Original Article

Lethal and sublethal effects of a methomyl-based insecticide in *Hoplobatrachus rugulosus*

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Abstract: The aim of this study was to investigate the lethal and sublethal toxicity of a methomyl-based insecticide in *Hoplobatrachus rugulosus*, as methomyl-based insecticides are applied in massive amounts and agrochemicals have effects on the decline in amphibian populations. To evaluate the toxic effects of methomyl from agricultural application, a methomyl-based insecticide containing 40% methomyl was selected. The median lethal concentration of 96 hours of methomyl exposure was 8.69 ppm for *H. rugulosus* tadpoles. The lethal concentration also produced severe histological damage in the livers and kidneys of the exposed tadpoles. The sublethal concentration used for methomyl was 144 ppb during the metamorphosis period. It was found that the sublethal concentration of the methomyl compound could decrease growth, metamorphosis time, and size, disturb biochemical parameters, and produce histological damage. In livers, methomyl effects increased oxidative stress and dramatically decreased the glycogen level of the treated froglets. Mononuclear infiltration, blood congestion, amorphous substances, and hepatocytes vacuolization were observed throughout liver tissue. The methomyl-based insecticide also increased oxidative stress and decreased nitric oxide levels in the kidneys of the exposed froglets. Renal tissue damage including blood congestion, amorphous substances, and Bowman's capsule spaces reduction were found in the methomyl exposure group. The methomyl compound also produced vacuoles in various stages of oocytes, but no histological damage was found in testicular tissue. Our results indicated strong toxic effects of the methomyl-based insecticide on *H. rugulosu*s, a broadly tolerant anuran. (DOI: 10.1293/tox.2016-0039; J Toxicol Pathol 2017; 30: 15–24)

Key words: methomyl, *Hoplobatrachus rugulosus*, LC$_{50}$, sublethal toxicity

Introduction

Methomyl, an insecticide of the carbamate type, has been classified as a potentially harmful chemical[1]. It has been applied to control many pests, such as insects, snails, and worms[2]. As a result of its broad effects, massive amounts of methomyl-based insecticides have been used without being recorded for almost half a century. Therefore, methomyl residue is frequently detected in agricultural products, soil, and natural water[3]. The primary effect of methomyl compounds is to interrupt the function of acetyl cholinesterase, causing vulnerability to damage in the nervous system[4]. Hence, contamination of the environment with methomyl-based insecticides might cause serious harm to nontarget organisms.

Pesticide contamination is considered an important factor that has been implicated for 20 years in the amphibian crisis[5]. Given the overlapping distribution of amphibians with important agricultural areas in many countries and the enormous amounts of pesticides utilized in such areas each year, most amphibians are suffering from pesticide contamination. Nowadays, population decline and species extinction have changed for the worse in anurans. Nearly 168 species have become extinct, and at least 43% of the rest have been decreasing globally[6]. In Southeast Asia, over 800 species of amphibians have been recorded[7], and large numbers of new species have continuously been discovered since 1997[8]. However, approximately 58,000 tons of pesticides are applied each year in the agricultural areas of the region[9]. For this reason, the population and diversity of amphibians in Southeast Asia have been continuously threatened by pesticide contamination.

Amphibians play roles in both terrestrial and aquatic ecosystems. Decrease of their populations could cause serious ecological problems, for example, outbreaks of insect pests and mosquitoes. In addition, freshwater fishes, small reptiles, and other amphibian predators are affected by the decline of amphibian populations.

Accordingly, pesticide toxicity in amphibian species is important. Unfortunately, study of the effects of methomyl contamination on nontarget species, especially amphibians, has been insufficient. Therefore, the present study aimed to evaluate the toxic effects of a methomyl-based insecticide

on the East Asian bullfrog (*Hoplobatrachus rugulosus*). This species is a representative of the amphibians widely distributed throughout Eastern and Southeast Asia[10], inhabiting various types of habitats and having a broad range of tolerance. Besides, *H. rugulosus* is a good source of protein for local people and also an economically important animal in Thailand.

Materials and Methods

Animal and rearing condition

The present study was performed with 7-day-old *H. rugulosus* larvae, the mean total length of which was 2.46 ± 0.034 cm. Newly hatched tadpoles were obtained from the Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Thailand. Larvae were transferred to plastic tanks containing 4 L of dechlorinated tap water and allowed to acclimatize to laboratory conditions for a week. They were fed a commercial tadpole diet, and the accumulated residues were removed twice daily. The present study was approved by the Institutional Animal Ethics Committee of Chiang Mai University, Thailand.

Since the commercial grade of methomyl is always applied to agricultural fields, a methomyl-based insecticide with 40% active ingredient was selected in the present study. The methomyl-based insecticide (Lannate 40% SP) was purchased from Dupont Co., Ltd., Thailand. All treatment solutions were made with dechlorinated tap water. The treated concentrations were calculated from the product with 40% methomyl. Therefore, we used the term methomyl for methomyl-based insecticide (Lannate 40% SP) in this paper.

Experimental design

Lethal toxicity: Two hundred and twenty tadpoles were randomly divided into eight groups. Each group was separated into two replicates and supplied 4 L of tested solutions. The control group was kept in pesticide-free water, and seven experimental groups were exposed to 3, 5, 7, 9, 10, 15, or 20 ppm of the methomyl-based insecticide. Tested solutions were renewed every 24 hours to maintain the pesticide concentration. Dead tadpoles, if present, were immediately removed, and mortality was recorded every 24 hours for 96 hours. At the end of the experiment, all tadpoles were anesthetized for histological examination.

Sublethal toxicity: One hundred and eighty tadpoles were divided into two groups, each with three replicates. Ninety tadpoles were kept in pesticide-free water to serve as a control group. Another group was exposed to a sublethal concentration of the methomyl-based insecticide. The chosen concentration, the LC_{50} (0.144 ppm), to serve as the sublethal concentration was obtained from a previous study[11].

During metamorphosis, each condition was renewed every three days. Snout-vent length, developmental stage, and mortality of the tadpoles were recorded. The complete metamorphosis of *H. rugulosus* was recorded as days to metamorphosis, and all of the tadpoles were sampled for histological and biochemical examinations.

Histological examination: Livers, kidneys, and gonads were carefully removed and fixed in 10% formalin. Hematoxylin and eosin staining of 5-μm-thick paraffin-embedded sections was examined under a compound microscope.

