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รายงานวิจัยฉบับสมบูรณ์

Final Report

"ผลของเมโธมิลต่อม้ามและการส่งสัญญาณภายในเซลล์เม็ดเลือดขาว"

Effects of Methomyl on Spleens and Cell Signaling in Leukocytic Cells

โดย นางสาวทิพิชา โปษยานนท์

Ms. Tipicha Posayanonda

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ผู้วิจัย นางสาวทิพิชา โปษยานนท์

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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ชื่อโครงการ:	ผลของเมโธมิลต่อม้ามและการส่งสัญญาณภายในเซลล์เม็ดเลือดขาว
ผู้วิจัย:	นางสาวทิพิชา โปษยานนท์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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เมโธมิล (methomyl) เป็นสารฆ่าแมลงในกลุ่มคาร์บาเมตที่มีการใช้กันอย่างแพร่หลายในประเทศไทยและประเทศเกษตรกรรมอื่น ๆ ทั่วโลก ความเป็นพิษจากสารฆ่าแมลงกลุ่มคาร์บาเมตนั้นเป็นที่ทราบโดยทั่วไปว่าเกิดจากการยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรส (acetylcholinesterase) อย่างไรก็ตาม จากการศึกษาวิจัยที่ผ่านมาของ Suramana et al. (2001) พบว่าเมโธมิลสามารถก่อให้เกิดความเป็นพิษด้านอื่นได้ด้วย เนื่องจากการศึกษาดังกล่าวพบว่าเมโธมิลสามารถก่อให้เกิดความผิดปกติที่ม้ามของหนูทดลองเมื่อตรวจสอบโดยใช้เครื่องมือ Fourier Transform Infrared Spectroscopy (FTIR) ดังนั้น งานวิจัยชิ้นนี้ จึงมีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ของกลไกการเกิดพิษอื่นๆ จากเมโธมิล โดยมุ่งเน้นไปที่การก่อให้เกิดพิษต่อม้ามและเซลล์เม็ดเลือดขาว โดยจากผลการศึกษาพบว่า เมื่อนำชิ้นส่วนของม้ามที่แยกออกมาจากหนูทดลองหลังจากให้เมโธมิลแบบให้ครั้งเดียวทางปากขนาด 8 มก./กก. ไปแล้วเป็นเวลา 24 ชั่วโมง มาศึกษาโดยใช้กล้องจุลทรรศอิเล็คตรอนแบบแสงผ่าน พบว่ามีการบวมของไมโตคอนเดรียเกิดขึ้นและมีการเปลี่ยนแปลงของไมโตคอนเดรียไปในทางเสื่อม โดยพบว่ามีการหายไปของคริสตีที่ผนังไมโตคอนเดรีย และยังพบเซลล์ตายจากการอ่านชิ้นเนื้อภายใต้กล้องจุลทรรศน์ธรรมดาที่เมโธมิลขนาดเดียวกันอีกด้วย ซึ่งจากการทดลองเพื่อศึกษาถึงผลกระทบของเมโธมิลต่อเมตาบอลิสมของพลังงานที่เกี่ยวข้องกับการทำหน้าที่ของไมโตคอนเดรียนั้นพบว่ามีการเพิ่มขึ้นอย่างมีนัยสำคัญของระดับ 2,3-DPG ในเลือดหนูที่ได้รับเมโธมิลทางปากขนาด 8 มก./กก. แบบให้ครั้งเดียวไปแล้วเป็นเวลา 24 ชั่วโมง อีกทั้งยังมีการเพิ่มขึ้นของการทำงานของเอนไซม์ NADH-DCIP reductase ในเม็ดเลือดแดงแบบมีความสัมพันธ์กันระหว่างขนาดและการตอบสนอง นอกจากนี้ เมื่อศึกษาในส่วนของการนำส่งสัญญาณภายในเซลล์เม็ดเลือดขาวเพาะเลี้ยงที่เกิดอะพอพโตซิส (apoptosis) จากการได้รับเมโธมิลแล้ว พบว่าอะพอพโตซิสที่เกิดขึ้นดังกล่าวนั้นบางส่วนสามารถถูกยับยั้งได้โดยสารอินเตอร์ลิวคิน-6 (Interleukin-6, IL-6)

