Inhibitory Effect of Carbamate on the Cell Division of Rat Bone Marrow

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Inhibitory Effect of Carbamate on the Cell Division of Rat Bone Marrow

A thesis submitted to the Faculty of Science of the United Arab Emirates University in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Science

by

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ABSTRACT

The cytotoxic effect of methomyl carbamate was studied in three groups (control group, 1% treated group and 10% treated group) and each group contain 2-3 White Wistar rats. The 1% treated group was received 1% of methomyl LD$_{50}$, while the 10% treated group was received 10% of methomyl LD$_{50}$. The administration was orally and repeated three times with 24 hr intervals time. The effect of methomyl was investigated in four experimental run, by analysing the change in cell count in bone marrow after methomyl treatment. It was found that methomyl treatment caused significant reduction in cell number in rat bone marrow indicating that methomyl inhibits cell division. The molecular mechanism of inhibition of cell division was investigated. Analysis of DNA in treated and control animals showed that DNA synthesis is not inhibited upon methomyl treatment. Further investigation of gene products of Ras growth signaling pathway showed that methomyl treatment inhibited the expression of genes that are considered as primary response element (eg. C-Fos) of activation of Ras growth signal transduction pathway. These results indicate that methomyl treatment inhibits cell division by inhibiting, at least partly, Ras-mediated growth signaling pathway. In addition, cytotoxic effect of carbamate on rat was studied by analyzing the expression of two key molecules, cytochrome P-450 and GST proteins which are considered as markers of toxic effect. It was found that cytochrome P-450 gene expression was induced significantly after methomyl treatment while GST level did not change significantly. This suggests that methomyl is a mild toxic agent for mammals and cytochrome P-450 is involved in its metabolism. It might be advisable to use methomyl as insecticide under special precautions.
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ABBREVIATIONS

DNA, Deoxyribonucleic acid
LD\textsubscript{50}, Lethal dose for 50\% of the group
IL, Interleukine
TH, T helper cell
NADPH, Nicotinamide adenine dinucleotide phosphate (reduced form)
GDP, Guanosine diphosphate
NCF, N-Nitroso derivative of carbofuran
PDGF, Platelet-derived growth factor
RTK, Receptor tyrosine kinase
GSH, Reduced glutathione
TBS, Tris buffer saline
PBS, Phosphate buffer saline
APS, Ammonium per sulphate
TCA, Trichloro acetic acid
TEMED, N-N-Tetra methyl ethylene diamine
NCM, Nitro cellulose membrane
\(\mu\)g, Microgram
Krpm, Kilo rotation per minute
UV, Ultra violet
RNA, Ribonucleic acid
CA, Chromosomal aberration
INF, Interferon
NK, Natural killer cell
IP3, Inositol triphosphate
GTP, Guanosine triphosphate
OH\textsuperscript{d}dG, 8-Hydroxy guanosine
NO\textsuperscript{−}, Nitroso derivative
EGF, Epidermal growth factor
GST, Glutathione-S\textsuperscript{−}transferase
GSSG, Oxidized glutathione
TBST, TBS with tween-20
SDS, Sodium dodecyl sulphate
TE, Tris EDTA
EPS, EDTA, proteinase, sarcosyl
BPB, Bromo phenol blue
\(\mu\)ci, Micro curi
Kg, Kilogram
KDa, Kilo dalton
O.D., Optical density
INTRODUCTION
INTRODUCTION
For a long time, man has waged a constant battle against many thousands of organisms (insects, fungi, microbes, weeds, etc.) termed pests. These pests either threaten health or compete directly for food and fiber. During this time man has developed many different weapons to control these pests. These weapons are termed pesticides. A pesticide is defined as any substance or mixture of substances applied for destroying or controlling the pest growth. The pests include: insects, rodents, nematodes, fungi, bacteria, weed and viruses.

The demands of a continually expanding population and the need to improve both human and animal health have caused man to rely increasingly upon the use of large quantities of synthetic organic chemicals for the control of plant pest. The agricultural sector is the largest consumer sector everywhere. These pesticides play an important role in protecting production of crops as well as in storage (Carey and Kutz, 1985).

1. Pesticide classification:

Pesticides are classified according to different categories, the major categories are described in Table 1.
Table 1: Classification of Pesticides:

<table>
<thead>
<tr>
<th>Class</th>
<th>Basis of classification</th>
<th>Group</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Pest type</td>
<td>-Insecticide.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Mulluscicide.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Fungicide.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Herbicide.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Rodenticide</td>
</tr>
<tr>
<td>II</td>
<td>Time of application:</td>
<td>-Protective: used before infection takes place.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Direct: used after infection takes place.</td>
</tr>
<tr>
<td>III</td>
<td>Active chemical groups:</td>
<td>-Inorganic.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Organic: divided into:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) Natural compounds: extracted from the plants.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Synthetic compounds: the most common are:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i) Organochlorine.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) Organophosphate.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii) Carbamate.</td>
</tr>
<tr>
<td>IV</td>
<td>the type of formulation</td>
<td>-Wettable powder.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Aerosols.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Solutions.</td>
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</table>
2. Environmental contamination due to pesticide application:

Agricultural workers (about three quarters of the labor force in the poorest countries of the world) face an occupational hazard from dealing with pesticides (Jeyaratnam et al., 1987). This occupational hazard also extends to the workers who are in contact with agricultural products such as production and transportation. Furthermore, pesticides have both environmental and health hazards. The environmental contamination is built up either due to the bioaccumulation of the pesticide in the plant, where it is transferred to the animals and human beings through the food chain or due to the accumulation of the pesticides residue in the environment.

Many different studies have shown that environmental contamination and occupational exposure have great impact on the health hazard of mankind (Jeyaratnam et al., 1982; Jeyaratnam et al., 1987; Carey and Kutz, 1985; Taha and Gray, 1993). Different factors play a vital role in the environmental contamination by pesticides, such as the nature of the compound, solubility, degree of biodegradation, nature of the soil, site of application, weather conditions and mode of application. Additionally, improper application of pesticides is important. Due to inappropriate application, for example reducing the interval time between the application and harvesting time leads to accumulation of pesticide residue in the plant. In the interval time, plants biotransform the pesticide to its metabolites and mostly excrete it. Most of pesticides are biodegraded by the microflora of the soil. Thus inappropriate application increased the environmental contamination risk.

The great issue in the environmental contamination is the quantity of pesticide residue in the environment. The pesticide residue depends on its nature and a standard
level is determined for each pesticide residue in food and drinking water. If the pesticide residue exceeds the permissible level it will adversely affect the quality of the environment and human health and consequently the economy of the country. The major routes of human exposure to pesticides are accidental exposure of pesticides drifted from the application site and chemical residues in crops and drinking water (Jeyaratnam et al., 1982; Wood and Kanagasabapathy, 1983; Carey and Kutz, 1985; Jeyaratnam et al., 1987).

3. Pesticide usage and health risk assessment:

With increasing awareness of the pesticide usage and health risk, improvement of the quality of the environment has become an important concern in developed countries. Most nations are aware of the potential hazards posed by the use of pesticides and each government independently must judge the potential costs and benefits to its citizen in deciding which pesticide is used. They therefore developed legislations for the application of pesticides. Some pesticides should be restricted according to their toxic potency and health risk. Currently developed countries build policies in controlling the health hazard by pesticides application and their experience for others should be considered. On the other hand, most of developing countries are not concerned the environmental quality and pesticides application, due to the poverty in the first interest, where the agriculture sector is the major production sector in these countries and due to uncontrolled increasing population.
The health risk by using pesticides is assessed through two main ways (Klassen, C., 1996):

I. Identification of hazard:
   a) Epidemiology
   b) Toxicology
   c) In vitro test
   d) Structure activity analysis

II. Characterization of risks:
   a) Potency
   b) Exposures
   c) Susceptibility

The toxicity of each pesticide should be determined before it is registered. Toxicological studies of pesticides include short-term exposure, long-term exposure, dermal/ocular effects, reproductive effects, developmental effects, immunotoxic effects, neurotoxic effects, carcinogenicity, and mutagenicity (USEPA: Office of drinking water health advisories, 1989; Baker and Wilkinson, 1990). These studies involved identification of the pesticide hazards. Most of the pesticides are considered as non-mutagenic in short term studies. New methods to determine the mutagenicity of the pesticides have become compulsory. Moreover, one should consider the long-term exposure to pesticides and its effects on the human body. That is why these studies should be done in vivo to have better understanding on its harmful effects. Carbamates group of pesticides are widely used in various parts of the world including U.A.E., but the mutagenic mode of action for many carbamates is not yet clearly known. Methomyl is one of the famous carbamates which is used in AL-Ain
and the significant mutagenicity of this carbamate was not proven yet. Therefore, it is important to investigate the biochemical bases of methomyl action and its potential toxicity.

4. Carbamates:

Although the first pesticidal carbamic ester was synthesized in the 1930's and was marketed as fungicide, it was not used as an insecticide until the mid-1950's. These insecticides (Figure 1) were developed in the early 1960's as biodegradable alternative of highly stable organochlorines such as dichlorodiphenyl trichloroethane (DDT) (Ecobichon, 1991; Ryan, 1971, Wootton et al, 1993). Many carbamate (Figure 1) pesticides were developed during the last decades. The most common carbamate pesticides used as insecticides are aldicarb, aminocarb, carbaryl, carbofuran, methiocarb, methomyl, propoxur and thiodicarb) (Baron, 1991, USEPA-ODW, 1989).

The common structure for carbamates is represented in Figure 1.

\[
\begin{align*}
&\text{O} &\text{CH}_3 \\
\text{R} &\text{O} &\text{C} &\text{N} &\text{R}'
\end{align*}
\]

Figure 1: carbamate common structure, where R is an alcohol, oxime, or phenol and R' is hydrogen or a methyl group (Ryan, 1971; Baron, 1991).
All the commonly used insecticidal carbamates are N-methyl carbamates (Ryan, 1971). The toxicological studies of these insecticides were stated and the mode of action on pest control was well known (Baker and Wilkinson, 1990; Baron, 1991; USEPA-ODW, 1989).

4.1. Carbamates and the Environment:

Pesticides application has environmental concerns due to the environmental problems which may have resulted from its application. These problems include water pollution, soil degradation, the appearance of resistant insects and destruction of native flora and fauna. In addition to these environmental problems, its effect on human health, mainly through the accumulation of its residues on crops (Dinham, 1993).

4.1.1. Pollution of Soil:

Soil is the highly contaminated part of the environment from application of pesticides. Carbamates are generally biodegradable component that is why they do not persist in the soil environment. The chemistry of carbamates is based on carbamic acid (NH2-COOH), which is unstable compound that decomposes to CO2 and ammonia. There are many factors that affect the degradation processes; including temperature, pH, soil moisture, organic matter, physical and chemical properties of soil and the microflora (Lorber et al., 1989; Mulbry and Kearney, 1991; Ali et al., 1979; Kiene and Capone, 1986; Racke and Coats, 1988).