Biochemical examinations

Glycogen assay: The method for estimation of glycogen in the liver was slightly modified from the method described by Seifter *et al.*[12]. Briefly, 0.05 g of liver was boiled in 30% (w/v) KOH for 10 min. Then 95% (v/v) ethanol was added into the cooled solution, which was subsequently boiled again. The mixture was then centrifuged at 3,000 rpm for 15 min. The pellet was redissolved in 1 mL of distilled water and mixed with 10 mL of anthrone reagent. The mixture was then boiled for 10 min and immediately placed in an ice bath. The absorbance of the solution was measured at 620 nm and calculated with reference to a glucose standard curve. Finally, the concentration of glycogen was expressed in mg/g wet weight.

MDA assay: The method for MDA measurement was slightly modified from that of Hritcu *et al.*[13] using tetramethoxypropane as a standard. The livers and kidneys were weighed and homogenized with phosphate buffer (pH 7.0) and centrifuged at 3,000 rpm for 10 min. Two hundred microliters of supernatant was mixed with 500 μL of 0.85% (w/v) NaCl, 1,000 μL of 100% (w/v) trichloroacetic acid in 0.06 M HCl, and 200 μL of 0.28 mM thiobarbituric acid. The mixture was boiled for 20 min and centrifuged at 3,000 rpm for 10 min. The supernatant was read at 532 nm. The results are presented in μmol/mg protein.

Nitric oxide assay: Nitric oxide was measured by its metabolites (nitrate and nitrite). The procedure was performed according to the method described by Kumar *et al.*[14] using $NaNO_3$ as a standard. Two hundred microliters of homogenized tissue or standard was mixed with 20 μL of NaOH and 30 μL of $ZnSO_4$. The sample was added into a solution containing 5×10^2 units of nitrate reductase, 20 μL of 0.2 M N-trimethylaminoethane sulfonic acid, and 20 μL of 0.5 M of sodium formate. The mixture was anaerobically incubated at room temperature for 20 min. After complete incubation, the mixture was added to 1 mL of distilled water and centrifuged at 260 rpm for 5 min. Supernatant was mixed with 20 μL of 1% (w/v) sulfanilamide in 15% (v/v) phosphoric acid and incubated at room temperature for 10 min. Finally, 20 μL of 0.1% (w/v) N-(1-naphthyl) ethylenediamine was added to the solution. The absorbance of each sample at 540 nm was recorded. The data were displayed in μmol/mg protein.

Protein assay: The level of protein content from each tissue was estimated following the method described by Bradford[15], and the bovine serum albumin (BSA) was used as a standard. Five milligrams of Coomassie Blue G were dissolved in 1 mL 25% (v/v) methanol containing 42.5% (v/v) H_3PO_4. One volume of prepared dye was diluted with four volumes of distilled water, and 0.04 mL of each sample was mixed with 2 mL of diluted dye. The absorbance was

read at 590 nm.

Statistical analysis: The obtained data were statistically analyzed with IBM SPSS Statistics version 20.0 for Mac OS X (IBM Corp., Armonk, NY, USA). Lethal concentration values were calculated using probit analysis. Student's t-test was used to analyze mortality, growth, metamorphosis traits, and biochemical values for chronic exposure.

Results

Lethal toxicity

Mortality: Mortalities were recorded only in the methomy-treated groups (Fig. 1). After 24 hours of exposure, mortalities were found in all treated groups, even the group with the lowest concentration (3.0 ppm). Mortalities increased with the increase in methomyl concentration, and 100% mortality was observed in the group exposed to 20.0 ppm methomyl.

The mortality of tadpoles in each group was calculated; the LC_{10}, LC_{50}, and LC_{90} and 95% confidence limits are presented in Table 1. It was found that the value of each LC point decreased with increasing exposure time. These results indicated that the mortality of tadpoles depends on the concentration and tim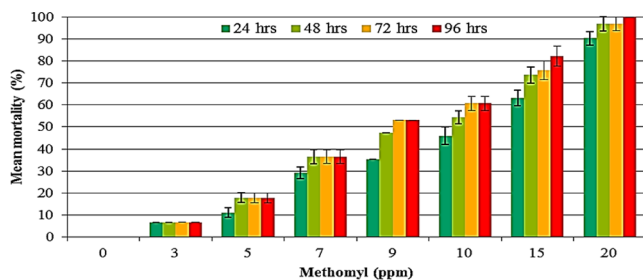e. Moreover, the present study found that the 96 hr LC_{50} value for the methomyl-based insecticide was 8.69 ppm for *H. rugulosus*.

Sublethal toxicity

Survivorship: The cumulative mortalities of *H. rugulosus* are displayed in Fig. 2. For the methomyl-exposed group, mortality first appeared on the 21st exposure day and continuously increased up to 55% at the end of experiment. Only a minority of dead tadpoles were observed in the control group.

Growth: Changes in snout-vent length (SVL) of tadpoles with time in both the control and treated groups are presented in Fig. 3. It was found that the SVL of tadpoles in both the control and treated groups continuously increased until almost the end of the experiment. Obviously, the SVL of the methomyl-exposed group of tadpoles was relatively lower than that of the control group throughout the experiment. In addition, the treated group began to show slight changes in SVL (within 43 days), which was later than in the control group (within 37 days). However, at the end of the treatment period, SVL was not significantly different between the two groups.

Metamorphosis: Days to metamorphosis and SVL at metamorphosis are presented in Table 2. Methomyl-exposed

Fig. 1. Mean percentages of mortalities of tadpoles with exposure to different concentrations of methomyl over the course of 96 hr.

Table 1. Lethal Concentrations of Methomyl (ppm) (95% Confidence Limits) with Exposure Time for *H. rugulosus*

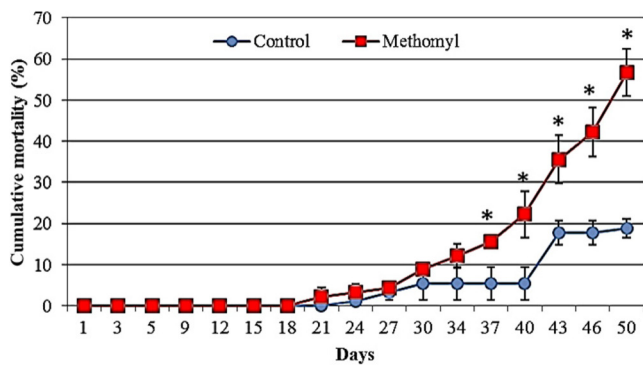| Point	Exposure times (hr)			
	24	48	72	96
LC_{10}	4.12 (2.22−5.41)	3.94 (1.83−4.85)	3.8 (1.85−4.62)	3.67 (2.11−4.73)
LC_{50}	10.88 (9.75−12.41)	9.72 (8.74−10.99)	8.87 (8.02−9.92)	8.69 (7.88−9.68)
LC_{90}	17.65 (15.47−21.21)	15.81 (13.95−18.83)	14.23 (12.67−16.74)	13.71 (12.24−16.05)

