

INCREASE IN LACTATE DEHYDROGENASE ISOENZYME-4 AND SPLENOCYTE TOXICITY IN METHOMYL-TREATED RATS

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The toxic effect of methomyl was studied in rats after a single or repeated oral administration. Rats treated with a single dose of methomyl (3, 5, or 7 mg/kg) showed significant increase ($P < 0.05$) in total lactate dehydrogenase (LDH) activity on day 1. The highest level of LDH activity was observed on day 3 in rats receiving 7 mg/kg of methomyl. The total LDH activity returned to normal on day 7 after dosing. Specific increases in LDH-3 and LDH-4 isoenzyme activities were observed. In rats treated with a single dose of 6 and 8 mg/kg of methomyl, spleen weight and splenocyte viability significantly dropped ($P < 0.05$) on days 1 and 3, respectively. Splenotoxicity was prevented by pretreatment with 60 mg/kg of N-acetylcysteine. The results suggest that the splenotoxic effect of methomyl is more likely directly related to oxidative cell injury than to cholinesterase inhibition. The significance of cytotoxic effects and the nature of cytotoxicity in relation to reactive oxidative damage deserve further investigation.

Key words: carbamates, cholinesterases, insecticides, splenotoxicity

Methomyl is a monomethylcarbamate insecticide, S-methyl N-(methylcarbamoyloxy)thioacetimidate, used on a wide range of crops worldwide. Although the recognised mode of action of methomyl is inhibition of acetylcholinesterase (AChE), similarly to other carbamate and organophosphorus insecticides, AChE inhibition by methomyl is reversed more rapidly. *Saiyed and co-workers* (1) reported that the total lactate dehydrogenase (LDH) activity significantly increased in spraymen and was accompanied by electrocardiogram changes after a 5-day use of methomyl in the field. *Gupta and co-workers* (2) indicated that the total LDH activity and the activities of all LDH isoenzymes significantly increased at various time intervals in the diaphragm and serum of rats after a single administration of 5 mg/kg of methomyl. *Gupta and co-workers* (3) reported that carbofuran, another monomethylcarbamate, produced characteristic alterations in creatine kinase (CK), LDH, and their isoenzyme patterns in brain regions, as well as in serum of rats given 1.5 mg/kg of methomyl subcutaneously. Spleen is a well-known target organ of methomyl toxicity (4).

This study was undertaken to assess the effects of methomyl on LDH activity and splenotoxicity in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (102.4 ± 6.3 g) were obtained from the National Laboratory Animal Center of the Salaya Campus, Mahidol University. The rats were housed three per cage in an air-conditioned room (temperature: 23 ± 2 °C) for at least a week before the experiment. The animals were supplied standard pelleted feed for rats and mice (C.P. Ltd., Thailand) and tap water *ad libitum*.

Chemicals

Methomyl of a purity above 98% was donated by DuPont Ltd, Thailand. The chemicals for the LDH assay were purchased from Helena Laboratories, USA. The SGPT Kit was purchased from Clinical Diagnostic Co., Thailand. All other chemicals were reagent-grade and were purchased from Sigma Chemical Co., USA.

Treatment protocols

Single dose administration of methomyl. After acclimatisation, 96 rats were divided in four groups; the control and those administered 3, 5, and 7 mg/kg of methomyl. Methomyl solution diluted in distilled water was given orally by gavage (0.1 ml). On days 1, 3, 5, and 7 after dosing, six rats from each group were anaesthetised for blood and the organ collection. The organs for histopathological examination included the liver, heart, spleen, and kidney.

Repeated dose administration of methomyl. Forty-eight rats were divided in two groups: the control group and the treatment group which was given 5 mg/kg of methomyl for five consecutive days by gavage. Sampling followed the same protocol as for the single dose.

