

Sex-, tissue-, and exposure duration-dependent effects of imidacloprid modulated by piperonyl butoxide and menadione in rats. Part I: oxidative and neurotoxic potentials

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Earlier research has evidenced the oxidative and neurotoxic potential of imidacloprid, a neonicotinoid insecticide, in different animal species. The primary aim of this study was to determine how metabolic modulators piperonyl butoxide and menadione affect imidacloprid's adverse action in the liver and kidney of Sprague-Dawley rats of both sexes. The animals were exposed to imidacloprid alone (170 mg kg⁻¹) or in combination with piperonyl butoxide (100 mg kg⁻¹) or menadione (25 mg kg⁻¹) for 12 and 24 h. Their liver and kidney homogenates were analysed spectrophotometrically for glutathione peroxidase, glutathione *S*-transferase, catalase, total cholinesterase specific activities, total glutathione, total protein content, and lipid peroxidation levels. Imidacloprid displayed its prooxidative and neurotoxic effects predominantly in the kidney of male rats after 24 h of exposure. Our findings suggest that the observed differences in prooxidative and neurotoxic potential of imidacloprid could be related to differences in its metabolism between the sexes. Co-exposure (90-min pre-treatment) with piperonyl butoxide or menadione revealed tissue-specific effect of imidacloprid on total cholinesterase activity. Increased cholinesterase activity in the kidney could be an adaptive response to imidacloprid-induced oxidative stress. In the male rat liver, co-exposure with piperonyl butoxide or menadione exacerbated imidacloprid toxicity. In female rats, imidacloprid+menadione co-exposure caused prooxidative effects, while no such effects were observed with imidacloprid alone or menadione alone. In conclusion, sex-, tissue-, and duration-specific effects of imidacloprid are remarkable points in its toxicity.

KEY WORDS: *catalase; cholinesterase; glutathione peroxidase; glutathione S-transferase; neonicotinoids; oxidative stress*

Neonicotinoids account for approximately one fourth of the world insecticide market (1). It is a novel insecticide group that has been replacing organophosphate, organochlorine, and pyrethroid insecticides (2) and is highly effective against piercing-sucking pests in the crops and in flea control

in cats and dogs (3). Imidacloprid (IMI; IUPAC name: *N*-{1-[(6-chloro-3-pyridyl) methyl]-4,5-dihydroimidazol-2-yl} nitramide; CAS no: 138261-41-3) is a systemic neonicotinoid insecticide that has first been registered in 1994 (4). Turkey alone used nearly 500 tonnes of it in 2012.

Imidacloprid and other neonicotinoids act on insect nicotinic acetylcholine receptors (nAChRs), and fundamental differences between the nAChRs of insects and mammals make them remarkably "safe" for the latter (5). However, what is not safe for mammals are IMI's metabolites, especially nitroguanidine and aminoguanidine derivatives (6-8). Mammalian CYP450s, (CYP3A4 in particular) and aldehyde oxidase (AOX; EC 1.2.3.1) are two important enzymes in its metabolism (9). CYP450s are responsible for the conversion of IMI to 5-hydroxy, olefin, nitrosoimine, guanidine, and urea derivatives (10), while AOX converts IMI to nitrosoguanidine and aminoguanidine metabolites (11).

Many studies have shown the oxidative and neurotoxic potential of IMI in a variety of animal species, including rats (12-19). However, none has investigated how biotransformation affects prooxidative and neurotoxic effects of IMI in mammals and whether it depends on sex, tissue, or exposure duration. Modulators such as piperonyl butoxide (PBO) and menadione (MEN) are very useful to understand how biotransformation affects the toxicity of a chemical under investigation (20, 21). PBO (IUPAC name: 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole; CAS no: 51-03-6) is a methylenedioxyphenyl compound used to enhance the potency of certain pesticides by inhibiting CYP450s and esterases (22-24). As Tomizawa and Casida (5) stated in their review that PBO synergized with IMI in toxicity to insects, we wondered what would be the toxicity (oxidative stress and cholinesterase activity) of this combination in mammals.

Menadione (IUPAC name: 2-Methylnaphthalene-1,4-dione; CAS no: 58-27-5) is a specific inhibitor of AOX and it can increase reactive oxygen species (ROS) levels as a redox active compound (25-27). However, according to Kerver et al. (28), it also lowers superoxide radical formation by inhibiting AOX-catalysed 3,3'-diaminobenzidine (DAB) oxidation in the liver and intestine of male Wistar rats and, according to Al-Omar and Al-Arifi (29), superoxide radical and hydrogen peroxide formation by inhibiting AOX-mediated oxidation in Dunkin-Hartley guinea pig liver. This is why we decided to also investigate the effects of MEN on IMI-induced oxidative stress.

By determining how these two metabolic modulators affect the oxidative and neurotoxic potential of IMI in rats, we hoped to get an insight

into the mechanisms behind IMI toxicity and their relation to sex, tissue, and exposure duration.

MATERIALS AND METHODS

Chemicals

Pure IMI and MEN crystals were purchased from Supelco and Sigma, respectively (Sigma-Aldrich Chemie GmbH). Technical grade (90 %) PBO was supplied from Aldrich (Sigma-Aldrich Chemie GmbH). All other chemicals used were of analytical grade.