Fig. 2. Cumulative mortality of *H. rugulosus* with exposure to 0.144 ppm methomyl during metamorphosis compared with that of the control group. *P<0.05 vs. control group (Student's t-test). Error bars represent the standard error of the mean.
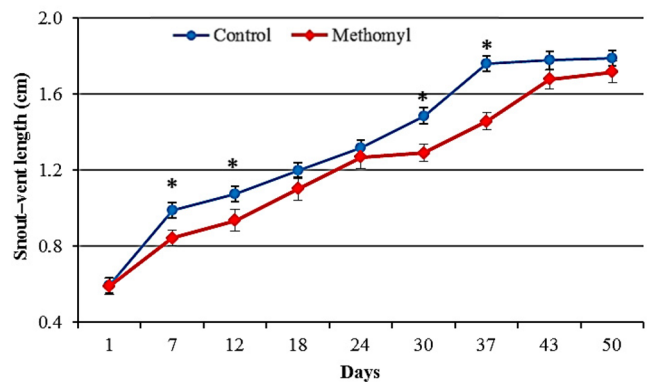

Fig. 3. Snout-vent length of *H. rugulosus* exposed to 0.144 ppm methomyl during metamorphosis compared with that of the control group. *P<0.05 vs. control group (Student's t-test). Error bars represent the standard error of the mean.

tadpoles reached the complete metamorphosis stage significantly earlier than the control group. However, the treated group had a shorter SVL than the control group.

Biochemical assays: Exposure of tadpoles to 1.44 ppm methomyl during metamorphosis produced many biochemical alterations (Table 3).

Liver: Biochemical analysis of the livers indicated that the MDA level was significantly higher in the methomyl-exposed group (1.84 + 0.04 µmol/mg protein) compared with the control group (0.66 + 0.04 µmol/mg protein). On the other hand, the glycogen level in the liver was dramatically higher in the control group (105.98 ± 7.30 mg/g) compared with that in the methomyl-treated group (4.51 ± 1.10 mg/g).

Kidneys: For the kidney, the level of MDA in the methomyl-treated group (0.68 + 0.06 µmol/mg protein) was significantly greater than in that of the control group (0.45 µmol/mg protein). Furthermore, the nitric oxide (NO) concentrations in this tissue were also statistically lower in the treated group (0.127 µmol/mg protein) compared with the control group (0.442 + 0.02 µmol/mg protein).

Histological examination

Lethal toxicity: To evaluate the histological effects of methomyl at a lethal concentration, livers and kidneys of tadpoles from the group treated with 20 ppm were examined and are shown in Figs. 4B and 5B.

Liver: In the control group (Fig. 4A), liver tissues consisted of hepatocytes arranged in hepatic cords intervening with hepatic sinusoids. Hepatocytes were typically hexagonal in shape with large, round central nuclei, and the cytoplasm was regularly distributed with very fine granules of glycogen and lipid droplets. Sinusoids were hepatic capillaries carrying erythrocytes in rows. In contrast, the livers of the group treated with 20 ppm methomyl (Fig. 4B) showed severe histological damage based on the number of large vacuoles in the hepatocytes with cytoplasmic disintegration.

Kidneys: In addition to the damage in the liver, 20 ppm methomyl also produced histological damage in the kidneys (Fig. 5B). Typically, kidney tissue of amphibians consists of a renal corpuscle, renal tubules, and interstitial hematopoietic tissue. Renal corpuscles are small capillary tufts covered in a Bowman's capsule. The renal tubules are characterized by a simple cuboidal epithelium as displayed in Fig. 5A. In the treated group, histological damage could be clearly observed in both the renal corpuscle and renal tubule of the kidney. Dilatation of the Bowman's capsule and renal tubule was observed throughout the tissue, as shown in Fig. 5B.

Sublethal toxicity: The sublethal experiment also showed histological alterations in the liver, kidneys, and gonads (Figs. 6B, 7B, 8B).

Liver: In the control group, hepatocytes were of normal shape and regularly arranged throughout tissue (Fig. 6A). In contrast, the methomyl exposed group showed various histological alterations in the livers (Fig. 6B). The hepatocytes appeared to be swollen and to have cytoplasmic disintegration accompanied by vacuolization. In addition, blood congestion was focally observed wherein amorphous substances accumulated inside sinusoids and blood vessels. Mononuclear infiltration observed throughout the tissue indicates liver inflammation.

Kidneys: Kidney tissue of control froglets (Gosner stage 46) is shown in Fig. 7A. Similar to 11-day-old tadpoles, no histological lesion was observed in the renal corpuscle, renal tubules, and interstitial hematopoietic tissue of untreated frogs. However, exposure to 144 ppb of methomyl produced histopathological evidence in the kidney tissue of tadpoles treated with methomyl (Fig. 7B). In addition, blood congestion and amorphous substances appeared in interstitial tissues. Moreover, blood congestion was also found inside the Bowman's capsules, and there was a reduction in space in the Bowman's capsules in the methomyl-exposed group (Fig. 7B).

Gonads: Similar to the liver and kidneys, gonadal tissue responded to the effects of methomyl. Histological examination revealed that methomyl showed toxicity in the ovarian tissue (Fig. 8B), whereas no histological damage was observed in testicular tissue (Fig. 9B). Of note, methomyl could induce histopathological changes in ovarian tissues after exposure during the metamorphosis period. A number of vacuoles were discovered in various stages of oocytes inside the ovary (Fig. 8B). On the other hand, no histopathological damage was observed in male gonadal tissue of either group (Figs. 9A and 9B).

Table 2. Metamorphosis Traits under Different Rearing Conditions

Groups	Days to metamorphosis (Gosner stage 46)	SVL at metamorphosis (Gosner stage 46)
Control	43.81 ± 0.62	1.86 ± 0.04
Methomyl	36.67 ± 1.37*	1.42 ± 0.04*

Values are shown as the mean ± SE. *P<0.05 vs. control group (Student's *t*-test).