Splenotoxicity study. Ninety-six rats were divided in four groups: the control group, the group treated with 6, and the group treated with 8 mg/kg of methomyl, and a group pretreated with 60 mg/kg of N-acetylcysteine (NAC) before administration of 8 mg/kg of methomyl. On days 1, 3, and 5 after dosing, eight rats from each group were anaesthetized for blood collection and spleen removal.

Determination of total LDH activity

Total LDH activity was determined according to the method of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (5). In short, pyruvate was reduced to lactate in the presence of LDH at pH 7.4 and 37 °C. The progress of the accompanying oxidation of NADH to NAD was monitored continuously by measuring the rate of absorption decrease at 339 nm in the spectrophotometer.

Determination of LDH isoenzyme activities

LDH isoenzymes were determined according to the method of Helena Laboratories (6). Each LDH isoenzyme was separated using the electrophoretic technique on cellulose acetate in tris-barbital-sodium barbital buffer. After the separation, the isoenzymes could be detected colourimetrically using the Helena LD-VIS isoenzyme reagent, when a tetrazolium salt was reduced with the formation of a coloured formazan.

Determination of splenotoxicity

Splenotoxicity in rats exposed to methomyl was investigated by comparing the spleen weight and the splenocyte viability of the treatment groups and the control group (7). Using the method of *Ellman and co-workers* (8), the respective erythrocyte and spleen AChE activities were determined to assess the effect of the methomyl-induced splenotoxicity on AChE.

Data analysis

Results are expressed as mean and S.E. for statistical comparison of total LDH activity, each LDH isoenzyme, and relative organ weight between the control and the methomyl-treated groups. The statistical analysis consisted of one-way or two-way ANOVA and *post hoc* Duncan's multiple range test. A probability level (P value) of less than 0.05 was regarded significant.

RESULTS

Clinical signs of methomyl toxicity

The severity of methomyl toxicity in rats, after a single oral administration of methomyl at doses of 3, 5, and 7 mg/kg, was dose-related. Like with other carbamates and organophosphorus insecticides, the acute toxicity was characterised by nicotinic and muscarinic signs which persisted from 30 minutes to one hour. The animals completely recovered within two hours after the methomyl administration. Methomyl caused no mortality in this study.

In the repeated treatment study, rats receiving methomyl in daily doses of 5 mg/kg for 5 days showed the same clinical signs of toxicity as observed in a single treatment study. However, not until the day 3 after dosing did the toxic signs start to decrease in severity.

Changes in the total LDH and LDH isoenzyme activities

The total LDH activity in the plasma of control rats ranged from 146–184 U/L (N=21) in the single-dose study to 130–177 U/L (N=24) in the repeated dose study (Tables 1 and 2). On day 1 after a single-dose administration, all treated groups (3, 5, and 7 mg/kg) showed a significant increase ($P < 0.05$) in the total LDH activity when compared to the control.

Table 1 Total lactate dehydrogenase activity at various time points after oral treatment of rats with various single doses of methomyl.

Time (after treatment)	Control	Methomyl 3 mg/kg	Methomyl 5 mg/kg	Methomyl 7 mg/kg
Day 1	161.4 ± 17.7	341.1 ± 8.9*	296.4 ± 10.7*	211.4 ± 37.1*
Day 3	146.4 ± 16.3	108.9 ± 11.8	242.9 ± 57.0	668.6 ± 527.8
Day 5	178.6 ± 27.4	137.1 ± 20.9	156.0 ± 22.2	137.2 ± 18.0
Day 7	184.3 ± 20.7	176.2 ± 6.8	148.6 ± 15.4	165.5 ± 30.2

Values are expressed as mean ± S.E. (N=3-6)

*Significantly different from the control values (P<0.05)

Table 2 Total lactate dehydrogenase activity at various time points after daily treatment with 5 mg/kg of methomyl for five days.

