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Full Length Research Paper

Subacute toxicity of piperonyl butoxide and resmethrin in mice

Selma Yazar Urek[1], Emine Baydan[1], Begüm Yurdakök[1], Yesim Akaydin[2], Tolunay Kozlu[2] and Ayse Tunca[3]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine of Ankara University, Ankara, Turkey.
[2]Department of Embryology and Histology, Faculty of Veterinary Medicine of Mustafa Kemal University, Hatay, Turkey.
[3]Dirimsel Outpatient Clinic, Ankara, Turkey.

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The subacute toxic effects of separate and combined use of piperonyl butoxide and resmethrin (cismethrin) on the liver and kidneys of male mice were investigated. It is known that when given alone, pyrethroids are toxic and their toxicity becomes more complicated when they are co-formulated with piperonyl butoxide. In the present study, macroscopic and microscopic changes were determined in the liver and kidney tissues. Furthermore, biochemical alterations and clinical neurotoxic effects were observed. Toxic effects were more evident in the group subjected to combined use. The results obtained demonstrated that, in mice, piperonyl butoxide and resmethrin are directly toxic to the liver and kidneys. The toxic effects and tissue degeneration were more widespread in the group subjected to combined use.

Key words: Piperonyl butoxide, resmethrin, sub-acute toxicity, histopathology, liver, kidney.

INTRODUCTION

Piperonyl butoxide (PBO), α-[2-(2-butoxyethoxy)ethoxy]-4, 5-methylene-dioxy-2-propyltoluene], is a well-known synergist of pyrethrins, pyrethroids, carbamates, organo-phosphates and many growth regulators. In commercial formulations, the activity of pyrethroids, such as resmethrin (RES), is usually increased by the addition of synergists, including PBO (Keane, 1999; Cox, 2002; Proudfoot, 2005; Muguruma et al., 2006). When given alone, the mode of action of pyrethroids is toxic and complex and becomes more complicated when these compouds are co-formulated with PBO (Bradberry et al., 2005). PBO is widely used in the control of insects which affect humans and animals (Keane, 1999). RES [5-(phenylmethyl)-3-furanyl] methyl 2, 2-dimethyl-3-(2-methyl-1-propenyl) cyclopropane-carboxylate is classified as a Type 1 pyrethroid. In mammals, it causes clinical signs referred to as the Type 1 or T (tremors) syndrome (INCHEM, 1996; Carr et al., 2006). RES is a racemic mixture of 4 optical isomers, namely, (1R, trans)-, (1R, cis)-, (1S, trans)- and (1S, cis)-. The (1R, cis)- isomer is known as cismethrin (INCHEM, 1989). Of the earlier mentioned 4 isomers, (1R, trans)- and (1R, cis)- have the strongest insecticidal activity, while (1S, trans)- and (1S, cis)- do not have such activity (INCHEM, 1996). Although, RES is not highly toxic to mammals, it is very effective on mosquitoes even at low doses. Literature reports available on acute toxicity indicates that, when used by the oral route, resmethrin is mildly toxic to mice, excluding the isomer cismethrin (Ueda et al., 1975; Ridlen et al., 1984; INCHEM, 1989, 1996; Carr et al., 2006).

The objective of the present study was to investigate the toxic effects of separate and combined use of PBO and RES (cismethrin) on the liver and kidney in male mice. As indicated earlier, it is accepted that the oral use

*Corresponding author: E-mail: selmayazar@hotmail.com, yazar@veterinary.ankara.edu.tr.

Abbreviations: PBO, Piperonyl butoxide; **RES,** resmethrin; **AST,** aspartate aminotransferase; **ALT,** alanine aminotransferase; **TLC,** thin layer chromatography; **LD_{50},** lethal doses.

Table 1. Treatment schedule of chemicals in the treatment and control groups.

Groups	Chemicals	Doses (mg/kg/day)	Administration Route	N
Group 1	PBO	1 g/kg (2 g/10ml in olive oil)	Intraperitoneal	10
Group 2	RES	1000 mg/kg/day	In feed	10
Group 3	PBO+RES	1 g/kg/day +1000 mg/kg/day	In feed + intraperitoneal	10
Group 4	No chemical	Control	Normal feed	10

Table 2. Comparison of the body weights measured on the first and last days of the study, different superscripts a, b within the same row indicates significant differences ($p \leq 0.05$).