Table 3. Biochemical Analyses in Livers and Kidneys of *H. rugulosus*

| Groups | Liver | | Kidney | |
	MDA (µmol/mg protein)	Glycogen (mg/g)	MDA (µmol/mg protein)	Nitric oxide (µmol/mg protein)
Control	0.66 ± 0.04	105.98 ± 7.30	0.45 ± 0.00	0.442 ± 0.02
Methomyl	1.84 ± 0.04*	4.51 ± 1.10**	0.68 ± 0.06*	0.127 ± 0.00*

Values are shown as the mean ± SE. *P<0.05 vs. control group (Student's *t*-test). **P<0.01 vs. control group (Student's *t*-test).
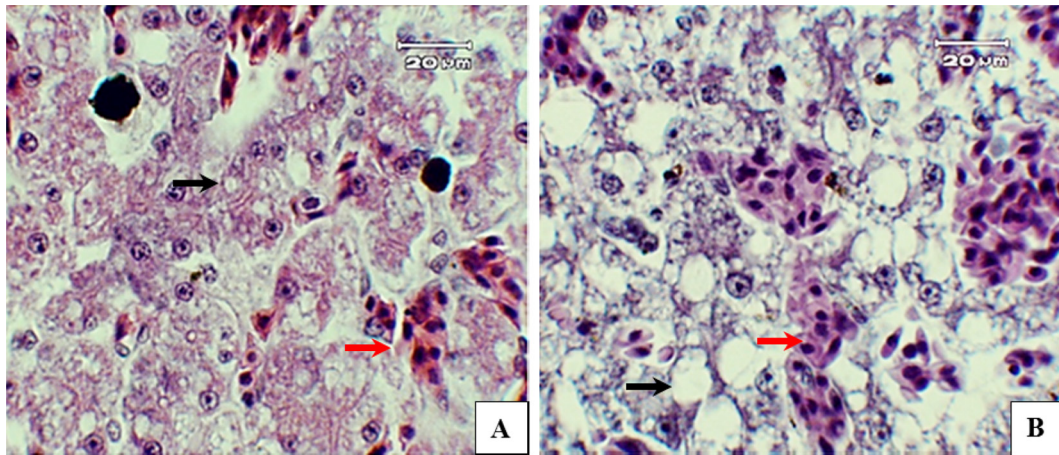
Fig. 4. Liver tissues of *H. rugulosus* tadpoles exposed to 20 ppm of methomyl (B) over the course of 96 hr compared with those of the control group (A). Symbols: black arrow, vacuole; red arrow, sinusoid. H&E staining. Magnification, 400×.
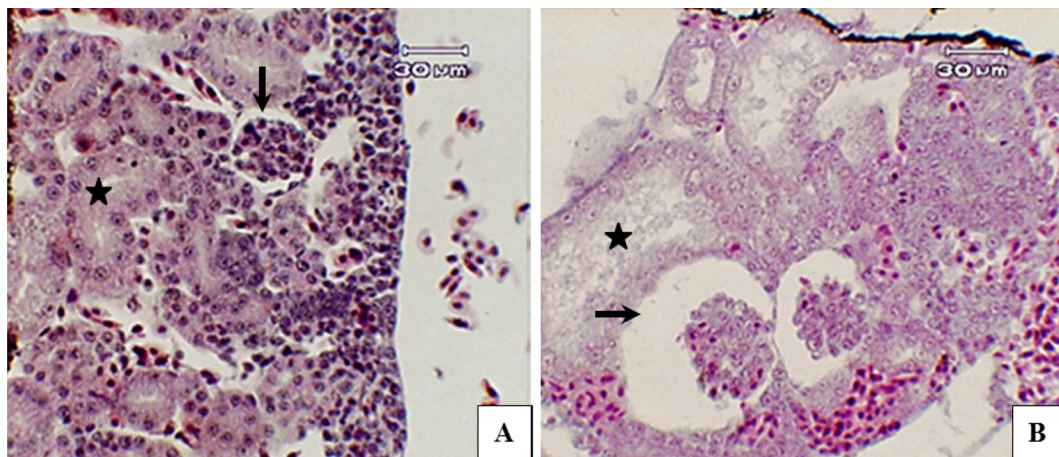

Fig. 5. Kidney tissue of *H. rugulosus* tadpoles exposed to 20 ppm methomyl (B) over the course of 96 hr compared with that of the control group (A). Symbols: black arrow, Bowman's capsule space; black star, lumen of renal tubules. H&E staining. Magnification, 200×.
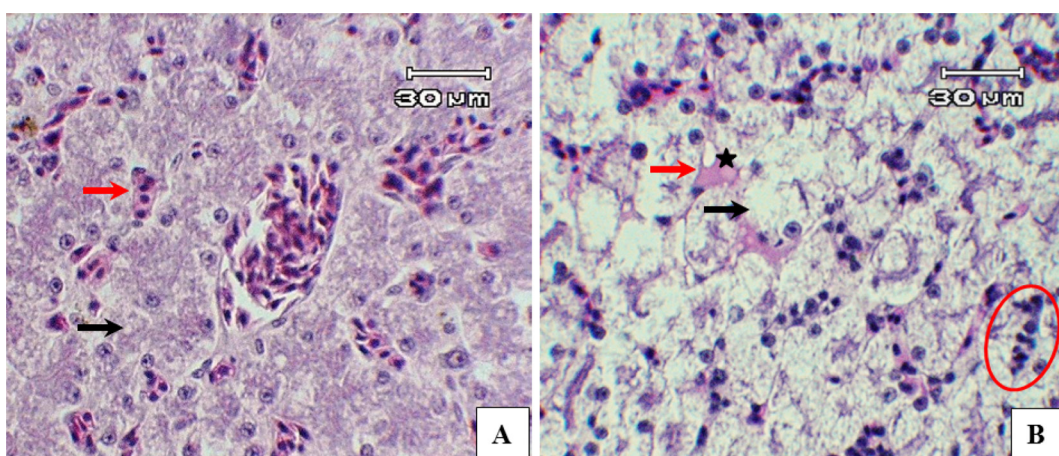