When the pesticide is attached to the soil particles it can be degraded to its metabolites by the various factors. The metabolic pathway depends upon the chemical nature of R and R’ which are attached to the carbamic acid. The metabolic end
product may be more toxic than the parent compound. The pesticide residue may be later volatilized to the air, or removed with crops (by absorption, wind erosion, and water erosion) or could be leached to the ground water (Ali et al., 1979; Kiene and Capone, 1986; Racke and Coats, 1988).

4.1.2. Pollution of Water:

Pesticide residues in soil leach to the ground water or erode by the wind which might subsequently be transferred to the surface water. In addition, pesticides may be applied directly for control of aquatic animals and plants. Therefore, water quality has become a key environmental topic. Many monitoring programs were built up to determine the pesticides or its metabolites in the aquatic systems. The persistence of pesticides in the aquatic system depends upon several factors such as solubility of carbamates, photodecomposition rate, pH, water velocity and organic matter (Miles et al., 1988; WHO, 1986; Pico et al., 1994).

Some monitoring studies reported the presence of some carbamate residues in the aquatic system and drinking water. Some of these levels exceeded over the tolerance limit which is fixed by FAO/WHO (Pico et al., 1994; WHO, 1986; Bushway et al., 1992; Kross, et al., 1992; Marade and Weaver, 1994; Dixit and Banerji, 1994).

4.1.3. Pollution of Plants:

Pesticides are often used on plants to protect them from pests. The contamination of vegetation occurs by pesticide either during spraying or by its migration through contaminated soil into the roots of plants. Even there is a recommended interval time between the application of pesticide and harvesting. Traces of pesticide residue may remain in the fruits. Furthermore, the improper
application of pesticide would increase the probability of pesticide residue persistence in fruits and vegetables. The persistence of pesticides on the plant leaves and fruits depends upon several factors such as the time of application (season), temperature, the application rate, ozone concentration, type of crop and method of irrigation (Reeve et al., 1992; WHO, 1996). Carbamate residues levels in the fruit and vegetables were reported in many studies (Wood and Kanagasabapathy, 1983; Banerji et al., 1993; Goldman et al., 1990; WHO, 1994; WHO, 1993, Dixit and Banerji, 1994).

4.1.4. Carbamates application and human health:

A) Occupational exposure:

Studies have been conducted to investigate the occupational pesticide poisoning among workers involved in spraying (Hussain et al., 1990; Jeyaratnam et al., 1987). In Sri Lanka, 24.9% of the observed cases were caused by occupational or accidental exposure to pesticides and carbamate accounted for 1.4% of these poisoned cases with one death case (Jeyaratnam et al., 1982).

In another study of pesticide poisoning in four Asian countries, carbamate accounted for 0.9% of the admitted cases in hospital in Indonesia, 10.7% in Malaysia and 15.9% in Thailand. Spraying, mixing or diluting of pesticides were the most frequently associated occupational activities resulting in poisoning. A significant proportion of pesticide users could not identify a specific source of information and this is correlated with the lack of legislative controls requiring pesticide users to be formally trained in safe work practices (Jeyaratnam et al., 1987).

In a study of determining the quantity of carbofuran exposure by the applicator, the dermal exposure accounted to be 99.8%, while the inhalation exposure
accounted to be 0.2%. Only 7% of the total exposing level was excreted in the urine (Hussain et al., 1990). The occupational hazard also includes those who manufacture the pesticides and transport it. The permissible level of exposure for some carbamates in the work place was determined by WHO. For example, the air concentration of carbaryl in the work place should not exceed 5 mg/m³, while the average levels of benomyl and carbendazim were less than 0.2 mg/m³, respectively (WHO, 1993; WHO, 1994).

B) Non-occupational exposure:

Due to the illegal use of some restricted pesticides, food poisoning cases were observed. In a study of pesticides food poisoning from contaminated water melons in California in 1985, 77% of the reported cases were classified as being possible carbamate (aldicarb) illness, where this carbamate can be incorporated in the flesh of the fruit (Goldman et al., 1990).

5. Metabolism of methomyl:

5.1. General Metabolic Reaction in Carbamate Biotransformation:

Carbamates, in common with other xenobiotics (that is, compounds foreign to the body), undergo a limited number of metabolic reactions in vivo. These can be classified as oxidation, reduction, hydrolysis, and conjugation. The most important organ involved in these reactions in mammals is the liver. Most of the responsible enzymes are found in the microsomal fraction of liver homogenate, a preparation derived from the endoplasmic reticulum of liver cells (Ryan, 1971).

The enzyme systems responsible for the oxidative metabolism of xenobiotics require oxygen and NADPH as cofactors. During metabolism one of molecular
oxygen is incorporated into the substrate. The enzymes responsible are classified as mono-oxygenases. The overall mechanism appears to operate through the reduction of a component of the microsomes by NADPH which then reacts with molecular oxygen to form an "active oxygen" intermediate and transfers oxygen to the substrate (Ryan, 1971). Exogenous compounds undergo metabolic transformation in vivo to less toxic and more polar metabolites that can be eliminated from the organism more readily. The phase I detoxification processes usually form reactive metabolites whereas phase II processes conjugate the polar phase I metabolites with some natural body substituent to form a product with enhanced water solubility and excretability (Ecobichon, 1991). Carbamate ester insecticides can undergo simultaneous attack at several points in the molecule depending on the nature of the substituents attached to the basic structure. Also the route(s) and the rate(s) of metabolism being highly species specific and dependent on the substituent chemical groups attached to the basic "back bone" structure of these esters (Ecobichon, 1991). Tissue enzymes of both phase I (oxidative, reductive, hydrolytic) and phase II (transfer or conjugative reactions with glutathione, glucuronic acid, glycine and so forth) types are found widespread in plant, invertebrate and vertebrate species and, indeed, responsible for some aspects of the species sensitivity and/or both natural acquired resistance to many of these insecticides (Ecobichon, 1991).

A study of several carbamates in rats showed that decarbamylation, i.e., hydrolytic removal of the carbamoyl group, was a major feature of metabolism because in ten carbamates (including N-methyl and N-dimethyl) labeled as O\(^{14}\)C (O)N, much of the \(^{14}\)C (typically 60-80%) appeared as \(^{14}\)CO\(_2\) (Jaglan and Arnold, 1984). In addition to the hydrolysis of the carbamate ester group by tissue
enzymes and the release of a substituted phenol, carbon dioxide and methyl amine, several oxidative and reductive reactions involving cytochrome P-450 related monooxygenases can proceed the ultimate products being considerably more polar than the potent insecticide (Ecobichon, 1991).

The extent of hydrolysis of carbamate ester insecticides varies greatly between species, ranging from 30 to 95 percent hydrolysis. The type of oxidative reactions observed with carbamate esters can be simplified into two main groups:

(1) direct ring hydroxylation

(2) oxidation of appropriate side chains;

resulting in the hydroxylation of N-methyl groups or methyl groups to form hydroxymethyl groups, N-demethylation of secondary and tertiary amines, O-dealkylation of alkoxy side chains, thioether oxidation and so forth. Phase II conjugative reactions can occur at any free reactive grouping with glucuronide and sulfate derivatives as well as GSH conjugates (mercapturates) being formed (Ecobichon, 1991).

5.2. Metabolism of methomyl:

The chemical formula of methomyl is shown in Figure 2.

![Chemical structure of methomyl](image)

Figure 2: Chemical structure of methomyl.
The decarbamylation of methomyl (Figure 2) is the first step in its metabolism (Jaglan and Arnold, 1984). Harvey et al. (1973) studied the metabolism of methomyl in the rat. They reported that ten percent or less of the original radio-label was found in the whole body and organs one day after treatment (Figure 3).

The radioactivity is rapidly eliminated in the ratio of 1 part $[^{14}\text{C}]$ carbon dioxide, 2 parts $[1-^{14}\text{C}]$ acetonitrile, and 1 part urinary metabolites. No methomyl or S-methyl N-hydroxy thioacetimidate was detected. $\beta$-Glucuronidase-aryl sulfatase treatment of the urinary metabolite fraction was without effect, indicating that there are none of the usual urinary type conjugates of these compounds present either. Finally, the urinary metabolite fraction was shown to be devoid of either the S-oxide or S-S-dioxide of methomyl (Harvey et al., 1973).

Harvey and Reiser (1973) studied the metabolism of methomyl in tobacco, corn, and cabbage. They found that tobacco plants growing continuously in a nutrient solution containing 10 ppm of radioactive methomyl absorbed 20-25 % of the available radioactivity over a 4-week period. The principal component, accounting for 14% of the absorbed dose, was identified as methomyl itself. In addition, small amounts of a polar radio-labeled fraction and of a non polar radio-labeled fraction dose, were also observed in leaf extract. Of the radioactivity taken up by the plant and evolved as volatile components, approximately one-half was shown to be $[^{14}\text{C}]$carbon dioxide and one-half as $[^{14}\text{C}]$acetonitrile. The material remaining in the nutrient solution at the end of the experiment was nearly all unchanged methomyl. The half-life of the methomyl in this experiment was between 3 and 7 days (Harvey and Reiser, 1973).
One week after treatment of the leaves of young cabbage plant with radio-labeled methomyl, 20% of the radioactivity had been lost from the plant as volatile metabolites. These volatiles were identified as $[^{14}\text{C}]\text{carbon dioxide}$ and $[^{14}\text{C}]\text{acetonitrile}$ in approximately equal amounts. A total of 74% remainder in or on the plant, and 2% had been washed onto the soil (Harvey and Reiser, 1973).

\[ \text{CH}_3\text{C}=\text{N-O-C-N} \]

\[ \text{CH}_3\text{S} \]

\[ \xrightarrow{?} \]

\[ \text{volatile} \]

\[ \text{CO}_2 \]

\[ \text{CH}_3\text{CN} \]

\[ \text{CH}_3\text{COOH} \]

Figure 3: Metabolism scheme of methomyl in rats.

When the growing shoots young corn plants were treated with radio-labeled methomyl, 47% of the original radioactivity was lost from the plants as volatile components within 10 days. A total of 49% remained on the plant and 12% was
washed onto the soil. Approximately four times as much as $^{14}$C-acetonitrile as $^{14}$C-carbondioxide was produced by the corn plant (Harvey and Reiser, 1973).

The extracts of the treated plants were all investigated specifically for the presence of these suspected metabolites of methomyl. One was S-methyl N-hydroxy thioacetimidate, the hydrolysis product of methomyl. The other two were the S-oxide and S-,S-dioxide derivatives of methomyl because biological oxidation of sulfur-containing compounds has been reported frequently in the literature (Harvey and Reiser, 1973).