Animals and treatment

This study was approved by the ethics committee of the Cukurova University Medical Sciences, Experimental Research, and Application Centre (no:11, date: 2 July 2010), which also supplied young adult, male and female rats (weighing about 200 g, regardless of sex) of the Sprague-Dawley strain (*Rattus norvegicus* var. albinos). They were separated by sex, kept in clean plastic cages, and acclimatised to laboratory conditions for seven days. Wood shavings in cages were changed every day. Laboratory temperature was 22 ± 1 °C and relative humidity was 65 ± 5 %. The rats were maintained under natural photoperiod and fed *ad libitum*.

Feeding ceased 24 h before toxicity experiments. The rats were randomly divided in six groups of six animals. One group received physiological saline (saline control) to determine the basal levels of the parameters tested. All the chemicals used in this study were dissolved in dimethylsulfoxide (DMSO) and were given intraperitoneally (*i.p.*). As DMSO has some biphasic effects, which can be antioxidant or prooxidant (30), we also included a solvent control, the DMSO group. Data obtained from treated groups, however, were compared with this group only instead of saline control to display the toxicity of the investigated chemicals against the solvent's effects. This is why we refer to the DMSO control as control.

One group received only IMI (IMI group) in the LC_{50} dose of 170 mg kg^{-1} (31). One group received 100 mg kg^{-1} of PBO only (PBO group). One received 25 mg kg^{-1} of MEN only (MEN group). Two groups received pre-treatment with either PBO or MEN 90 min before receiving IMI in the same doses as

described above (IMI+PBO group and IMI+MEN group, respectively).

None of the rats died during the experiment. The rats were killed by cervical dislocation after 12 h or 24 h of exposure. Liver and kidney tissues were dissected on an ice plate, washed in physiological saline to remove blood residues, blotted dry, weighed, and stored at -80°C until biochemical analysis.

Biochemical analyses

All biochemical analyses were done using a Shimadzu UV1800 UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan). Liver and kidney tissues were homogenised using a glass-teflon homogeniser (WiseStir HS30E, Daihan Scientific, Seoul, Korea) in an ice-containing bucket with 50 mmol L^{-1} pH 7.4 phosphate buffer that contained 1.17 % KCl and centrifuged at $10500 \times g$ at 4°C for 30 min (Universal 320R, Hettich, Tuttingen, Germany). The obtained supernatants from each homogenate were used to analyse glutathione peroxidase (GPx), glutathione *S*-transferase (GST), catalase (CAT), total cholinesterase (tChE), total glutathione (tGSH), thiobarbituric acid reactive substances (TBARS), and total protein content. Aliquots of liver or kidney homogenates were mixed with 10 % 5-sulphosalicylic acid (1:0.5 v/v), then centrifuged at $10,500\text{ g}$ at 4°C for 5 min, and the obtained supernatants used to measure tGSH content based on the recycling reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) (32). The incubation medium contained 0.3 mmol L^{-1} of NADPH in stock buffer (143 mmol L^{-1} of sodium phosphate/ 6.3 mmol L^{-1} of Na-EDTA, adjusted to pH 7.5), 6 mmol L^{-1} of DTNB, 50 U/mL of GR, and $25\text{ }\mu\text{L}$ of sample. 5-thio-2-nitrobenzoic acid formation was monitored continuously at 412 nm for 3 min. The tGSH content was determined by comparison with a standard curve generated using GSH and expressed as $\mu\text{mol L}^{-1}$ per mg of protein.

Glutathione peroxidase activity was assayed at 37°C and 340 nm using GSH and *t*-butylhydroperoxide as substrates (33). The reaction medium contained 100 mmol L^{-1} of GSH, 10 U mL^{-1} of GR, 2 mmol L^{-1} of NADPH, and 7 mmol L^{-1} of *t*-butylhydroperoxide. Specific activity was expressed as U/mg protein using the molar extinction coefficient of $6.22\text{ mmol L}^{-1}\text{cm}^{-1}$.

Catalase activity was determined according to Beutler (33) as a decrease in absorbance of $10\text{ mmol L}^{-1}\text{ H}_2\text{O}_2$ at 37°C for 2.5 min. The reaction was initiated by adding the supernatant. The degradation

rate of H_2O_2 by CAT was measured at 230 nm . Specific activity was expressed as U/mg protein using the molar extinction coefficient of $0.071\text{ mmol L}^{-1}\text{cm}^{-1}$.

Glutathione *S*-transferase activity was measured according to Habig et al. (34), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Enzyme activity was determined by monitoring changes in absorbance at 340 nm , which reflects the rate of CDNB (1 mmol L^{-1}) conjugation with GSH (1 mmol L^{-1}) at 30°C for 2 minutes. Specific activity was expressed as U/mg protein using the molar extinction coefficient of $9.6\text{ mmol L}^{-1}\text{cm}^{-1}$.

