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Another Mode of Action of Temephos Against *Aedes aegypti* Larvae: A Stomach Poison Investigation

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ABSTRACT

Introduction: *Aedes aegypti* is a key vector for the spread of several severe arboviral infections. The Indonesian Ministry of Health launched Temephos as a national effort to limit the *Aedes aegypti* larvae population. The old theory has been passed down for generations that the principle of the mechanism of action of temephos as a neurotoxin. The main aim of this study was to investigate the mechanism of action of temephos as a stomach poison by using histopathology study. **Method:** There are two treatments with three replications: a container containing only 100 ml of water with tween 20 and a container containing 100 ml of water with 1 ppm of temephos 8G. The 20 third-instar Ae. aegypti larvae in containers containing 100 ml of water with 1 ppm of temephos 8G were compared with those in 100 ml of water containing Tween-20. The experiment was done in three replications. The number of dead larvae was recorded after 24 hours of treatment. Histological sections of the larval midgut were prepared and stained with hematoxylin-eosin (HE). Light microscopy was used to examine changes in the length of the midgut lumen and the epithelium. Data were analyzed using a one-way ANOVA. The appearances of the nucleus of the epithelial cell and the degree of damage were qualitatively observed. **Results:** The results showed that no dead larvae were found in the control group, however, 100% mortality was found in the temephos group. The changes in midgut lumen length and in the epithelium length were significantly different from those in the control group ($p<0.05$). Nuclei of epithelial cells were lost and midgut cells were damaged in the temephos group. **Conclusions:** This study reports the first discovery of the mechanism of action of temephos other than a neurotoxin, namely stomach poison.

Key words: Temephos, *Ae. aegypti*, Midgut, Histopathology.

INTRODUCTION

More than half of the world's population is at risk of infection spread by mosquitoes.[1] The *Ae. aegypti* mosquito (Culicidae) is an important mosquito vector in Indonesia because it can transmit a variety of mosquito-borne diseases, such as dengue, zika, chikungunya, and yellow fever. *Ae. aegypti* has a distinctive morphology, namely mesepimeron with two separate patches of white scales; white lyre-shaped mark on black scutum and a pair of submedian-longitudinal white lines; the anterior part of the midfemur has a longitudinal white stripe, and clypeus on head with two separate patches of white scales.[2] Increased spread and successful transmission of mosquito-borne diseases may be facilitated by improved transportation systems, urbanization, climate change, and vectorial invasive behavior.[2-6] Dengue and chikungunya infections are two important health mosquito-borne viral disease in Indonesia. Dengue hemorrhagic fever has become more common in Indonesia over the last 50 years. Indonesia has one of the highest dengue burdens in the world.[7] Java Island has the greatest average number of Dengue Hemorrhagic Fever (DHF) cases each year. In recent years, the biggest instances have been in Bali and Borneo (Kalimantan). Over the previous 50 years, the yearly incidence rate of DHF in Indonesia has risen rapidly, from 0.05 cases per 100,000 person-years in 1968 to 77.96 cases per 100,000 person-years in 2016.[8] Dengue fever is associated with significant economic expenditure, with the Asia Pacific region accounting for more than half of the global cost.[7] Chikungunya virus (CHIKV) outbreaks were reported in several Indonesian regions, from 2000 to 2011, and then again in 2015 and 2016.[9,10] Because therapies are expensive and mostly supportive, and vaccines are still being investigated, routine vector control programs remain the most effective preventive tool for arthropod-borne diseases.[11,12] Vector control is the main strategy to minimize the incidence of dengue and chikungunya infections through eliminate immature vector.[13] In community, vector control is very dependent on larvicide and adulticide.[12] Larvicides are chemicals used to suppress mosquito populations while they are still immature in the aquatic environment. Adulticides are chemicals that are designed to rapidly reduce adult mosquito populations.[14]