Fig. 6. Histological features of livers from the control (A) and group treated with 0.144 ppm methomyl (B). Symbols: black arrow, vacuole; red arrow, sinusoid; red circle, mononuclear cell infiltration; black star, amorphous substance. H&E staining. Magnification, 200×.
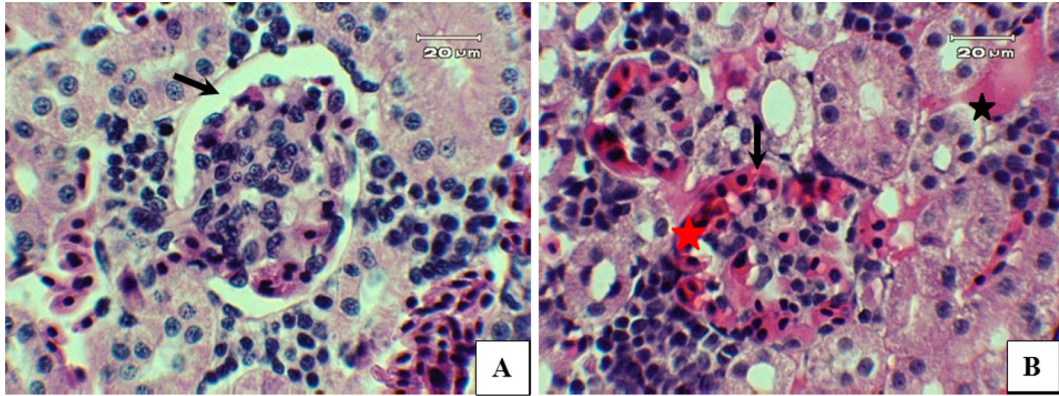
Fig. 7. Kidney tissue of *H. rugulosus* exposed to 0.144 ppm of methomyl (B) during metamorphosis as compared with that of the control group (A). Symbols: black arrow, Bowman's capsule space; red star, blood congestion; black star, amorphous substances. H&E staining. Magnification, 400×.
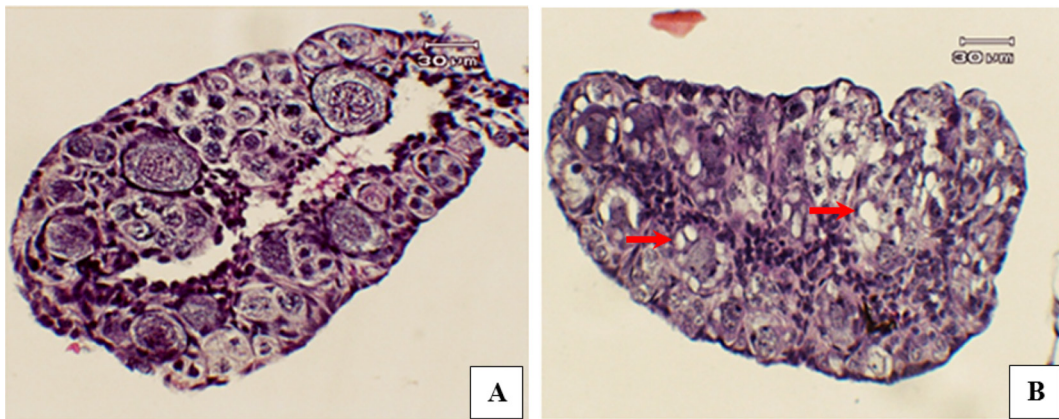

Fig. 8. Ovarian tissue of *H. rugulosus* exposed to 1.44 ppm methomyl (B) during metamorphosis compared with that of the control group (A). Symbols: red arrows, vacuole. H&E staining. Magnification, 200×.
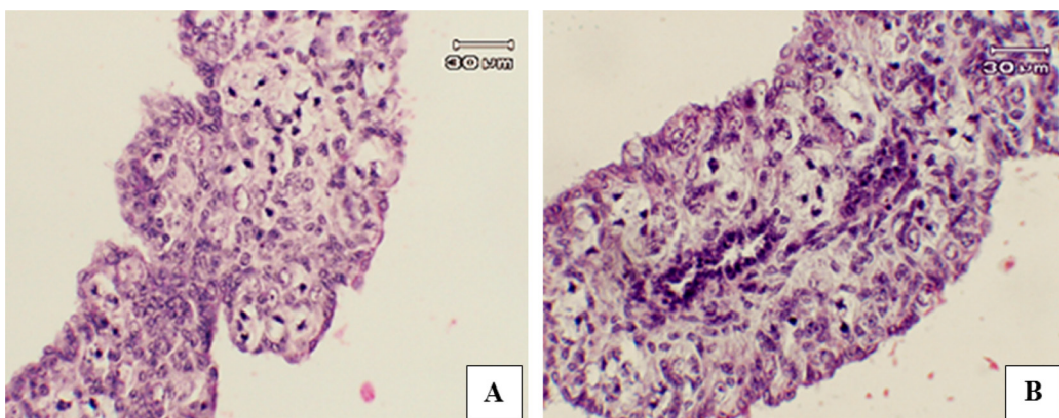

Fig. 9. Testicular tissue of *H. rugulosus* exposed to 1.44 ppm methomyl (B) during metamorphosis compared with that of the control group (A). H&E staining. Magnification, 200×.

Discussion

Lethal toxicity

Mortality: The results of the present study suggest that methomyl could have a substantial effect on the mortality of *H. rugulosus*. This is the first report of the toxicity of methomyl in *H. rugulosus*. Although *H. rugulosus* was previously classified as a broad tolerant species[10], our experiment demonstrated that this species is sensitive to exposure to methomyl [LC_{50} = 10.88 (24 hr); 9.72 (48 hr)] when compared with other species of anurans. For example, the LC_{50} values of methomyl were 23 ppm (24 hr) in *Bufo vulgaris formosus*[16], 27.16 ppm (48 hr) in *Rana limnocharis*[17], and 40 ppm (48 hr) in *Bufo bufo japonicus*[18]. However, information about methomyl toxicity in amphibians is still insufficient. Most of the previous research mainly focused on the toxicity of methomyl in mammals and freshwater fishes. Furthermore, methomyl has been indicated as very highly toxic to aquatic organisms and highly toxic to terrestrial species. The LC_{50} values for the topmouth gudgeon (*Pseudorasbora parva)* and Nile tilapia (*Oreochromis niloticus*) were reported to be 0.425 ppm[19] and 1.81 ppm[20], which are considered to be very low and to result in high toxic effects. On the other hand, the LC_{50} values of other freshwater fishes were reported to be 7.7 ppm in the bluegill (*Lepomis macrochirus*) and 6.8 ppm in the cutthroat trout (*Oncorhynchus clarki*)[21]. Although the LC_{50} value of *H. rugulosus* in this study (8.69 ppm) was higher than those previously reports for freshwater fishes, the difference in pesticide formation and life stage of this bullfrog could place the LC_{50} values into the same range as freshwater fishes in previous experiments. In amphibians, not only species differences[22] but also differences in the ages of the tadpoles and chemical concentration affect the LC_{50} value. Ezemonye and Tango[23] showed that the LC_{50} value (96 hr) of an atrazine herbicide for the broad-banded grassland frog, *Ptychadena bibroni*, increased with the increase in tadpole age. In addition, the LC_{50} of 95% methomyl in the largemouth bass, *Micropterus salmoides*, was 1,250 ppb, while the LC_{50} of 24% methomyl was 760 ppb[24]. Due to *H. rugulosus* being defined as a broad tolerant species, methomyl has a high possibility to have a stronger effect against other anuran species.

Sublethal toxicity

Survivorship: The survival effect at the sublethal concentration confirmed the strong toxicity of methomyl in *H. rugulosus*. Although the tested concentration in this phase was about 60 times lower than the LC_{50} value, less than half of the *H. rugulosus* tadpoles were able to survive. In this study, the lowest observable adverse effect concentration (LOAEC) of methomyl for the early life stage of *H. rugulosus* was lower than 144 ppb; meanwhile, the LOAEC value of methomyl in the freshwater fathead minnow (*Pimephales promelas*) during the early life stage was 117 ppb[24]. Even the LC_{50} value of methomyl was obviously higher for *H. rugulosus* (8.69 ppm) than for other species of freshwater fishes, but the chronic toxicity was quite similar. Our results for

the lethal and sublethal toxicities of methomyl in this study indicated strong effects on survivorship of *H. rugulosus* and revealed a trend regarding the methomyl toxicity concentration that could harm other amphibians as well.

