

Effects of piperonyl butoxide on the toxicity of the organophosphate temephos and the role of esterases in the insecticide resistance of *Aedes aegypti*

Boscolli Barbosa Pereira^[1], Jean Ezequiel Limongi^[1], Edimar Olegário de Campos Júnior^[2], Denis Prudencio Luiz^[2] and Warwick Estevam Kerr^[2]

[1]. Departamento de Saúde Ambiental, Universidade Federal de Uberlândia, Uberlândia, MG. [2]. Departamento de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, MG.

ABSTRACT

Introduction: The effects of piperonyl butoxide (PBO) on the toxicity of the organophosphate temephos (TE) and the role of esterases in the resistance of *Aedes aegypti* to this insecticide were evaluated. **Methods:** *A. aegypti* L4 larvae susceptible and resistant to TE were pre-treated with PBO solutions in acetone at concentrations of 0.125, 0.25, 0.5, 1, and 2% for 24h and subsequently exposed to a diagnostic concentration of 0.02mg/L aqueous TE solution. The esterase activity of the larvae extracts pre-treated with varying PBO concentrations and exposed to TE for three time periods was determined. **Results:** At concentrations of 0.25, 0.5, 1, and 2%, PBO showed a significant synergistic effect with TE toxicity. High levels of esterase activity were associated with the survival of *A. aegypti* L4 larvae exposed to TE only. **Conclusions:** The results of the biochemical assays suggest that PBO has a significant inhibitory effect on the total esterase activity in *A. aegypti* larvae.

Keywords: Temephos. Insecticide resistance. *Aedes aegypti*. Esterases.

INTRODUCTION

Dengue fever, a major viral disease, is a serious public health problem, especially in tropical countries where the climate and urban culture provide optimal conditions for the development and proliferation of its main vector, the mosquito *Aedes aegypti*¹.

The control of *A. aegypti* is primarily based on the use of chemical and biological products that are integrated with environmental management programs. Public programs aimed at controlling mosquitoes are characterized by the use of commercial insecticides, including organophosphates and pyrethroids. The organophosphate temephos (TE) was widely used in Brazil to control *A. aegypti* larvae until recently; however, due to reports of vector resistance to this insecticide, this method was replaced by the National Dengue Control Program. Nevertheless, TE remains on the list of insecticides to control dengue in Brazil and may be used again in the future^{2,3}.

The resistance to insecticides has contributed to an increase in *A. aegypti* populations and the incidence of dengue cases in Asia, and Central and South America, especially in Brazil^{2,4}.

The biochemical mechanisms involved in the development of resistance to insecticides include detoxification metabolism processes. Several enzymes and enzyme systems participate in these processes, including oxidases, esterases, and transferases⁵. These enzymes allow the insect to convert an insecticide to a non-toxic form or to rapidly eliminate the compound from the body.

Synergists such as piperonyl butoxide (PBO) have been employed in an attempt to overcome resistance to insecticides. Synergists are known to act as an alternative substrate, competing with the insecticide and interfering with detoxification. Synergists also act allosterically to inhibit the binding sites of esterases and multifunction oxidases (MFOs), minimizing the amount of insecticide needed to control insects and the levels of environmental contamination by pesticide residues⁶.

Several studies have demonstrated the successful use of synergists for insect control through their inhibitory action on esterase activity and MFOs in resistant strains^{7,8}; however, studies investigating their action in *A. aegypti* are rare. The aim of the present study was to evaluate the effects of PBO on the toxicity of the organophosphate pesticide TE and the involvement of esterases in the resistance of *A. aegypti* to this compound.

METHODS

Samples

Aedes aegypti-susceptible and -resistant strain samples were provided by the Center for Zoonosis Control of Uberlândia (CZCU). The research was conducted by studying

Address to: Dr. Boscolli Barbosa Pereira. Dept de Saúde Ambiental/UFU. Av. João Naves de Ávila 2121, 38400-902 Uberlândia, MG, Brasil.
Phone: 55 34 3291-5989; **Fax:** 55 34 3291-4169
e-mail: boscolli86@hotmail.com; boscolli@ig.ufu.br
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natural resistant populations of *A. aegypti* collected in five neighborhoods with high rates of vector infestation according to the Health Department of Uberlândia. The Rockefeller population, maintained for ten generations in the laboratory, was used as the susceptible population of reference. L4 larvae were used in the experiments as recommended by the World Health Organization⁹.

Aedes aegypti strains

To confirm resistance in the strains, the larvae were submitted to a diagnostic dose of 0.012mg/l of TE, according to standardized methodology recommended by the World Health Organization¹⁰. When the mortality rate was higher than 98%, the population was considered susceptible. Populations that showed mortality rates of 80 to 98% underwent repeat testing for confirmation, and populations in which the mortality rates were equal to or lower than 80% were considered resistant.