For TBARS measurement, supernatants were mixed with 10 % trichloroacetic acid, vortexed, and then centrifuged at 11 g for 10 min. After the addition of thiobarbituric acid, samples were vortexed and held in a water bath at 100°C for 10 min. Absorbance of the samples was measured at 535 nm , and these values were converted to TBARS concentration using the standard graphic prepared with 1,1',3,3'-tetramethoxypropane (35). TBARS concentration was expressed in nmoles per mg of protein.

Total protein content was measured using the Folin-phenol reagent according to Lowry et al. (36). The absorbance was measured at 750 nm and converted to concentration using bovine serum albumin as the standard. The concentration was expressed in mg in per mL of supernatant.

Since no inhibitor was used to differentiate cholinesterase types, we determined the total ChE activity in the supernatants. Briefly, supernatants were incubated with 10 mmol L^{-1} DTNB in pH 8 phosphate buffer for 5 min. Activity was started by adding 15 mmol L^{-1} acetylthiocholine iodide as substrate and monitored for 5 min at 412 nm (37). Specific activity was expressed as nmol L^{-1} of thiocholine formed per min per mg of protein using the molar extinction coefficient of $13.6\text{ mmol L}^{-1}\text{cm}^{-1}$.

Statistical analyses

The results are expressed as mean \pm standard error of mean (SEM) of six samples. The groups were compared using the Mann-Whitney *U*-test and the Kruskal-Wallis rank test of variance. The data were analysed using the Statistical Package for Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 17. The level of significance was set at $P < 0.05$. We did not perform the statistical analysis considering exposure durations in the same tissue because of the significant differences between control values.

Therefore, the results for different exposure durations for the same tissue are given in separate tables.

RESULTS AND DISCUSSION

Oxidative stress is a part of IMI toxicity that has been evidenced by earlier studies (16-19). Our study has added to this knowledge by showing that IMI toxicity varies with sex, tissue, and exposure duration.

Sex- and duration-specific effects of IMI in rat liver and kidney

In the first 12 h of exposure, we found no effect on oxidative stress parameters and tChE regardless of sex and tissue (Tables 1 and 2), except for a decrease in liver tGSH in male rats (Table 2). The highest oxidative toxicity and neurotoxicity was observed in the kidney of male rats after 24 h of exposure: tGSH, TBARS, and tChE increased, but protein content decreased (Table 3). The only effect found in female rats was increase in kidney GST activity after 24 h of exposure (Table 3). Kidney is the target organ for most xenobiotics, as proximal tubular cells are able to accumulate slightly acidic and basic compounds, amino acid conjugates, and quaternary compounds at toxic levels (38). The duration-dependent effect of IMI in our study is most probably related to its metabolism. Broznić et al. (39) reported that IMI was mostly cleared from the liver of BALB/C mice within 24 h and from the kidney within 48 h. In rats, kidney clearance is generally completed within 24 h after oral or intravenous administration (40).

Oxidative effects of IMI were more pronounced in both tissues of the male rats regardless of exposure duration. It is likely that different metabolic rates resulted in sex-related toxicity differences in the IMI-exposed rats. Induced CYP450 activities by nicotine enantiomers lead to oxidative stress conditions in Chinese hamster ovary K1 cell line (41). Kyerematen et al. (42) reported faster metabolism of nicotine in the kidney and liver of male than female Sprague-Dawley rats. Being a nicotine-like compound, IMI is metabolised more effectively in males than in females (40), and its faster metabolism could result in higher ROS production.

Imidacloprid is metabolised mainly by CYP3A4, and by CYP2C19, CYP2A6, CYP2C9, CYP2D6, CYP2E1, CYP1A2, and CYP2B6 (10). Kammerer et al. (43) reported that protein kinase inhibitor ML3403

was metabolised *in vitro* by CYP450 isoenzymes (CYP3A4, CYP2C19, CYP1A2, and CYP2D6) much faster in male than in female Wistar rats. Sundseth and Waxman (44), in turn, found that clofibrate-induced liver and kidney CYP4A expression was higher in Fischer 344 male rats than in female, probably under hormonal influence.

Aldehyde oxidase functions as a ROS source (45) and is affected by sex hormones such as oestradiol, which may account for sex-specific differences in IMI toxicity, even though we did not analyse this enzyme's activity. Huh et al. (46) reported that Fe²⁺-induced lipid peroxidation was higher in male than in female Sprague-Dawley liver, and that oestradiol, possibly by the way of AOX inhibition, alleviated lipid peroxide formation compared to progesterone and testosterone. Ventura and Dachtler (47) suggested that androgens increase AOX activity in the liver of male and female C57BL/6J mice, and that oestradiol decreases it in males.