Time (after treatment)	Control	Methomyl
Day 1	130.0 ± 17.3	124.3 ± 25.6
Day 3	154.3 ± 9.6	140.0 ± 6.0
Day 5	177.2 ± 19.8	232.9 ± 99.2
Day 7	177.2 ± 11.4	204.3 ± 54.9

Values are expressed as mean ± S.E. (N=3-6)

Table 3 The number of rats with increased total lactate dehydrogenase (LDH) activity treated with a single or repeated doses of methomyl

Time (after treatment)	Levels of increased total LDH activity	Single dose treatment			Repeated 5-day treatment
		3 mg/kg	5 mg/kg	7 mg/kg	5 mg/kg/day
Day 1	1	1/4	2/4	2/4	1/6
	2	3/4	-	-	-
	3	-	-	-	-
	4	-	-	-	-
Day 3	1	-	-	-	-
	2	-	1/6	-	-
	3	-	1/6	-	-
	4	-	-	1/6*	-
Day 5	1	-	1/6	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	1/6**
Day 7	1	-	-	1/6	1/6
	2	-	-	-	-
	3	-	-	-	1/6
	4	-	-	-	-

*1 = total LDH > 1.5-2 times the normal values; 2 = total LDH > 2-2.5 times the normal values;

3 = total LDH > 2.5-3 times the normal values; 4 = total LDH > 3 times the normal values.

*The highest total LDH activity in rats treated with a single dose of methomyl of 2779 U/L.

**The highest total LDH activity in rats repeatedly treated with methomyl for five days of 664 U/L.

One out of six rats treated with the highest dose (7 mg/kg) of methomyl showed a great increase in the total LDH activity on day 3 (Table 3). In terms of LDH isoenzymes, rats receiving an oral dose of 7 mg/kg of methomyl showed a significant increase in LDH-3 and LDH-4 ($P < 0.05$) on day 3 (Table 4). However, after the repeated treatment, no significant difference was observed between the control and the treated animals.

Table 4 Lactate dehydrogenase (LDH) isoenzymes in the plasma of rats treated with an oral dose of methomyl (3, 5, and 7 mg/kg) at various time points after dosing

Time (after treatment)	Group	Total LDH activity (%)				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Day 1	C	13.8 ± 1.6	5.6 ± 3.2	5.4 ± 2.6	6.4 ± 2.1	68.9 ± 8.6
	M3	9.0 ± 2.0	1.9 ± 0.6	2.8 ± 1.6	5.2 ± 1.8	81.1 ± 3.7
	M5	6.8 ± 1.8	2.7 ± 1.1	5.1 ± 2.2	6.8 ± 3.1	78.7 ± 7.1
	M7	9.2 ± 2.3	1.2 ± 0.0	3.0 ± 1.6	6.3 ± 1.4	80.3 ± 5.1
Day 3	C	17.8 ± 2.8	3.5 ± 1.6	4.7 ± 1.9	5.2 ± 1.8	68.0 ± 5.6
	M3	21.6 ± 3.5	2.4 ± 0.8	1.2 ± 0.1	1.8 ± 0.2	73.3 ± 4.2
	M5	14.0 ± 2.6	2.3 ± 0.2	1.6 ± 0.7	4.6 ± 1.3	77.6 ± 1.6
	M7	8.8 ± 3.6	2.0 ± 0.7	10.0 ± 2.7*	14.7 ± 4.5*	63.0 ± 5.3
Day 5	C	12.2 ± 1.9	3.6 ± 1.5	5.3 ± 2.0	8.3 ± 2.5	70.9 ± 5.1
	M3	17.9 ± 2.7	3.1 ± 1.5	2.5 ± 0.8	4.2 ± 0.9	72.3 ± 3.5
	M5	14.8 ± 1.6	4.6 ± 1.7	3.9 ± 1.9	6.0 ± 1.5	70.7 ± 3.4
	M7	12.7 ± 0.1	2.4 ± 1.0	2.7 ± 1.0	5.5 ± 1.3	76.7 ± 3.0
Day 7	C	11.5 ± 2.1	3.8 ± 1.1	2.3 ± 1.3	2.8 ± 0.5	79.5 ± 3.1
	M3	11.3 ± 1.0	2.4 ± 0.7	2.0 ± 0.5	6.0 ± 1.4	78.4 ± 2.4
	M5	12.2 ± 1.9	3.2 ± 1.3	5.2 ± 2.0	6.8 ± 2.5	72.7 ± 4.0
	M7	12.2 ± 1.2	7.3 ± 4.1	1.6 ± 0.3	5.3 ± 0.4	73.5 ± 3.9

