

Estrous Cycle and Early Pregnancy of White Mice Exposed to Methomyl

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ABSTRACT: Methomyl is an oxime carbamate pesticide that is widely used in the Philippines. This insecticide is known to be an endocrine disrupting chemical and a potent genotoxic in mammalian cells. However, limited studies were conducted specifically on its direct effects on estrous cycle and its teratogenic effect. This study aimed to (a) assess the effect of methomyl on the body weight and on the estrous cycle of mice, and; (b) examine the teratogenic effect of methomyl on the progeny of the female albino mice. Five week-old experimental mice in three treatment setups were used in the study for both independent experiments. The treatment schedule for pregnant mice was administered during organogenesis (day 6 to 15 of gestation). Results showed that the average gained weight of the mice of both high dose (HD) and low dose (LD) groups were lower as compared to the average gained weight of the control group but did not show any statistical significant differences ($p=0.562$). For the experiment 1, methomyl significantly ($p=0.013$) affect the estrous cycle of the mouse especially in LD group. For experiment 2, results revealed that there was a significant difference among the treatment set-ups ($p=0.0001$) in terms of fetal morphometric measurement. Furthermore, abnormality and high number of resorption was also observed in both LD and HD treatment groups. Therefore, methomyl significantly affect the body weight, estrous cycle and fetal morphometry. This further confirm that methomyl is an endocrine disrupting and genotoxic chemical that affects the estrous cycle and causes teratogenic effect.

Keywords: Morphology, pesticide, Philippines, teratogenic.

INTRODUCTION

Pesticides are substance added to the environment to injure or kill pest. Most of these chemical compounds show effective results in their intended use, but some could

cause direct and/or indirect harmful effects in human and wildlife health (FAO, 2003 as cited by Mokhtar et al., 2013). Pesticide toxicology attracts some concerns because of their effect on the morphology and physiology of the parent animals and their progenies (Rull et al., 2006; Paganelli et al.,

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2010). Methomyl is an example of oxime carbamate pesticides which is commonly used to kill a variety of arthropods such as lepidopterous, coleopterous and some hemipterous insects (Shalaby et al., 2010). Since this pesticide has been widely used for agriculture and for industrial purposes, it has been available in general public for retail sale. Compared to other types of pesticides, methomyl is highly toxic to humans, livestock, pets and wildlife (Buchholz et al., 2002). According to acute toxicity test, it covers all toxicity categories such as; category I, the highest category, through fecal route and eye irritation, category II for inhalation, category III for dermal effects and category IV for skin irritation (Wilson et al., 2006). Although it is registered for commercial or professional use, United States federal agency for conducting research experiments which is the National Institute for Occupational Safety and Health (NIOSH), part of Centers for Disease Control and Prevention (CDC) advised the public, especially the farmers to utilize it properly with extra precaution (Mineau, 2002). Because of its high solubility in water and its soil half-life is 33 days, it may contaminate the groundwater and could also contaminate drinking water (Mortensen & Serex, 2014). It is also an endocrine disrupting chemical and a potent genotoxic in mammalian cells (Andersen, 2002). Additionally, it has an adverse effect on non-target animals such as birds, fish and mammals. Human poisoning incidences were also possible (Tsatsakis et al., 2001; Driskell, Groce & Hill, 1991; Miyazaki et al., 1989). According to Snelder et al. (2008), methomyl is often used in Visayas region of the Philippines for agricultural crop augmentation to control pest.

Despite of these harmful effects in both humans and animals, there were only limited studies conducted on this pesticide, more specifically to clarify its direct effects on estrous cycle and to clear out its teratogenic effect. Study of Mokhtar et al. (2013)

exposed the rat before pregnancy and they observed its significant teratogenic effects in three different doses (2 mg/kg b.wt 1 mg/kg b.wt and 0.67 mg/kg b.wt corresponding to 1/10 and 1/20 and 1/30 LD50 methomyl, respectively). Results of their study revealed an increased number of resorptions, skeletal malformations and loss of fetal weight by high 1/10 LD50 dose. However, their study did not expose the rat samples to methomyl during pregnancy. Moreover, Shalaby et al., (2010) conducted a study on the effect of methomyl on the reproductive toxicity in male rats and it was manifested by lowered fertility index, decreased weight of the testes, seminal vesicles and prostate glands and lowered semen quantity and quality. This study is consistent to the data of Mahgoub and Mednay (2001) who reported hormonal changes and testicular damage after chronic exposure of male rats to insecticide methomyl.