The polar metabolite fractions from cabbage and corn were subjected to enzymolysis with $\beta$-glucosidase. The reaction failed to liberate any S-methyl N-hydroxy thioacetimidate, confirming the absence of this compound even as a sugar conjugate in the polar fraction (Harvey and Reiser, 1973). Evidence for the presence of radio-labeled tartaric acid as a metabolite has been obtained on the thin-layer chromatographic plates as well as an indication of labeling in the plant sugars (Harvey and Reiser, 1973).

In conclusion, in all crops studies and by either foliar or root application, methomyl itself is the only terminal residue resulting from methomyl application (Harvey and Reiser, 1973). Thus from these studies two main things can be concluded:

1. methomyl can be transferred from the plant to the human being without any change.
2. carbamoyl group is one metabolite of the methomyl metabolism due to the decarbamylation reaction.
6. Hematopoiesis:

Hematopoiesis is the dynamic process of blood cell production and development. The hematopoietic system continuously maintains a cell population through a complex network of tissues, organs, stem cells and controlling factors. This network is responsible for the maturation and division of undifferentiated cells into the operational cell lines that perform immune functions, transport oxygen and carbon dioxide, and maintain hemostasis (Harmining, 1992).

6.1. Cell Division in Bone Marrow:

In kinetic terms, bone marrow is a mixture of three kinds of cells. Some cells are continuously dividing, proceeding from one mitotic division to another. The second population is comprised of cells that have left the cell cycle and then conclude differentiation. The third sort of cells is one that has left the cell cycle temporarily and has entered a dormant state. These resting stem cells are often denoted G0 cells and regarded as a reserve component, where they activate the cycling if the marrow cell compartment requires expansion replenishment (Jandl, 1987).

When the hematopoietic cells are stimulated by the hematopoietic growth factors, they undergo a continuous cell cycle in which the cells divide, differentiate, or remain dormant. The bone marrow contains cell populations in all phases of cell development. Cells have two mitotic divisions, and the cell cycle is generally described as the cell activity between the mitotic divisions. After the first mitotic division, the cell enters a resting or a dormant phase (G0). When the cell is suitably activated, it enters a postmitotic rest period (G1), which is a phase directly preceding DNA synthesis. The cell proceeds into the S phase of active DNA synthesis, where
the DNA content is doubled. The next phase is premitotic rest period (G1) as the cell prepares to enter the mitotic period (M) (Harmining, 1992).

6.2. B cell as Hematopoietic Cell Model:

The stimulation of resting B cell to proliferate involves at least two steps: (i) The signals involved in activating B cells from a quiscent state to enter the G1 phase of the cell cycle (ii) The signals which are required for activated cells to progress through the G1 phase into the S phase. Binding of surface immunoglobulin by antibodies activates arrested B cells. B cells activated with modest doses of anti-immunoglobulin are induced to increase RNA synthesis, but DNA synthesis does not occur. Progression from the activated state to the S phase requires the presence of B cell growth factor. In murine B lymphocytes, the two step stimulation of B lymphocytes can be accomplished by a combination of modest doses of anti mouse immunoglobulin plus cytochalasins (Buckler et.al.; 1988).

Macrophage plays an important role in the activation of B cell growth. It secretes one or more factors that are absolute requirement for B cell activation. The only characterized factor and perhaps the only one is called interleukin-1, or IL-1 (Darnell et. al, 1990). In the absence of this factor, mature B cells will not grow even if high concentrations of multivalent antigen are present.

The achievement of B cell activation is also assisted by helper T cells. A protein carrier is first bound and interlized by macrophages which degrade it to peptides, and display it on its surface. The function of TH cell is to recognize the degradation products of specific antigens and to secrete protein factors that stimulate
the various other cells involved in the immune response. Some of the secreted factors promote antibody production from B cells. This stimulation is needed. The interaction of antigens with antibodies on the surface of B cells is insufficient to stimulate B cell growth and secretion of soluble antibody. The TH cell produces a factor called B cell growth factor, which activates the B cell to proliferate (Darnell et al., 1990).

Activation of TH cells induces them to secrete another important protein, interleukin-2 (IL-2). This protein auto-stimulates TH cell causing them to proliferate. For TH cells, regulation is achieved by control of IL-2 receptor. That receptor is only present on TH if they have bound to their macrophage processed antigen. Thus in the presence of a TH antigen, the TH cell makes IL-2 plus the IL-2 receptor then proliferates (Darnell et al., 1990).

The initial stages of B cell activation are thought to be mediated by the inositol phospholipid signaling pathway (Alberts et al.; 1989). When antigen bound to the specific receptor complex, it is followed by a sequence of events which induces activation of a GTP-binding protein and breakdown of membrane lipid by a phospholipase with the release of two second messengers, inositol triphosphate (IP3) and diacylglycerol (DG). While IP3 increases the concentration of Ca\textsuperscript{2+} in the cytosol, the DG is important to activate a specific protein kinase, which can then phosphorylate a number of proteins with different functions in the target cell. The enzyme activated by DG is called protein kinase C (C-kinase), and this enzyme is thought to phosphorylate and thereby activate the plasma membrane Na\textsuperscript{+} H\textsuperscript{+} exchanger that controls intracellular pH. The resulting increase in intracellular pH may help signal some cells to proliferate (Alberts et al.; 1989).
6.3. Role of Interferon in lymphopoiesis:

Interferons (IFNS) are well known both as antiviral proteins and as potent regulators of cell growth and differentiation (Yamada et al., 1991). IFNs were found to induce IRF-1 (IFN regulatory factor 1) gene expression (Fujita et al., 1989).

Activated T cells produce γ-interferon and its production is increased by IL-1 and IL-2. Released γ interferon has a number of effects. (i) It increases the number of IL-2 receptors on T cells and increases the cytotoxic efficacy of T cells and NK cells. (ii) It activates macrophages to secrete more IL-1, which participates in the positive feedback loop for the production of more γ-IFN. (iii) It develops greater resistance to viral invasion.

IL-7 played a key role in the proliferation of mature pre-B-cell stage while the pro-B-cell stage is independent of the cytokine IL-7. It has been shown that the murine IL-7R gene contains IRF-1 site within its promoter region. IL-7R is expressed in pre-cells, but not on mature B cells. Thus, over expression of IRF-1 might affect the expression of murine IL-7R (Yamada et al., 1991).

On the other hand, interferons inhibit cell growth. Yamada and his colleagues (1991) reported that IRF-1 causes B-cell depletion, but no significant alterations in T cell population and function were observed. The mechanism of B cell depletion is not clear. The IRF-1 protein might exert its function for negative cell growth through regulating the expression of cellular genes such as IFN-β and IFN-α genes whose expression levels may depend on cell types.
Kumar et al. (1994) suggested that variability in the expression of IFN-inducible genes and/or responsiveness to IFN-α treatment may be due to changes in the relative proportion of cells in different phases of the cell cycle and the levels of constitutive expression of IFN-induced genes. The cells in the G1/S phase and cells between the G2/M and G1 phase may constitute the major population of IFN-α-responsive cells and non-responsive cells, respectively in a synchronized culture.

6.4. Effect of Pesticide on Cell Proliferation and Growth Factors:

Bernier et al. (1990) have administered three different doses of aminocarb to female mice, which were: 5.0, 0.31, and 0.08 mg/kg body weight. They found that the B cell subpopulation at the intermediate and lower doses, aminocarb appeared to decrease the number of pre-cells. While the higher dose did not show a significant effect. A decrease in the cell number in G0/G1 was associated with an increased number of cells entering the S phase. Thus, the pesticide induced an augmentation of premitotic events in the bone marrow but not at the level of the mitotic rate. Olson et al. (1987) reported that aldicarb administered in mice at 1-1000 ppb concentrations for 14-34 days significantly suppressed B cell responses. However, Dean et al. (1990) reported that aldicarb has indirect effect on T cells and the mechanism of modulation may indirectly be mediated through IL-1 secretion by the macrophages. They suggest that aldicarb treated macrophages could produce decreased amounts of IL-1 which is critical for T cell activation.

Casale et al. (1993) reported that pesticides used extensively throughout the world, inhibit serine hydrolysis by carbamylating or phosphorylating a serine residue
at the catalytic site of kinases. These insecticides are viewed as potential inhibitors of serine hydrolase-dependent immune functions including interleukin 2 (IL-2) signaling. They observed that carbaryl produces a marked concentration dependent inhibition of IL-2 driven: (i) proliferation of CTLL2 cells (ii) proliferation of human natural killer (NK) cells (iii) enhancement of target cell killing by human NK cells.

Serine hydrolase activity appears to be integral to diverse immune functions including: (i) antigen stimulated Ca\(^{2+}\) signaling in cytolytic cells (ii) IL-2 signaling in lymphocytes (Casale et al., 1993). They suggested that the potential of pesticides is to inhibit IL-2 driven proliferation of T cells, an immune function dependent on serine hydrolase activity. Carbaryl inhibited T cell proliferation at concentrations compatible with little or no cholinergic toxicity; and in the absence of T cell death.

Both ethyl or methyl carbamates could affect the cell growth in different organisms. In case of ethyl carbamates, McCully and Vezeridis (1989) reported that ethyl carbamate is a carcinogenic compound and can cause a pulmonary tumor. Bojan (1976), has studied the effect of urethane (ethyl carbamate) on the reproduction of Trypanosoma equiperdum. Urethane administered in the first experiment 30 min after infection at 1 mg /g of body weight and 2 mg /g of body weight dose level inhibited the reproduction of the Trypanosoma for period of approximately 25 and 50 hr, respectively. Methyl carbamates also affects the cell cycle through affecting the essential molecules. Dechacin et al. (1991), reported that the higher concentrations of aminocarb tested on algae namely 5, 10 and 50 \(\mu\)g /ml had not only dramatically affected macromolecular synthesis but also some cell cycle events. While bentiocarb showed a significant decrease in algae growth. Visual observation with a light
microscope clearly showed that the release of autospors was inhibited when 5 μg/ml aminocarb was added at G1 or S phase, or 50 μg/ml were added at S phase of synchronous cultures of *Chlamydomonas segnis* (Dechacin et al. 1991).

Carbamates could affect cell proliferation through inhibiting the cytoplasmic division. This was due to interactions with cell microtubules. Carbendazim and benomyl (fungicide carbamates) interfere with the formation and/or functioning of microtubules, causing a mitotic arrest. Benomyl has been found to bind to fungal tubulin, aneuploidy and polyploidy were observed in cells where microtubules were affected by carbamate treatment. From this point of view benzimidazole compounds were developed as anticancer drug because they act as spindle poisons (Zelesco et al. 1990; Davidse and Flach, 1977; Brabander et al. 1976; Nicollof and Kappas, 1987; Albertini, 1989; WHO, 1993).