Our results suggest that increased tChE activity could be related to increased lipid peroxidation and tGSH levels and decreased protein content in the kidney of male rats after 24 h of exposure (Table 3). In a study by Abu-Donia et al. (13), IMI increased AChE activity in newborn Sprague-Dawley rats via maternal exposure, and the authors suggested that this induction occurred due to apoptotic events via cellular Ca²⁺-uptake. In another study (48), neonicotinoid insecticides acetamiprid and IMI induced cellular Ca²⁺ uptake via α_7 nAChRs in Sprague-Dawley rat cerebellar cell line, and Ca²⁺ uptake was inhibited by α -bungarotoxin, a specific α_7 nAChR antagonist. Yeboah et al. (49) suggest that α_7 nAChRs, which are constitutively expressed in the kidney of Sprague-Dawley rats, are highly permeable to Ca²⁺ ions. Duzguner and Erdogan (16) believe that higher plasma Ca²⁺ concentrations could be responsible for the induction of ROS-generating enzymes xanthine oxidase and myeloperoxidase in the liver and brain of female Wistar rats treated with IMI intravenously. Kimura-Kuroda et al. (48) have suggested that mammalian ACh receptors are affected by IMI exposure because of conformational changes of the receptor induced by ACh. We therefore believe that higher tChE activity that lowers ACh-assisted IMI-binding may be an adaptive response to oxidative stress induced by elevated Ca²⁺ uptake. This is supported by Bond et al. (50), who found that AChE release in rat astroglial cells increased in response to *t*-butylhydroperoxide-induced oxidative stress.

Table 1 Prooxidative and neurotoxic effects of IMI modulated by PBO and MEN in the kidney after 12 h of exposure in male and female Sprague-Dawley rats

KIDNEY 12 h							
Sex	Saline Control	DMSO Control	IMI	PBO	MEN	IMI+PBO	IMI+MEN
tGSH							
Male	1.40±0.06	1.32±0.07	1.33±0.07	1.41±0.10	1.25±0.07	1.39±0.11	1.45±0.24
Female	2.55±0.09	2.30±0.13	2.39±0.07	2.56±0.05	2.30±0.05	2.49±0.12	2.25±0.05
GPx							
Male	5.59±1.08	3.92±0.22	3.87±0.22	3.97±0.32	3.59±0.08	3.52±0.24	3.43±0.17
Female	3.50±0.09	3.30±0.23	2.88±0.19	3.32±0.24	2.91±0.12	2.89±0.23	2.79±0.10
CAT							
Male	149.68±1.37	163.53±7.62	159.56±4.82	148.56±6.32	153.95±5.90	159.97±7.80	132.88±12.93
Female	148.95±5.70	158.50±14.49	145.61±10.43	138.98±10.80	122.99±10.51	137.47±5.81	133.46±9.81
GST							
Male	224.17±23.23	220.63±19.23	216.77±18.20	222.67±17.83	206.69±16.16	210.22±3.94	223.53±11.77
Female	83.51±7.76	94.33±7.94	80.27±3.94	93.25±7.56	96.53±5.66	88.26±6.88	90.58±5.34
TBARS							
Male	367.03±10.03*	242.85±16.93	251.03±25.82	292.63±17.99	326.60±27.22	301.50±16.55	250.68±14.29
Female	393.80±16.31	255.45±70.63	331.50±22.23	316.75±33.10	362.10±23.78	338.19±25.73	358.24±18.81
Total protein							
Male	6.64±0.17	7.22±0.25 ^{ab}	7.36±0.28 ^a	6.71±0.19 ^b	7.24±0.29 ^{ab}	7.32±0.29 ^{ab}	8.15±0.18 ^c
Female	5.00±0.17	5.14±0.28	5.35±0.18	4.79±0.20	5.31±0.11	5.26±0.33	5.15±0.17
tChE							
Male	1.84±0.13	2.26±0.23 ^{ab}	1.88±0.22 ^a	2.36±0.29 ^{ab}	2.77±0.24 ^b	3.40±0.21 ^c	2.96±0.34 ^{bc}
Female	2.39±0.24	2.61±0.14 ^a	2.71±0.15 ^a	2.87±0.09 ^a	2.14±0.09 ^b	2.71±0.05 ^a	2.54±0.10 ^a

The results are presented as mean±standard error of mean. Asterisk (*) denotes significant difference between the saline and DMSO control groups. Otherwise, saline control was not compared statistically to other groups. Different superscript letters denote significant differences in the same line (P<0.05; N=6)

Table 2 Prooxidative and neurotoxic effects of IMI modulated by PBO and MEN in the liver after 12 h of exposure in male and female Sprague-Dawley rats

