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Comet assay in *Aedes aegypti* (Diptera: Culicidae): genotoxic effect of chemicals on neuroblasts

Ensaio Cometa em *Aedes aegypti* (Diptera: Culicidae): efeitos genotóxicos de químicos em neuroblastos

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Aedes (*Stegomyia*) *aegypti* is the main vector of the viruses that cause dengue, chikungunya, urban yellow fever, and Zika viruses, whose control is carried out mainly via chemical insecticides. Studies on the genotoxic effect of insecticides on these mosquitoes are hampered due to the lack of detection of genomic lesions. As such, we used the comet assay method in larvae of *A. aegypti* treated with concentrations of 1, 2 and 3% of dimethyl sulfoxide (DMSO), 0.002, 0.005 and 0.007 µg/mL of temephos (TE), 0.05, 0.5 and 1.0 mM of cyclophosphamide (CPA) for 4 h, for detection of possible DNA damage. Our results demonstrated that this test is effective in *A. aegypti* neuroblasts, which indicates possibilities for further studies using new candidate substances for the control of this important vector of arboviruses.
Keywords: arbovirus, genotoxicity, mosquito.

Aedes (*Stegomyia*) *aegypti* é o principal vetor dos vírus causadores da dengue, chikungunya, febre amarela urbana e Zika, cujo controle é feito principalmente por meio de inseticidas químicos. Estudos sobre o efeito genotóxico de inseticidas nesses mosquitos são dificultados pela falta de detecção de lesões genômicas. Em vista disso, utilizamos o método do ensaio cometa em larvas de *A. aegypti* tratados com concentrações de 1, 2 e 3% de dimetil sulfóxido (DMSO), 0,002, 0,005 e 0,007 µg/mL de temefós (TE), 0,05, 0,5 e 1,0 mM de ciclofosfamida (CPA) por 4 h, para detecção de possíveis danos no DNA. Nossos resultados demonstraram que este teste é eficaz em *A. aegypti*, o que indica possibilidades de novos estudos utilizando novas substâncias candidatas para o controle desse importante vetor de arbovírus.
Palavras-chave: arbovírus, genotoxicidade, mosquito.

1. INTRODUCTION

Dengue is the most notable arbovirus to affect humans, and it is transmitted mainly by the bite of the female *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) mosquito after it has been infected with the virus of the genus Flavivirus of the family Flaviviridae. A total of four different serotypes are known to circulate (DEN-1, DEN-2, DEN-3 and DEN-4). Other arboviruses transmitted by *A. aegypti*, such as Zika virus (ZIKV) and yellow fever (YFV), which belong to the Flaviviridae family, genus *Flavivirus*, and Chikungunya virus (CHIKV), Togaviridae family, genus *Alphavirus*, also have epidemiological importance [1].

In recent decades, populations of *A. aegypti* in Brazil have been subjected to insecticide selection pressures of varying levels and from varying sources. Due to their wide use in *Aedes* mosquito control programs, in some cases, the insects have developed resistance to the chemical insecticides temephos and deltamethrin [2]. Thus, using larvicidal tests [3], there have been intense searches for alternatives for vector control, especially those of botanical origin [4].

Considering that among many living beings the DNA molecule has similarity in its structure, function and repair mechanisms, these characteristics allow the use of the single cell gel electrophoresis assay (SCGE), or the comet assay, for genotoxicity studies of chemical substances. The comet assay is a powerful tool in the analysis of genotoxicity and DNA repair at the cellular level [5] of vertebrates [6] and invertebrates [7].

The present study presents a protocol for the *in vivo* application of the comet assay in neuroblasts of larvae of *A. aegypti* from Manaus, Amazonas. The chemicals dimethyl sulfoxide (DMSO), temephos (TE) and cyclophosphamide (CPA) were used to validate the comet assay method, and enable the study of this genotoxicity parameter in chemical agents, which will serve as a basis for other studies involving mosquitoes.

2. MATERIALS AND METHODS

2.1 Acquisition of *A. aegypti* larvae

Larvae of *A. aegypti* were captured in the center of the city of Manaus, capital of Amazonas state, Brazil (03° 08' 33.5" S, 60° 01' 13.5" W) in March 2019, according to the method proposed by Santos et al. (2020) [8]. The collection was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and the Biodiversity Authorization and Information System (SISBIO), through the permanent license (No. 32941) (May 21st, 2012). The mosquitoes were identified with aid of taxonomic identification keys.