Growth and metamorphosis: Our study showed that methomyl could decrease the body size at metamorphosis as well as the time to metamorphosis of *H. rugulosus*. This was therefore evidence of the endocrine-disrupting effect of methomyl. Clearly, metamorphosis of amphibians is directly regulated by thyroid hormones[25, 26]. Moreover, the levels of thyroid stimulating hormone (TSH), thyroxine (T4), and 3,3′,5-triiodothyronine (T3) affected the progression of metamorphosis events as well as the ratio of T3 and T4[27]. The disruption of thyroid hormone receptor gene expression and/or thyroid stimulating hormone could interrupt the whole metamorphosis event. However, the mechanism of methomyl in hormonal disruption is still unclear. The effect of methomyl on the thyroid gland was noticed earlier in mammals. For instance, Busey[28] found a significant increase in thyroid gland weight in Charles River rats treated with 200–400 ppm methomyl by oral administration. Meanwhile, Porter *et al.*[29] discovered that the thyroxine level increased in Sprague-Dawley rats exposed to a methomyl mixture. Besides the effects on body size at metamorphosis and time to metamorphosis, methomyl also influenced the growth of *H. rugulosus.* The growth rate of the anuran tadpole is normally greater in the premetamorphosis period (Gosner stages 21–30), and body length slightly increases during the premetamorphosis period (Gosner stages 31–40)[30]. Therefore, exposure to methomyl not only decreased time to metamorphosis but also reduced the premetamorphosis period, resulting in smaller sizes in the treated tadpoles throughout almost the entire experiment (Fig. 3). On the other hand, no evidence of interaction of methomyl with the thyroid pathway and no developmental endpoints or thyroid histopathological changes were observed in the amphibian metamorphosis assay[31]. Differences in species, stage of exposure, and stage of termination could affect the results. The levels of TSH, thyroid hormone (TH), corticotrophin-releasing hormone (thyrotropin-releasing hormone), and TH receptors were different in each developmental stage[32, 33]. Some TH receptors have been reported to be expressed in very early stages of life[32]. However, our results were insufficient to strongly guarantee the TH-disrupting effect of methomyl. Further studies are necessary to substantiate information about the endocrine-disrupting effect of methomyl.

Organ toxicity

Liver: In *H. rugulosus*, exposure to both lethal and sublethal concentrations of methomyl could produce severe liver damage. The livers of methomyl-exposed tadpoles exhibited a number of large vacuoles in the hepatocytes along with cytoplasmic disintegration. This result is in agreement with previous reports on histological lesions in livers of methomyl-treated organisms. Methomyl-treated mice previously showed cytoplasmic vacuolization[34].

Blood congestion was also reported in methomyl-exposed Sprague-Dawley rats[35]. A dilated central vein and sinusoids have been reported in some methomyl treated-rodents[34, 36, 37]. However, other histological responses to methomyl in anurans and rodents could possibly be different depending on their histology and physiology. The substance found in hepatic vacuoles of methomyl-treated tadpoles could not be identified in this study; however, abnormal tissue could alter liver function and cause failure of the homeostatic mechanism, leading to death. Crucially, all of these toxic effects at lethal concentrations were observed in the bullfrogs within 96 hours of exposure. In addition, exposure to a sublethal concentration of methomyl increased the MDA level in the liver and kidney of *H. rugulosus*. MDA is one of the important biochemical compounds used to indicate reactive oxygen species (ROS) generated from lipid peroxidation[38]. In general, ROS, which can be neutralized by a variety of antioxidants, are generated by cellular metabolism. However, excessive ROS would damage various chemical and biological membranes and have been suggested as a cause of toxicity in several organs[39, 40]. Furthermore, oxidative stress has also been shown to affect organ dysfunction and tissue damage[41]. In the present experiment, organ dysfunction and histological damage were observed in livers and kidneys of the methomyl-treated group. The livers of the treated frogs had dramatically low levels of glycogen, indicating failure to preserve energy for basic activities. Additionally, histological examination revealed severe damage in the liver leading to liver dysfunction. The hepatotoxicity of methomyl was previously reported to result in increasing levels of MDA together with histological damage[34, 37]. Furthermore, various liver enzymes were reported to be affected by exposure to methomyl[37]. Since the liver performs a variety of vital functions (nutrient metabolism, detoxification, and homeostasis), liver damage could lead to serious pathology and death.

Kidneys: In addition to the liver, methomyl also rapidly produced histological damage in the kidneys of *H. rugulosus*. The dilation of renal tubules and Bowman's capsule space indicated the accumulation of fluid inside the kidney in treated frogs at lethal concentration. Fluid retention is related to kidney damage and acute renal failure[42, 43]. Histological damage of the kidney, in addition to the liver, has been reported in the literature. Congestion of glomerular capillaries, endothelial cell swelling, and degenerated tubules have been observed in several species of methomyl- treated rodents[36, 44, 45]. Moreover, exposure to a sublethal concentration also changed the histological features and biochemical parameters in the kidneys of the methomyl-treated frogs. The increase in MDA level was hypothesized as a cause of histological damage and NO reduction. Similar to the liver, ROS has been reported to exert histological toxicity in the kidneys[46]. Moreover, the level of NO in the kidneys of the methomyl-treated frogs was lower than that in the control group of frogs. A decreasing NO level in the kidneys has been reported to be an obvious marker for chronic kidney disease in many models[47–49], and ROS has been suggested to be involved in the progression of kidney disease through depletion of NO[47, 50]. Excessive ROS and reduction of NO have also been reported as a cause of the change in renal histopathology and biochemical reaction resulting in chronic kidney disease[51].

Gonads: For anurans, gonadal differentiation can be clearly observed in 4- to 5-week-old tadpoles (Gosner stage 27–28)[52]. Therefore, the effects of methomyl on *H. rugulosus* gonads could not be defined in the lethal toxicity test. However, histological damage in the ovary was found in the sublethal experiment. In the pesticide-treated group, the presence of vacuoles in oocytes could affect fertilization and/or embryonic development. Methomyl has previously been reported to have effects on reproductive toxicity in both male and female mammals. Mokhtar *et al.*[53] stated the toxic effects found in the ovary of female rats treated with methomyl, and Shanthalatha *et al.*[54] reported about the presence of vacuoles and hemorrhage in follicular cells including degenerated oocytes in female rats after treatment with sublethal doses of methomyl. The fact that the testes of froglets lacked any histological lesions in the present study is also of interest. Shalaby *et al.*[35] revealed that a sublethal concentration of methomyl induced histological damage in rat testes and revealed the toxic effect on sex glands and the testosterone level. Furthermore, histological and biochemical alteration in methomyl-treated male mice has also been reported[34]. In the testes of adult rodents, spermatogenesis was clearly observed, including pathological features. However, the histological findings for froglets in the present study (Gosner stage 46) showed immature testes without spermatogenesis inside. Therefore, the difference in histological effect of methomyl on testes observed in *H. rugulosus* might depend on the species, maturity of the testes, and/or chemical doses.