LIVER 12 h							
Sex	Saline Control	DMSO Control	IMI	PBO	MEN	IMI+PBO	IMI+MEN
tGSH							
Male	6.61±0.75*	4.30±0.40 ^a	2.73±0.25 ^b	4.68±0.28 ^a	7.04±0.77 ^c	0.97±0.07 ^d	1.16±0.02 ^c
Female	5.63±0.45*	4.09±0.15 ^a	3.78±0.34 ^{ab}	3.06±0.24 ^b	4.50±0.37 ^a	2.62±0.39 ^b	3.50±0.05 ^b
GPx							
Male	2.45±0.22	2.47±0.08 ^a	2.23±0.15 ^{ab}	2.17±0.13 ^{ab}	2.15±0.09 ^b	1.86±0.08 ^b	2.14±0.11 ^b
Female	7.25±0.28	6.74±0.39	6.79±0.20	7.37±0.44	7.00±0.35	6.85±0.34	6.41±0.13
CAT							
Male	199.17±13.61	210.57±11.84	211.71±4.12	215.12±6.03	208.49±6.54	215.57±6.62	199.55±26.85
Female	170.83±5.45	142.94±10.20	136.14±9.39	141.18±9.63	142.05±6.49	134.85±13.52	116.59±4.29
GST							
Male	181.00±29.02	217.54±29.39	219.13±23.87	263.74±36.00	228.93±19.49	171.51±29.64	155.61±22.37
Female	236.62±13.91	239.40±20.86 ^{ab}	175.00±16.92 ^b	260.26±22.81 ^a	252.42±27.74 ^a	197.44±24.84 ^{ab}	283.42±16.31 ^a
TBARS							
Male	316.32±10.77	340.30±13.90	327.88±12.58	298.55±9.65	308.38±13.17	305.19±13.42	308.57±11.59
Female	300.77±33.65	310.51±25.02 ^a	275.58±14.24 ^a	261.13±20.79 ^{ab}	279.02±12.09 ^a	220.35±18.19 ^b	214.82±20.20 ^b
Total protein							
Male	23.24±0.83	24.06±0.61	24.33±0.64	24.69±0.73	24.94±0.68	24.84±0.43	23.21±0.91
Female	23.42±0.79	25.49±0.24	24.61±0.35	26.77±1.26	25.59±0.48	24.12±1.29	25.82±0.39
tChE							
Male	1.75±0.06*	1.93±0.05 ^a	1.64±0.11 ^{ab}	1.68±0.13 ^{ab}	1.61±0.08 ^b	1.53±0.03 ^b	1.61±0.18 ^{ab}
Female	3.86±0.23	4.47±0.30 ^a	4.35±0.55 ^{ab}	4.71±0.40 ^a	3.09±0.22 ^b	3.31±0.22 ^b	2.09±0.07 ^c

The results are presented as mean±standard error of mean. Asterisk (*) denotes significant difference between the saline and DMSO control groups. Otherwise, saline control was not compared statistically to other groups. Different superscript letters denote significant differences in the same line (P<0.05; N=6)

Furthermore, Yang et al. (51) found that *d*-tubocurarine, which is an nAChR antagonist, prevented diisopropylphosphorofluoridate to increase ACh-related ROS and lipid peroxidation in the muscle of male Sprague-Dawley rats.

While the normal range of tChE activity in our study was 1.75-2.30 nmol L⁻¹ per min per mg of protein in male liver and 3.64-3.86 nmol L⁻¹ per min per mg of protein in female liver (Tables 2 and 4), literature reports a wide variety of inconclusive findings, and our results were not far from some of these (52-58).

Obregon et al. (59) found that DMSO mixed with different brain region homogenates of male Wistar rats inhibited ChE activity at all tested concentrations. However, in our study DMSO had no effect on the liver and kidney tChE after *i.p.* injection, except for a transitory elevation in the liver of male rats at 12 h. Similarly, Carr and Nail (60) reported no DMSO effect on ChE activities of different brain regions in Sprague-Dawley pups of either sex, while the serum activity increased significantly. However, this increase did not suffice to protect against chlorpyrifos treatment, just as in our study higher tChE in the DMSO control group did not suffice to protect against IMI+PBO in male rats. At 12 h, IMI+PBO significantly lowered tChE activity compared to either saline or DMSO control.

In our study, GPx activity increased and CAT activity decreased in the liver of male rats after 24 h of exposure (Table 4). CAT and GPx compete for H₂O₂ detoxification (61). In a study by Duzguner and Erdogan (16), IMI lowered GPx activity in the liver and increased it in the brain of female Wistar rats, while CAT was not affected. The authors suggested that GPx could be more important than CAT in countering IMI-induced ROS formation.

Table 3 shows that GST activity in our study increased in the kidney of female rats after 24 h of exposure without a change in TBARS levels. GST catalyses the conjugation of GSH with xenobiotics, including lipid peroxidation products (62), and according to the WHO (31) this reaction is not important in IMI biotransformation. The level of GST subunits 3 and 4 in females are 3-4 times higher than in males, while subunits 1 and 2 are 1.5-2 times higher in the kidney of male rats (63). Gender-specific subunit levels may be related to this increase and further research should look into which GST subunit is responsible for IMI effect in the kidney of female rats.

Effects of PBO on IMI toxicity

Piperonyl butoxide can be valuable to determine the effects of CYP450-mediated biotransformation on IMI toxicity (10).

We observed no change in oxidative stress parameters in the kidney regardless of sex after 12 h of exposure (Table 1). However, tGSH content was more pronounced in the liver of male rats treated with IMI+PBO than IMI alone (Table 2). IMI+PBO co-exposure decreased liver GPx activity compared to control, but it did not differ significantly from IMI and PBO alone groups. This lower GPx activity may be associated with lower tGSH, which is a substrate for the enzyme.