ดังนั้น ผู้วิจัยจึงสรุปได้ว่า นอกจากจะมีผลโดยตรงกับการยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรสอย่างที่ทราบโดยทั่วไปแล้ว เมโธมิลยังสามารถก่อให้เกิดการตายของเซลล์เม็ดเลือดขาวได้อีกด้วย โดยคาดว่ามีกลไกมาจากการรบกวนที่การนำส่งสัญญาณที่เกี่ยวข้องกับ IL-6 และในขณะเดียวกันยังก่อให้เกิดผลกระทบหรือความผิดปกติต่อม้ามโดยการทำให้เกิดการเปลี่ยนแปลงของไมโตคอนเดรียไปในทางเสื่อม ซึ่งผลกระทบต่างๆ เหล่านี้จะส่งผลให้เกิดการรบกวนต่อเมตาบอลิสมของพลังงาน และมีแนวโน้มที่จะก่อให้เกิดผลกระทบต่อเซลล์และระบบต่างๆ ในร่างกายมนุษย์ได้ โดยเฉพาะเซลล์ที่เกี่ยวข้องกับระบบภูมิคุ้มกันของร่างกาย

ผู้วิจัยเสนอว่าควรมีการศึกษาเพิ่มเติมทางระบาดวิทยาอย่างเป็นระบบในส่วนของเกษตรกรหรือผู้ทำงานที่ได้รับสัมผัสกับเมโธมิลหรือสารฆ่าแมลงกลุ่มคาร์บาเมตเป็นประจำอย่างต่อเนื่อง โดยเฉพาะการตรวจวัดระดับเม็ดเลือดและความผิดปกติในเรื่องของระบบภูมิคุ้มกันซึ่งยังไม่เคยมีการศึกษาอย่างเป็นระบบในขณะนี้

คำหลัก: เมโธมิล, ม้าม, เม็ดเลือดขาว, การส่งสัญญาณ

Abstract

Project Code:	BGJ/27/2544
Project Title:	Effects of Methomyl on Spleens and Cell Signaling in Leukocytic Cells
Investigator:	Ms. Tipicha Posayanonda, Faculty of Pharmaceutical Sciences, Chulalongkorn University
E-mail Address:	noopnob@hotmail.com
Project Period:	September 2001 – June 2002

Methomyl is a methyl carbamate insecticide widely used in Thailand and many agricultural countries for crop protection. Toxicity of carbamate insecticides is commonly known as acetlcholinesterase inhibition. However, Suramana et al. (2001) reported another toxic effect of methomyl that could be generated by other mechanisms as spleen cell abnormality was detected by Fourier Transform Infrared Spectroscopy (FTIR). Therefore, this research was aimed to study other possible mechanisms of methomyl induced toxicity focusing on spleen and leukocytic cells. From this study, transmission electron microscopic sections of spleens collected from rats after the 24 hour-oral exposure to single dose of methomyl at 8 mg/kg illustrated the mitochondrial swelling and degenerative changes with cristae loss. Cell death was also shown under light microscope at the same dose of methomyl. Energy metabolism related to mitochondrial function was further investigated, and the results showed a significant increase in level of 2,3-DPG in rat blood at twenty-fourth hour after an oral exposure to a single dose of methomyl at 8 mg/kg. An increase in red blood cell NADH-DCIP reductase activity in a dose-response relationship manner was also reported. A further study on cell-signaling pathways of leukocytic cell lines undergone apoptosis by methomyl exposure found that interleukin-6 (IL-6) could partly prevent methomyl-induced apoptosis in these cell lines.

Therefore, it is concluded that in addition to its direct effect on acetylcholinesterase, methomyl can also cause leukocytic cell death through a mechanism related to an interference in IL-6-related signaling pathways. At the same time, it is suggested that methomyl could lead to abnormality of spleens by causing mitochondrial degeneration. These observations propose that energy metabolism and cells in different systems of human body, especially those in the immune system, tend to be affected or interfered by methomyl exposure.

It is recommended that systematic epidemiological studies among farmers or workers continually exposed to methomyl or other carbamate insecticides specifically focused on blood cells and abnormalities of immune system are needed for further investigation.

Keywords: Methomyl, Spleen, Leukocyte, Signaling

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Chapter 1
Introduction

Background

Methyl carbamates are insecticides extensively used worldwide because they are considered to be relatively safe and non-persistent. Methomyl is classified as a methyl carbamate, which is widely used in Thailand and many agricultural countries for crop protection.