Because of these reasons, the researchers wanted to know if there would be a teratogenic effect of methomyl in pregnant albino mice as there are still no existing studies on the pesticides effect on the animal, more specially during pregnancy. Specifically, this study aimed to (a) assess the effect of methomyl on the body weight of mice; (b) determine the effect of methomyl on the estrous cycle of mice, and; (c) examine the teratogenic effects of two dosages of methomyl on the head length, head-rump length, tail length, number of resorption and the presence of abnormalities on the progeny of the female albino mice. The study was only limited into teratogenic effects of methomyl as well as its effects during estrous cycle of female albino mice. Histological basis is not included in this study. The results of this study will contribute in designing a strategic program which aims to responsible use of methomyl during farming period as well as minimize its hazardous effects, most specifically in maternal toxicity for both humans and animals.

MATERIALS AND METHODS

Five (5) five-week old healthy male and thirty (30) virgin female mice were used in the study in which it includes two (2) independent experiments. The experiment one (1) is the effects of methomyl in estrous cycle and experiment two (2) is the teratogenic effects of methomyl on mice. For both experiments, three treatment set-ups were prepared; control (C) set-up treatment, low dose (LD) set-up treatment and high dose (HD) set-up treatment. They were purchased from the breeding laboratory in UP Manila, Manila City (15 females); and from the breeding laboratory of Food and Drug Administration in Alabang, Muntinlupa City (15 females and 5 males). The animals were housed in Animal Biology Division animal house wherein there were 5 mice/cage under controlled hygienic condition at room temperature and photoperiod of 12h D/12h N. The animals were maintained on the standard laboratory pelleted food and drinking water, and libitum throughout the period of experimentation. All steps and preparation in the experiments were in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines for testing of prenatal development toxicity studies in rodents (2001).

Methomyl was obtained from the University of the Philippines Los Banos, College Laguna in the form of pure blue crystal powder. All mice were chosen randomly and their weights were measured. The mean weight of each group and the desired dose was used for measuring the concentration.

In computing the dose, it follows the calculation below;

$(\text{Mean body weight} \times 10 \text{ mg/kg})/10 = \text{doses to be given to mice for higher dose}$

$(\text{Mean body weight} \times 10 \text{ mg/kg})/30 = \text{doses to be given to mice for lower dose}$

In Experiment 1, the estrous cycle of mice was evaluated. After five days of acclimatization, evaluation of each mouse'

estrous cycle was done to determine if all the individuals follow the normal reproductive cycle (4-5-day cycle). A two-week cycle assessment was done prior to treatment. Females in set-up treatment 1 served as the controls (0.25 ml of distilled water solution) as adapted and modified from Madu (2015). In set-up treatment 2, female mice were given methomyl orally diluted in distilled water solution at dose of equivalent to 1/30 LD50 methomyl, while in Treatment 3 they were given equivalent to 1/10 LD50. Phases of the reproductive cycle were assessed for each mouse once a day for the duration of 28 days by viewing vaginal smears using compound microscope (100X and 400X). Average duration of the estrus cycle was determined by counting the number of the days per cycle (e.g. proestrus- last day of diestrus means 1 cycle).

The mice in Experiment 2 were used to assessed the teratogenic effect of methomyl. The estrous cycle of each mouse were assessed until, proestrus was determined. Each proestrus female was placed in a cage with a male from 4:00 pm to 8:00 am the following day in a 2:1 ratio. Mating was confirmed by the presence of sperm cell/plugs. The presence of spermatozoa is recorded as day zero of pregnancy and a daily increase in weight is further confirmation of pregnancy. Administration of methomyl was done during organogenesis (day 6 to 15 of gestation) based on Organization for Economic Co-operation and Development (OECD) guidelines (2001). Animals were observed for signs of toxicity like weaknesses during the experiment. The animals were sacrificed on day 20 of the gestation period by cervical dislocation, and then caesarean section was performed, in order to obtain the fetuses. Presence or absence of resorption was recorded and the number of dead or live fetuses were also counted. The fetuses were weighed and examined by a hand lens, to checked any

malformation on its face and muzzle, bilateral eye bulges, buccal cavity, limbs and tail. Morphometric measurements of head, crown-rump, and tail length was done on the fetus using Vernier caliper.