IMI+PBO decreased total protein content in the liver of male rats after 24 h of exposure (Table 4). GPx activity dropped, while CAT remained within the control range. This suggests that GPx is more involved in IMI-induced oxidative stress than CAT. Lower CAT activity in the liver of female rats also supports this hypothesis because no change was observed in tGSH, TBARS, and total protein content (Table 4). Since PBO inhibits CYP450 enzymes, the accumulation of the parent IMI compound may provide more substrate for other biotransformation pathways such as AOX. This elevation in AOX activity could explain the oxidative effects in the liver of male rats. A similar explanation was suggested by Valentovic et al. (64), who said that increased liver toxicity of 3,5-dichloroaniline given with PBO could be related to the metabolites of the parent compound produced by metabolic processes other than CYP450.

Unlike the liver, the kidney of male rats was partly protected against IMI toxicity by PBO pre-treatment; it had no effect on increased tGSH and decreased total protein content in the kidney of male rats after 24 h of exposure (Table 3). However, TBARS levels, which increased in the IMI and PBO alone groups, did not increase significantly in respect to control. Baliga et al. (65) found that PBO provided protection against H₂O₂-induced cytotoxicity in proximal tubular cell line LLC-PK1 and they suggested that CYP450 may have served as a source of catalytic iron in ROS production pathways.

Co-exposure to IMI+PBO lowered liver tGSH and TBARS in females after 12 h of exposure (Table 2); while there was no change in the IMI alone group. These changes seem to be related to PBO pre-treatment. Murguruma et al. (66) reported that PBO increased microsomal ROS production in the liver.

Table 3 Prooxidative and neurotoxic effects of IMI modulated by PBO and MEN in the kidney after 24 h of exposure in male and female Sprague-Dawley rats

KIDNEY 24 h							
Sex	Saline Control	DMSO Control	IMI	PBO	MEN	IMI+PBO	IMI+MEN
tGSH	Male 1.24±0.06	1.14±0.03 ^a	1.49±0.02 ^b	1.44±0.06 ^{bc}	1.45±0.03 ^b	1.41±0.11 ^{bc}	1.30±0.05 ^c
	Female 2.05±0.20*	1.67±0.04	1.74±0.06	1.69±0.11	1.76±0.15	1.67±0.07	1.69±0.07
GPx	Male 2.53±0.08	2.69±0.21 ^{ab}	2.82±0.23 ^{abc}	2.74±0.10 ^a	2.73±0.08 ^a	2.13±0.16 ^{bc}	2.07±0.05 ^c
	Female 3.86±0.27*	3.01±0.09	3.41±0.19	3.81±0.27	3.58±0.17	3.50±0.17	3.86±0.26
CAT	Male 132.00±6.69	129.42±4.33 ^a	140.58±3.44 ^{ab}	153.31±6.90 ^b	150.60±3.24 ^b	152.21±6.87 ^b	147.38±3.90 ^b
	Female 129.21±3.22	116.09±6.40	120.38±5.54	105.31±4.90	125.64±8.57	127.65±4.64	122.67±2.04
GST	Male 68.11±3.93*	113.46±12.35	120.40±5.74	119.47±10.67	126.92±12.85	113.44±10.41	123.58±8.87
	Female 63.13±3.62	53.97±1.64 ^a	69.15±1.06 ^b	36.60±0.68 ^c	87.44±5.01 ^d	106.74±7.35 ^d	100.37±4.67 ^d
TBARS	Male 279.16±13.38	255.72±11.26 ^a	302.25±2.73 ^b	308.22±19.09 ^b	252.71±3.99 ^a	247.87±19.05 ^{abc}	203.38±11.32 ^c
	Female 465.48±29.67*	327.86±26.26 ^{ab}	384.79±28.09 ^{abc}	301.93±25.99 ^b	366.23±28.06 ^{abc}	377.70±17.63 ^{ac}	408.62±14.82 ^c
Total protein	Male 7.98±0.20	8.37±0.23 ^a	6.94±0.14 ^b	7.20±0.17 ^{bc}	6.88±0.11 ^b	7.14±0.29 ^{bc}	7.74±0.15 ^{ac}
	Female 5.63±0.30*	6.68±0.14	6.34±0.24	6.77±0.21	6.42±0.29	6.28±0.23	6.14±0.19
tChE	Male 1.72±0.07	1.74±0.04 ^a	2.23±0.06 ^b	1.60±0.14 ^a	1.65±0.13 ^a	2.88±0.22 ^c	2.75±0.32 ^{bc}
	Female 1.69±0.10	1.78±0.09 ^{ab}	2.07±0.14 ^a	1.95±0.08 ^a	1.76±0.15 ^{ab}	1.96±0.08 ^a	1.65±0.06 ^b

The results are presented as mean±standard error of mean. Asterisk (*) denotes significant difference between the saline and DMSO control groups. Otherwise, saline control was not compared statistically to other groups. Different superscript letters denote significant differences in the same line (P<0.05; N=6)

Table 4 Prooxidative and neurotoxic effects of IMI modulated by PBO and MEN in the liver after 24 h of exposure in male and female Sprague-Dawley rats

