ARTIGO ORIGINAL

Aedes Aegypti (Diptera, Culicidae): a new system to study impaired biological effects of phenobarbital

Aedes Aegypti (Diptera, Culicidae): um novo sistema para o estudo dos efeitos biológicos do fenobarbital

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Abstract Larval eclosion, developmental time, mortality in larval and pupal stages and adult longevity were analyzed in *Aedes aegypti* from the municipalities São José do Rio Preto (SJ), São Paulo State, and Goiânia (GO), Goiás State, Brazil, submitted to treatment with phenobarbital (PB). Treatments were carried out using eggs or L1 larvae placed to develop in 0.2mg/mL of PB aqueous solution. Significant delay in the developmental time was observed in the mosquitoes from SJ. In addition, both populations showed significant increase in mortality and significant decrease in adult longevity. Drastic effects were observed at the pupal stage: about 50% of the treated imagoes from SJ and about 21% of those from GO could not emerge from the pupal case. The present observations indicated that PB treatment affects the development of *A. aegypti* impairing important biological features. Because this organism is easy to manipulate in laboratory it may be considered a promising system for studies on side effects of this barbiturate intensively used in the treatment of epilepsy.

Keywords Mosquitoes, Phenobarbital, Development, Longevity

Resumo Eclosão, tempo de desenvolvimento, mortalidade em larvas, pupas e adultos e longevidade foram parâmetros analisados em *Aedes aegypti* de São José do Rio Preto (SJ), e de Goiânia (GO), Brasil, submetidos ao tratamento com fenobarbital (PB). Os testes foram realizados colocando-se ovos ou larvas L1 para se desenvolverem em solução aquosa contendo 0.2mg/mL de PB. Foi observado nos mosquitos de SJ um atraso significante no tempo de desenvolvimento. Além disso, ambas as populações tratadas mostraram aumento na mortalidade e diminuição na longevidade dos adultos. Efeitos drásticos foram observados nas pupas: cerca de 50% e 21% dos imagos tratados de SJ e GO, respectivamente, não puderam emergir do casulo pupal. As observações indicaram que o tratamento com PB afeta o desenvolvimento normal de *A. aegypti*. O fato de que este organismo pode ser facilmente criado em laboratório, o torna um sistema útil para se estudar os efeitos desse barbitúrico amplamente utilizado no tratamento da epilepsia.

Palavras-chave Mosquitos, Fenobarbital, Desenvolvimento, Longevidade

Introduction

In the last years, the barbiturate Phenobarbital (PB) has been intensively used in studies of insecticide resistance, in several organisms. This substance increases tolerance to insecticides, allowing study the mechanisms involved in the development of resistance. PB acts basically inducing the synthesis of the enzymatic system cytochrome P450 that is involved in insecticide detoxification, consequently decreasing insecticide toxic effects [1, 2, 3, 4]. With this aim, a study was carried out in our laboratory using PB treatment in the mosquito *Aedes aegypti* that is vector of the human diseases dengue, dengue hemorrhagic fever and yellow fever. Parallel to the observations on tolerance increase, impaired biological effects were also observed in the PB treated mosquitoes.

The barbiturate PB is a medicine that has been used in the treatment of the epilepsy since 1912. It is an important cure agent, which still today is frequently administered for long periods to adults and children [5]. However, several studies in literature, using as subjects man, mice and rats have mentioned side effects of PB administration. Disturbances of the psychomotor function, of the learning and memory were the main negative effects observed [6, 7, 8]. These impaired effects of PB are, in most cases, dependent on

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the doses administered, being generally not observed in therapeutic doses [8, 9, 10].

Other prejudicial effects mentioned in the literature include (1) cell division disturbance causing a 50% decrease in the number of cells in culture of mouse neurons and disruption of the normal interaction between neurones and glia that is required for survival of neurones [11]; (2) hepatic lesions involving increased DNA synthesis and cell proliferation, and decreased apoptosis [12]; or (3) apoptosis induction [13, 14, 15].

Such damaging effects have produced a concern about PB administration mainly because it is also used in pregnant mothers and neonates with seizures. The present observations of harmful developmental effects of PB treatment in *Aedes aegypti* indicate that this organism, which is easy to manipulate in laboratory, may be an useful system to study more deeply the mechanisms and the consequences of that damage.