Methomyl is an acetylcholinesterase inhibitor, and its reported toxicity is mostly related to cholinergic symptoms. However, some of its toxicity may not be related to its action on acetylcholinesterase.

Some studies reported that cardiotoxicity generated by methylcarbamates is not totally mediated through their action on acetylcholinesterase enzyme. Futagawa et al. (2000) reported that the developed depressor response, leading to a decrease in cardiac contractility and/ or vascular resistance, observed in methylcarbamates-exposed rabbits was the direct effect of methylcarbamates on cardiac and vascular smooth muscle contraction.

Klotz, Arnold, and McLachlan (1997) reported that methomyl, when given alone, had a weak activity on estrogen and progeterone to activate both estrogen and progesterone-responsive reporter genes in breast and endometrial cells. However, when given together with either estrogen or progesterone, methomyl inhibited activities of these hormone by acting through pathways independent of competition for receptor binding. This study suggested methomyl as a general endocrine modulator in mammalian cells.

In addition, some *in vivo* studies showed that methomyl could generate oxidative stress. Lohitnavy and Sinhaseni (1998) reported that rats treated with methomyl showed a significant decrease in the spleen cell viability. The methomyl-induced splenotoxicity was protected by pretreating rats with N-acetylcysteine (NAC), a free radical scavenger which promotes glutathione antioxidant capacity. Therefore, the study suggested that methomyl might generate oxidative stress, which could consequently contribute to the splenotoxicity.

The possibility of oxidative damage by methomyl was also reported by Rannug and Rannug (1984) that enzyme involved in the defense against harmful reactive oxygen species (ROSs) such as superoxide dismutase, catalase, and glutathione transferase are inhibited by carbamate pesticides. This is supported by a metabolite of methomyl, which gets through glutathione conjugation, causes glutathione depletion, therefore can generate oxidative stress in biological systems (International Programme on Chemical Safety [IPCS], 1996).

Reactive oxygen species (ROSs) have long been known to be potent mediators of apoptosis in various animal and plant systems. They participate in signaling pathways during the induction phase of apoptosis and are one of several apoptogenic consequences of mitochondrial permeability transition (PT) pore, which is a critical part of apoptosis (Jabs, 1999).

Apoptosis is a major form of cell death that is used to remove excess, damaged or infected cells throughout life. It is important in normal cell turnover, the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and also in chemical-induced cell death (Arends and Wyllie 1991; Ellis, Yuan, and Horvitz, 1991; Cohen et al., 1992). Loss of control of the apoptotic programme contributes to many diseases (e.g. cancers, and autoimmune diseases) (Normal and Lodwick, 1999; Bratton and Cohen, 2001).

Effects of methomyl on apoptosis modification in plant have been reported. Male sterile maize expressing high levels of URF13 protein in its inner mitochondrial membrane was shown susceptible to methomyl. The *in vitro* studies showed that the interaction of methomyl with this protein caused pore generation in inner mitochondrial membrane, rapid swelling of the mitochondria, stimulation of NADH oxidation, inhibition of malate-driven respiration, uncoupling of oxidative phosphorylation, and the leakage of small molecules and ions (Chaumont et al., 1995; Rhoads et al., 1994).

Because of its direct effects on mitochondrial modifying apoptosis in plant and its *in vivo* reported oxidative stress in spleen cells, other mechanisms of toxicity generated by methomyl is investigated in this study focusing on the system related to immune response as the immune suppression from pesticides can be particularly significant in many developing countries where infectious diseases are a major cause of deaths.

Objectives

To investigate effects of methomyl on spleen and signaling in leukocytic cells in order to support the explanation of its mechanisms of toxicity.

Contributions of the Study

Better understanding of the toxicity of methomyl which may not be directly related to acetylcholinesterase inhibition.

Materials and Methods

In vivo

Male Wistar rats (obtained from the National Laboratory Animal Center of Salaya Campus, Mahidol University, each weighed 100-120 g) were divided into different groups (n=6) and treated with the following conditions for 6 and 24 hours: 1) Control; 2) Methomyl 4 mg/kg body weight (single dose, p.o.); and 3) Methomyl 8 mg/kg body weight (single dose, p.o.). After 6 hours and 24 hours, rat spleens were collected and prepared for viewing in the transmission electron microscope with accelerating voltage 100 KV using a method of the Scientific and Technological Research Equipment Center, Chulalongkorn University. After 24 hours, rat blood from the similar *in vivo* treatments was collected for determination of 2,3-DPG levels by measuring the decrease in absorbance at 340 nm (Sigma Diagnostics, Inc., 2000). Since the decrease is caused by the oxidation of NADH to NAD, it could reflect the amount of 2,3-DPG originally present in blood.