Temephos ($C_{16}H_{20}O_6P_2S_3$), also known as O,O,O′O′-tetramethyl O,O′-thiodi-p-phenylene bis(phosphorothioate), is a non-systemic organophosphorus (OP) larvicide that is used to control mosquitoes, midges, black flies, and other insects in public health.[15,16] Temephos remains the main chemical for controlling immature stages of *Ae. aegypti* throughout much of Southeast Asia.[17] Temephos is widely used to control larval stage populations of *Ae. aegypti* in Indonesia. Temephos is commercially available in a range of formulations, including granules, diluted solutions, emulsifiable concentrates, and slow release formulations, and can be applied in a variety of methods depending on the site and rate of application.[18] Temephos is one

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of the most frequently used larvicides in the world due to its ease of use, community acceptance, high effectiveness, cheap operational cost, reasonably long residual life, low mammalian toxicity, and specificity for mosquito larvae.[17,19-21] The use of temephos has been established as a national program by the Indonesian government to control larvae since 1970s.[22] Temephos has been used in Indonesia for 53 years.

Insecticides can be classified according to their mode of action, namely: physical poison, respiratory poison, stomach poisons, protoplasmic poison, nerve poison, and growth inhibitors.[23-25] The old theory has been passed down for generations that the principle of the mechanism of action of temephos as a neurotoxin, which has the target of inhibiting acetylcholinesterase (AChE) activity.[16,26-32] Acetylcholinesterase (AChE) in insects hydrolyzes the neurotransmitter acetylcholine (ACh) to end neuronal excitement at the postsynaptic membrane.[31] Insecticides inhibit AChE action, resulting in many acetylcholine deposits in nerve cells, resulting in paralysis and dead cells.[30,33,34]

The digestive tract of *Ae. aegypti* is divided into three sections: the foregut, midgut, and hindgut.[35] The midgut of the mosquito larva has the main functions of digestion, synthesis of digestive enzymes, ion transport, absorption, and osmoregulation processes.[36] The midgut is a target for insecticides that act as stomach poisons. Plant extracts and metabolites are essential for the degeneration of insect midgut epithelium. They have been shown to have a negative impact on digestive epithelial cells and to slow arthropod growth.[36] Previous research on the toxic effects of temephos on the midgut was still limited to testing *Culex quinquefasciatus* mosquitoes through histopathology, electron microscopy observations, and protein profiles.[33,37] Observations on *Ae. aegypti* larvae were only observed at the external morphology level with photomicrography. The limitations of this research have not been carried out by histopathological studies.[6] In fact, more observations of mosquito midgut damage were observed due to exposure to botanical insecticide extracts, such as *Averrhoa bilimbi*, *Asarum heterotropoides, Annona squamosa, Annacardium occidentale, Brucea javanica, Magnolia denudata, Passiflora foetida*, and *Brucea javanica.*[38-40] This suggests that temephos is not only work as a neurotoxin, but it also works as a stomach poison. To date, how temephos overcomes the midgut is poorly understood, despite being an important subject matter. We hypothesized that temephos can cause damage the midgut of *Ae. aegypti* larvae based on histopathological effects. Because there has been little research on the effect of temephos as a stomach poison, this study aimed to investigate the mechanism of action of temephos as a stomach poison by histopathology.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Ethical Committee of Universitas Ciputra's School of Medicine in Surabaya, Indonesia, as detailed in Ethical Clearance No. 036/EC/KEPK- FKUC/ II/ 2023.

Larvae rearing and colonization

The eggs of *Ae. aegypti* were provided by the Laboratory of Entomology, Institute of Tropical Diseases, Universitas Airlangga, Surabaya, East Java province, Indonesia. The eggs hatched into larvae and were reared into third-instar larvae. The water was cleaned by removing food residue every day. This generation was kept in ideal conditions, such as 65–80% room humidity and a water temperature of 28-30°C.

Larvicidal test

The larvicidal assay of the extract was evaluated according to the WHO guidelines for laboratory and field testing of mosquito larvicides.[41] There are two treatments with three replications: a container containing only 100 ml of water with tween 20 and a container containing 100 ml of water with 1 ppm of temephos 8G. The 20 third-instar *Ae. aegypti* larvae in containers containing 100 ml of water with 1 ppm of temephos 8G were compared with those in 100 ml of water containing Tween-20. The number of dead larvae was recorded after 24 hours of treatment.