7. Genotoxicity of carbamates

Many studies were proved the genotoxicity of carbamates. These studies were done in different organ systems, either *in vitro* or *in vivo*. According to these studies, different hypothesis appeared to explain the genotoxicity and mode of action of carbamates.

Many carbamates have shown a chromosomal aberrations (CA). Methomyl and the technical formulation "Lannate 25" were tested on whole blood human lymphocyte cultures. The pure methomyl showed, after 48 hr of incubation, a significant dose-dependent increase of chromatid type and total aberration were observed at 0.02-0.18 M dose range while "lannate 25" showed a high clastogenic activity and a significant, dose-related increase in chromosome-type aberrations.
Moreover, dicentric chromosomes were observed in lannate-treated lymphocytes (Bonatti et al., 1994).

Marshall, another carbamate, and its effective ingredient carbosulfan induced chromosomal aberration in cultured human lymphocytes. Carbosulfan induced the formation of CA at concentrations $10^{-6}$, $5 \times 10^{-6}$, $10^{-5}$, and $5 \times 10^{-5}$ v/v treated for 12, 24 and 48 hr. While marshall induced CA at concentration $10^{-5}$, and $5 \times 10^{-3}$ v/v at all previous treatment time. The CA appeared as different forms; chromatid break, chromosome break, fragment, ring, sister union, dicentric chromosome and translocation. The chromatid breakage and fragmentation are the most frequent abnormality observed. Both tested substances showed a substational increase in the number of gaps. But no relationship was established between the number of gaps and increasing treatment duration and concentration, during the 12 and 24 hr treatment times. Beside that, both test substances caused chromosome contractions. In the longer treatment periods, the cells could decrease the effects of the pesticides with their own repair mechanisms. Abnormal cells also were detected in this study. In carbosulfan treated cells, the abnormal cells appeared as tetraploid cells, while in marshall treated cells, the abnormal cells appeared as triploid cells, tetraploid cells and pentaploid cells. The tetraploid cells are the most frequent type of abnormal cells with both compounds (Topaktas and Rencuzogullar, 1993). Huda and Sinha (1991), reported that Roger (carbamate pesticide) induced clastogeny in bone marrow cells of mice. The dose was 0.6 μl/kg b wt./day and the treatment lasted for ten days. This clastogeny is represented by various types of chromosome/chromatid abnormalities. It appeared as chromatid gaps, Iso-chromatid gaps, chromatid breaks, Iso-chromatid
breaks, minute fragments, acentric fragments, metacentric chromosomes, ring chromosomes, and chromatid interchange.

Carbamates pesticides are positive in short-term mutagenicity assays in different strains of *Salmonella typhimurium*, *Saccharomyces cerevisiae* and in *Escherichia coli* (Nelson et al., 1981; Klopman et al., 1985; Albertini, 1989). The N-nitroso derivatives of carbofuran and its metabolites (3-hydroxy carbofuran and 3-keto carbofuran) were synthesized by reaction with nitrite under acidic condition. Mutagenicity was determined by the Ames assay method with *S. typhimurium* strain TA100 (Nelson et al; 1981). Maximum mutagenicity occurred at 5 μg per plate for 3-OH NCF and at 10 μg for NCF and 3-K NCF. The three nitrosocompounds also caused large increments of chromosome aberrations in Chinese hamster ovary cells. At 10⁻⁴ M, 3-K NCF induced aberrations in 92.4% of the metaphase analysed. Both 3-OH NCF and NCF demonstrated significant clastogenic activity above control levels at concentrations of 5x10⁻⁴M. Both NCF and 3-OH NCF induced large numbers of SCEs in CHO cells (Nelson et al; 1981).

The possibility that the N-nitroso form of CF or other carbamates could be formed in the human stomach exists; the pesticides are used routinely on a variety of crops, nitrite is a common component of the human diet and is present in human saliva., and nitrosation of carbamates under conditions simulating those in the human stomach has been demonstrated (Nelson et al., 1981).

In vitro, lannate 25 but not methomyl, was able to induce DNA damage in lymphocyte culture. This was measured as a single-strand breaks or alkali-sensitive sites, by the alkaline elution technique. Also it caused an oxidative damage. This was
measured by detecting 8-hydroxy guanosine (OH$_8^\text{dG}$), one of the oxidative damage metabolites, through HPLC-EC. (Bonatti et al., 1994).

The genotoxicity of methomyl was studied also *in vivo*. DNA damage was evaluated by the alkaline elution assay in liver and kidney cells of Swiss CD1 mice, by the HPLC-EC assay for the detection of 8-hydroxy guanosine (OH$_8^\text{dG}$) and by the $^{32}$P-post labelling technique in liver cells. The alkaline elution assay was for liver and kidneys of Swiss CD1 mice treated intraperitoneally with a single dose of methomyl (5 mg/kg) or lannate 25 (20 mg/kg, corresponding to 5 mg/kg methomyl). This study showed a significant increase in the elution rate constant after 4 hr of treatment with the technical formulation or its active ingredient, methomyl. Also OH$_8^\text{dG}$ was determined after 4, 8 and 24 hr of administration of methomyl and its technical formulation. The dose was 55 mg/kg body weight. There was a significant increase in oxidative damage after 4 hr of treatment for both compounds due to the formation of OH$_8^\text{dG}$ (Bolognesi et al., 1994). The formation of OH$_8^\text{dG}$ is considered to be a relevant factor for DNA damage and potential genotoxic and carcinogenic effects due to reactive oxygen species (Mathews and van Holde, 1996; Niesink et al., 1996; Sahu, 1991). Bolognesi and his colleagues (1994) suggested that the oxidative damage of DNA by methomyl could be due to inhibiting of some critical enzymes involved in the defense against harmful oxygen species, such as superoxide dismutase, catalase, and glutathione transferase.

In order to investigate the capability of methomyl to form DNA adducts, mice were expose to methomyl or "lannate 25" up to the highest tolerated doses. Liver DNA was analyzed using the nuclease P1 version of the $^{32}$P DNA post labelling technique. No DNA adducts were detected in liver DNA from mice treated with 5
mg/kg analytical grade methomyl by using $^{32}$P post labelling. But it is detected in hepatic DNA from mice exposed to single doses of lannate 25 (2.5; 5; 10 mg/kg, corresponding to 0.62; 1.25; 2.5 mg/kg methomyl), where it showed six adduct spots in the chromatograms indicating that "lannate 25" is a specific DNA adducts (Bolognesi et al., 1994).

The frequencies of sister chromatid exchange were increased by treatment of human lymphocyte culture with propoxur and NO-propoxur at doses of 100 and 200 µg/ml of both chemicals. Both chemicals also induced an increase in the number of micronuclei at doses of 50, 100, and 200 µg/ml for propoxur, while at doses 100 and 200 µg/ml for NO-propoxur (Cid et al., 1990).

8. Regulation of cell division:

There are two main classes of genes that are involved in the regulation of cell division: Proto-oncogenes and Tumor suppressor genes. Proto-oncogenes are genes that stimulate multiplication of normal cells (Becker et al., 1996; Alberts et al., 1989), while Tumor suppressor genes are genes which suppress cell growth. The balance in these groups of genes function is required for normal cell and tissue growth (Becker et al., 1996; Alberts et al., 1989). Imbalance may occur due to activating mutation of proto-oncogene (s) and/or inactivating mutation of tumor suppressor gene (s) that can cause neoplastic transformation. An agent that causes neoplastic transformation is called carcinogen. A carcinogen may cause changes in behavior of cell by the following ways:

a) It contributes an oncogene to the cell.
b) It activates a proto-oncogene residing in the cell's genome by altering the gene's structure or its expression.

c) Diminish the function of a tumor-suppressor gene(s) in the cell.

Based on the location of oncogene (proto-oncogene) product, they are classified into four groups:

Class I: Oncogenes that code for growth factor such as sis oncogene in which the encoded product is part of platelet-derived growth factor (PDGF).

Class II: Oncogenes that code for growth factor receptors such as erb B oncogene which encode for erythrocyte growth factor receptor (EGF receptor).

Class III: Cytoplasmic oncogenes, which is responsible for regulating intracellular signal transduction, such as Ras and c-Raf-1 oncoproteins.

Class IV: Nuclear oncogenes: are present in the nucleus, such as c-Jun, c-Fos, and c-Myc.

8.1. Ras proteins:

The Ras proteins are 21 KDa plasma-membrane proteins that bind guanine nucleotides. The three forms of the protein, N-, H-, and Ki-Ras are involved in transmitting cellular signals from the membrane to the nucleus. A Ras protein is active when bound to GTP while inactive when bound to GDP. The functions of Ras proteins are regulated in two ways: (1) by an increase in their intrinsic GTPase activity thereby producing an inactive GDP-bound form and (2) by an increase in the rate of guanine nucleotide exchange which results replacement of GDP by GTP. Ras proteins remain in the activated GTP-bound form after cells have been stimulated by the ligand (Moodei and Wolfman, 1994).
In a variety of mammalian cells, Ras proteins regulate differentiation, progression into S phase and immunological responses (Moodei and Wolfman, 1994). Ras was identified as a critical determinant of cellular differentiation. Microinjection of antibodies against Ras into mammalian cells blocks the DNA synthesis induced by receptor tyrosine kinase (RTK) and most non-nuclear oncoproteins. This is due to the fact that Ras-activated kinase cascade completes a sequence of biochemical reactions that links the signals initiated by mitogenic receptors to the cytoskeletal and transcriptional targets that mediate cell division or differentiation (Avruch et al., 1994). Moodei and Wolfman (1994) suggested that there are at least two cell cycle-specific requirements for Ras function: the first, upon the exit from and entry into Go to G1 and the second, as cell enter S phase. This may reflect a difference in the requirements for Ras activation by competence and progression factors.

8.2. Raf kinases:

Raf kinases are signal-integrating enzymes that have the ability to switch tyrosine kinase signaling to serine/threonine phosphorylation, and connect growth factor receptors with transcription factors. The connection involves a cascade of protein kinases that is essential for cellular proliferation and differentiation of species ranging from worms to humans. This cascade also mediates transformation by most oncogenes (Daum et al., 1994). Raf kinase represents the critical transformation effector of Ras and Raf was essential for AP-1/ets-mediated transcription induced by Ras (Daum et al., 1994). Raf with MEK and MAPK are forming what is known as the cytoplasmic kinase cascade which represents an essential pathway of mitogenic signal transduction in many cell types (Daum et al., 1994).
8.3. The mitogen-activated protein kinase (MAPK):

The MAPK cascade is a major signaling system which transduces extracellular ones into intracellular responses. The JNK protein kinases are one of the MAPK subgroups and were first identified as a protein kinase activity that phosphorylates the transcription factor c-jun. A lower level of JNK activation is also observed following Ras activation. Importantly, Ras activation potentiates the effects of other stimuli on JNK protein kinase activity. This potentiation represents a mechanism that allows JNK to integrate the inputs from more than one signal transduction pathway. The functional significance of the phosphorylation of c-jun by JNK is an increase in transcriptional activity (Davis, 1994; Rausch and Marshall, 1997).

c-Fos and c-jun proteins are required for cell cycle progression and for entry into the S phase (Kovary and Bravo, 1991; Fujita et al., 1992; Mayo et al., 1994). Kovary and Bravo (1991) reported that fos and jun proteins are essential for serum stimulated cells to reach the S phase, as injection of antibodies to a single jun protein or fos protein inhibits DNA synthesis by 80% or more.