Same treatments of control and methomyl on 3 groups of Male Wistar rats were conducted. After 6 hours, blood was collected (with 1.5 mg/ml EDTA as an anti-clotting agent) for determination of NADH-DCIP reductase activity by measuring the absorbance change at 600 nm over 5 minutes (adapted from Zurbriggen and Dreyer, 1996). The decrease in absorbance overtime indicated an increase in NADH-DCIP reductase activity. The difference between the absorbance at 5 minutes and 0 minutes in the treated group was compared to that of control. Result was shown as x-fold increase.

In vitro

Role of interleukin-6 (IL-6) in methomyl induced apoptosis was determined by incubation of MonoMac 6 (MM6), THP-1, and Jurkat cells (all were provided by the MRC Toxicology Unit, University of Leicester, U.K.) with IL-6 (50 ng/ml) and methomyl (0, 6, 12, 18, 24, and 30 mM) for 6 hours prior to flow cytometric analysis with tetramethylrhodamine ethyl ester (TMRE) (Piqué et al., 2000).

Data Analysis

- The 2,3-DPG levels of treated and control groups were compared by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant.

- The increase in NADH-DCIP reductase activities of the treated groups (expressed as numbers of fold-increased) was compared with controls by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant.

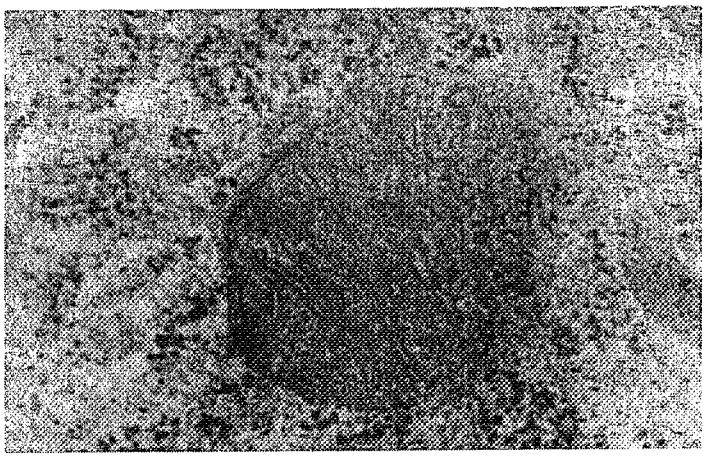
- Flow cytometric results were expressed as the mean percentage of apoptotic cell death of treated cells $\pm$ S.E. compared to that of the control by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant. The same statistical analysis was used in the comparison of percentage of cells with the reduced $\Delta\Psi m$ and of percentage of cells in the sub-G_1 area between the control and treated groups.

- Percentage of cell death at the same dose of methomyl exposure between cells treated and untreated with IL-6 was compared by using the unpaired student t-test, p-value of less than 0.05 was regarded as significant.

Results

Effect of Methomyl on Spleens

(A)



(B)

(C)

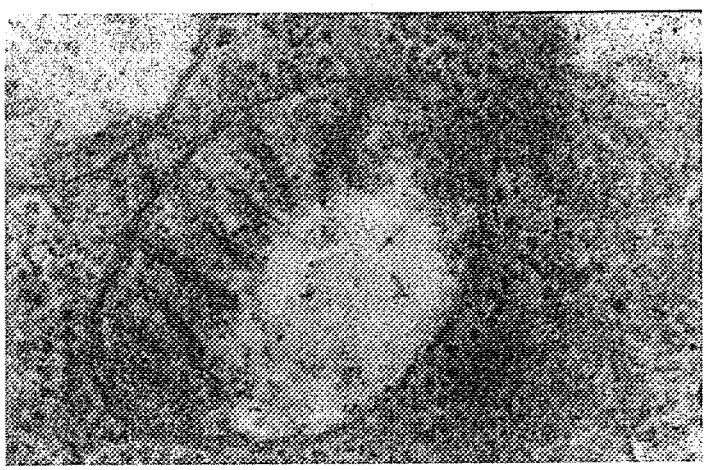


Figure 1 Electron microscopy of spleen cells collected from rats exposed to methomyl. (A) Control; (B) After 6 hours of methomyl treatment at 8 mg/kg body weight (oral, single dose); and (C) After 24 hours of methomyl treatment at 8 mg/kg body weight (oral, single dose). The arrows show mitochondria with cristae loss after methomyl exposure.