Histopathology study evaluation

Six larvae from each treatment were fixed in 5 mL of 10% formaldehyde, and then they were transferred to the Biosains Institute, Universitas Brawijaya, Malang, East Java Province, Indonesia. They were placed in a 10% Formalin solution for 24 hours at room temperature while still alive. Following this interval, they were washed thoroughly with PBS (Phosphate Buffer Saline) to remove any residue. Dehydration was accomplished by immersing larvae in a variety of escalating ethanol concentrations for 15 minutes, beginning with 50%, 70%, 80%, 90%, and 95%, followed by 100% ethanol (2 times) for 30 minutes, then overnight. The larvae were embedded in paraffin (Sakura) using Tissue-Tek TEC 5 Sakura (embedding and Cryo Module). The embedding cast was made in Stainless steel molds (10 mm; Sakura), and each block contained one larva positioned lengthwise. The molds were kept at 62°C for at least 30 minutes for ensure the molds hot enough for the process. The longitudinal sections of larval midgut (3 μm) were cut using a manual microtome (Accu-Cut SRM Sakura) with disposable blades (MX35 Ultra Thermo Scientific). These slides were kept for at least 24 hours in a Hotplate (Sakura) at 40°C. Finally, for histopathological investigation, the slices were stained with Mayer's hematoxylin and eosin Y. An Olympus microscope was used to view and photograph the histology slides (BX53). ImageJ software version 1.53t was used to calculate the scale bar. Midgut damage observed was determined by the length of the epithelium cell, the length of the lumen, appearance of the nucleus of epithelium cell, and the degree of damage.

Statistical analysis

Statistical analysis was done with SPSS version 26. The normality test will be analyzed using the Shapiro-Wilk test, while the homogeneity test will be analyzed using the Levene test. Changes in midgut larvae, such as the length of the epithelium cell, the length of the lumen, were analyzed using a one-way ANOVA, and a post hoc test, namely the LSD test, to find out the differences between each group. Using + and - symbols, the appearance of the nucleus of the epithelium cell and the degree of damage were qualitatively assessed.

RESULTS

There were no dead larvae in the control group, however, 100% mortality larvae in the temephos group. The effects of the control group and the temephos group on the histopathological changes of the midgut are presented in Table 1.

The mean value of midgut lumen length in the two treatment exposures showed that temephos administration produced the highest midgut lumen length compared to control group. The results of the one-way ANOVA statistical test showed that larvae in the control group and those exposed to temephos had significantly different midgut lumen lengths ($p = 0.030 < 0.05$). Using the LSD test, post hoc analysis revealed that the larvae group given temephos had an average of 8.24 (b), which was significantly different from the control group.

Based on Table 1, the length of the epithelium in the control group had a mean of 1.25 + 0.09 μm with the lowest value being 1.15 μm and the highest value being 1.31 μm. The epithelial length of dead larvae exposed to temephos for 24 hours had an average of 2.58 + 0.18 μm with the lowest value being 2.37 μm and the highest value being 2.70 μm.

Figure 1 depicts the abnormalities that occur in midgut cells after a 24-hour exposure to temephos: the lumen is dilated and filled with food boluses; epithelial cells are not tightly packed and are separated from the basal membrane, the nucleus of epithelial cells is lost or damaged;

Table 1: Histopathological change in the midgut of *Ae. aegypti* larvae.