Fos proteins and jun proteins can be induced by different stimuli such as: cytokines, certain cytotoxic stimuli, ionizing radiation, cellular stress (such as hypoxia) and some chemotherapeutic agents (Bogoyevitch et al., 1996; Manome et al., 1993; Kharbanda et al., 1990; Sherman et al., 1990; Oguro et al., 1997; Kick et al., 1996). c-Fos and c-Jun gene products form a heterodimeric complex (AP-1), that in turn regulates target gene expression by binding to a specific DNA sequence (TGACTCA) in the promoter region (Fujita et al., 1992). Few studies reported the induction of c-Jun and c-Fos expression by carbamates. Kick et al. (1996), reported
that photodynamic treatments of human epithelial Hela cells can decrease AP-1 DNA binding of other strong inducers, such as the antioxidant pyrrolidine dithiocarbamate (PDTC).
Aims of Present Investigation

Methomyl, a commonly used agricultural insecticide in U.A.E., may have a health risk and cause environmental pollution. Therefore, it is important to investigate the biochemical consequences of methomyl in mammalian cells to evaluate the potential risk for human health. In this study, the effect of methomyl was analyzed at the molecular level in rat model. The whole investigation was aimed to investigate:

A) the effect of methomyl treatment on cell division: Highly proliferative bone marrow cells were chosen as a target to study the molecular mechanism by which cell growth is affected.

B) the effect of methomyl treatment on DNA synthesis of rat bone marrow cells: Carbamoyl group is a by-product of carbamate metabolism in vivo and this group interferes with nucleotide biosynthesis, where carbamoyl phosphate is a critical precursor in pyrimidine biosynthesis. Thus any interference of carbamate metabolite may affect the synthesis of DNA.

C) cytotoxic effect of methomyl: Glutathione-S-transferase (GST) and cytochrome P-450 of liver cells of methomyl treated rats were studied by immunoblotting.
MATERIALS AND METHODS
1. Treatment of Rats With Methomyl:

1.1. Reagents:

a) Lannate 20 formulation (90% methomyl) was obtained from Al-Ain Municipality.

b) $^3$H- methyl Thymine (40-60 Ci per mmol) was obtained from Sigma Chemical Company.

1.2. Animals:

White Wistar male or female rats (4-12 weeks old) weighing (40-300 g) were obtained from Desert and Marine Research Centre, AL-Ain, and used for all the experiments.

1.3. Preparation of Methomyl:

The LD$_{50}$ of methomyl for rats equals 17 mg/ Kg of body weight (Baron, 1991). A 10% of the LD$_{50}$ was prepared. This preparation was made by dissolving 18.89 mg of lannate (90% methomyl) in 100 ml of normal saline.

1.4. Administration Procedure:

To test the effect of methomyl on cell growth, three groups (2-3 animals in each) of animals were used. The first group was treated with 1% LD$_{50}$ of methomyl while the second group was treated with 10% LD$_{50}$ of methomyl. The pesticide was administered orally and was repeated three times for each group of animals with 24 hr intervals between each dose. The control group (third group) received normal saline in the same manner of the pesticide administration procedure. With the third administered dose, 20 µCi of $^3$H- methyl thymine were also injected intraperitoneally to the three groups (control, methomyl treated 1% and methomyl treated 10% animals).
2. Determination of the Role of Methomyl on the Cell Division of Rat Bone Marrow:

2.1. Reagent:

a) PBS (phosphate buffer saline).

b) Formamol 20%.

c) acidic toluadin dye

2.2. Isolation of Bone Marrow Cells:

To accelerate the proliferation of lymphocytes in rat bone marrow, during the treatment, the rat tail was cut from the first day of treatment. The animals were weighed and sacrificed in chloroform jar after 48 hr of the final treatment. Both ends of humerus and femur were cut, then bone marrow was flushed out and suspended in 10 ml of phosphate buffer saline (PBS). The flushing was by using a medical syringe (needle was 21 Gaute × 1.5 length). The bone marrow suspensions were aliquoted as following: a) 0.5 ml for cell counting, b) 2.5 ml for TCA precipitation and c) 7 ml for DNA extraction.

2.3. Determination of the Number of Cells in Rats Bone Marrow:

The number of cells in bone marrow suspension was determined by using hemocytometer, and for each sample, the number of cells was counted three times and an average was taken.
**Neubauer Hemocytometer:**

The most commonly used counting chamber is the Neubauer hemocytometer, which has two identical grids. When the cover slip is in the correct position, the chamber has a depth of 0.1 mm. The cell suspension is introduced into the chamber with a pasture pipet. Depending on the number of cells present, the cells may be counted on the central grid where squares are surrounded by triple lines. The area of this grid is 1 mm² and therefore, the volume is 0.1 mm³. If all the cells in this central area are counted (N):

\[ N = \text{the number of cells in } 0.1 \text{ mm}^3 \]

Therefore, the number of cells in 1 cm³ = N \times 10⁴.

**2.4. Counting Procedure:**

A 0.5 ml of the bone marrow suspension was added to 0.5 ml of 20% formamol and 0.2 ml of acidic toludin dye was also added to stain the nucleated cells. The cells were counted using hemocytometer.

3. Isolation and Characterization of DNA of Methomyl Treated Rat Tissues:

3.1. Isolation of DNA from Bone Marrow Cells:

**A) Reagents:**

a) TNE(0.01 M tris.HCl pH 8, 0.1 M NaCl, 0.001 M EDTA).

b) 1% SDS.

c) Proteinase K.

e) absolute isopropanol.

f) EPS (0.25 M EDTA pH 8, 100 μg/ml proteinase K, 0.5% sarcosyl).

g) phenol.

h) absolute ethanol.

i) TE (Tris pH 8, 0.001 EDTA).

B) Extraction of DNA:

Two methods were used for the isolation of DNA throughout the work:

I. Seven milliliters of bone marrow suspension was centrifuged. The precipitate was resuspended in 3 ml of TNE containing 1% SDS and proteinase K (400 μg/ml). The mixture was incubated overnight. An equal volume of phenol was added and the mixture was rocked using a shaker for 2 min. Then it was centrifuged for 15 min. at 3000 rpm. The upper aqueous phase was taken and this step was repeated. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the extracted aqueous phase and rocked using a shaker for 2 min. Then centrifuged at 3000 rpm for 15 min. and the aqueous phase was taken. This step was repeated twice. 2.5 volume of absolute ethanol was added to the aqueous phase and mixed gently until a white cottony clump appeared. The white cottony clump was isolated by using a glass rod and redissolved in TE buffer (Bothwell et al. 1990).

II. Seven milliliters of bone marrow suspension was centrifuged for 10 min. and then 1 ml of EPS buffer was added to the precipitate, thereafter incubated at 37°C for 2 hr. After incubation, 3 ml of WBC lysis buffer (0.3 M NaCl, 10 mM Tris HCl pH 7.4, 10 mM EDTA, 7 M Urea, 2% SDS) was added to the mixture and mixed. Four milliliters of phenol / chloroform / isoamyl alcohol (25:24:1) were added to the
mixture and vortexed. The mixture was centrifuged at 4K rpm for 10 min., then the upper aqueous phase was taken. Four ml of chloroform : iso amyl alcohol (24:1) were added to the aqueous phase, vortexed, then centrifuged at 4K rpm for 7 min. The aqueous phase was aspirated and then a 2.5 volume of absolute ethanol was added gently, mixed until white precipitate appeared. The samples were centrifuged at 4K rpm for 10 min. to isolate the DNA. The isolated DNA was dried for 5-10 min and dissolved in TE buffer.

3.2. Characterization of DNA:

A) Estimation of DNA in each sample:

The DNA concentration was measured by spectrophotometer at 260 nm. The amount of ultraviolet light absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. At this wavelength the absorbance (A260) of 1.0 corresponds to 50 μg of double-stranded DNA per 1 ml (Schleif and Wensink, 1981). In this study, the absorbance of DNA was estimated using DU-70 (Beckman) spectrophotometer at 260/280 nm and the amount of DNA was calculated for each sample of the cell count.

B) Analysis of DNA by agarose gel electrophoresis:

A) Reagents:

a) BPB (bromo phenol blue).

b) Agarose gel.

c) TBE (Tris borate EDTA).

d) Ethidium bromide.
B) Sample preparation:

1 μl of BPB (bromo phenol blue) was added to 10 μl of the extracted DNA and mixed.

C) Agarose Gel Preparation:

0.8 % of agarose gel was prepared by dissolving 0.8 g of agarose in 100 ml of TBE buffer (1X) and boiled until agarose is dissolved completely. 5 μl of ethidium bromide (10 mg/ml) was added to the dissolved agarose. Then the mixture was poured to the gel casting tray and kept at room temperature to solidify.

D) Agarose gel electrophoresis:

The gel was transferred to the electrophoresis tank containing 1X TBE and ethidium bromide (0.5 mg/ ml). Ten microliters of the sample were loaded onto the gel, and 5 μl of lambda Hind III (size marker) were loaded to one well, then the gel was electrophoresed at 100 volts for 90 min.

E) Visualization of DNA in agarose gel:

The agarose gel was exposed to UV light source (transilluminator, flowgen-TFX-20M San Gabriel, CA.) and a picture was taken by using a Polaroid camera (DS34 with EP H6 Hood).

4. Determination of Incorporation of $^3$H- Methyl Thymine in Rat Bone Marrow Cells:

This was assessed into two steps. The first step was precipitation of the incorporated tritiated thymine by using 5 % TCA, while the second one was through counting the radioactivity in the extracted DNA.
4.1. Reagents:
   a) 5% TCA (Trichloro acetic acid).
   b) Methanol.
   c) NaOH.
   d) HCL.
   e) Scintillation liquid.

4.2. Determination of Radioactivity Present in TCA Precipitation:

   Precipitation with trichloroacetic acid (TCA) is widely used to separate radioactive oligomeric nucleic acids from radioactive nucleosides and nucleotides (Schleif and Wensink, 1981). Then 2.5 ml of bone marrow suspension was added to 2.5 ml of 5% TCA at -4°C and kept at this temperature over night. To separate the precipitate, samples were centrifuged at 3000 rpm for 5 min., then the supernatent was discarded. The cells were washed again with 5% TCA. The cells were rewashed with methanol and the samples were centrifuged at 3000 rpm for 5 min.. Then the cells were dissolved in 0.5 M NaOH, and neutralized with HCl. Finally 4 ml of scintillation liquid was added to the samples to count β-radiation using scintillation counter as count per 5 min..