Effect of Methomyl on 2,3-DPG Levels in Blood

Table 1 Levels of 2,3-DPG in blood collected from rat after single dose oral-treatments of methomyl for 24 hours. (* $p<0.05$, compared to control)

Methomyl Treatment (mg/kg body weight) (n=6)	Blood Levels of 2,3-DPG (Mean ± S.E.)
Methomyl 0 mg/kg (Control)	3.18 ± 0.321
Methomyl 4 mg/kg	3.46 ± 0.236
Methomyl 8 mg/kg	3.70 ± 0.224*

A significant increase in the level of 2,3-DPG in rat blood at twenty-fourth hour after an oral exposure to a single dose of methomyl at 8 mg/kg.

Effect of Methomyl on Red Blood Cell NADH-DCIP Reductase Activity

Table 2 NADH-DCIP reductase activities in red blood cells collected from rat after single dose oral-treatments of methomyl for 6 hours. (* $p<0.05$, compared to control)

Methomyl Treatment (mg/kg body weight) (n=6)	Number of folds increased in red blood cell NADH-DCIP reductase activities compared to control (Mean ± S.E.)
Methomyl 0 mg/kg (Control)	1.00 ± 0.000
Methomyl 4 mg/kg	1.151 ± 0.124*
Methomyl 8 mg/kg	1.181 ± 0.175*

An increase in red blood cell NADH-DCIP reductase activity from methomyl in a dose-response relationship manner.

Effect of IL-6 on Methomyl-Induced Cell Death

Interleukin-6 (IL-6) could partly prevent methomyl-induced apoptosis in MM6, THP-1 and Jurkat cells as shown in Tables 3-5.

Table 3 Effects of 50 nM IL-6 on the reduction of mitochondrial transmembrane potential in MM6 cells detected by TMRE after methomyl exposure for 6 hours.
(* p<0.05, comparison between IL-6-treated- and -untreated-group at the same dose of methomyl)

Treatments	% Cells with $\downarrow\Delta\Psi$ (Mean ± S.E.)
Methomyl 0 mM (Control)	12.48 ± 3.828
Control + IL-6 50 ng/ml	10.94 ± 2.46
Methomyl (M) 6 mM	11.6 ± 3.517
M 6 mM + IL-6 50 ng/ml	10.18 ± 1.785
M 12 mM	11.23 ± 3.659
M 12 mM + IL-6 50 ng/ml	10.68 ± 4.235
M 18 mM	48.04 ± 7.061
M 18 mM + IL-6 50 ng/ml	47.07 ± 13.18
M 24 mM	84.03 ± 2.035
M 24 mM + IL-6 50 ng/ml	77.19 ± 3.925 *
M 30 mM	96.33 ± 1.233
M 30 mM + IL-6 50 ng/ml	89.45 ± 3.029 *

Table 4 Effects of 50 nM IL-6 on the reduction of mitochondrial transmembrane potential in THP-1 cells detected by TMRE after methomyl exposure for 6 hours.
(* p<0.05, comparison between IL-6-treated- and -untreated-group at the same dose of methomyl)

Treatments	% Cells with $\downarrow\Delta\Psi$ (Mean ± S.E.)
Methomyl 0 mM (Control)	14.65 ± 5.82
Control + IL-6 50 ng/ml	9.6 ± 1.039
Methomyl (M) 6 mM	15.23 ± 6.234
M 6 mM + IL-6 50 ng/ml	9.433 ± 3.128
M 12 mM	27.28 ± 5.562
M 12 mM + IL-6 50 ng/ml	25.68 ± 6.087
M 18 mM	44.36 ± 4.401
M 18 mM + IL-6 50 ng/ml	32.26 ± 4.907
M 24 mM	74.34 ± 10.33
M 24 mM + IL-6 50 ng/ml	66.45 ± 7.092
M 30 mM	97.61 ± 9.576
M 30 mM + IL-6 50 ng/ml	79.14 ± 8.761 *

Table 5 Effects of 50 nM IL-6 on the reduction of mitochondrial transmembrane potential in Jurkat cells detected by TMRE after methomyl exposure for 6 hours. (* $p<0.05$, comparison between IL-6-treated- and -untreated-group at the same dose of methomyl)