2.2 *Aedes aegypti* larvae toxicity bioassay

The compounds dimethyl sulfoxide - DMSO (Merck Millipore), temephos - TE (Fersol 500 CE) and cyclophosphamide - CPA (Endoxan®) were evaluated via a bioassay. The experiment, which was performed in triplicate, was carried out in 100 mL plastic cups, which contained ten 3rd instar *A. aegypti* larvae, for each treatment, totalling 300 larvae. For four hours, the samples were subjected to the concentrations of 1, 2 and 3% of DMSO, 0.002, 0.005 and 0.007 µg/mL of TE, and 0.05, 0.5 and 1.0 mM of CPA, or the negative control (NC), which contained filtered water. After exposure, the larvae of each group were transferred to cups containing 20 mL of Ringer's solution (Equiplex®) (Figure 1).

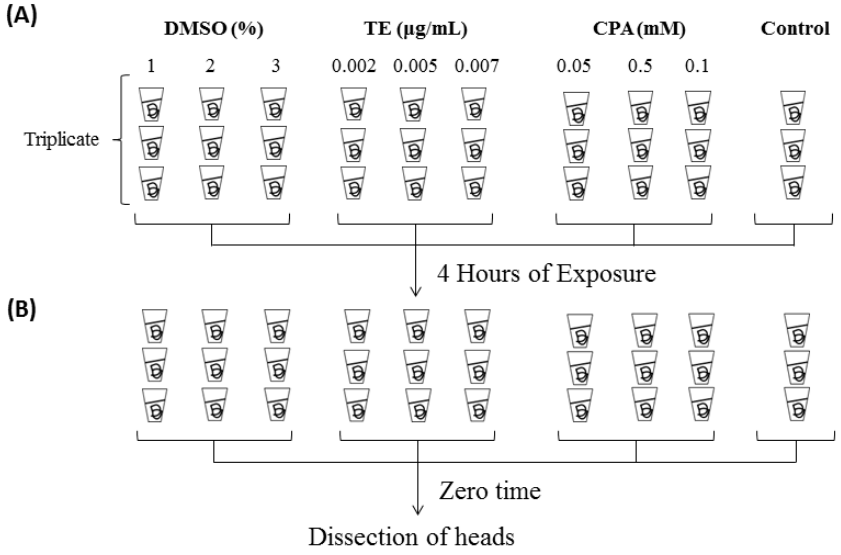

Figure 1: Toxicity test of dimethyl sulfoxide (DMSO), temephos (TE) and cyclophosphamide (CPA), using ten 3rd instar Aedes aegypti larvae for each treatment, in triplicate, for four hours (A), which were then transferred to Ringer's solution (B).

2.3 Dissection and cytological preparations of the neuroblasts of the *A. aegypti* larvae

The heads of the *A. aegypti* larvae were dissected (Figures 2 A and 2 B) and placed in a Kline plate containing 50 µL of Ringer's solution. The brain ganglia (Figure 2 C) were dissected and transferred to 2 mL microtubes containing 300 µL of Ringer's solution, and then homogenized. The cell suspension (50 µL) was mixed with 120 µL of low melting point agarose (0.5%) and spread on glass slides coated with normal melting point agarose (1.5%) and maintained at 4 ºC for 5 minutes. Immediately after, the glass slides were immersed in a freshly prepared lysis solution consisting of 2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100 and 10 mM Tris, pH 10, for 60 min at 4 ºC.

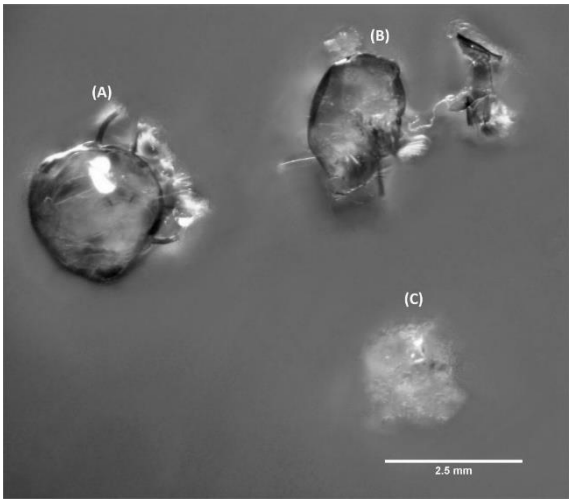

Figure 2: Head of third-instar larva of Aedes aegypti (A, B). Brain ganglion of third-instar larva of Aedes aegypti (C).