Materials and Methods

Aedes aegypti mosquitoes from São José do Rio Preto (SJ), São Paulo State and Goiânia (GO), Goiás State, (Brazil) were used. *Aedes* are holometabolous insects. Their development includes the stages of egg, larvae (with four sub-stages: L1, L2, L3 and L4), pupae and adults. Mosquitoes in larval and pupal stages were collected in tyres and other artificial containers by SUCEN (Superintendência de Controle de Endemias do Estado de São Paulo), in SJ, and studied in the first generation produced in the Vector Laboratory (IBILCE/UNESP). The mosquitoes from Goiânia were supplied by Dr. Ionizete Garcia da Silva from the Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás. A difference between both populations is that GO was considered resistant to insecticides [16] and SJ was considered susceptible [17, 18]. The present study was carried out from 1999 to 2000.

The mosquitoes are maintained in laboratory in cages with walls of fine mesh. One of the walls contains an opening for handling. Into the cages are put: (1) a vial containing sugar solution and a thread of hydrophilic cotton partially immersed in it, used to feed the adults; (2) a glass half filled with tap water containing a strip of filter paper in the limit of the water level for oviposition; and (3) a glass containing water and a cone of filter paper having the basis immersed in the water, for maintaining humidity inside the cage. Once or twice a week, a little mouse immovable in a metallic mesh is put into the cage for feeding females with blood. Blood meals are necessary for development of their oocytes.

The treatment and the control experiments for samples from SJ started with 776 and 674 eggs, respectively, while those for samples from GO started with 200 eggs in both cases. The treatments with PB (5-Ethyl-5phenyl-2, 4, 6 (1H, 3H, -5H)-pyrimidinetrione) were carried out by putting eggs or L1 larvae of *A. aegypti* to develop in a 0.2mg/mL PB aqueous solution. 0.3mg/ml of fish food was daily added for feeding larvae Plastic containers (12x12x5cm) with 150ml of the medium were used with transference to new medium in alternate days. The control tests were prepared using the same protocol of the treated tests, without PB. Larval eclosion, mortality during development, developmental time and adult longevity were studied. The analyses were performed daily till the death of the last adult produced in the experiments.

Statistical analysis for comparison of treated and control experiments as to production and mortality of mosquitoes involved the use of the hypothesis test for proportion of two samples [19]. The Student's t test was also used for comparison of the experiments with respect to longevity [20]. Differences between tests were measured at 1% significance level.

Results

Table 1 shows the results of tests started with eggs. The number of ecloded larvae and the developmental time were recorded.

Table 1. Percentage of eclosion and mean time at onset of each stage (in hours), for PB treated (T) and control (C) mosquitoes from São José do Rio Preto (SJ) and Goiânia (GO).

				Mean time in hours at onset of each stage (minimum and maximum values)					
Origin	**Test**	**n eggs**	**Eclosion (%)**	**egg-L1**	**L2**	**L3**	**L4**	**P**	**A**
SJ	T	776	566 (73)	24	60	93	144 *	226*	276*
					(48-144)	(72-144)	(96-216)	(192-264)	(240-336)
	C	674	473 (70)	24	48	76	108	164	236
						(72-96)	(96-144)	(144-192)	(192-288)
GO	T	200	154 (77)	36	60	96	120	204	264
				(24-48)	(48-72)	(72-120)	(96-144)	(168-240)	(216-312)
	C	200	129 (64.5)	24	60	84	108	192	252
					(48-72)	(72-96)	(96-120)	(144-240)	(192-312)

L1= first instar larvae; L2= second instar larvae; L3= third instar larvae; L4= fourth instar larvae; P= pupae; A= adult; *= Significant differences between T and C mosquitoes tested at level P<0.01.

Table 2. Number (N) and percentage (in parentheses) of female (F) and male (M) imagoes produced and mortality during development (Mo) in PB treated tests (T) started with L1 larvae from São José do Rio Preto (SJ) and Goiânia (GO), and control tests (C).

			Imago production			**PEI**			
Origin	Test	N L1	Total	F	M	Total	F	M	Mo
SJ	T	225	85 (38)*	45	40	84 (37.3)	32	52	140 (62)*
	C	143	131 (92)	54	77				12 (8)
GO	T	60	44 (73)*	22	22	12 (20)	5	7	16 (27)*
	C	47	47 (100)	24	23				0

PEI= partially emerged imagoes; *= Significant differences between T and C mosquitoes tested at level P<0.01.