LIVER 24 h							
Sex	Saline Control	DMSO Control	IMI	PBO	MEN	IMI+PBO	IMI+MEN
tGSH	Male 1.10±0.11	1.06±0.11 ^{ab}	0.89±0.06 ^a	1.24±0.08 ^b	1.02±0.05 ^{ab}	1.11±0.08 ^b	1.34±0.13 ^b
	Female 3.36±0.20	3.52±0.21	2.98±0.32	3.90±0.22	3.62±0.30	3.48±0.15	3.35±0.07
GPx	Male 3.85±0.16	3.76±0.09 ^{ab}	4.01±0.08 ^c	3.47±0.25 ^{abcd}	4.03±0.12 ^{ac}	3.19±0.07 ^d	3.33±0.13 ^{bd}
	Female 6.06±0.32	5.79±0.26	6.45±0.31	6.19±0.20	5.33±0.21	5.57±0.21	6.04±0.33
CAT	Male 178.03±2.67*	146.11±4.09 ^a	122.24±2.92 ^b	178.10±13.55 ^c	149.37±4.88 ^{ac}	167.35±7.84 ^{ac}	163.97±6.96 ^c
	Female 165.68±7.83	144.80±8.23 ^a	138.12±9.85 ^{ab}	134.16±7.74 ^{abc}	146.20±13.30 ^{ab}	111.22±2.95 ^c	119.59±2.12 ^{bc}
GST	Male 208.07±25.83	209.05±24.41	215.53±9.41	240.88±16.93	215.48±21.17	221.81±14.80	201.22±18.38
	Female 335.05±12.15	294.94±24.26	341.59±11.66	348.60±14.69	342.43±32.42	348.88±15.91	325.98±19.22
TBARS	Male 472.05±49.68	409.06±17.88 ^{ab}	462.78±24.02 ^a	454.11±24.85 ^a	386.37±14.50 ^b	360.00±2.71 ^b	393.14±39.57 ^{ab}
	Female 210.55±20.94	197.68±22.46	243.37±19.45	284.11±11.88	266.85±27.59	247.82±7.70	215.72±20.94
Total protein	Male 24.31±0.66*	27.59±0.57 ^a	26.37±0.80 ^{ab}	25.23±1.15 ^{ab}	24.30±0.39 ^{bc}	24.98±0.37 ^{bc}	25.05±0.75 ^{ac}
	Female 19.49±0.56	20.86±0.85	24.61±0.35	26.77±1.26	25.59±0.48	24.12±1.29	25.82±0.39
tChE	Male 2.30±0.14	2.17±0.04 ^a	2.55±0.17 ^a	2.22±0.12 ^a	1.85±0.05 ^b	1.72±0.07 ^b	2.38±0.13 ^a
	Female 3.64±0.36	2.78±0.11 ^a	2.72±0.23 ^{ab}	3.61±0.09 ^c	3.17±0.38 ^{abc}	2.54±0.28 ^{ab}	2.28±0.04 ^b

The results are presented as mean±standard error of mean. Asterisk (*) denotes significant difference between the saline and DMSO control groups. Otherwise, saline control was not compared statistically to other groups. Different superscript letters denote significant differences in the same line (P<0.05; N=6)

This suggests that lower TBARS levels in the present study could be attributed to lower tGSH levels that act against ROS production.

Furthermore, co-exposure to IMI+PBO displayed tissue-specific effect of IMI on tChE activity. In the kidney of male rats, it was higher than in control, IMI, and PBO groups after 12 and 24 h of exposure (Tables 1 and 3). We believe that increased tChE activity may be an adaptive response against oxidative stress, as we discussed earlier. In the liver of male rats, however, tChE activity dropped significantly compared to control, IMI, and PBO groups after 24 h of exposure (Table 4). IMI+PBO also lowered tChE activity after 12 h of exposure compared to control (Table 2). Liver tChE activity also dropped in females compared to control, IMI, and PBO groups after 12 h (Table 2), while it remained within the control range after 24 h of exposure. PBO alone increased tChE activity (Table 4). While some authors (14, 15) reported that IMI lowered ChE, tissue-specific effects of IMI on tChE activity require further investigation.

Effects of MEN on IMI toxicity

Interestingly, MEN as a modulator of IMI metabolism has revealed similar tissue-specific effect of IMI on tChE activity as observed in PBO co-exposure. MEN lowered tChE activity in the kidney female rats (Table 1). Similarly, Torda and Wolff (67) reported that MEN decreased ChE activity in human serum. However, IMI+MEN co-exposure resulted in no significant change, probably due to the antagonistic effect of IMI against MEN. In contrast, IMI+MEN co-exposure lowered liver tChE activity in female rats compared to control, IMI, and MEN alone groups in both exposure durations (Table 2 and 4). Therefore, we are convinced that IMI, whose metabolism was inhibited by PBO or MEN, inhibits liver tChE activity.

Menadione co-exposure proved to be useful against oxidative stress in the kidney of male rats after 24 h of exposure (Table 3). It countered the TBARS-increasing and protein-lowering effects of IMI. It also lowered IMI-induced tGSH, while GPx decreased and CAT increased. Kitamura et al. (68) reported that AOX catalysed fenthion metabolism to fenthion sulphoxide, and that this transformation was inhibited by MEN and β -oestradiol in the liver of male Sprague-Dawley rats. Similarly, Honda et al. (8) reported that MEN decreased AOX activity in mouse fibroblast M10 cells. These findings suggest that MEN co-exposure may have inhibited AOX-mediated biotransformation of IMI in our study and therefore the oxidative effects of

its metabolites. Elevated tChE activity in this group may also have contributed to the protection against oxidative stress as discussed above.