Variable	Control		PBO		RES		Combination	
Days	1st day	14th day	1st day	14th day	1st day	14th day	1st day	14th day
Body weight (Median ± SD, g)	32 ± 2	35 ± 5	39 ± 2[a]	29 ± 3[b]	35 ± 3	35±4	35 ± 1[a]	23 ± 4[b]
N	10	10	10	10	10	10	10	8

The results are presented as median ± SD, $p \leq 0.05$.

of cismethrin is non-toxic in mice (INCHEM, 1996). Therefore, it was aimed to draw attention to the toxic effects of these chemicals and to warn both producers and consumers.

MATERIALS AND METHODS

PBO (technical grade) and the cismethrin -(1R,cis-isomer)- isomer of resmethrin (14.5% w/w)- were used in this study. PBO was provided by cooperation (Biyoteknik, Istanbul, Turkey) and RES was obtained from a chemical company (Bayer, Istanbul, Turkey). Four-week-old, forty healthy Swiss albino male mice, each of approximately 30 to 42 g weight were used in this research. Mice were obtained from the Refik Saydam Hygiene Centre in Ankara, Turkey. Prior to the start of the trial, the mice were kept under laboratory conditions for 7 days for acclimatization. This study was conducted in accordance with the national and institutional guidelines (Ankara University Ethics Committee Guidelines) for animal experiment. The management of the animals complied with the guidelines of the Committee on the Care and Use of Experimental Animals of Ankara University, Faculty of Veterinary Medicine, Turkey. Mice were housed at a temperature of 22 ± 2°C and a humidity level of 60 ± 10% and were provided with *ad libitum* standard laboratory feed and tap water. The study was partially funded by the Department of Pharmacology and Toxicology of Ankara University Faculty of Veterinary Medicine. In previous studies, different doses of PBO and RES were used for different purposes and in this study, lethal doses (LD_{50}) were prepared. LD_{50} values were determined based on previous sub-acute toxicity studies conducted in mice (for RES: Miyamota, 1976, for PBO: INCHEM, 1965; Fujitani et al., 1993a). Four groups were established. Chemical agents were administered to three of the groups, whilst one group was maintained for control purposes. Each group was comprised of 10 healthy male mice. PBO (1 g/kg: 2 g/10 ml in olive oil) was injected intraperitoneally to the first group, 1000 mg/kg/day of RES was given in feed to the second group and a combination of both compounds was given to the third group (Table 1). The study continued for 14 days. After mixing RES into the powdered diet, pellets were formed and fed to the mice. Each lot of the basal diet was analysed and confirmed to be free of pesticide and aflatoxin contamination by thin layer chromatography (TLC). During the trial period, the animals were observed daily. All clinical symptoms, food intake, water consumption and mortality were monitored. The weight gains of the mice were recorded at the beginning and end of the study (Table 2) and their sera were collected for biochemical analyses. Although, no early deaths were observed, towards the end of the study, 2 deaths were encountered in the third group given the combination of both compounds. In these cases, body weights were recorded and blood samples were taken before death. At the end of the trial period, all 4 groups were necropsied. Selected organs (the liver and kidneys) were evaluated for histopathological examination under the light microscope. Blood was taken directly from the heart without using anaesthetic substances and collected into tubes by a fine tipped injector. Blood samples were centrifuged at 3000 rpm for 10 min within 1 h after sampling and stored at –18°C until analyzed. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine analyses were performed by an autoanalyzer. These results are summarized in Table 3.

At the end of the study, all the mice were sacrificed with diethyl ether. Each liver and kidney obtained from the animals was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 µm thickness and stained with hematoxylin and eosin (H and E). Subsequently, all the H and E preparations were examined under the light microscope.

The statistical package SPSS for Windows (release 11.0) was used for data analysis. Numerical values are given as median ± SD. For statistical analyses, the Mann-Whitney U test was used. A p value ≤ 0.05 was accepted as statistically significant.

RESULTS AND DISCUSSION

During the trial period, hyper-excitability, unsteadiness and irritability were the most evident symptoms for the study groups, particularly, for the combined group. Furthermore, body weight gain was markedly depressed in the PBO and combined groups (Table 2).