Groups	The mean midgut lumen length (μm)	The mean epithelial cell length (μm)	Cell nucleus is lost or damaged	Midgut cell damage
Control	5.71 ± 0,73*	1.25 ± 0,09*	-	-
Temephos 8G	8.24 ± 1,44*	2.58 ± 0,18*	+	+

The values were expressed using mean ± SEM
LSD (* = Significantly different at 5% level of significance)
-: no damage, +: there is damage

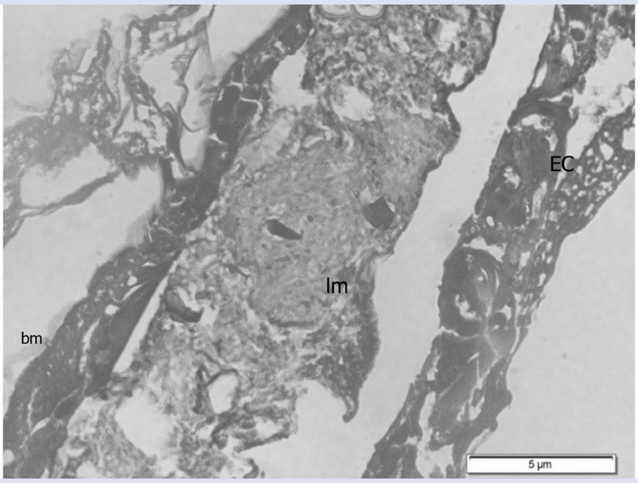

Figure 1: Longitudinal section part of 3rd instars larvae of *Ae. aegypti* larvae midgut exposed to 1 ppm temephos for 24 hours, stained with H&E (magnification, ×400). lm= lumen, EC: epithelial cells, bm: basal membrane.

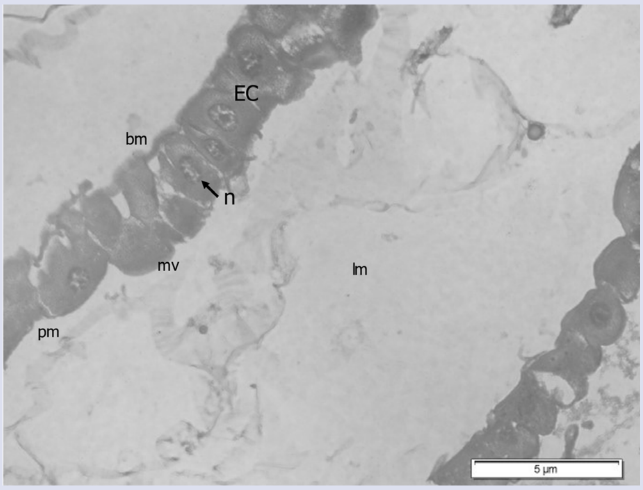

Figure 2: Longitudinal section part of the midgut of the control group, stained with H&E (magnification, ×400). lm= lumen, EC: epithelial cells, n: nucleus of epithelial cells, bm: basal membrane, mv= microvilli, pm= peritrophic membrane.

microvilli damage; the cytoplasm protrudes; peritrophic membrane damage; basal membrane damage. On the other hand, the normal midgut is shown by the larvae in the control group (Figure 2).

DISCUSSION

The larvae of the midgut are divided into four categories i.e., cardia, gastric caeca, anterior, and posterior midgut. In all of these areas, the epithelium is composed of a single layer of columnar digestive cells with apical microvilli, a nucleus with polytene chromosomes, and cytoplasm with many mitochondria. The midgut epithelium is separated from the swallowed food by a well-developed peritrophic matrix.[28] The peritrophic membrane serves as a barrier against diseases and poisons.[39] The brush border of microvilli on the apical membrane (facing the gut lumen) and irregularly twisted basolateral membrane infoldings of most gut epithelial cells are compatible with their role in the synthesis of digestive enzymes and nutrition assimilation.[42]