2.4 Comet assay

After lysis, the slides were placed in a horizontal electrophoresis tank containing 300 mM NaOH and 1 mM EDTA (pH > 13) for DNA denaturation during 20 minutes. Electrophoresis was performed at 1 V/cm (25 V and 300 mA) for 15 min. The slides were subsequently immersed in a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 10 min. After drying at room temperature, the slides were fixed in ethanol PA for 10 minutes and stored for further analysis.

2.5 Analyses of the comets – microphotography

Each cytological preparation was stained with 30 µL of ethidium bromide (20 µL/mL) and immediately analyzed under an epifluorescence microscope (Axio Imager A2 Zeiss) using the Cy3 filter at a wavelength of 510-575 nm. A total of 100 comets per slide were captured and analyzed at a magnification of 400 x, which equated to 500 structures per treated group. The photos of the comets were transferred to Comet Score 2.0 software. The following parameters were used: tail length (mm), tail DNA (%) and tail moment (arbitrary units).

Subsequently, descriptive statistical analysis of the data was performed and the Kruskal-Wallis test was applied. Significance was noted in this test, and Fisher's minimum significant difference test with Bonferroni correction was applied to the rank means of the treatment in comparison with the control. A 5% probability level was used and the analyses were performed in the R software (Version 4.1.0). The value $p<0.05$ was considered statistically significant.

3. RESULTS

A dose-dependent increase in DNA damage was observed when compared to the control, as evidenced by a statistically significant increase in the parameters of the comet ($p<0.001$), i.e., tail length, tail DNA (%) and tail moment.

Regarding the analyses of the variable tail length, with the exception of the 1% DMSO treatment, the other treatments differed significantly from the control, thus indicating an increase in DNA damage (Figure 3 A). On the other hand, the 0.007 μg/mL TE and 1.0 mM CPA treatments had the highest mean; however, they did not differ significantly from 0.5 mM CPA, 3% DMSO, 0.002 μg/mL TE and 0.005 μg/mL TE treatments. The treatments 0.05 mM CPA and 2% DMSO have intermediate values, and the first of these two does not differ significantly from the 0.5 mM CPA, 3% DMSO, 0.002 μg/mL TE and 0.005 μg/mL TE treatments, and the second does not differ significantly from the control or 1% DMSO treatment. For the variable tail DNA, the control differed from all the other treatments. It is worth noting that the 1% DMSO treatment did not differ from the 3% DMSO and 0.002 μg/mL TE treatments (Figure 3 B). Nonetheless, 2% DMSO, 0.005 μg/mL TE, 0.007 μg/mL TE, 0.05 mM CPA, 0.5 mM CPA, 1.0 mM CPA presented the highest values of tail DNA, thus indicating an increase in genotoxicity in the mitotic nuclei that were submitted to these concentrations.

Regarding the variable tail moment, when compared to the control group, the 1% DMSO treatment did not induce any significant DNA damage in the cells of the organisms exposed to treatment, which contrasts with all other treatments since they showed significant difference in relation to the control (Figure 3 C). However, 1% DMSO treatment does not differ significantly from 2% DMSO treatment. Conversely, the treatments 0.007 μg/mL TE and 1.0 mM CPA have the highest values for this variable; however, they do not differ significantly from 0.005 μg/mL TE, 0.05 mM CPA, 0.002 μg/mL TE, 0.5 mM CPA and 3% DMSO.