Biochemical analyses demonstrated that ALT values

Table 3. Comparison of the biochemical values of the treatment groups with each other and with those of the control group (Mann-Whitney U test). Different superscripts a, b within the same column indicate significant differences ($p \leq 0.05$).

Groups	N	Urea (mg/dl) (median ± SD)	Creatinine (mg/dl) (median ± SD)	AST (U/L) (median ± SD)	ALT (U/L) (median ± SD)
Control	10	54 ± 12	0.37 ± 0.5	105 ± 61	35 ± 3
PBO	10	39 ± 8	0.20 ± 0.05	116 ± 71	35 ± 11
Control	10	54 ± 12	0.37 ± 0.5	105 ± 61	35 ± 3[a]
RES	10	54 ± 6	0.37 ± 0.08	166 ± 12	50 ± 20[b]
Control	10	54 ± 12	0.37 ± 0.5	105 ± 61[a]	35 ± 3[a]
Combination	8	42 ± 11	0.48 ± 0.10	241 ± 93[b]	58 ± 11[b]
PBO	10	39 ± 8	0.20 ± 0.05[a]	116 ± 71[a]	35 ± 11[a]
Combination	8	42 ± 11	0.48 ± 0.10[b]	241 ± 93[b]	58 ± 11[b]
RES	10	54 ± 6	0.37 ± 0.08	166 ± 12	50 ± 20
Combination	8	42 ± 11	0.48 ± 0.10	241 ± 93	58 ± 11

The results are presented as median ± SD, $p \leq 0.05$.

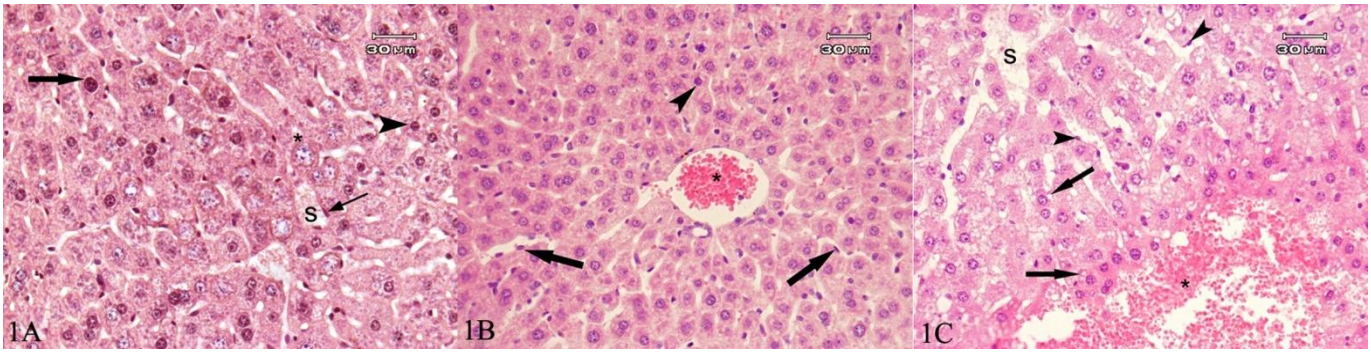

Figure 1. A, Microscopic findings of the liver, given RES; s: sinusoid; thin arrow: increase in the number of cells of the sinusoidal wall; asterisk, arrowhead and thick arrow: Nuclei in the different stages of pyknosis; B, Microscopic findings of the liver, given PBO; asterisk: hyperaemia, arrows: significant alterations in the cells of the sinusoidal wall, arrowhead: pyknotic nuclei; C, Microscopic findings of the liver, given combination; s: enlarged sinusoid, arrows: vacuolization, arrowheads: significant alterations in the cells of the sinusoidal walls.

were significantly higher in the RES group when compared to the controls. In comparison to the control group, AST and ALT values were significantly higher in the combined group. Creatine, AST and ALT values were significantly higher in the combined group when compared to the PBO group (Table 3).

At the time the mice were sacrificed, macroscopic examination revealed colour changes in the organs. The livers were pale and the kidneys were black in colour with these changes being more pronounced in the combined group.