Body weights were measured every day and the results obtained were expressed as mean \pm SD. The treatments were compared by means of using one-way analysis of variance (ANOVA) using SPSS version 20. $P < 0.05$ was considered statistically significant.

RESULTS & DISCUSSION

The results show that the average gained weight of the mice of both HD and LD groups were lower as compared to the average gained weight of the control group (Table 1). However, the result was not statistically significant ($p=0.562$). At first, fluctuations of weight were observed but weight were continuously decreasing in continuous administration of methomyl.

Table 1. Effects of methomyl administration on the changes of the weights (grams/g) and the duration (days) of estrous cycle in female mice

Dosage group	CHANGES Initial weight (g)	ON Final weight (g)	WEIGHTS Gained weight (g)	Cycle days
HD	21.94 \pm 1.54	23.56 \pm 3.79	1.62 \pm 4.80	4.04 \pm 1.63
LD	20.4 \pm 0.50	22.12 \pm 1.66	1.72 \pm 1.54	3.03 \pm 1.60
C	20.93 \pm 0.52	25.27 \pm 1.07	4.33 \pm 1.55	4.43 \pm 1.35

The result was also observed from the study of Mokhtar et al. (2013) where the administration of methomyl decreases the gained weight of rats. These findings could be attributed to the decreased in the food intake by disruption in the normal hormonal production and/or direct cytotoxic effect of methomyl insecticide as mention by Al-Shinnawy (2008) in their study on male albino rats. Moreover, another reason of the lower body weight gain is the loss of appetite and/or metabolic disturbance due to administering methomyl. Although food and water intake has not been measured in this study, this may be one of the reasons for low weight gain of the treated mice.

Meanwhile, results showed that methomyl affects the estrous cycle of the mouse at both HD and LD concentrations by shortening its duration relative to the control. The duration of the cycle was significantly different among the test groups ($p=0.013$) where there was a relatively shorter estrous cycle of mice in LD group. In addition, result showed that

cyclic changes of the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity. The results obtained in the study indicate that the control mice exhibited regular 4–5 days' estrous cycle. Mice treated with methomyl causes a significant increase of the duration of diestrus phase. Moreover, the result showed a decreased duration of proestrus and estrus stages in in both HD and LD groups. This may indicate that the methomyl can affect the estrous cycle of white mice. If there were disruptions in these stages it may affect the normal reproductive processes of mice.

Bretveld et al. (2006) stated that some pesticides may interfere with the female hormonal function, which may lead to negative effects on the reproductive system through disruption of the hormonal balance necessary for proper functioning and this statement might include methomyl as observed in our experiment. This was supported by Stamati et al. (2007), which he stated the prolonged stage of estrous cycle in

mice has evidently seen and observed. The observation of the present study was also observed from other studies involving pesticides (Asmathbanu & Kaliwal, 1997; Dhondup & Kaliwal, 1997; Sortur & Kaliwal, 1999). The prolonged diestrus and the shortening of proestrus and estrus phases could be the result from the disruption of the hypothalamo-pituitary gonadal axis. It has been already reported that the pesticides could decrease GnRH release which directly influence the gonadotropin synthesis, then further influence the normal feedback mechanisms of FSH and LH (Baligar & Kaliwal, 2002). Similarly, Ferguson et al. (1984) have suggested that the treatment with carbamate pesticide carbofuran inhibits acetylcholinesterase, resulting in alterations in the pituitary gonadotropins and could influence on gonadal function directly through the effect on the pituitary acetylcholinesterase in rats. This may cause imbalance in gonadal steroids which are essential for normal functioning of the gonads (Carter et al., 1984). Although not tested in the present study, gonadotropin imbalance due to methomyl toxicity is likely the most plausible explanation of the disruption of estrous cycle through arresting estrogen production and/or affecting the folliculogenesis by disrupting estrogen progesterone ratio (Baligar & Kaliwal, 2002).

Table 2 showed the mean weight and lengths of the litter as compared to control and treated with methomyl. The result showed that there was a statistically significant differences on the head length

($p=0.0001$), crown-rump length ($p=0.0001$), tail length ($p=0.0001$) and weight ($p=0.0001$) of the fetuses among the test groups, where there were lower of these measurements of the fetuses in LD group. This is contrary to the recent study of Mokhtar et al. (2013) where embryo toxicity was more prominent in high dosage groups in rats. It should be noted that the latter's experiment involves methomyl treatment before the animals were impregnated, which could also be considered as an important factor why such differences on the data between the two studies was observed. There was a reduction of the food intake observed of pregnant mice in HD and LD groups which could cause the lower weight and sizes of the fetuses.

