

INFLUENCE OF METHYL AND ISOPROPYL N-METHYL ANTRANILATES ON CARBON TETRACHLORIDE-INDUCED CHANGES IN RAT LIVER MORPHOLOGY AND FUNCTION[†]

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Abstract. *The aim of the present study was to examine potential protective effects of methyl N-methylantranilate (M) and isopropyl N-methylantranilate (I) in a rat model of acute intoxication with carbon tetrachloride (CCl₄) by tracking the changes in liver morphology and function. Serum transaminase and bilirubin were significantly elevated in animals treated with CCl₄ alone. A pretreatment with M and I prior to the administration of CCl₄ significantly prevented the increase of serum levels of liver damage markers. Histopathological evaluation of the livers of the test animals also revealed that M and I reduced the incidence of liver lesions. Our experiments showed that both M and I possess protective effect in CCl₄-induced liver damage in rats. The results are of interest due to the presence of natural or synthetic M in the human diet.*

Key words: *carbon tetrachloride, methyl and isopropyl N-methyl anthranilates, liver, transaminases*

1. INTRODUCTION

Methyl anthranilate and methyl N-methylantranilate (M) are flavoring esters added to many food products such as ice cream, candy and chewing gum [1]. The first ester is also

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very important in perfume industry and is additionally used as a flavoring for nonalcoholic beverages (methyl anthranilate is one of the four important odorants identified in *Pinot noir* wines as well) [2]. Recently published results show that M and isopropyl *N*-methylantranilate (I), both occurring naturally in medicinal and edible plants, produce a dose-responsive antinociceptive activity in chemical and thermal models of nociception in mice [3]. Also, anxiolytic, antidepressant and diazepam-induced sleep modulating activities were observed in animals that received M and I [4], suggesting a polypharmacological versatility of these compounds.

Carbon tetrachloride (CCl₄) is a synthetic industrial chemical used as a solvent that can cause hepatotoxicity and nephrotoxicity in workers exposed to it, as well as in experimental animals [5]. Inhalation, ingestion, skin absorption or intraperitoneal administration to experimental animals causes necrotic damage to cells and tissue, resulting in leakage of their enzymes into the blood stream [6]. Apart from liver, CCl₄ can cause disorders in kidneys, lungs, testes and brain, as well as in blood, generating free radicals [7,8]. Trichloromethyl free radicals (-CCl₃) are generated from CCl₄ via a biotransformation by hepatic microsomal Cytochrome P450 [9]. Furthermore, these radicals can form covalent bonds with macromolecules and oxidize them to form lipid, protein, or nucleic acid adducts [10], and to initiate and propagate lipid peroxidation in biological membranes [11].

The aim of the present study was to examine the potential protective effects of M and I in a rat model of acute intoxication with CCl₄ by tracking changes in liver tissues morphology and function.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Methyl and isopropyl *N*-methylantranilates (M and I, respectively) were synthesized following a previously reported procedure [3]. The anesthetic used, ketamine (Ketamidor 10%), was obtained from Richter Pharma AG, Wels, Austria.

2.2. Animals

Adult male and female Wistar rats weighing 200-250 g were maintained under standard laboratory conditions - 22±2 °C, 60% humidity and 12/12 (light/dark) cycle, with food and water available *ad libitum*. All experimental procedures were conducted in accord with the principles of care and use of laboratory animals in research and approved by the local Ethics committee. The approval of the committee (number 01-7289-11) was given on 14th October, 2011. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.3. Experimental design

Animals were randomly divided into four experimental groups of six animals each and were kept individually during the entire experiment. Groups one and two were treated daily with M and I (in a dose of 200 mg/kg) for seven days and on the seventh day, 2 h after the application of the last dose of M or I, they received CCl₄ dissolved in olive oil

(1:1), at a dose of 1 ml/kg. The control group received olive oil (at 1 ml/kg) for seven days. In animals of the remaining group (CCl₄ group) acute organ damage was produced by the administration of CCl₄ (1 ml/kg) dissolved in olive oil (1:1, v/v). All substances were administered daily by an intraperitoneal injection (*i.p.*). Twenty-four hours after a CCl₄ injection, rats were sacrificed by an overdose of ketamine. The animals and their livers were weighted and corresponding tissue samples were collected for histological analyses. Blood samples, withdrawn by a cardiac puncture, were used for the evaluation of biochemical parameters and were kept at -80 °C until use.