The differences of the total number of ecloded larvae between the PB treated and the control tests were not significant in both populations. In relation to the time at onset of the developmental stages, the treated tests showed delay in comparison with the control tests. For mosquitoes from SJ, the differences were significant in the stages L4, pupae and adults. Differences between treated and control tests were greater in SJ than in GO population; in GO none difference was statistically significant.

Data on tests started with L1 larvae are in Table 2. The number of imagoes (or adults) produced was significantly smaller in the treated than in the control tests, in both populations. In the treated experiments, about 50% of the imagoes from SJ and about 21% from GO could not emerge completely from the pupal case and died in this condition (Figure 1). The number of males partially emerged was almost twice the number of females, in SJ population. Mortality during development was higher in the treated tests, in mosquitoes from both localities.

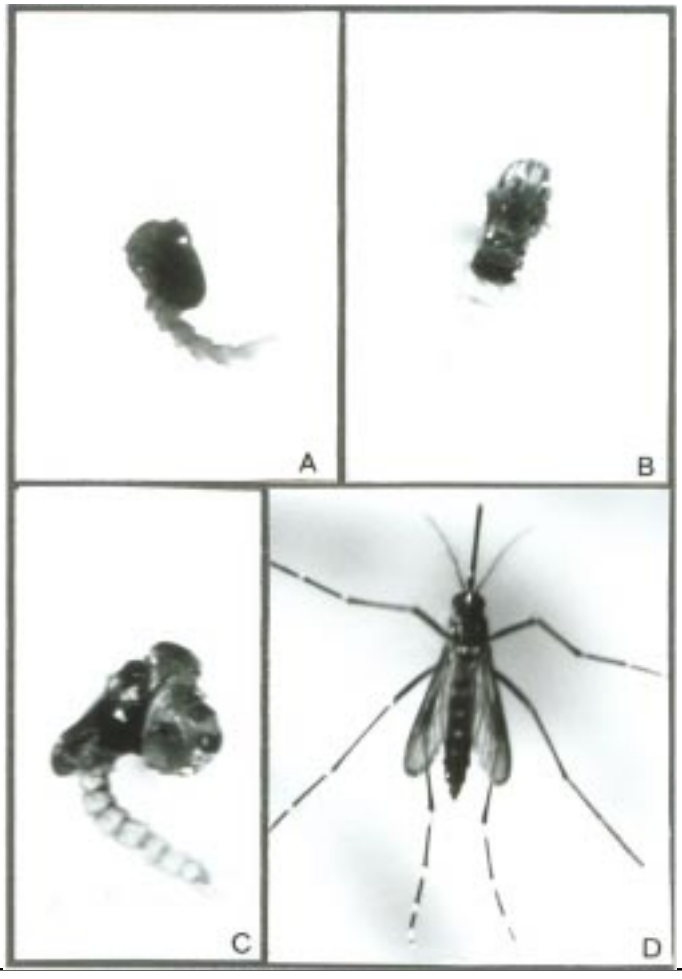
As to the adult longevity (Table 3), *Aedes* females generally live longer than males. PB treatment decreased the longevity of both sexes. Differences between treated and control tests were significant for mosquitoes from both localities. The treated imagoes that failed to emerge, even when they were carefully taken off the pupal case with the help of a fine paintbrush, died within 24 hours. The treated imagoes that emerged normally survived from 1 to 13 days.

Table 3. Adult longevity (in days) for mosquitoes from São José do Rio Preto (SJ) and Goiânia (GO).

		Adult longevity							
		N		Minimum		Maximum		Mean	
Origin	Test	F	M	F	M	F	M	F	M
SJ	T	14	24	1	1	8	4	2.5*	1.3*
	C	21	41	8	9	53	51	44.6	41.5
GO	T	17	12	1	1	13	3	4.1*	1.2*
	C	10	11	47	26	77	58	65.7	39.3

T= PB treated tests; C= control tests; N= number of females (F) and males (M). *= Significant differences between T and C mosquitoes tested at level P<0.01.

Figure 1 A-D. A- Normal pupae; B-C. Two different views of a PB treated mosquito, partially emerged from the pupal case; D- Recently emerged female of *Aedes aegypti*.