The histopathological examinations of the liver in the RES group revealed the presence of enlarged hepatocytes, oedema, leukocytes, a vacuolated cytoplasm, nuclei in the different stages of pyknosis, an increase in the number of cells composing the sinusoidal wall and hyperaemia (Figure 1A). The PBO group displayed the same degenerations, but these were diffused (Figure 1B). In the combined group, there were widespread degenerations and also dissociated Remark cords (Figure 1C).

The histopathological examination of the kidneys in the RES group produced a panoramic image of the oedematous cortical region and also revealed the presence of haemorrhagic areas in the medulla (Figure 2A). Similar but widespread findings were determined in the PBO group as well as, dilatation of the distal tubules and hyperaemia of the glomerular capillaries (Figure 2B). Similar findings were observed in the combined group yet degeneration was widespread. Additional findings were the enlargement of the Bowman's space, dilatation of the distal tubules, and hyperaemia in the glomerular capillaries and blood capillaries (Figure 2C).

Pyrethrins are commonly used for the control of pests in agriculture and households. PBO is a well-known synergistic chemical used in combination with pyrethrins. RES is a pyrethroid insecticide and is used to kill a variety

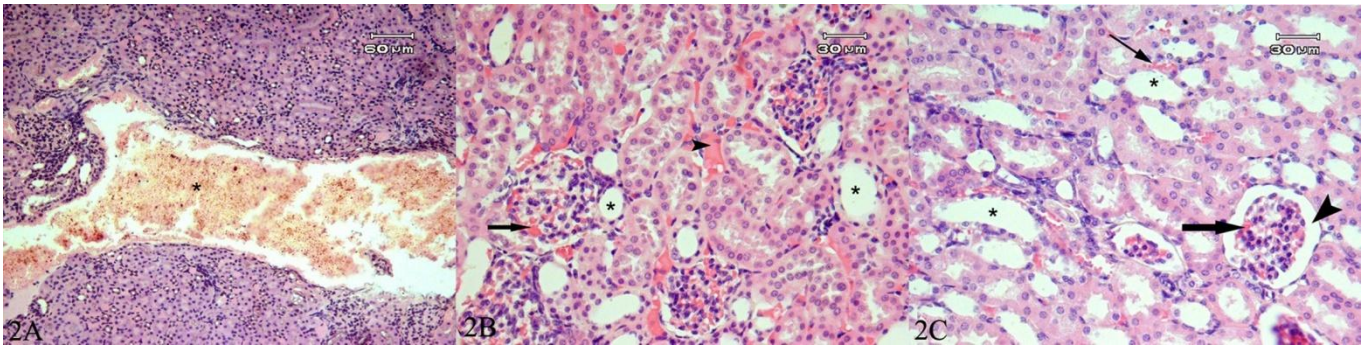

Figure 2. A, Microscopic findings of the kidney, given RES; a panoramic image of the oedematous cortical region; asterisk: hyperaemia; B, Microscopic findings of the kidney, given PBO: asterisk: dilatation of the distal tubules, arrow: hyperaemia in the glomerular capillaries, arrowhead: haemorrhage; C, Microscopic findings of the kidney, given combination; arrowhead: enlarged Bowman's space, asterisk: dilatation of the distal tubules, arrow: hyperaemia in the glomerular capillaries, thin arrow: hyperaemia in a blood capillary.

of flying and crawling insects, including mosquitoes (Cox, 2004; Bradberry et al., 2005). It is a mixture of 4 optical isomers and one of them, the (1R, cis)-isomer is cismethrin (Cox, 2004). Acute toxicity data presented in previous studies indicated that the oral uses of RES are mildly toxic, but one of its isomers, cismethrin, which was used in the present study is agreed to be non-toxic to mice. Furthermore, previous studies have shown that PBO causes similar damage to the liver and kidneys (Maekawa et al., 1985; Fujitani et al., 1993a, 1993b). However, to the authors' knowledge, no previous study exists on the combined use of RES and PBO in mice. This is the first study conducted on this subject.

Although, human environmental exposure to these compounds, which are found together in the environment, is most likely to occur via the oral route, in the present study PBO and RES were administered by different routes (RES-Cismethrin-by oral, PBO by IP) to prevent any possible chemical interactions between the two agents.