The present study clearly demonstrates the toxic effects of a methomyl-based insecticide on mortality, growth, histological and biochemical changes, and probability of endocrine-disrupting effects in *H. rugulosus*. The effects on histopathological changes of the ovary could be a serious problem affecting amphibian populations. Moreover, the extremely low level of liver glycogen indicates potential effects on living activities: preying, escaping predators, and mating. Our results might raise awareness about methomyl-based insecticide toxicities in amphibians and others nontageted species.

Disclosure of Potential Conflicts of Interest: The authors declare that there are no conflicts of interest.

References

1. Tamimi M, Qourzal S, Barka N, and Assabbane A. Methomyl degradation in aqueous solutions by Fenton's reagent and the photo-Fenton system. Separ Purif Tech. **61**: 103–108. 2008. [CrossRef]

2. Fernández-Alba AR, Hernando D, Agüera A, Cáceres J, and Malato S. Toxicity assays: a way for evaluating AOPs efficiency. Water Res. **36**: 4255–4262. 2002. [Medline] [CrossRef]

3. EPA Environmental Protection Agency of the United States. Registration Eligibility Decision (RED); Methomyl. 1998, from http://www.epa.gov/oppsrrd1/-REDs/0028red.pdf

4. IPCS International Program on Chemical Safety. Methomyl. Environmental Health Criteria 178. World Health Organization Geneva. 1986.

5. Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J, and Tsui M. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ Health Perspect. **114**(Suppl 1): 40–50. 2006. [Medline] [CrossRef]

6. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, and Waller RW. Status and trends of amphibian declines and extinctions worldwide. Science. **306**: 1783–1786. 2004. [Medline] [CrossRef]

7. Frost DR. Amphibian species of the world: an online reference. Version 5.2 American Museum of Natural History, New York. 2009, from http://research.amnh.org/herpetology/amphibia/ind ex.php.

8. Bain RH, Truong NQ, and Kien DV. New herpetofaunal records from Vietnam. Herpetol Rev. **38**: 107–117. 2007.

9. FAO Food and Agriculture Organization of the United Nations. 2014. Available from http://faostat3.fao. org/browse/E/EP/E.

10. IUCN International Union for Conservation of Nature. The IUCN Red List of Threatened Species. 2015, from http://www.iucnredlist.org

11. Trachantong W, Saenphet S, and Saenphet K. Toxicological effects of three pesticides on histological alteration in East Asian Bullfrog (*Hoplobatrachus rugulosus*). Abstacts book of The 38th congress on science and technology of Thailand (STT 38): science for the future of mankind. 323. 2012.

12. Seifter S, Dayton S, Novic B, and Muntwyler E. The estimation of glycogen with the anthrone reagent. Arch Biochem. **25**: 191–200. 1950. [Medline]

13. Hritcu L, Ciobica A, Stefan M, Mihasan M, Palamiuc L, and Nabeshima T. Spatial memory deficits and oxidative stress damage following exposure to lipopolysaccharide in a rodent model of Parkinson's disease. Neurosci Res. **71**: 35–43. 2011. [Medline] [CrossRef]

14. Kumar L, Rajput N, and Majumdar S. Nitric oxide metabolites in induced sputum: a noninvasive marker of airway inflammation in asthma. Indian Pediatr. **42**: 329–337. 2005. [Medline]

15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. **72**: 248–254. 1976. [Medline] [CrossRef]

16. Nishiuchi Y. Toxicity of formulated pesticides to fresh water organisms. LXXIV. Suisan Zoshoku. **8**: 107–112. 1980.

17. EPA Environmental Protection Agency of the United States. Environmental Effects Database (EEDB). ECOTOX Database System. 2001, from http://www.epa.gov/Ecotox

18. Hashimoto Y, and Nishiuchi Y. Establishment of bioassay methods for the evaluation of acute toxicity of pesticides to aquatic organisms. J Pest Sci. **6**: 257–264. 1981. [CrossRef]

19. Li H, Jiang H, Gao X, Wang X, Qu W, Lin R, and Chen J. Acute toxicity of the pesticide methomyl on the topmouth gudgeon (*Pseudorasbora parva*): mortality and effects on four biomarkers. Fish Physiol Biochem. **34**: 209–216. 2008. [Medline] [CrossRef]

20. Bakoulia P, Karadima C, Rouvalis A, and Iliopoulou-Georgudaki J. Acute toxicity evaluation of an insecticide used in potato cultures with the use of bioassays. Fresenius Environ Bull. **17**: 1002–1006. 2008.

21. Erickson W, and Turner L. Methomyl analysis of risks to endangered and threatened salmon and steelhead. Environmental Field Branch, Office of Pesticide Programs. 2003.

22. Trachantong W, Promya J, Saenphet S, and Saenphet K. Effects of atrazine herbicide on metamorphosis and gonadal development of *Hoplobatrachus rugulosus*. Maejo Int J Sci. **7**: 60–71. 2013.

23. Ezemonye LIN, and Tongo I. Lethal and sublethal effects of atrazine to amphibian larvae. Jordan J Biol Sci. **2**: 29–36. 2009.

24. Mayer FL, and Ellersieck MR. Manual of acute toxicity: Interpretation and database for 410 chemicals and 66 species of freshwater animals (resource publication no. 160). United States Fish and Wildlife Service. Washington DC. 1986.

25. Dodd MHI, and Dodd JM. The biology of metamorphosis. In: Physiology of the Amphibia, volume III, B Lofts (ed). Academic Press, New York. 1976.

26. Shi YB. Amphibian Metamorphosis: from Morphology to Molecular Biology. Jonh Wiley & Sons Inc. New York. 1999.

27. Miyata K, and Ose K. Thyroid hormone-disrupting effects and the amphibian metamorphosis assay. J Toxicol Pathol. **25**: 1–9. 2012. [Medline] [CrossRef]

28. Busey WM. 22-Month dietary feeding study in rats with Lannate(R) methomyl insecticide. Unpublished Report No. 201-164 from Hazleton Laboratories, Inc., Falls Church, VA., USA. Submitted to WHO by E.I. du Pont de Nemours and Co., Inc. Wilmington. 1968.