C=Control; M3=Methomyl 3 mg/kg; M5=Methomyl 5 mg/kg; M7=Methomyl 7 mg/kg.

Values are expressed as mean ± S.E. (N=3–6)

*Significantly different from the control values ($P < 0.05$).

Splenotoxicity and acetylcholinesterase activity

Rats given a single dose of methomyl (6 or 8 mg/kg) showed early signs of dose-related splenocyte toxicity. The protective effect against methomyl-induced splenotoxicity was observed in rats pretreated with 60 mg/kg of N-acetylcysteine (NAC). On day 1 after dosing, the absolute and the relative spleen weight and splenocyte viability in rats receiving 6 or 8 mg/kg of methomyl were significantly lower ($P < 0.05$) than in the control animals. No significant toxic effects were observed in NAC-pretreated rats (Table 5).

Table 5 *Acetylcholinesterase (AChE) activity, absolute and relative spleen weight, and splenocyte viability in rats treated with 6 and 8 mg/kg of methomyl, rats pretreated with 60 mg/kg of N-acetylcysteine followed by 8 mg/kg of methomyl, and the control groups at various time points after dosing*

Day(s) after treatment	Group	AChE activity (U/g Hgb)	Spleen weight		Splenocyte viability(%)
			(g)	(% of body weight)	
1	C	8.34 ± 1.77	0.995 ± 0.112	0.48 ± 0.05	84.8 ± 3.7
	M6	7.15 ± 1.18	0.904 ± 0.035*	0.45 ± 0.03	63.5 ± 9.5**
	M8	7.58 ± 1.73	0.877 ± 0.051*	0.44 ± 0.04	57.2 ± 10.8**
	NAC+M8	7.69 ± 1.62	1.004 ± 0.104	0.49 ± 0.04	81.5 ± 5.9
5	C	9.41 ± 3.45	1.043 ± 0.160	0.49 ± 0.06	80.9 ± 1.8
	M6	8.21 ± 1.55	0.917 ± 0.067	0.43 ± 0.03*	73.5 ± 7.0
	M8	8.57 ± 2.99	0.927 ± 0.047	0.44 ± 0.02	69.3 ± 4.9
	NAC+M8	9.64 ± 2.18	1.012 ± 0.062	0.48 ± 0.02	81.4 ± 2.3
7	C	8.01 ± 1.86	1.082 ± 0.176	0.45 ± 0.06	82.1 ± 4.5
	M6	7.60 ± 1.62	0.977 ± 0.135	0.42 ± 0.04	80.7 ± 1.7
	M8	7.47 ± 1.31	0.965 ± 0.118	0.43 ± 0.06	76.5 ± 7.9
	NAC+M8	8.20 ± 2.79	1.012 ± 0.041	0.44 ± 0.03	80.1 ± 3.2

C = control; M6 = methomyl 6 mg/kg; M8 = methomyl 8 mg/kg; NAC = N-acetylcysteine 60 mg/kg
Values are expressed as mean ± S.D. (N=4-8)

Significantly different from the control values: *P<0.05; **P<0.01 (Student's t-test).

DISCUSSION

Oral administration of even the lowest dose of methomyl (3 mg/kg) caused marked cholinergic signs within five minutes after dosing. However, these toxic effects were soon annulled by rapid metabolism and elimination of methomyl and spontaneous reactivation of acetylcholinesterase. After a repeated administration of methomyl, no cumulative toxicity or signs of toxicity were observed and the rats recovered before the following administration.