Pretreatment with PBO and TE exposure

Susceptible and resistant L4 larvae were pre-treated with PBO diluted in acetone at concentrations of 0.125, 0.25, 0.5, 1, and 2% for 24h. For each test, 10 groups of 10 larvae were placed in Petri dishes with 40ml of 0.02mg/l aqueous TE solution, while the control groups were placed in 40ml of water only. The larval mortality was assessed following 2h of exposure according to the procedure by Bezzerra et al⁴.

Esterase activity

The esterase activity of the larvae extracts pretreated with PBO and exposed to TE for three exposure times (30, 60, and 120min) was determined using α -naphthyl acetate as the substrate in microtiter plates, according to the technique developed by Devonshire¹¹ with modifications by Pruet et al¹². The protein content was estimated in triplicate using Bradford's method (Bradford, 1976)¹³ with bovine serum albumin as the standard.

The living larvae were centrifuged (4°C, 15,000 × g, 15 min) with 100ml of extraction buffer (20mM sodium phosphate, pH 7.0). The obtained supernatant was collected and diluted 1:50 in extraction buffer. The diluted 50 μ l samples were incubated with 150 μ l of extraction buffer containing 0.5mM α -naphthyl acetate for 30min at 30°C in darkness.

Fifty microliters of extraction buffer containing 0.15% o-dianizidina and 1.75% sodium dodecyl sulfate were added. The optical density values were measured in an enzyme-linked immunosorbent assay (ELISA) reader (Titertek Multiskan Plus MKII, Flow Laboratories, McLean, VA, USA) using 450 nm filters and converted to micromoles of naphthol per min per mg of protein (mmol/mg/min protein), according to a standard curve using the Microplate Manager PC v. 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

The mortality rates of the susceptible and resistant larvae strains were compared using the Chi-square test of heterogeneity between the populations. An analysis of variance (ANOVA), followed by Tukey's and Kramer HSD tests, were used to compare the rates of esterase activity between the susceptible and resistant strains in different profiles of exposure to the five concentrations of PBO tested. The differences were considered significant if $p < 0.05$.

RESULTS

Susceptibility and resistance to temephos: the effects of PBO on the toxicity of TE

The results of 2h exposure to 0.02 mg/l TE in the susceptible and resistant strains of *A. aegypti* larvae in the control test (without pre-treatment with PBO) and experimental tests (with pretreatments of different concentrations of PBO) are shown in **Table 1**.

No mortality was observed in the water-only control in either of the studied strains. The larvae of the susceptible strain exposed to the diagnostic dose of 0.012mg/l TE without pre-treatment with PBO showed 98% mortality, while the mortality of the resistant strain under the same conditions was 51.3%.

The mortality rates in the susceptible larvae pre-treated with varying concentrations of PBO and exposed to 0.02mg/l TE showed no significant differences compared with the control group. The resistant group larvae with PBO pre-treatment at concentrations of 0.25, 0.5, 1, and 2% showed a significantly higher mortality ($p < 0.05$) compared with the resistant control group, with the highest mortality obtained by 1% PBO pre-treatment.

TABLE 1 - Percent mortality of *Aedes aegypti* larvae exposed to different concentrations of piperonyl butoxide.

Strain	Tests						
	Controls		TE (0.02mg/l) after pre-treatment with PBO (%)				
	Water	TE (0.02 mg/l)	0.125	0.25	0.5	1	2
Mortality (M)	M	M	M	M	M	M	M
Susceptible	0	98.0	100.0	96.0	100.0	100.0	98.0
Resistant	0	51.3	52.0	67.3 ^c	78.6 ^c	90.6 ^d	77.3 ^c

PBO: piperonyl butoxide; **TE:** temephos; **M:** mortality of larvae (%). ^cDifference at $p < 0.05$. ^dDifference at $p < 0.01$. $n = 140$. χ^2 test of heterogeneity between the populations.

Effects of PBO on esterase activity

The esterase activity in the larvae of the susceptible and resistant strains treated with five concentrations of PBO and the respective control groups treated only with acetone at three exposure times (30, 60, and 120min) are shown in **Table 2**.

In the control groups, no difference ($p>0.05$) was observed in esterase activity in either the susceptible or resistant strain regardless of the exposure time. In the treated groups, esterase activity in the resistant larvae exposed to 0.25, 0.5, and 1% PBO was significantly lower than in the control group when the exposure time was extended to 60 and 120min ($p<0.05$). Regarding the susceptible larvae, only 1 and 2% PBO at both 60 and 120min exposure times were associated with significantly reduced esterase activity ($p<0.05$).