2.4. Biochemical measurements

The blood was centrifuged at 1500 rpm at 4 °C for 15 minutes to obtain the serum in which aspartate transaminase (AST), alanine transaminase (ALT), cholesterol (Cho), total (TB) and direct bilirubin (DB) were assayed by Olympus AU680 Chemistry-Immuno Analyzer.

2.5. Histopathological observation

The liver tissue specimens separated for histopathological examination were fixed in buffered formaldehyde solution (10%, w/v). The fixed tissues were then dehydrated with ethanol solutions of differing concentration (50-100%, v/v), embedded in paraffin, cut into 4-5 µm thick sections, stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) and further examined with an Olympus BH2 light microscope. The extent of CCl₄-induced liver damage was evaluated based on morphological changes in sections stained with HE. A score system was used for the histopathological examinations. The meaning of grades for liver (mononuclear cell infiltration, vacuolar degeneration, congestion and necrosis) is as follows: absent (-), mild (+), moderate (++) and severe (+++).

2.6. Statistical analysis

Results were expressed as the mean ± SD. Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (Graph pad Prism version 5.03, San Diego, CA, USA). Probability values (p) less than 0.05 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

Serum levels of ALT, AST, TB and DB in animals treated with the substance M and CCl₄ were significantly elevated when compared to the control group (vehicle), whereas the same parameters were significantly reduced when compared to the CCl₄ group. Such elevations are indicative of liver injury, especially the rise of ALT level [12]. Pretreatment of animals with the substance I did not have any effect on CCl₄-induced increase of AST and ALT levels. Serum levels of TB and DB in animals from the group I + CCl₄ were significantly elevated when compared to the control group but significantly lower in comparison with the animals treated with CCl₄ alone. An effective control of DB and TB levels points towards an early improvement in the secretory mechanism of hepatic cells.

In addition, no significant difference was found in serum cholesterol levels between the experimental groups (Table 1).

Table 1 Serum biochemical parameters and statistical comparison of the groups of animals after different experimental treatments

Groups	AST (U/L)	ALT (U/L)	Cho (mmol/L)	TB (μ mol/L)	DB (μ mol/L)
Control	176 \pm 32	37 \pm 2	1.5 \pm 0.3	2.1 \pm 0.1	0.4 \pm 0.1
CCl ₄	3070 \pm 513	1306 \pm 147*	1.9 \pm 0.2	11 \pm 3*	8.9 \pm 1.8 [‡]
M+CCl ₄	528 \pm 55 ^{†#}	321 \pm 22 ^{**#}	1.6 \pm 0.4	5.7 \pm 0.7 ^{*#}	2.4 \pm 0.9 [‡]
I+CCl ₄	2985 \pm 124	1119 \pm 373*	1.6 \pm 0.3	7.6 \pm 0.9 ^{*#}	4 \pm 1 ^{†#}

Values are presented as means \pm SD, n=6. One-way ANOVA followed by Tukey's test.

*p<0.001; **p<0.05; †p<0.01 vs. Control, #p<0.001 vs. CCl₄

The trichloromethyl radical binds to tissue macromolecules, induces peroxidative degradation of membrane lipids and disturbs Ca²⁺ homeostasis which leads to tissue injury and/or depletion of antioxidant defenses. Thus, the antioxidant activity or the inhibition of free radicals generation could be an important asset of the anthranilates in the protection against CCl₄-induced tissue damage [13]. Another possibility is that the reversal of increased serum enzyme levels in CCl₄-induced liver damage by these substances may be due to the prevention of leakage of intracellular enzymes by their membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [14]. The efficacy of any hepatoprotective drug is essentially dependent on its capability to either reduce the harmful effects or maintain the normal hepatic physiological mechanisms altered by a hepatotoxin [15].