Regarding the changes in the total LDH activity and LDH isoenzymes, the increase in the total plasma LDH activity in rats which received a single dose of methomyl was observed on days 1 and 3 after administration. The enzyme activity returned to the normal level on days 5 and 7. The findings indicate that the effects of methomyl are reversible. The enzymatic recovery may be explained by the short half-life of LDH, the cessation of effects, and/or the rapid excretion of methomyl.

On day 3, acetylcholinesterase activity was not significantly reduced due to a rapid spontaneous reactivation of the carbamylated enzyme. However, the significant increase in LDH-3 and LDH-4 was noted on day 3 at the dose level of 7 mg/kg of body weight.

There is a limited number of studies that deal with the distribution of LDH isoenzymes in various organs of rats. Further studies should focus on the precise sources of LDH-4 and on the effects of methomyl on other organs. In rats, LDH-4

was predominant in the spleen (7) and pulmonary vascular endothelium (9). The spleen and/or vascular endothelial cells are possible targets of methomyl toxicity.

There were slight changes in the liver and spleen of rats treated with methomyl. These alterations may involve injury which also reflects in significant changes in LDH-3, LDH-4, and in the decrease in relative weight of rat liver. However, there were no differences from the controls. Furthermore, there was no change in the transaminase (SGPT) activity in any of the groups, which suggests that methomyl may not be involved in the hepatic cell injury (unpublished data).

Rats orally treated with a single dose of 6 and 8 mg/kg of methomyl manifested a decrease in both spleen cell viability and spleen weight. There is increasing evidence that the oxidative stress and free radical damage in the cellular components is a consequence of toxic exposure. Reactive oxidative damage appears to participate in either genotoxic or non-genotoxic mechanisms such as down regulation of cell to cell communication, increased proliferation, or decreased apoptosis. Busey (10) observed glutathione reduction in human lymphocyte exposed to methomyl *in vitro*, as well as an increase in micronuclei and chromosomal aberrations. Methomyl induced chromosomal aberrations in the mouse spleen cell within 24 hours after intraperitoneal injection of 1 mg/kg of methomyl (11).

Our later and yet unpublished experiments suggest that methomyl appears to produce such chromosomal effects through cytoskeleton impairment.

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Sažetak

AKTIVNOSTI IZOENZIMA LAKTAT DEHIDROGENAZE I UČINCI NA SLEZENU ŠTAKORA TRETIRANIH METOMILOM

Istraživani su učinci karbamatnog insekticida metomila u štakora. Nakon jednokratne peroralne doze od 3, 5 ili 7 mg/kg nađeno je značajno povećanje sveukupne aktivnosti laktat dehidrogenaze (LDH) nakon prvog dana tretmana. Najveća je aktivnost utvrđena trećeg dana nakon tretmana metomilom u dozi od 7 mg/kg. Enzimska aktivnost postepeno se smanjivala i sedmoga se dana normalizirala. Posebno je zapaženo povećanje aktivnosti izoenzima LDH-3 i LDH-4.

U posebnim je pokusima utvrđeno značajno smanjenje težine slezene i vitalnosti splencita u štakora tretiranih sa 6 i 8 mg/kg metomila i to nakon prvog i nakon trećeg dana tretmana. Toksični učinci na slezenu mogli su se spriječiti pretretiranjem sa 60 mg/kg N-acetilcisteina. Prema rezultatima ovih pokusa čini se da bi toksični učinak metomila na slezenu mogao biti izravan učinak metomila na stanice slezene a ne putem kolinergičnog mehanizma. Važnost ovakvog citotoksičnog učinka i mehanizme citotoksičnosti i njihove povezanosti s reaktivnim oksidativnim procesima pri staničnom oštećenju valja još istraživati.

Ključne riječi:
insekticidi, karbamati, kolinesteraza, slezena

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