DISCUSSION

In insects, organophosphate insecticides are detoxified by enzymes such as carboxylesterase, transferases, and oxidases that act by hydrolysis or by reducing the number of free organophosphate molecules. This detoxification metabolism is probably the most studied mechanism of insect pesticide resistance. Synergists have been employed to overcome resistance in various pests and vectors¹⁴⁻¹⁶. The present study demonstrated that 0.25, 0.5, 1, and 2% PBO increased the toxicity of TE to a resistant strain of *A. aegypti* larvae, with the maximum effect observed at the 1% concentration, which was associated with a 39.3% higher mortality than observed in the control group.

The results of the analysis of the esterase activity in the resistant strains suggest that the synergistic effect of PBO on the toxicity of TE was, at least partially, due to a decrease in the general esterase activity of these populations. The inhibitory effect of the esterase activity was not shown to be dose- or time-dependent in either larval strain. Moreover, as the resistance was not completely overcome, mechanisms in addition to esterase activity may be involved in the resistance of these populations.

The larvae of the resistant strain pretreated with 2% PBO showed a lower mortality than the strain pretreated with 1% PBO. This reduction may be explained by an antagonistic effect of PBO, which is likely due to the activation of cytochrome P450. This activation may have inhibited PBO because cytochrome P450 enzyme isoforms are known to be responsible for the inhibition of organophosphates and PBO¹⁷.

PBO acts as an inhibitor of P450, suppressing the bioactivation of TE and reducing its toxicity. Many arthropod species possess multiple isomers of P450, and all are substrate-specific¹⁸. In this study, PBO reduced the toxicity of TE but only at the highest concentration tested. Thus, although PBO had no significant effect on the toxicity of TE at a concentration of 0.125%, synergism increased its toxicity at concentrations ranging from 0.25% to 1%. The effect of 0.5% and 1% PBO was higher for the resistant strain but decreased at the 2% concentration.

TABLE 2 - Esterase activity of susceptible and resistant strains of *Aedes aegypti* larvae exposed to piperonyl butoxide.

Time (min)	PBO (%)	Strain	
		Resistant M \pm SD ^a {mortality}	Susceptible M \pm SD ^a {mortality}
30	0	339 \pm 164 {0}	410 \pm 144 {0}
	0.125	436 \pm 89 {0}	402 \pm 67 {0}
	0.25	367 \pm 91 {0}	398 \pm 84 {0}
	0.5	353 \pm 51 {0}	377 \pm 54 {0}
	1	337 \pm 77 {0}	363 \pm 49 {0}
	2	331 \pm 62 {1}	349 \pm 78 {1}
60	0	395 \pm 231 {0}	315 \pm 103 {1}
	0.125	321 \pm 87 {0}	279 \pm 51 {0}
	0.25	277 \pm 61 ^b {0}	277 \pm 32 {0}
	0.5	281 \pm 36 ^b {0}	201 \pm 90 ^b {0}
	1	263 \pm 66 ^b {1}	198 \pm 101 ^b {1}
	2	329 \pm 85 {0}	292 \pm 56 {0}
120	0	422 \pm 156 {0}	424 \pm 129 {0}
	0.125	375 \pm 58 {0}	431 \pm 69 {0}
	0.25	310 \pm 53 ^b {0}	375 \pm 71 {0}
	0.5	303 \pm 71 ^b {2}	302 \pm 58 ^b {0}
	1	246 \pm 55 ^b {1}	271 \pm 43 ^b {0}
	2	355 \pm 96 {0}	360 \pm 72 {0}

PBO: piperonyl butoxide; ^aMean \pm standard deviation (mmol/mg/min protein). ^bDifference at a significance level of 5%. n=10. Analysis of variance (ANOVA); Tukey-Kramer test.

Resistance based on cytochrome P450 explains the synergistic effect of PBO with TE toxicity at the lower concentrations (0.25, 0.5, and 1%) and inhibition at the highest concentration (2%).

The differential effects of PBO on the toxicity of insecticides in resistant strains observed in these studies may be attributed to the inhibitory effects of PBO to different P450 isomers involved in the activation and detoxification of organophosphate molecules. According to this hypothesis, at 2%, the effects of isomers responsible for the bioactivation of TE increased, offsetting the effect of the isomers involved in metabolic detoxification¹⁸⁻²².

For the susceptible strain, pre-treatment with PBO did not confer altered levels of toxicity; therefore, the individuals in this population may express tolerance to the insecticide⁶.

Toxicology assays and investigations of esterases conducted in resistant mosquito strains have indicated that the survivability of these insects is associated with the increased expression of esterases²⁰. Similar results have been observed in assays involving other classes of organophosphates^{22,23}.

Effects similar to the findings presented in this study have been found in various assays showing synergism between low concentrations of PBO and organophosphates in insect species^{14,15,20,24,25}.

The high level of synergism of 1% PBO in the larvicidal effect of TE has important implications for the control of *A. aegypti* larvae. TE formulations with this concentration of PBO may be effective in the control of both susceptible and resistant strains of *A. aegypti* larvae.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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