4.3. Determination of Radioactivity Present in Purified DNA:

   As described previously, 100 μl of the extracted DNA was added to 3.9 ml of scintillation liquid and β-radiation was counted as described previously.
5. Characterization of Protein by Gel Electrophoresis:

5.1. Reagents:

   a) Homogenizing buffer (0.1 M Mono potassium phosphate, 0.15 M Potassium chloride pH7.5).
   b) 1.5 M Tris. HCl pH 8.8.
   c) 10% SDS.
   d) Acryl amide / bis (30%).
   e) 10% APS (ammonium per sulphate).
   f) TEMED (N-N-tetra methyl ethelene diamine).
   g) 0.5 M tris pH6.8.
   h) 1X running buffer.
   i) Protein low range molecular weight size marker (Bio-Rad).
   j) Bradford reagent (Bio-Rad).
   k) Bovine serum albumin.

5.2 Preparation of Sample Buffer:

Sample buffer was prepared by mixing the following chemicals:

\[
\begin{align*}
H_2O & \quad 4 \text{ ml} \\
0.5 \text{ M Tris-HCl, pH 6.8} & \quad 1 \text{ ml} \\
\text{Glycerol} & \quad 0.4 \text{ ml} \\
10\% \text{ Sucrose} & \quad 0.4 \text{ ml} \\
10\% \text{ SDS} & \quad 1.6 \text{ ml} \\
2-\beta\text{-mercaptoethanol} & \quad 0.4 \text{ ml} \\
0.05\% \text{ Bromophenol blue} & \quad 0.2 \text{ ml}
\end{align*}
\]
5.3 Liver Samples preparation:

About 500 mg of the liver tissues were homogenized in homogenizing buffer by using a homogenizer. The homogenized suspension was centrifuged and the supernatant was collected. The protein quantity was estimated by Bradford method (Bradford, 1976). Ten μl of the protein sample were added to 10 μl of sample buffer and vortexed (to dissolve protein ingredient). Finally, the samples were heated at 95 °C for 5 min.

5.4. Bone marrow samples preparation:

Three hundred μl of sample buffer were added to the extracted tissues and mixed well. Samples were heated at 95°C for 5 min. and centrifuged, then protein quantity was estimated. The amount of protein was made equal by appropriate dilution with sample buffer.

5.5. Estimation of protein concentration:

A standard protein solution, 10% of bovine serum albumin was prepared by dissolving in distilled water. Three different concentrations (10, 50, and 100 μg/ml) of bovine serum albumin were added to 1 ml of Bradford reagent (Bradford, 1976). Solutions were mixed well, then kept at room temperature for 10 min. The absorbance of each sample was determined at 595 nm using DU-70 (Beckman) spectrophotometer. A standard curve was made by plotting O.D. (Y axis) versus protein concentration (X axis). The absorbance of liver and bone marrow samples were estimated in a similar way and the protein samples concentration were determined by using the standard curve (Figure 4).
5.6. Preparation of acrylamide gel:

Protein gel electrophoresis has two phases of gel: the separating and the stacking gel. The separating gel was first prepared by mixing: 3.35 ml of deionized distilled water, 2.5 ml of 1.5 M Tris HCl, pH 8.8, 100 µl of 10% SDS, 4 ml of Acrylamide / bis (30%), 50 µl of 10% APS (ammonium per sulphate), and 5 µl TEMED (N-N-tetra methyl ethelene diamine). The prepared gel was poured smoothly, in the clamp assemblies until the permissible level was reached and kept at room temperature for 2 hr to polymerize. Then the stacking gel was prepared by mixing: 6.1 ml of deionized water, 2.5 ml of 0.5 tris HCl pH 6.8, 100 µl of 10% SDS, 1.3 ml of Acryl/bis (30%), 50 µl of 10% APS (ammonium per sulphate), and 10 µl of TEMED (N-N-tetra methyl ethelene diamine). The prepared stacking gel was poured smoothly to the polymerized separating gel, then the comb was inserted and kept until the gel polymerized. Finally, the prepared gel was transferred to the electrophoresis tank which contains running buffer (60 ml of 5X running buffer + 240 ml H2O).

5.7. Preparation of 5X running buffer:

The following amount of chemicals were taken and dissolved completely in 600 ml of distilled water: 9 g of Trizma base, 43.2 g of Glycine and 3 g of SDS.

5.8. Protein gel electrophoresis

The prepared protein samples (10-20 µl) were loaded in the gel in order. Then the electrophoresis was run at 200 volt for 45 min. After running the electrophoresis, the gel was removed gently and soaked in CB stain (Coomassie blue) for 15 min. The stained gel was destained in destaining solution (200 ml methanol + 50 ml acetic acid + 250 ml H2O) for 0.5 hr. By destaining the protein bands were made visible (Bio-rad Laboratories, 1988).
6. Western blotting / immuno characterization:

6.1. Reagents:

   i) Preparation of Towbin’s buffer: 6.06 g of Tris and 28.8 g of glycine were dissolved in 800 ml of distilled water and 400 ml of methanol were added to the solution. Then the volume was made to 2 liters by distilled water.

   ii) Preparation of TBS (Tris buffer saline): The following chemicals: 0.8 g Tris base, 6.85 g Tris HCl, and 8.9 g sodium chloride were dissolved in 1000 ml of distilled water.

   iii) Preparation of TBST (Tris buffer saline with Tween-Twenty): One milliliter of Tween-20 was added to 1000 ml of TBS solution to get TBST.

   iv) Preparation of 5%LFM (low fat milk): 1.25 g of low fat milk was dissolved in 25 ml of TBS.

6.2. Western blott procedure:

   After finishing the protein electrophoresis, the proteins were transferred from gel to the nitrocellulose membrane. The transferring was applied in the holding cassette in this order: fiber pad, filter paper, gel, nitrocellulose membrane, filter paper, fiber pad. These should be soaked in Towbin transfer buffer prior to the assembly of gel holding cassette. The transferring process was run at 50 volts for 5 hr (limit = 0.21) in the fridge. NCM (nitrocellulose membrane), after transferring was soaked in 5% LFT (low fat milk) TBS (blocking step), over night. NCM was rinsed with TBS for 10 min. (two changes), then treated with the primary antibody for 2-3 hr (see Table 2). Later on, the NCM was washed with TBST (20 min., 4 changes), then rinsed with TBS for 10 min. (2 changes). NCM was treated with the secondary antibody for 30
min. at 37°C, then washed with TBST (10 min., 2 changes), and rinsed with TBS for 10 min.(2 changes). The NCM was treated with another link antibody (Avidine) for 1 hr at 37°C . The NCM was washed with TBST (10 min., 2 changes) followed by washing with TBS (10 min, 2 changes). Finally, the color development was carried out by using paraoxidase system. This system prepared by dissolving 1 DAB tablet in 20 ml TBS and filtered, then 20 μl of hydrogen peroxide were added just before starting the color developing step. The NCM should be washed with water after this step.

Table 2: List of samples, primary and secondary antibodies used in immunoblots.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>c-Raf-1, rabbit polyclonal</td>
<td>anti rabbit</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>c-fos, mouse monoclonal</td>
<td>anti mouse</td>
</tr>
<tr>
<td>liver</td>
<td>c-jun, rabbit polyclonal</td>
<td>anti rabbit</td>
</tr>
<tr>
<td>liver</td>
<td>P-450, rabbit polyclonal</td>
<td>anti rabbit</td>
</tr>
<tr>
<td>liver</td>
<td>αGST, mouse monoclonal</td>
<td>anti mouse</td>
</tr>
</tbody>
</table>
Figure 4: Protein standard curve for estimation of protein.

Three different concentrations (10, 50 and 100 µg) of bovine serum albumine solution (10%) were diluted and added to 1 ml of Bradford reagent. The absorbance of each sample was determined at 595 nm. The standard curve was made by plotting O.D. against concentration of protein (mg/ml).
9. Statistical analysis:

One way anova with unequal variance following t-distribution was used to detect the significant differences between all groups in all runs. This method was subjected to the cell count ×10⁶ cell / ml and the amount of DNA / 10⁶ cells.

The regression equation was derived between the animal size and the log cell count, and the equality in the resulted slope for each group was also examined by comparing two slopes-test for parallelism (Kleinbaum and Kupper, 1978).
RESULTS
Effect of Methomyl Treatment on the Cell Division of Rat Bone Marrow:

The effect of methomyl on bone marrow nucleated cell number was determined by two approaches as described below:

I. By counting the number of nucleated cells from the bone marrow:

In this study three groups of rats (control, treated with 1% and 10% of methomyl) were used to investigate the effect of the oral administration of methomyl on the cell division of rat bone marrow. Each group contains 2-3 rats. The treatment was repeated three times with 24 hr intervals, and beside the third administered dose, tritiated thymine was injected intraperitoneally. Animals were sacrificed on the fifth day of starting the treatment, bone marrow was collected and the nucleated cells number was counted by using hemocytometer. The average cells count for each group was calculated. This experiment was repeated four times. There was significant (P < 0.005) decrease in the number of cells in methomyl treated (both 1% and 10%) animals (Table 3).

A decrease in the cell count was observed in all runs, the data are illustrated in Figure 5. The decrease in the cell count in 1% treated group was more, compared to the 10% treated group (except in run #3). It may be possible that inhibition in cell division reached to the saturation level at 1% of methomyl. Therefore, further increase in inhibition of cell division was not observed with 10% methomyl.

II. By determining the relationship between log cell count and the animal size:

The relationship between the animal size (weight) and the log of cell count was evaluated by using the regression equation, to determine the slope for each
equation which represents the rate of bone marrow cells frequency. Figures 6, 7, and 8, which represent the relationship between the log of bone marrow cell count and the weight of rats in each group. In all cases, regression analysis indicated that there is a linear relationship between these variables. The slope represents the rate of the nucleated cells frequency in bone marrow. A reduction in the slope value represents a depletion on the cell cycle where the cell count was decreased. There was insignificant decrease in the slope value of 10% treated group compared with control group. Although 1% treated group has the highest slope value, this did not represent an increase in the rate of nucleated cells frequency in bone marrow. That is due to the absence of this group in the last experimental run.

However, methomyl treatment showed a significant reduction in cell count, indicating that methomyl inhibits cell division in rat bone marrow.

Effect of Methomyl on the Synthesis of DNA in Rat Bone Marrow Cells:

To study the effect of methomyl on the synthesis of DNA in rat bone marrow cells, the DNA of bone marrow cells was extracted from 1% and 10% methomyl treated animals as well as from the control group taking approximately the same number of cells from each group. Extracted DNA was estimated spectrophotometrically.