Treatments	% Cells with $\downarrow\Delta\Psi$ (Mean ± S.E.)
Methomyl 0 mM (Control)	14.08 ± 3.554
Control + IL-6 50 ng/ml	14.64 ± 3.319
Methomyl (M) 6 mM	18.01 ± 4.185
M 6 mM + IL-6 50 ng/ml	15.56 ± 2.869
M 12 mM	31.23 ± 3.659
M 12 mM + IL-6 50 ng/ml	28.68 ± 4.235
M 18 mM	44.36 ± 4.401
M 18 mM + IL-6 50 ng/ml	32.26 ± 4.907*
M 24 mM	72.58 ± 3.104
M 24 mM + IL-6 50 ng/ml	64.06 ± 3.746*
M 30 mM	94.03 ± 2.134
M 30 mM + IL-6 50 ng/ml	77.61 ± 4.710 *

Discussion and Conclusion

The *in vivo* results showed under the light microscope that spleens collected from rats after 24 hour-oral exposure to a single dose of methomyl at 8 mg/kg body weight revealed cell death. At the same dose of methomyl, the transmission electron microscope illustrated the the mitochondrial swelling and degenerative changes with cristae loss. These effects seen could be caused by the collapse of $\Delta\Psi$ and PT pore opening, a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, the matrix space expansion, swelling, and rupture of the outer mitochondrial membrane (Green and Reed, 1998; Bratton and Cohen, 2001). Therefore, it was possible that methomyl could interfere with mitochondrial function leading to an interference with energy metabolism and consequently apoptotic cell death. This mitochondrial effect was substantiated by a further investigation showing the increasing levels of 2,3-DPG in blood, which are an indicator of the amount of oxidized NADH; and the increase in red blood cell NADH-DCIP reductase activity, which indicated methemoglobinemia and oxidative stress. These indicators refer to changes in energy metabolism and oxidative stress related to mitochondrial function.

The *in vitro* results reported that methomyl could reduce mitochondrial transmembrane potential ($\Delta\Psi$) and induce apoptosis in MM6, THP-1, and Jurkat cell lines at different concentrations. The mechanisms as to how methomyl reduces $\Delta\Psi$ leading to apoptosis are not known. There are many possibilities:

(i) ROSs generation: Methomyl can deplete an important free radical scavenger, glutathione (IPCS, 1996), therefore ROSs are generated. It is well-established that ROSs have two roles in the apoptotic process, the inducers of PT pore and the consequences of PT pore (Jabs, 1999).

(ii) Genotoxicity: The capability of methomyl to cause damages in DNA via ROSs was shown in a previous study (Bonatti et al., 1994). The p53 protein is a key sensor of DNA damage. Once the p53 is activated, it induces cell-cycle inhibition and apoptosis (Bratton and Cohen, 2001)

(iii) Role of methomyl as a sulfhydryl group-binding agent may explain the observed effects on mitochondria and apoptosis. The modification of a critical cysteine residue at adenine nucleotide translocator (ANT), an important protein that controls the opening of PT pore, by sulfhydryl reacting agents can lead to the $\Delta\Psi$ collapse and apoptosis (Belzacq et al., 2001; Moreno et al., 2001). Therefore, ANT modification can be affected by methomyl and may be the cause of apoptosis seen.

(iv) From the studies in male sterile maize, methomyl may interfere with the activity of mitochondrial aldehyde dehydrogenase (mtALDH) enzyme, whose activity is required to inhibit mitochondrial PT pore generation (Irwin et al., 2002). In addition, the enzyme is important in the control of NADH, which is the key to cell death protection, within the cells. The interference with the enzyme will cause the NADH depletion and cell death. Therefore, it is possible that methomyl might induce PT pore generation and cell death by the mechanism concerned with energy metabolism.

The result also demonstrated that apoptosis in the leukocytic cell lines induced by methomyl was related to interleukin-6 (IL-6) since IL-6 could partly block the apoptosis observed.

There are various pathways involved in the IL-6-regulation of cell growth, survival, and differentiation: JAK/STAT pathway; Ras/MAPK; Src family tyrosine kinases; and phosphatidylinositol-3-kinase (PI-3 kinase) pathway. However, how IL-6 is related to the apoptosis seen is still unclear, and should be clarified. In addition further studies of possible effects of methomyl on immunological system and its inter-relation to toxicity or abnormality in spleens observed *in vivo* should be explored.