Fujitani et al. (1993a) investigated the sub-acute toxicity of PBO in male mice for a trial period of 20 days. The researchers determined that PBO was toxic primarily to the liver. They reported that, histopathologically enlarged hepatocytes, anisonucleosis, multinucleated cells and single cell nucleosis were detected in the liver and that depressed body weight gain was observed in comparison to the control group. In agreement with this study, hepatic toxicity and depressed body weight gain were also observed in the present study.

Muguruma et al. (2006) reported similar results. These researchers investigated the toxic effects of 6000 ppm of PBO on the liver in 1 to 4 and 8 weeks. They found that body weight gain was significantly inhibited at week 4 and enlarged hepatocytes due to an increase in their cytoplasm was also observed.

Takahashi et al. (1997) administered different doses of PBO to male and female mice for 52 weeks. They investigated the chronic effects of PBO on the liver and kidneys and performed biochemical analyses. There was a dose-dependent reduction in body weight gain in the PBO-treated groups throughout the trial period. Hepatocellular hyperplasia as well as, hyperplastic and neoplastic nodular lesions were observed in the liver. The kidneys presented dose-dependent, black-coloured haemorrhages. The macroscopic findings of the murine kidneys in the present study were similar. Takahashi et al. (1997) reported AST and ALT values to have increased greatly in both sexes. In the present study, when administered alone, PBO led to increased ALT levels but with no statistically significant difference. According to the authors, PBO may be more acutely toxic to mice than rats and exhibit stronger toxic effects in male mice. The time required for the development of hepatic tumours is shorter in mice. The results of Takahashi et al. (1997) are in line with the opinion of Fujitani et al. (1993a) suggesting that liver damage could occur as little as one week after exposure to PBO.

The Tokyo Metropolitan Research Laboratory investigated the effects of PBO on the kidneys (Cox, 2002). In a three month- feeding study conducted on rats, they determined that kidney damage including atrophy and the dilation of renal structures occurred with all tested doses (Fujitani et al., 1993b). Although, the present study was conducted on mice, similarly, severe kidney damage and severe dilatation of the distal tubules were also observed.

PBO and RES are both neurotoxins. According to the data obtained by WHO, overdoses of PBO and RES cause hyper-excitability, unsteadiness and irritability (INCHEM, 1996; WHO, 1996). In the present study, similar clinical findings were obtained with LD_{50} doses.

When rats were fed RES in the diet at a dose of up to 6000 mg/kg for 14 days, mortality was observed at the highest doses, reduced body weight and food consumption were also noted at doses of 1500 mg/kg and above (WHO, 1996). We studied mice not rat and RES

dosage was 1000 mg/kg/day. When RES was administered at this dose, body weight reduction was statistically insignificant and mortality was not observed.

According to the results of a short-term study published by the WHO (1996), when RES was administered to rats at a dose of 5000 mg/kg diet, decreased body weight, toxic effects on the liver and kidneys and increased ALT levels were observed. In the present study, a statistically significant increase was observed in the ALT levels of the RES group. Furthermore, histopathological changes were detected in the kidneys.

To the authors' knowledge, no previous study exists on the investigation of the effects of the combined use of PBO and RES, but reports are available on the combination of PBO with different chemicals. In these reports, the authors agreed that the toxic effects of PBO were greater in the event of its combined use (Friedman and Eaton, 1978; Fujii and Epstein, 1979).

In the present study, it was observed that both liver toxicity and biochemical and histopathological alterations were more pronounced in the case of combined use. Furthermore, in the combined group, both appetite and weight loss observed in the mice were statistically significant. It is considered that these findings could be the result of the synergistic effects of PBO and RES.

In conclusion, toxicology studies have shown that PBO often affects the liver (Cox, 2002). PBO aggravates nephrotoxicity and renal cysteine depletion (Song et al., 1999). PBO is the fourth most commonly using pesticide (Cox, 2002). Pyrethrin and PBO combinations are generally used in liquid, gel and shampoo forms. The effects of PBO on human health and the environment depend on its concentration as well as on the duration and frequency of exposure. Nevertheless, all the potential health effects of this chemical have not been fully investigated. The activity of pyrethroids, including RES, is usually increased by the addition of synergists such as PBO. However, researches conducted on combinations particularly with RES are limited in number. We suggest that these insecticides and particularly, the combined use of RES and PBO could be toxic to mammals. Therefore, further studies are required on this subject.

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