The most common types of damage found in the larval midgut of *Ae. aegypti* treated with *A. bilimbi* fruit extract were columnar cell vacuolization, epithelial nuclei crossing the midgut lumen, microvilli disruption, and basement membrane damage.[40] Histological alterations in all parts of the *Culex quinquefasciatus* midgut, such as cracks in food bolus and peritropic membrane and irregular forms of epithelial layer, epithelial cell, and microvilli after exposure to temephos, malathion, cypermethrin, and deltamethrin.[33] Midgut damage results in abnormal midgut functions such as food digestion, nutrient absorption, and defense against pathogenic microorganisms.[43] Most gut epithelial cells have brush borders on the apical membrane (facing the gut lumen) and irregularly twisted basolateral membrane infoldings, which is consistent with their role in digestive enzyme synthesis and nutrition assimilation.[42] A recent study found seven proteins involved in protein catabolism as a reaction to the insecticide in the midgut of *Cx. quinquefasciatus* larvae. Three proteins associated to energy metabolism in the midgut of *Cx. quinquefasciatus* larvae, arginine kinase, isocitrate dehydrogenase, and ATP synthase/vacuolar ATPase, have been demonstrated to be downregulated by temephos.[37]

The epithelium is a rectangular, homogeneous layer of cubical and cylindrical cells that emphasize the brush boundary.[44] An epithelial cell layer defines every interface between the inner world of the mosquito body and the outside world of the external environment. The two general roles of the epithelium are to relay exterior stimuli to the internal world and to protect the internal environment from unfavorable abiotic conditions.[42] The nuclei of epitel cell are central and globular.[44] The epithelial lining becomes lysed and causes the bolus of food to spread across the lumen.[39] The epithelial cells protruded in the midgut of *Culex pipiens* larvae, with the brush border entirely disorganized and thinning down after 24 h exposure with ar-turmerone (200 ppm).[45] Fiaz *et al*. discovered, similarly to this work, that a damaged epithelial layer was associated with cell debris discharged in the midgut lumen, cytoplasm vacuolization followed by cell breakdown, and cell debris release into the midgut lumen.[46]

The peritrophic membrane, also known as the peritrophic matrix (PM), is a non-cellular substance, a semi-permeable and fibrous layer created by midgut epithelial cell secretion, that separates the ingested food from the midgut epithelium. Inside the midgut, the structured PM surrounds the food bolus. The permeability of PM is composed of chitin, proteins, and proteoglycans. PM's primary functions include preventing tissue damage, regulating digestive enzyme secretion, and forming a protective barrier against pathogens and toxin.[47,48] Damage or detachment of the peritrophic membrane results in loss of its function.[44]

There are currently no vaccines available. Therefore, the risks associated with mosquito-borne diseases can only be managed through the control of the vector populations and the monitoring of potential arbovirus infections in humans. Temephos is one way to control vector populations, especially at immature stages in the water. The application of temephos in the field is by sowing it in a bath filled with water or a water reservoir. Temephos is predicted to enter the body of the larvae when consumed with food in the medium.[49] Temephos is a lipophilic substance that penetrates quickly through cuticular surfaces and spiracles.[32] A chemical with a higher lipophilicity can pass through lipid cell membranes, the blood-brain barrier, and protein binding.[50] This lipophilic property is predicted to enable temephos to penetrate the mouth or cuticle and eventually enter the midgut cells. This study is still limited to histological studies. In the future, it will be necessary to develop toward molecular, bioinformatics or molecular docking, and proteomic study. Finally, from this study it was agreed that the mechanism of action of temephos apart from being a neurotoxin, it also works as a stomach poison.[23,25]

CONCLUSION

The control group had no dead larvae and the temephos group had 100% mortality. Temephos has a mechanism of action as a stomach poison. Temephos can cause damage the midgut of *Ae. aegypti* larvae based on histopathological effect. This study has revealed that temephos was a stomach poison.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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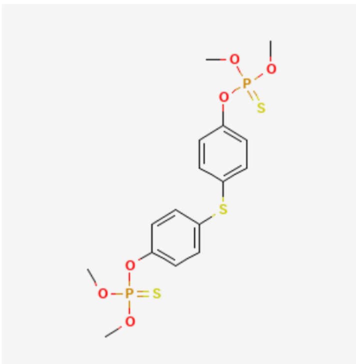
GRAPHICAL ABSTRACT



Temephos

Larvicidal Test

Histopathology Evaluation

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