Recommendations for Future Studies

It is recommended that following studies should be further investigated:

1. How spleen abnormality detected from methomyl exposure is related to effects of methomyl on mitochondrial degeneration and on apopototic signaling of leukocytes as well as the immunological system.
2. Systematic epidemiological studies specifically focused on blood cells and abnormalities of immune system of farmers or workers who continually exposed to methomyl or other carbamate insecticides.

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Outputs

Pulbications and Presentations at International Level

1. **Posayanonda, T.**, Suramana, T., Nuntharatanapong, N., Schwaeble, W., Snowden, R., Dusitsin, N., Sindhuphak, R., and Sinhaseni, P. 2004. Effects of methomyl on cell cycle and apopotosis in leukocytic cells; cited in Cholinergic Mechanisms: Function and dysfunction. Silman, I., Soreq, H., and Anglister, L. Great Britain. pp. 616.

2. **Posayanonda, T.**, Suramana, T., Nuntharatanapong, N., Lohitnavy, O., Snowden, R., Schwaeble, W., Dusitsin, N., Sindhuphak, R. and Sinhaseni, P. 2002. Effects of methomyl on spleen and apoptosis. XIst International Symposium on Cholinergic Mechanisms-Functions and Dysfunctions and 2nd Misrahi Symposium on Neurobiology, St. Moritz, Switzerland, May 5-9, 2002.

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Appendix

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122. Effects of methomyl on cell cycle and apoptosis in leukocytic cells

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Methomyl is a methyl carbamate insecticide widely used in Thailand and many agricultural countries for crop protection. The effects of methomyl on modifying apoptosis in plant have been reported to induce a 0.8–1.5 nm sized mitochondrial pore generation and cell death in male sterile maize expressing high levels of URF13 protein in its inner mitochondrial membrane.[1,2]

A study by Klotz et al.[3] suggested methomyl as an endocrine modulator. In addition, its cardiotoxicity from the developed depressor responses may not be related to the anticholinesterase action.[4,5] Lohitnavy and Sinhaseni[6] suggested that methomyl could generate oxidative stress, as shown by the blocking effect of N-acetylcysteine on methomyl-induced cell death in spleen cells. This oxidative damage induced by methomyl is in agreement with oxidative stress generated by glutathione depletion from a metabolite of methomyl in the biological systems.[7]

In this study, rats orally exposed to methomyl at 8 mg kg^{-1} body weight for 6 h showed lymphocyte apoptosis. The apoptosis induced by methomyl was also shown with different sensitivities in leukocytic cell lines. In these cell lines, methomyl reduced mitochondrial transmembrane potential ($\Delta\Psi m$) and induced apoptosis in MM6, THP-1, and Jurkat cells at different concentrations of 18, 12, and 12 mM, respectively, after 6-h exposure; and 12, 6, and 6 mM, respectively, after 24-h exposure. Methomyl was not shown to induce nuclear apoptosis in Raji cells since DNA fragmentation was not detected, instead, the Raji cells showed a cell cycle arrest in the G_0/G_1 phase.

The apoptosis that occurred could be blocked by zVAD-fmk, which indicated a caspase-dependent process. In addition, IL-6 could partly prevent methomyl-induced apoptosis in these cell lines, suggesting that the apoptosis was partially related to either the reduction of IL-6 or the interference in IL-6-related signaling pathways.

Transmission electron microscopic sections of spleens collected from rats after 24-h oral exposure to a single dose of methomyl at 8 mg kg^{-1} illustrated mitochondrial swelling and degenerative changes with loss of cristae. Cell death was shown under light microscopy at the same dose of methomyl. Energy metabolism related to mitochondrial function was investigated. The results showed that the increasing levels of 2,3-DPG in blood and the increase in red blood cell NADH-DCIP reductase activity were affected in a dose-response relationship.

It is concluded that methomyl affects mitochondrial function via interference with energy metabolism. It can induce apoptosis at high dose treatments both in vitro and in vivo. In addition, its effects on spleen show mitochondrial degeneration and cell death. Mechanisms of these effects are postulated as a possible relationship to the reduction of IL-6 or the interference with IL-6-related signaling pathways. These changes may suggest further investigation into possible modulatory effects of methomyl on IL-6-related signals in the immune response.

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