The result of the present study was unusual because teratogenic effects usually are prominent in high dosage group but the present study showed abnormality was observed in low dose group. It was observed that the number of litters and the number of resorption was higher on the high dosage group which could suggest that the high dosage is already lethal to the fetuses thus the teratogenic action of methomyl could only be observed in low dosage. The most probable explanation is the individual-based maternal characteristics. This could suggest that other factors such as *in utero* position of the fetus and the uterine vasculature inside the mother could have affected the sizes and weights of the fetuses (Niebyl & Simpson, 2008).

Table 2. Summary of the mean lengths (millimeters/mm) of head, crown-rump and tail length and weight (grams/g) of the litters in different dosage as compared to control

Dosage groups	Head length (mm)	Crown-rump length (mm)	Tail length (mm)	Weight (g)
High Dose (n=18)	9.71 ± 2.37	26.82 ± 3.03	9.64 ± 1.07	1.51 ± 0.14
Low Dose (n= 20)	7.49 ± 1.97	21.53 ± 6.22	7.84 ± 3.65	1 ± 0.62
Control (n=37)	9.70 ± 1.15	28.02 ± 2.76	11.36 ± 1.58	1.34 ± 0.26

Table 3. Effects of methomyl in the number of resorption, dead fetuses and abnormalities observed upon administration to the pregnant mice

Group (No. of pregnant mice)	Number of Implantation	Resorption (%)	No of Dead fetuses	Number of viable fetus	Abnormalities Observed
Low Dose (3)	30	33.33	-	20	Left eye is not bulging
High Dose (3)	34	47.05	-	18	-
Control (4)	39	0	2	37	-

Table 3 shows that there is presence of resorption in the treated mice. As compared to the study of Mokhtar et al. (2013) on rats, result of the present study showed the same in terms of the presence of resorption in treated mice. There are high numbers of resorptions in the HD and LD groups as compared to the control group. In terms of the presence of abnormalities, methomyl has no effect as there was only 1 abnormality observed from the fetuses of the treated mothers, contrasting to the study of Mokhtar et al. (2013) in rats.

The abnormality detected is most likely not the result of methomyl, as newborn infants might have major malformations at birth or can be detected later in life (Van Gelder et al., 2010) even without the presence of teratogens. In the study of Mokhtar et al. (2013), different kinds of morphological abnormalities were observed such as cleft palate, limb abnormalities, tail defects (tailless), and dermal edema. Thus, the study supports earlier reports that methomyl could not cause fetal abnormalities. However, the observed high number of resorption, more prominently in HD group could indicate that methomyl is embryo toxic. The weight of the pregnant treated animals may decrease due to resorption which was initially observed in the present study.

Resorption is the disintegration of a dead fetus inside which is partially or completely reabsorbed in the uterus. The death of the fetuses inside the uterus of the treated groups were possibly due to the methomyl's capability to cause an

overproduction of reactive oxygen species (Garg et al., 2009) which could indicate that oxidative stress due to methomyl could play an important role on the death of the fetuses (El-Shenawy et al., 2010). Moreover, in the study of Mokhtar et al. (2013), the activities of the antioxidant enzymes such as SOD and CAT in the rat ovaries were significantly decreased. It has been known that these antioxidants function as a primary defense against oxidative damages. The depletion of the enzymes could promote the production of free radicals which cause oxidative damages on cells (Salama et al., 2005).

CONCLUSION

Pesticide such as methomyl are considered to be an endocrine disruptor that affects the developmental process and reproductive cycles even on the non-target organisms. Based on the result of the study, the weight of the treated mice significantly decreases during the treatment. Furthermore, the estrous cycle of mice treated with methomyl significantly affected. As observed, there was prolonged diestrus phase and shortened estrous cycle. In addition, present of resorption was observed in both treated group while abnormality was observed in one the fetus in low dose group. Therefore, this study supports that methomyl is an endocrine disruptor and toxic chemical that affects the body weight, the estrous cycle and fetal morphometry. Moreover, this study suggests that methomyl has teratogenic/embryotoxic effect to pregnant mice.

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