Both Table # 4 and Figure 9 illustrate the average amount of DNA of the nucleated bone marrow cells from control and treated groups. The amount of DNA was higher in the treated groups than the control. The value for the 1% treated group was higher than that of the 10% treated group (except in run #1). Statistical analysis
using the one way analysis of variance indicates an insignificant (P value > 0.05) increase of the amount of DNA cells with the treatment, except run # 4, where the increase was significant. Thus, methomyl treatment did not inhibit the synthesis of DNA in rat bone marrow cells.

**Effect of Methomyl Treatment on the Incorporation of the $^3\text{H}$-Methyl Thymine in the DNA:**

$^3\text{H}$-methyl thymine incorporation in DNA of rat bone marrow cells was studied in either control and methomyl treated animals by TCA precipitation and DNA isolation methods. There was little incorporation of $^3\text{H}$-methyl thymine in DNA in either control and experimental groups (not shown). This may be because of the relatively lower amount of nucleotide compared to the endogenous nucleotides. Therefore, the difference in $^3\text{H}$-methyl thymine incorporation was insignificant. Cell culture system, where higher amount of nucleotide could be introduced, might be a useful way to investigate the role of methomyl on DNA synthesis.

**Effect of Methomyl on the Structure of DNA:**

To study the effect of methomyl treatment on DNA structure, a similar amount of the isolated DNA was analyzed by agarose gel electrophoresis. DNA was then stained with ethidium bromide and visualized under UV light. It was found that there were no differences in the electrophoretic mobility of the extracted DNA between control and treated groups in all cases (Fig. 10), indicating that methomyl treatment does not affect the size or structure of DNA.
Table 3: The Effect of Methomyl on the Number of Rat Bone Marrow Cells

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Average cell count (± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>0.542×10^6 (± 0.03)</td>
</tr>
<tr>
<td>2</td>
<td>0.887×10^6 (± 0.17)</td>
</tr>
<tr>
<td>3</td>
<td>1.108×10^6 (± 0.17)</td>
</tr>
<tr>
<td>4</td>
<td>0.290×10^6 (± 0.08)</td>
</tr>
</tbody>
</table>

The effect of methomyl treatment on rat bone marrow cells division was studied in three groups: control, treated 1% of methomyl LD₅₀ and treated 10% of methomyl LD₅₀. Bone marrow was extracted and cell number was determined by using hemocytometer. Each column in the above table represents a run of experiment and each number represents the average of 2-3 rats samples cell count. P value was estimated using one way anova test (P value <0.005). ND represent “not done”.
Figure 5: Effect of Methomyl Treatment on Bone Marrow Cells.

The cell numbers were plotted in a histogram for all groups in all experimental runs. 1) is the first experimental run, 2) is the second experimental run, 3) is the third experimental run, and 4) is the fourth experimental run. Each bar represents the value obtained from three animals.
Animals were weighed in all experimental runs. The log for the extracted bone marrow cells count was calculated for all animals. These results were statistically analyzed by regression equation, where X axis represents the animal average weight for the experimented groups, while Y axis represents the log of the average bone marrow cells count. The regression equation for control group was $Y = 5.24 + 0.0038X$, the regression equation for 1% treated group was $Y = 4.21 + 0.0056X$, while the for 10% treated group $Y = 5.12 + 0.0036X$. 

**Figure 6: Fitted Line of the Regression Equation for the experimented groups.**
Table 4: Effect of Methomyl on DNA Synthesis of Rat Bone Marrow Cells.

<table>
<thead>
<tr>
<th>Experimental Run Number</th>
<th>Control (µg/10^6 cell)</th>
<th>Treated 1% Group (µg/10^6 cell)</th>
<th>Treated 10% Group (µg/10^6 cell)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.39 (± 1.95)</td>
<td>69.15 (± 43.45)</td>
<td>87.56 (± 5.26)</td>
<td>0.078</td>
</tr>
<tr>
<td>2</td>
<td>20.13 (± 7.2)</td>
<td>67.72 (± 74.60)</td>
<td>32.35 (± 12.63)</td>
<td>0.065</td>
</tr>
<tr>
<td>3</td>
<td>18.31 (± 2.11)</td>
<td>26.72 (± 4.72)</td>
<td>21.89 (± 14.79)</td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>246.83 (± 85.56)</td>
<td>ND</td>
<td>318.63 (± 74.3)</td>
<td>0.023</td>
</tr>
</tbody>
</table>

DNA was extracted from the bone marrow cells of all groups (control, treated 1%, and treated 10% group). In the above table each column represents a run of experiment and each average number represents the amount of DNA for 2-3 rats after normalization with the extracted bone marrow cells number. ND represents “not done”.
Figure 7: Effect of Methomyl Treatment on the Synthesis of DNA in Rat Bone Marrow Cells.

The results presented in Table 4, on the study of methomyl treatment on DNA synthesis of rat bone marrow cells, were plotted in a histogram. 1) the first experimental run; 2) the second experimental run; 3) the third experimental run; and 4) the fourth experimental run.
The extracted DNA from rat bone marrow cells for both control and treated groups, which quantified as mentioned before, was analyzed by 0.8% agarose gel electrophoresis. DNA was then stained with ethedium bromide and visualized under UV light. 1) 10% treatment, 2) 1% treatment, 3) control, 4) 10% treatment, 5) 1% treatment, 6) control and M is the λ Hind III marker. Lanes 1-3 represent DNA from first experiment while 4-6 from second experiment.
Effect of Methomyl on Rat Bone Marrow Protein Synthesis:

Total proteins were extracted from rat bone marrow cells for both control and treated 10% group and estimated spectrophotometrically using Bradford method.

For these extracted cells, there was 35% reduction in cell count with methomyl treatment and 42.5% reduction of total proteins with 10% methomyl treatment (Table. 5 and Figure 11) in bone marrow. This reduction in total protein amount of rat bone marrow cells further suggests inhibition of cell division in bone marrow upon methomyl treatment. The total proteins were visualized by coomassie blue staining (Fig. 12). For all samples there was no difference in the pattern of protein, both in intensity and number of bands.

Effect of Methomyl Treatment on Protein Synthesis in Liver Cells:

Total proteins were extracted from a similar amount of liver (500 mg) for control and treated 10% group and estimated spectrophotometrically using Bradford method. There was no significant change in total liver proteins with methomyl treatment (Table 5 and Figure 11). The proteins were further investigated by SDS-Poly acrylamide gel electrophoresis and visualized by coomassie blue staining (Fig. 12). For all samples, there were no significant differences in the pattern of protein, both in intensity and number of bands. Therefore, Coomassie blue staining method is not sensitive enough to detect a change in protein molecule(s) if there is any.
Table 5: Determination of Total Protein Amount Extracted from Rat Bone Marrow and Liver

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>Total protein (mg)</th>
<th>Liver</th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>4</td>
<td>C1</td>
<td>31.6</td>
</tr>
<tr>
<td>C2</td>
<td>6.4</td>
<td>C2</td>
<td>31.2</td>
</tr>
<tr>
<td>C3</td>
<td>5.2</td>
<td>C3</td>
<td>32.4</td>
</tr>
<tr>
<td>T1</td>
<td>4.8</td>
<td>T1</td>
<td>32.8</td>
</tr>
<tr>
<td>T2</td>
<td>2</td>
<td>T2</td>
<td>33.6</td>
</tr>
<tr>
<td>T3</td>
<td>2.4</td>
<td>T3</td>
<td>32.4</td>
</tr>
</tbody>
</table>

Total protein was extracted from rat bone marrow and liver cells from both control and 10% methomyl treated group. Protein concentration was determined by Bradford method, using a standard curve plotted for bovine serum albumin. C1, C2 and C3 represent protein from control animals while T1, T2 and T3 represent protein from 10% methomyl treated animals.
Figure 9: Total Protein Amount Extracted from Rat Bone Marrow and Liver

Total protein was extracted from rat bone marrow and liver from both control and 10% methomyl treated group. Protein concentration was determined by Bradford method, using a standard curve plotted for bovine serum albumin.
The total proteins which were extracted from both rat bone marrow and liver cells were separated by SDS-polyacrylamide gel electrophoresis and stained with coomassie blue stain. It shows the expression level of total cellular proteins. C1, C2 and C3 represent proteins from control animals while T1, T2 and T3 represent proteins from 10% methomyl treated animals. M represents molecular weight size marker.
Effect of Methomyl Treatment on the Expression of c-Fos, c-Raf-1 and c-Jun in Rat Bone Marrow Cells:

The extracted proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using specific antibodies. It was found that c-Fos and c-Raf-1 in bone marrow cells were low in all animals when treated with 10% methomyl (Figures 13 and 14). This explains the molecular mechanism of inhibition of cell division upon methomyl treatment. These results suggest that inhibition of cell division of bone marrow cells is due to at least inhibition of Ras mediated growth signaling pathway. The intensity of bands in immunoblots was weak because of small amount of protein loaded on gel.

By immunoblotting, the expression level of c-Jun in liver cells was similar in the control group as well as in the 10% methomyl treated animals (Figure 15).

Effect of Methomyl Treatment on the Expression of Glutathione-S-Transferase α (GST) and Cytochrome P-450 (1A1) in Liver Cells:

Total proteins of liver cells were extracted and estimated using Bradford method for both control and 10% methomyl treated animals. Then these proteins were separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted using specific antibodies. There were no changes in the expression of α GST protein in both control and 10% methomyl treated animals (Figure 16).