Body and liver weight changes of rats in each experimental group noted during the treatments are shown in Table 2. A decrease in body weights was observed in rats that were treated with substances M and I in combination with CCl₄ compared to both the positive and negative control groups (Table 2). A significant increase of both absolute and relative liver weight (p < 0.05) was observed for the CCl₄-treated rats in comparison to the control group, whereas significant decreases in absolute liver weight were found in animals from the groups treated with a combination of M or I and CCl₄ (Table 2).

The application of CCl₄ resulted in a significant increase in relative and absolute liver weights when compared to the control group. Increase in liver weight could be due to an increased blood content, to the dilatation of central veins and sinusoids, swelling of hepatocytes, due to increase in water transport in cells and fatty liver or due to the increase in accumulation of fat in hepatocytes [16]. All these changes were obvious on histopathological examination of our tissue sections. Relative liver weights of animals from groups that received the combination of the anthranilates with CCl₄ were similar to the weights of animal livers from the control group. This result may be the consequence of both a decrease in total body weight as well as the protective effect of substances on CCl₄-caused damage.

Table 2 Body and liver weight of rats after different experimental treatments

Groups	Initial body weight (g)	Final body weight (g)	Percent change in body weight (%)	Absolute liver weight (g)	Relative liver weight (% to body weight)
Control	238±24	267±30	12±4	9.2±0.3	3.4±0.3
CCl ₄	253±28	258±29	3.4±0.1*	11.2±0.3 [†]	4.3±0.5*
M+CCl ₄	226±22	172±15 ^{†#}	-25±4 ^{†#}	6.8±0.6 ^{†#}	4.0±0.4
I+CCl ₄	243±26	181±16 ^{†#}	-28±5 ^{†#}	6.1±0.3 ^{†#}	3.8±0.4

Values are presented as means ± SD. *p<0.05; [†]p<0.001 vs. Control, [#]p<0.001 vs. CCl₄

Histopathological analyses of liver sections of the control and experimental animals are shown in Figure 1. The livers of control rats revealed characteristic hepatic architecture where the central veins, portal tracts, hepatocytes and sinusoids appear normal (Figure 1A). Histological examination of livers of rats that received CCl₄ showed massive fatty degeneration and extensive vacuolation with the disappearance of nuclei, gross necrosis, broad infiltration of lymphocytes and Kupffer cells around the central vein as well as the loss of cellular boundaries (Figure 1B). The treatment with M plus CCl₄ resulted in lobules having a normal configuration and a reduction of both degenerative lipid droplets around the centrilobular vein and vacuolated cells when compared to the CCl₄ group. However, inflammatory cells were still present (Figure 1C). The improved histology of liver as seen in histopathological observation for animals treated with the substance M as compared to that seen in animals administered only with CCl₄ indicated the possibility that this substance induces an acceleration of regeneration. The treatment with I plus CCl₄ also produced necrotic areas in the central lobe area as well as an inflammatory infiltrate; however, the necrotic areas were less extensive than in the livers of rats treated with CCl₄ alone, which is in good agreement with the results of the serum AST and ALT levels. Thus, the administration of I limited the damaged areas to the central zone of lobules. The histomorphological changes were graded and summarized in Table 4. Biochemical findings correlated well with the histological examination. There was a broad infiltration of lymphocytes and Kupffer cells around the central vein coupled with the loss of cellular boundaries (Table 3).

Table 3 Degrees of histopathological lesions of liver in rats after different experimental treatments

Groups	Liver sections		
	Hydropic and vacuolar degeneration	Mononuclear cell infiltration	Necrosis
Control	–	–	–
CCl ₄	+++	+++	+++
M + CCl ₄	+	++	+
I + CCl ₄	++	++	++

Scoring was done as follows: absent (–), mild (+), moderate (++) and severe (+++)

