate the risk that they would affect the results of VEGFR2 and NF-κB. The livers were obtained and weighed. The livers were homogenized in Phosphate Buffered Saline (PBS) - a phosphate-buffered saline solution devoid of calcium and magnesium ions (PAA Laboratories GmbH, Pasching, Austria) in the proportion of 200 mg of homogenized liver tissue per 0.5 ml of buffer using a mechanical homogenizer type Omni Th (Omni International, Kennesaw, GA, USA). The homogenates were centrifuged in a centrifuge (Sigma1-6P centrifuge, Polygen, Engelwood, NY, USA) 700 x g for 10 minutes at room temperature. Then, after centrifugation, the supernatant was separated. The supernatants obtained in this way were divided 200 µl in eppendorf tubes (0.5 ml) (Medlab Products, Karlsruhe, Germany) and stored at -75 °C (Platinum Angelantoni 500, Massa Martana, Italy) until enzymatic determinations were performed. The concentrations of VEGFR2 and NF-κB were tested in the obtained supernatant tubes.

The experiment used a "sandwich" version of the *enzyme-linked immunosorbent assay* (ELISA) method. Before beginning the determination of the concentrations of the test substances, the samples and ELISA kits were properly prepared. The determinations were performed using a wavelength λ = 450 nm using a BIO-RAD type ELISA microplate reader (Microplate Leader, Wuxi,China). The computer program connected to the reader, on the basis of the obtained absorbance of light from the wells with the established concentration standards, automatically determined the standard curves, on the basis of which it calculated the concentrations of the determined proteins in the tested samples. The obtained results were automatically multiplied by the appropriate dilution factor. The commercial ELISA kits were used: ELISA kit for NF-κB and ELISA kit for VEGFR2 (Cloud-Clone Corp. Katy, TX, USA).

Statistical analysis was performed with the use of Statistica v.13.0 (StatSoft, Cracow, Poland). Results were shown as mean±SD. The p value <0.05 was considered statistically significant.

RESULTS

The results were shown in Table 1. Body mass on female mice on the last day of the experiment was significantly lower (22.53g±1.1) when compared to control females (25.63±1.0) ($p<0.05$). No such difference was seen in males. There was no statistically significant differences in liver me mass neither between controls and LCH females nor between controls and LCH males. There was a statistically significant increase in NFκB concentration in the livers of female mice exposed to LCH when compared to control females (6.12ng/mL *vs* 3.27ng/mL; $p<0.05$). There was no such difference in males. There was also a significant increase in VEGFR2 concentration in the liver of females exposed to LCH in comparison to controls (173.81ng/mL *vs* 83.28ng/ml; $p<0.05$).

Tab. 1

The effect of subacute poisoning with LCH on body mass, liver mass, NF-κB in the livers and VEGFR2 concentrations in mice livers.

Group	Body mass [g] Mean±SD	Liver mass [g} Mean±SD	NF-±κB [ng/mL] Mean±SD	VEGFR2 [ng/mL] Mean±SD
Control females	25.63±1.0	1.28±0.1	3.27±0.7	84.28±16
Control males	27.84±1.5	2.02±0.1	5.49±0.35	170.61±25
LCH females	22.53±1.1*	1.33±0.1	6.12±1.5*	173.81±31*
LCH males	27.21±1.6	2.04±0.1	5.27±0.3	170.06±21

*$p<0.05$ *vs* control females.

DISCUSSION

LCH is an example of an insecticide that reaches a compromise between efficacy and toxicity: it is effective against insects and was considered to be safe for humans [7]. It is a type II pyrethroid with high activity against a wide range of insects from the order *Lepidoptera, Diptera, Hemiptera* and *Coleoptera*. LCH has found widespread use in public places as well as pet and farm animal health applications where it effectively controls a wide spectrum of insects and ectoparasites including cockroaches, flies, ticks and lice [46,47].

LCH is stable at a pH below 8, while under alkaline conditions it hydrolyzes by the action of the hydroxyl ion and the result is a cyanohydrin which then breaks down into aldehyde and hydrogen cyanide [50]. In laboratory studies, the degradation of LCH in soil was mainly due to biodegradation, as indicated by the rapid loss of LCH in non-sterile soil compared to sterile soil [51. In living organisms LCH is metabolized hepatic microsomal cytochrome P450 (CYP) and carboxylesterase (CES) enzymes and by cytosolic CES enzymes. The pyrethroids also metabolized by human liver microsomes and cytosol [48]. At the same time liver can be damaged by LCH.

The lipophilicity of LCH these compounds facilitates their rapid access and has a detrimental effect on various tissues [49,50]. Except for its primary neurotoxic effect it damages other organs too. Both *in vitro* and *in vivo* experiments with peripheral blood of rats have shown that LCH causes imbalances in the pro-oxidant-antioxidant relationship in erythrocytes, and also changes the fluidity of the cell membrane and affects hemolysis [51,52].