Discussion

Data in literature has shown that the organisms respond to PB treatment by the induction of gene expression. Brun et al. [21] showed that the expression of cytochrome P450 gene in *Drosophila melanogaster* under the effect of that substance increases by about 15-fold in some tissues. Cytochrome P450 metabolizes a variety of endogenous and exogenous chemicals including aromatic compounds, steroids, fatty acids, etc., and is also involved in insecticide resistance.

In the present study, some biological effects of PB treatment were analyzed in samples of two Brazilian populations of *Aedes aegypti*: GO, from Goiânia (State of Goiás), considered resistant to OP insecticides, and SJ, from São José do Rio Preto (State of São Paulo), considered susceptible to the same compounds [16, 17, 18]. The use of these two populations was due to the finding by Fuchs et al. [4] that PB treatment affects differently resistant and susceptible *Drosophila* flies, the first showing a lower inducibility of the cytochrome P450 system than the second.

The percentage of larval eclosion, the developmental time, the mortality from L1 larval stage till the emergence from pupal case and the adult longevity were analyzed in the present study. The percentage of eclosion was apparently not affected by PB at the concentration used (0.2 mg/mL), but the development was impaired, showing delay mainly at the onset of the L4 and pupal stage and in the emergence of the adults. Although this effect has occurred in mosquitoes from SJ and GO, the differences between control and treated tests were significant only in SJ, reinforcing data obtained by Fuchs et al. [4] that insecticide resistant mosquitoes have some additional protection against PB action on the characteristics studied. PB induced tolerance by PB treatment was also greater in mosquitoes from SJ than in those from GO [22].

The treated experiments yielded a smaller number of adults than the controls in mosquitoes from both localities. In addition to the increased mortality between L1 and adult stages, longevity was also significantly reduced in the treated experiments.

The impairing effect of PB at the pupal stage occurred by blockade of the imago emergence from pupal case. A relatively high percentage of imagoes died during emergence, with the body partially out of the pupal case. Even if they were carefully helped to get out from the pupal case, they died in a short period of time (24 hours or less). This observation suggests that the impossibility to emerge is not the cause of their death but an indication that a deeper problem is involved. The slower movements of the adults from the treated experiments compared to the control suggest that this problem may involve the nervous system. This suggestion is supported by data in rodents and man, present in the literature. As mentioned, several studies indicated an impairing effect of PB on development of the nervous system. Loegering & Johnson [11] showed that, in rat, PB at 30mg/mL reduced significantly the percentage of neurons with dendrites, the number of primary dendrites per neuron and the average length per dendrite. In rat cultures, PB at 15mg/ml also affected dendritic development and maintenance of dendritic morphology [23].

Sousa-Polezzi & Bicudo [22] observed significant differences between treated and control tests in the frequency of expression, and in the activity degree of two esterase enzymes: a carboxylesterase (EST-1) and a cholinesterase (EST-13). The finding of brain acetylcholinesterase (AchE) inhibition following pretreatment with monooxygenase inducers such as PB [24] and the finding that PB in high doses can decrease the release, alter the rate of synthesis and change the concentration of acetylcholine [5] reinforce the possibility of involvement of the EST-13 in the impaired effects on development of *A. aegypti*. The carboxylesterase EST-1 is also an important esterase because it is present in every mosquito and in every developmental stage. Its expression is generally very high in adults but decreases in PB treated mosquitoes. This low synthesis may also be part of the problem caused by PB treatment.

Data in literature showed that agents that potentiate the action of g-aminobutyric acid (GABA) (such as PB), as well as those that decreases brain levels of GABA (such as allylglycine) affects brain development. Larvae of *Drosophila melanogaster* fed with allylglycine pupated and matured apparently normally, but viable adults failed to emerge [25]. In the present study, *A. aegypti* emergence was affected by PB, reinforcing the indication that also in this case the nervous system is primarily impaired.

Because PB is used as an anticonvulsant in both pregnant mothers and neonates with seizures, there has been an increased concern over its potential adverse effects on the development of the nervous system [11, 15]. The present results in *A. aegypti*, showed that important biological processes are affected in the treated mosquitoes. They are also indicative that *Aedes* is a promising organism to study more deeply the side effects of PB administration and their mechanisms.

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