29. Porter WP, Green SM, Debbink NL, and Carlson I. Groundwater pesticides: interactive effects of low concentrations of carbamates aldicarb and methomyl and the triazine metribuzin on thyroxine and somatotropin levels in white rats. J Toxicol Environ Health. **40**: 15–34. 1993. [Medline] [CrossRef]

30. Moore JA. Physiology of the Amphibia. Academic Press. London. 1964.

31. EPA Environmental Protection Agency of the United States. EDSP: weight of evidence analysis of potential Interaction with the estrogen, androgen or thyroid pathways chemical: methomyl. 2015, from https://www.epa.gov/sites/production/files/2015–06/documents/methomyl-090301_2015–06–29_tx r0057182.pdf

32. Furlow JD, and Neff ES. A developmental switch induced by thyroid hormone: Xenopus laevis metamorphosis. Trends Endocrinol Metab. **17**: 40–47. 2006. [Medline] [CrossRef]

33. Korte JJ, Sternberg RM, Serrano JA, Thoemke KR, Moen SM, Lillegard KE, Hornung MW, Tietge JE, and Degitz SJ. Thyroid-stimulating hormone (TSH): measurement of in-

tracellular, secreted, and circulating hormone in Xenopus laevis and Xenopus tropicalis. Gen Comp Endocrinol. **171**: 319–325. 2011. [Medline] [CrossRef]

34. Manawadi S, and Kaliwal BB. Methomyl induced alteration in mice hepatic-oxidative status. Int J Biotechnol Appl. **2**: 11–19. 2010. [CrossRef]

35. Shalaby MA, El Zorba HY, and Ziada RM. Reproductive toxicity of methomyl insecticide in male rats and protective effect of folic acid. Food Chem Toxicol. **48**: 3221–3226. 2010. [Medline] [CrossRef]

36. Radad K, Hashim A, El-Sharqawy EEG, and El-Din Youssef MS. Histopathological effects of methomyl on Sprague-Dawley rats after repeated application. Bulg J Vet Med. **12**: 149–157. 2009.

37. El-Demerdash F, Attia AA, and Elmazoudy RH. Biochemical and histopathological changes induced by different time intervals of methomyl treatment in mice liver. J Environ Sci Health A Tox Hazard Subst Environ Eng. **47**: 1948–1954. 2012. [Medline] [CrossRef]

38. Nourazarian AR, Kangari P, and Salmaninejad A. Roles of oxidative stress in the development and progression of breast cancer. Asian Pac J Cancer Prev. **15**: 4745–4751. 2014. [Medline] [CrossRef]

39. El-Missiry MA, Fayed TA, El-Sawy MR, and El-Sayed AA. Ameliorative effect of melatonin against gamma-irradiation-induced oxidative stress and tissue injury. Ecotoxicol Environ Saf. **66**: 278–286. 2007. [Medline] [CrossRef]

40. Liu C, Yu K, Shi X, Wang J, Lam PKS, Wu RSS, and Zhou B. Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*). Aquat Toxicol. **82**: 135–143. 2007. [Medline] [CrossRef]

41. Ebaid H, Ajarem J, and Taweel GA. Acute endotoxemia during gestation induces organ dysfunction and tissue damage in mouse offspring. Pak J Zool. **44**: 765–776. 2012.

42. Moore RJ, Hall CB, Carlson EC, Lukaski HC, and Klevay LM. Acute renal failure and fluid retention and kidney damage in copper-deficient rats fed a high-NaCl diet. J Lab Clin Med. **113**: 516–524. 1989. [Medline]

43. Mehta RL, and Bouchard J. Effects of Fluid Overload on Outcome. Controversies in Acute Kidney Injury, Vol. 174. Karger, 200–211. 2011.

44. El-Demerdash F, Dewer Y, ElMazoudy RH, and Attia AA. Kidney antioxidant status, biochemical parameters and histopathological changes induced by methomyl in CD-1 mice.

Exp Toxicol Pathol. **65**: 897–901. 2013. [Medline] [CrossRef]

45. El-Fakharany II, Massoud AH, Derbalah AS, and Allah MSS. Toxicological effects of methomyl and remediation technologies of its residues in an aquatic system. J Environ Chem Ecotoxicol. **3**: 332–339. 2011.

46. Mansour SA, Mossa AT, and Heikal TM. Effects of methomyl on lipid peroxidation and antioxidant enzymes in rat erythrocytes: in vitro studies. Toxicol Ind Health. **25**: 557–563. 2009. [Medline] [CrossRef]

47. Mendoza MGA, Castillo-Henkel C, Medina-Santillan R, Jarillo Luna RA, Robles HV, Romo E, Rios A, and Escalante B. Kidney damage after renal ablation is worsened in endothelial nitric oxide synthase -/- mice and improved by combined administration of L-arginine and antioxidants. Nephrology (Carlton). **13**: 218–227. 2008. [Medline] [CrossRef]

48. Tain Y-L, Muller V, Szabo AJ, Erdely A, Smith C, and Baylis C. Renal cortex neuronal nitric oxide synthase in response to rapamycin in kidney transplantation. Nitric Oxide. **18**: 80–86. 2008. [Medline] [CrossRef]

49. Feliers D, Lee D-Y, Gorin Y, and Kasinath BS. Symmetric dimethylarginine alters endothelial nitric oxide activity in glomerular endothelial cells. Cell Signal. **27**: 1–5. 2015. [Medline] [CrossRef]

50. Beckman JS, and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. **271**: C1424–C1437. 1996. [Medline]

51. You Z, Xin Y, Liu Y, Han B, Zhang L, Chen Y, Chen Y, Gu L, Gao H, and Xuan Y. Protective effect of Salvia miltiorrhizae injection on N(G)-nitro-D-arginine induced nitric oxide deficient and oxidative damage in rat kidney. Exp Toxicol Pathol. **64**: 453–458. 2012. [Medline] [CrossRef]

52. Haczkiewicz K, and Ogielska M. Gonadal sex differentiation in frogs: how testes become shorter than ovaries. Zoolog Sci. **30**: 125–134. 2013. [Medline] [CrossRef]

53. Mokhtar HI, Abdel-Latif HA, Elmazoudy RH, Abdelwahab WM, and Saad MI. Effect of methomyl on fertility, embryotoxicity and physiological parameters in female rats. J Appl Pharm Sci. **3**: 109–119. 2013.

54. Shanthalatha A, Madhuranath BN, and Yajurvedi HN. Effect of methomyl formulation, a carbamate pesticide on ovarian follicular development and fertility in albino mice. J Environ Biol. **33**: 33–37. 2012. [Medline]