In this study we focused of LCH's hepatotoxicity. Other authors also investigated the subject. Aouey et al. tested pyrethroid hepatotoxicity in a rat model. In their study, adult male rats were orally exposed to 6.2 and 31.1 mg / kg body weight LCH for 7, 30, 45 and 60 days, respectively. Histopathological changes and changes in main parameters related to oxidative stress and inflammatory response in the liver were assessed. Moreover, metabolites of LCH (CFMP, 4-OH-3 PBA and 3-PBA) were identified in the liver tissues and then were quantified. Results showed that exposure to LCH significantly increased hepatic oxidative stress markers in a time-dependent and dose-dependent manner, accompanied by accumulation of CFMP and 3-PBA in liver tissues. Moreover, the expression levels of the tumor necrotic factor α (TNF-α) gene and the expression of interleukins (IL-6 and IL-1β) were significantly increased in the liver of the tested rats as compared to the control group. Taken together, this study provided new evidence that liver damage is likely to be caused by increased oxidative stress and inflammation under conditions of acute and subchronic exposure to LCH [52].

In the study of Martinez et al. where LCH's hepatotoxicity was investigated LCH at the doses of 1, 2, 4 and 8mg/kg bw was administered orally to rats for 6days. It increased, in a dose-dependent manner, hepatic activities of ethoxyresorufin O-deethylase, methoxyresorufin O-demethylase, pentoxyresorufin O-dependentylase , testosterone 7α- (CYP2A1), and lauric acid 11- and 12-hydroxylase. Similarly, LCH at the higher doses increased significantly hepatic CYP1A1, 1A2, 2A1, 2B1, 2B2, 2E1, 3A1, 3A2 and 4A1 mRNA levels and IL-1β,

NFκB gene expressions [53].

The significance of pyrethroid poisoning markers was clearly shown in numerous publications [54-58]. All of them confirm widespread exposure of the human population to pyrethroids without significant age nor sex differences. Klimowska and Wilegomas described a new method for pyrethrid metabolites detection in the urine [59]. Interestingly, it was shown that the levels of pyrethroid metabolites were higher in city dwellers than in people living in rural areas [60]. Rodzaj et al. suggest that the widespread exposure of Polish population to pyrethroids is from non-dietary sources [61]. Jurewicz et al. provided evidence that widespread use of pyrethroids affects male fertility [62]. All these publications indicate that there is a need for good markers of pyrethroid toxicity in mammals. In our experiments animals were intended to be a model in the search for such a marker in humans

The results of the study by Fetoui et al. Showed that the administration of LCH leads to the generation of oxidative stress in rat erythrocytes, which increases the level of reactive oxygen species (ROS), protein carbonyl (PCO), aldehyde malondialdehyde (MDA) and nitric oxide (NO) [63]. Moreover, the mutagenic effect of lambda-cyhalothrin was investigated by means of a micronucleus test carried out with the use of peripheral rat blood. It should be emphasized that chemicals can induce the formation of micronuclei in dividing cells [63-65].

The research problem we undertake is aimed at deepening the detailed knowledge on the influence of LCH intoxication on the liver.

In our study, we showed that after intoxication of female mice with LCH, there was a statistically significant decrease in animal body mass and an increase in NF-κB and VEGFR2 concentration.

The role of VEGF in maintaining microcirculation in the internal organs has been investigated in a number of animal models. In the ischemia-reperfusion model of renal injury, the production of VEGF in the kidney is not increased, but there is a redistribution of already produced VEGF to the kidneys and increased expression of VEGFR-2 mRNA [66,67]. Ischemia and reperfusion lead to oxidative stress [68] and so does intoxication with pyrethroids [25]. There are reports suggesting that mesenchymal stem cells act to protect against organ damage in an ischemia-reperfusion model not through cell regeneration, but through paracrine mechanisms. VEGF is one of the most important factors in these mechanisms [69]. Studies in rats have demonstrated chronic renal dysfunction and a decrease in the number of capillaries in the renal glomeruli and the periurethral space associated with a decrease in renal VEGF expression [70]. The administration of VEGF in this model had a protective effect on the vascular endothelium and allowed the inhibition of the progression of renal dysfunction, as well as scarring of tissue damage regardless of blood pressure, proteinuria or macrophage infiltration [71].

It has been proved that VEGF transcription is regulated by estrogens, the secretion of which is mediated by stimulation of the estrogen receptor [72]. It may explain why in controls the level of VEGFR2 is lower than in control males. Intoxication with LCH produces Oxidative stress and disrupts the protective mechanism in females.

The NF-κB can be activated by many factors. It is worth noting that the processes in which it is involved may also affect the metabolism of xenobiotics and the activity of liver enzymes [73,74]. NF-κB binding sites have been identified in the promoters of the genes of some xenobiotic metabolizing enzymes [75]. LCH at a dose of 4 mg/kg body weight increased the expression of NFκB specific mRNA by 1.37 times [53]. In our study LCH also increases the level of NF-κB but only in females, which is probably connected with their sensitivity to the chemical.

The NF-κB is a key regulator of inflammatory processes in the liver. It is required for hepatocyte survival and liver homeostasis. The functions of fibrogenesis-active hepatic cells and myofibroblasts are also regulated by NF-κB. The key role of NF-κB in regulating cell death, inflammation and wound healing makes it an important modulator of the progression of NF-κB liver disease and a potential link between chronic liver damage, fibrosis and hepatocellular carcinoma, which may be important in planning therapy these conditions and targeting this transcription factor. In murine models genetic ablation of NF-κB regulators leads to spontaneous liver damage, fibrosis and hepatocellular carcinoma [76,77].

CONCLUSION

The NF-κB and VEGFR2 can be used as markers of liver damage after subacute poisoning with LCH on female mice. Females are more sensitive to LCH than males.

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