In female rats, MEN co-exposure also revealed the oxidative effects of IMI in both tissues. Liver TBARS and tGSH levels were significantly lower than in the control, IMI, and MEN alone groups after 12 h of exposure (Table 2). Higher kidney GST activity and TBARS levels were observed in IMI+MEN group after 24 h of exposure (Table 3). In the liver of male rats, tGSH and GPx activity dropped, but total protein content and lipid peroxidation levels did not change after 12 h of exposure (Table 2). In the liver of male Wistar rats, Sidorova and Grishanova (69) found that MEN increased total CYP450 content, GST, and a variety of CYP450 enzyme activities, including CYP1A2, the enzyme that catalyses nitroimine reduction of IMI. This suggests that, CYP450-mediated metabolism of IMI could be accelerated by MEN co-exposure and contribute to IMI-related oxidative effects, especially in female rats, despite possible inhibition of AOX activity. Because of the opposite effects of MEN co-exposure (AOX inhibition and CYP450 induction), further *in vivo* studies with the same doses of IMI and MEN should investigate the mechanisms that affect CYP450 and AOX activities.

CONCLUSIONS

This is the first study that shows tissue-, exposure duration-, and sex-differences in the prooxidative and neurotoxic potential of IMI in rats. IMI's oxidative and neurotoxic effects after 24 h of exposure were probably related to its metabolism. The most pronounced prooxidative effect was observed in the kidney of male rats, and it may be related to hormonal effects on the enzymes that participate in IMI metabolism. However, further investigation is needed to evaluate the effects of sex hormones on CYP450s and AOX activity in the presence of IMI. IMI has also shown a tissue-specific effect on tChE activity, which was clarified by metabolic modulators PBO and MEN. Interestingly, PBO and MEN co-exposure exacerbated the prooxidative effects of IMI in the liver of male rats, while no change or a decrease was found in tChE activity. This points to a synergy between IMI and PBO in the liver, which could be more toxic to mammals in terms of oxidative stress and neurotoxicity. In contrast, PBO and MEN co-exposure alleviated the

prooxidative effects of IMI in the kidney, as evidenced by a significant rise in tChE activity in these groups. This calls for further investigation of the protective role of cholinesterase against IMI-induced oxidative stress. MEN co-exposure was also useful in revealing the oxidative effects of IMI in female rats in both tissues because IMI alone and MEN alone had no effects in females.

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Sažetak**Djelovanje imidakloprida pod utjecajem piperonil butoksida i menadiona ovisno je o spolu, tkivu i trajanju izloženosti. I. dio - oksidativno i neurotoksično djelovanje**

Rezultati ranijih istraživanja pokazali su oksidativni i neurotoksični potencijal imidakloprida, neonikotinoidnog insekticida, u različitim životinjskih vrsta. Primarni je cilj ovog istraživanja bio utvrditi kako modulatori metabolizma piperonil butoksid i menadion utječu na nepovoljne učinke imidakloprida na jetra i bubrege muških i ženskih štakora soja Sprague-Dawley. Životinje su 12 h odnosno 24 h bile izložene samo imidaklopridu (170 mg kg^{-1}) ili njegovoj kombinaciji s piperonil butoksidom (100 mg kg^{-1}) odnosno menadionom (25 mg kg^{-1}). U homogenatima jetara i bubrege spektrofotometrijski su utvrđene razine glutation peroksidaze, glutation S-transferaze, katalaze, specifične aktivnosti ukupne kolinesteraze, ukupni glutation, ukupni proteini te razine lipidne peroksidacije. Imidakloprid se pokazao prooksidativnim i neurotoksičnim uglavnom u bubrežima muških štakora nakon 24-satne izloženosti. Naši rezultati upućuju na to da su razlike u prooksidativnom i neurotoksičnom djelovanju imidakloprida povezane sa spolnim razlikama. Predtretmanom piperonil butoksidom odnosno menadionom (90 min prije davanja imidakloprida) otkriveno je da imidakloprid djeluje na ukupnu aktivnost kolinesteraze specifično za pojedina tkiva. Povišena aktivnost kolinesteraza u bubrežima mogla bi odražavati prilagodbu na oksidativni stres uzrokovan imidaklopridom. Piperonil butoksid odnosno menadion u jetrima muških štakora samo su pogoršali toksičnost imidakloprida. U ženki je djelovanje imidakloprida s menadionom bilo prooksidativno; takvo se djelovanje nije vidjelo nakon primjene samo imidakloprida odnosno samo menadiona. Vjerujemo da je promjenjivo djelovanje imidakloprida s obzirom na spol, tkivo i trajanje izloženosti važno za daljnja istraživanja njegove toksičnosti.

KLJUČNE RIJEČI: *glutation peroksidaza; glutation S-transferaza; katalaza; kolinesteraza; neonikotinoidi; oksidativni stres*

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