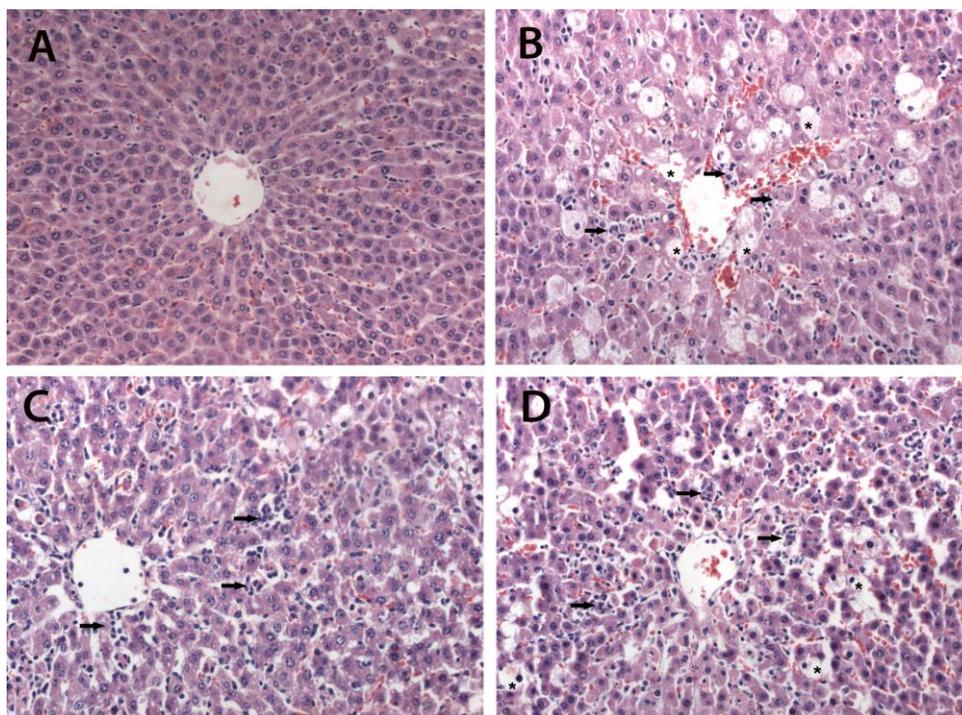


Fig. 1 Histopathological observations of liver sections stained with HE (x 200). (A) control rats (vehicle); (B) CCl₄-treated rats; (C) represents a liver section from rats treated with M plus CCl₄ and (D) represents a liver section from rats treated with I plus CCl₄. * necrotic cells, ➡ inflammatory cells

Besides their direct damaging effects on tissues, free radicals are able to trigger the accumulation of leukocytes in the tissues involved and thus cause tissue injury indirectly through activated neutrophils. It has been shown that activated neutrophils secrete enzymes (e.g., myeloperoxidase, elastase, and proteases) and liberate oxygen radicals [17]. These findings have been confirmed in our study as mononuclear cell infiltration was observable in liver tissue sections (Figure 1).

4. CONCLUSION

Our experiments showed that both methyl and isopropyl *N*-methylantranilates possess protective potential in CCl₄-induced liver damage in rats. This activity was observed by means of standard biochemical and histopathological analysis. The results are of interest due to the presence of natural or synthetic methyl *N*-methylantranilate (M) in human diet.

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UTICAJ METIL- I IZOPROPIL-N-METILANTRANILATA NA UGLJEN-TETRAHLORIDOM IZAZVANE PROMENE U FUNKCIJI I MORFOLOGIJI JETRE PACOVA

U ovom radu ispitivano je potencijalno protektivno dejstvo metil N-metilantranilata (M) i izopropil N-metilantranilata (I) u modelu akutne intoksikacije pacova sa ugljen-tetrahloridom (CCl₄) praćenjem promena u morfologiji i funkciji jetre. Kod životinja tretiranih sa samo CCl₄ značajno su bile povećane vrednosti transaminaza i bilirubina u serumu. Pretretman sa M i I značajno je umanjio porast serumskih markera oštećenja jetre. Patohistološka analiza tkiva jetre pokazala je da M i I smanjuju stepen oštećenja jetre. Ovaj eksperiment je pokazao da i M i I imaju protektivno dejstvo kod oštećenja jetre izazvanog CCl₄. Ovi rezultati su značajni zbog prisustva prirodnog ili sintetskog M u ljudskoj ishrani.

Ključne reči: ugljen-tetrahlorid, metil- i izopropil-N-metilantranilati, jetra, transaminaze