In this work the effect of methomyl treatment on GST expression was studied only on GST α family and there were no changes in GST α expression with methomyl treatment in rat liver cells. It might be important to check whether methomyl induces expression of other members of GST.
The expression of P-450 (1A1) was also investigated in methomyl treated animals (Figure 17). The increase in cytochrome P-450 (1A1) expression in rat liver cells with methomyl treatment suggests that cytochrome system is involved to metabolize methomyl and methomyl is a cytotoxic compound.
Figure 11: Expression of c-Fos Protein in Rat Bone Marrow Cells by Immunoblotting:

Total proteins of rat bone marrow cells were extracted for both control and 10% methomyl treated group (each contains three rats), and separated by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to nitrocellulose membrane and treated with c-fos antibodies. Arrow shows the position of c-fos protein (62 KDa). C1, C2 and C3 represent control while T1, T2 and T3 represent 10% methomyl treated protein extract.
Total proteins of rat bone marrow cells were extracted for both control and 10% methomyl treated group (each contains three rats), and separated by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to nitrocellulose membrane and treated with c-raf-1 antibodies. Arrow shows the position of c-raf-1 protein (74 KDa). C1, C2 and C3 represent control while T1, T2 and T3 represent 10% methomyl treated protein extract.
Figure 13: Expression of c-Jun Protein in Rat Liver Cells by Immunoblotting:

Total proteins of rat liver cells were extracted for both control and 10% methomyl treated group (each contains three rats), and separated by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to nitrocellulose membrane and treated with c-jun antibodies. Arrow shows the position of c-jun protein (45 KDa). C1, C2 and C3 represent control while T1, T2 and T3 represent 10% methomyl treated protein extract.
Total proteins of rat liver cells were extracted for both control and 10% methomyl treated group (each contains three rats), and separated by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to nitrocellulose membrane and treated with $\alpha$ GST antibodies. Arrow shows the position of $\alpha$ GST protein (28KDa). C1, C2 and C3 represent control while T1, T2 and T3 represent 10% methomyl treated protein extract.
Total proteins of rat liver cells were extracted for both control and 10% methomyl treated group (each contains three rats), and separated by SDS-polyacrylamide gel electrophoresis. Then proteins were transferred to nitrocellulose membrane and treated with cytochrome P-450 (1A1) antibodies. Arrow shows the position of cytochrome- P-450 (1A1) protein (55KDa). C1, C2 and C3 represent control while T1, T2 and T3 represent 10% methomyl treated protein extract.
DISCUSSION
Methomyl (Figure 2) is a carbamate pesticide with insecticidal properties, commonly used around the world. Poisoning with this compound does not produce serious complications and requires only a moderate medical surveillance. However, it is important to know its effect on mammalian cells.

In the present study, an inhibitory role of methomyl was studied in mammalian cells. Methomyl carbamate treatment showed a significant decrease in cell growth where the decrease was higher in 1% methomyl treated group compared with 10% methomyl treated group. Most probably 1% methomyl reached saturation level. Therefore, further inhibition with 10% treated group was not observed. There are insignificant differences between the treated groups and the control group in the rate of accumulation of nucleated cells in bone marrow. An increase in the DNA concentration was upon treatment. It might be interesting to investigate the reason for the increase in DNA content in the methomyl treated cells. One possibility is that blockage of subsequent steps after DNA synthesis where methomyl treatment may cause accumulation of DNA in the cell.

One of the possible mechanisms in the interference of carbamates in the cell cycle may be through inhibition of the cytoplasmic division. This inhibition is mainly through direct attachment to the microtubules or disruption of the spindle fibre assembly. The diploid and polyploid cells are commonly seen in this case (Zelesco et al., 1990; Davidse and Flach, 1977; Brabander et al., 1976; Nicollof and Kappas, 1987; Albertini, 1989; WHO, 1993). The significant increase in DNA amount with a significant decrease in cell count strengthened this possible mechanism of inhibition.

Bernier and his colleagues (1990) assessed the effect of aminocarb on DNA content of bone marrow cell population, mice were exposed by cavage to sublethal doses (5.00,
DISCUSSION
and S phase of cell cycle for aminocarb treated groups, as compared with the control group. They suggest that there is a shift in the cell frequency in aminocarb exposed animals for cells entering the S phase, and the pesticide affects the DNA in lymphoid and/or nonlymphoid cells in bone marrow. Carbendazim and benomyl (fungicide carbamates) interfere with the formation of and/or functioning of microtubules, causing a mitotic arrest. Benomyl has been found to bind to fungal tubulin. Aneuploidy and polyploidy were observed in these cells, where microtubules were affected by carbamate treatment (Zelesco et al., 1990; Davidse and Flach, 1977; Brabander et al., 1976; Nicollof and Kappas, 1987; Albertini, 1989). The polyploidy cells were also observed in cultured human lymphocytes treated with marshal and carbosulfan (Topaktas and Rencuzogullar, 1993).

The early biochemical markers of methomyl treatment are enzyme induction, activation of oncogenes and markers of oxidative damage (Poulsen and Loft, 1995). The change in the expression level of oncogene by methomyl treatment was examined for three oncoprotein: c-Raf-1, c-Jun and c-Fos in bone marrow and liver cells. Raf kinases are signal-integrating enzymes that have the ability to switch tyrosine kinase signalling to serine/threonine phosphorylation and connect growth factor receptors with transcription factor. The connection involves a cascade of protein kinases that is essential for cellular proliferation and differentiation of species ranging from worms to human. Inhibition ofraf expression will stop cell proliferation and development, where its involvement is known in mitogenic signal transduction in many cell types (Daum et al., 1994). The resulted blot of the bone marrow cells showed a low level of c-Raf-1 and c-Fos expression with treatment, indicating inhibition of Ras mediated
growth signal in these cells. This is the first study on the molecular mechanism of inhibition of cell growth by carbamates.

Ras proteins in cell cycle progression are required at least at two stages, the first upon the exit from and entry into G0-G1, and the second, as cells enter S phase (Moodie and Wolfman, 1994). Since Raf kinase represents the critical transformation effector of Ras (Daum et al., 1994; Avruch et al., 1994), the expression of Raf-1 protein will be in the same line with Ras protein in cell cycle progression. The activation of Ras protein in cell proliferation and differentiation are followed by activation of Raf-kinases, where it is together with MEK (mitogen extracellular receptor-regulated kinase) stimulate MAPK signal, which in turn induces c-Fos and c-Jun to form AP-1 complex which allows the cells to enter S phase (Daum et al., 1994). In bone marrow cells, c-Fos was reduced in treated group compared with the control group. In liver cells c-Jun induction was not affected by the treatment with methomyl.

Microsomal cytochrome P-450 dependent monooxygenase are recognized as key factors in insecticide metabolism. These enzymes are bound to endoplasmic reticulum membrane and are considered a good marker in detecting the cytotoxicity of compounds (Darnell et al., 1990; Stryer, 1988; Madan et al., 1995; Poulsen and Loft, 1995). The induction of P-450 in liver cells with methomyl treatment was expected where this enzyme system plays a role in methomyl metabolism. Even some transformation of xenobiotic by P-450 could convert it to highly reactive electrophiles that bind covalently to DNA (Poulsen and Loft, 1995), the biotransformation of methomyl by P-450 does not convert it to highly reactive electrophile as reported by Harvey et al. (1973).
In this study, there was no change in GST induction with methomyl treatment. This is due to the fact that this enzyme has different families and does not interfere in methomyl metabolism. Harvey et al. (1973) reported that no methomyl or S-methyl N-hydroxy thioacetimidate was detected in the metabolism of methomyl in rat, and the urinary metabolite was showed to be devoid of either S-oxide or S-dioxide methomyl. Other studies reported the depletion of glutathione by diethyl carbamate, this depletion of GSH was due to conversion of GSH to GSSG (Stefan et al., 1996; Kleener and Alexander, 1986; Heikkila et al., 1976).

As it was suggested earlier, there is a possible damage to the DNA as a result of the interference of carbomoyl group with the nucleotide synthesis. To investigate the later point, the rate of DNA synthesis was examined using tritiated thymine. No incorporation was resulted due to the fact that this thymine is not necessarily used in thymidine biosynthesis due to the presence of very low proportion of \(^3\)H-methyl thymine compared to endogenous thymine. The oxidative DNA damage by methomyl was observed in mice treated intraperitoneally with single dose of methomyl (5mg/kg). The DNA damage was determined by the detection of OH\(^8\)dG in liver DNA. The formation of OH\(^8\)dG is considered to be a relevant factor for oxidative damage to DNA. Bonatti and his colleagues (1994) suggested that methomyl may indirectly cause oxidative damage to DNA by formation of hydroxyl radicals. These hydroxyl radicals may arise due to enzyme inhibiting activities. The inactivation of glutathione reductase by carbamate was reported, this inhibition was due to carbamylating of the enzyme (Jochheim and Baillie, 1994).

In view of the present results, methomyl caused a significant decrease in bone marrow cell count. The disruption of cell cycle by methomyl treatment may be due to
blocking the cytoplasmic division. Where there was a reduction of c-Raf-1 and c-Fos expression in the methomyl treated bone marrow cells. It is not clear how methomyl inhibits the expression of c-Raf-1 and c-Fos. It might be interesting to investigate the molecular mechanism of inhibition of cell division by methomyl to have a clear picture of methomyl effect in mammalian cell. Methomyl was found to be a mild cytotoxic chemical for mammals.
Conclusion

The effect of methomyl (carbamate) was investigated in a rat model system.

The following results were observed in this study:

1. Methomyl was found to inhibit rat bone marrow cell division.

2. Methomyl treatment did not inhibit the synthesis of DNA.

3. Expression of c-fos and c-raf-1 in rat bone marrow cells was decreased after methomyl treatment.

4. Expression of Cytochrome P-450 (1A1) in rat liver cells was significantly increased after methomyl treatment.

5. Expression of Glutathione-S-transferase in rat liver cells did not change significantly after methomyl treatment.

6. Methomyl is a mild cytotoxic compound.
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تم دراسة السمية الخلية للكرابامات ميثومايل على الفئران، و ذلك بتحليل التغيير في عدد خلايا نخاع العظم بعد معالجتها بالميثومايل. فوجد أن المعالجة بالميثومايل تسبب ارتفاعاً معنوي في عدد خلايا نخاع العظم للفئران، دالاً بذلك إلى تثبيط لانقسام الخلايا. تحليل الحمض النووي المتقصي الأكسجين (DNA) للحيوانات المعالجة والغير معالجة بالميثومايل دلت أن الميثومايل لا يثبط تثبيت هذا الحمض النووي. كذلك فحص تأثير الميثومايل على النواتج الجينية الخاصة بطرق نقل إشارات النمو داخل الخلية (Ras)، حيث وجد أن المعالجة بالميثومايل تثبط تمثيل الجينات التي تعتبر عنصر رئيسي في الفعل الأولي لتنشيط طريقة نقل إشارات النمو في الخلايا (c-Ras). هذه النتائج أظهرت أن المعالجة بالميثومايل تثبط انقسام الخلايا عن طريق تثبيط، و لو بشكل جزئي، نقل إشارات النمو داخل الخلية. سمية الكرابامات على الفئران تم اختيارها بتحليل تمثيل بروتين السينيكروم 450 و بروتين GST و الذان يمثلان مؤشرًا للتأثير السمي. لقد وجد أن المعالجة بالميثومايل نتجت عن التمثيل الجيني لـ بروتين السينيكروم 450 - و بروتين GST، والذي يمثل نوعًا من إشارات النمو داخل الخلية و أن المعالجة بالميثومايل أظهرت في الاستنتاجات الجينيًا تثبيط بروتين السينيكروم 450 و كلاً من GST و P- عن طريق التثبيط سمية. و هذا دليل بأن الميثومايل مركب ذو سمية متوسطة على النباتات و أن السينيكروم 450 يساهم في أيضًا هذا المركب. و من ثم يفضل استخدام مبيد الميثومايل تحت احتياجات خاصة.
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