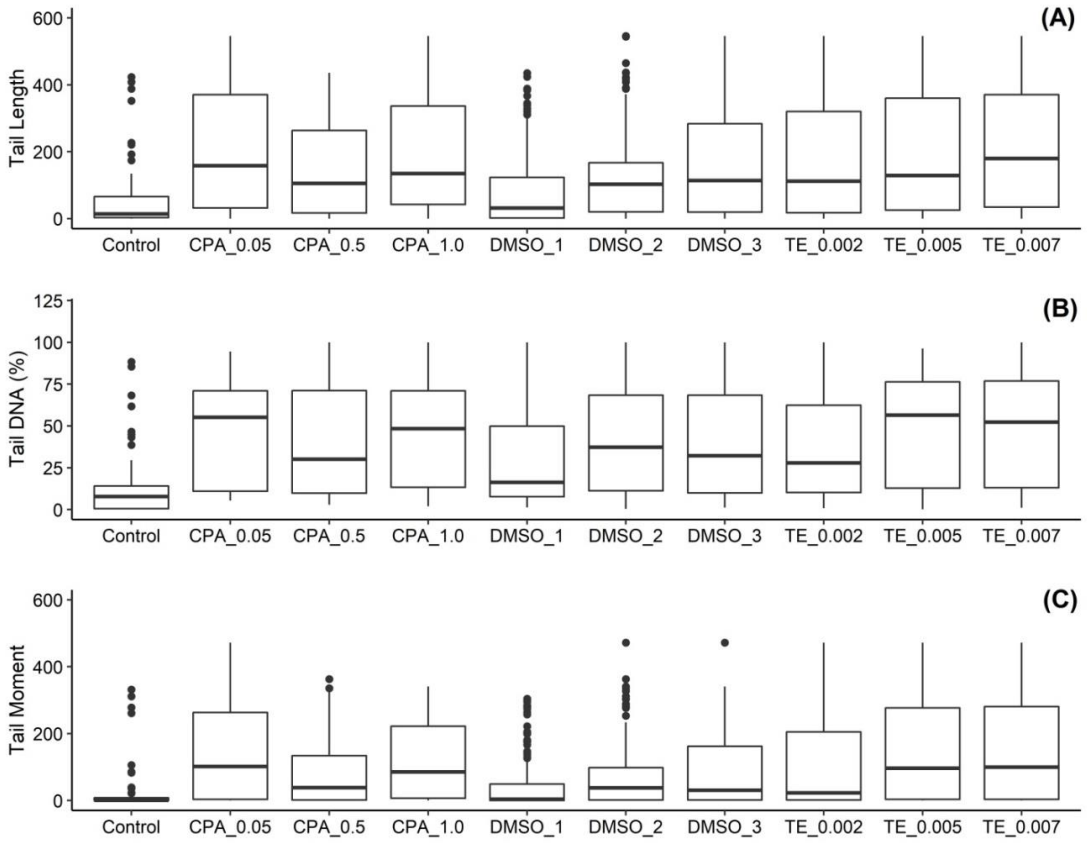

Figure 3: Genotoxic effect of dimethyl sulfoxide (DMSO), temephos (TE) and cyclophosphamide (CPA) via comet assay, using the parameters (A) tail length, (B) tail DNA, and (C) tail moment in third-instar larvae of Aedes aegypti.

4. DISCUSSION

In general, one of the peculiarities found in the results of this study is the fact that there was elevated DNA damage in the negative control, in the tail length and tail DNA parameters. Similar results, only in the tail moment parameter, were reported in the negative control in larvae of *D. melanogaster* [9]. Another interesting aspect was the registration of cells of different sizes in all phases of the cell cycle, which made the analysis difficult. A reduced number of cells per field under the microscope was also observed, thus corroborating the shortage of neuroblasts in each slide, which is commonly reported in this mosquito.

The adaptation of the comet assay in order to evaluate genomic lesions in insects is relatively recent [10], and the first reports were in *D. melanogaster* [11] and in adult males of *A. aegypti* [12]. The present comet assay study is a pioneer for larvae of *A. aegypti* subjected to the effect of chemicals that are widely used in genotoxicity research.

DMSO is a universal solvent that, in addition to being routinely used in experimental and biological research, is considered to be non-toxic in concentrations up to 10% [13]. However, its application in cytogenetic studies deserves attention because, in the data of the present study, DMSO at 2 and 3% induced DNA damage in neuroblasts of larvae of *A. aegypti*. This finding corroborates those of Calkins and Piermarini (2015) [2], who showed its toxic effect in *A. aegypti* when over 2%. In research with *A. aegypti* and *A. albopictus*, DMSO at concentrations of 0.5% showed no toxicity [14].

Studies using the micronucleus test on neuroblasts of larvae and oocytes of adult female *A. aegypti* recorded that TE at concentrations of 0.002, 0.005 and 0.007 μg/mL, for 4 hours, caused the formation of micronuclei, nuclear buds and nucleoplasmic bridges and chromosomal breaks in these nuclei [15]. Studies regarding genotoxicity caused by temephos in insects are still scarce, despite the wide use of this larvicide in the control of *A. aegypti* in the period 2003-2014 in Brazil. Nonetheless, in this study, DNA alterations were similar to those observed in *A. aegypti* after treatment with TE.

In this study, at all concentrations, CPA was genotoxic to *A. aegypti*. Another study, involving *D. melanogaster*, with low concentrations of CPA in acute (0.1 mg/mL, for 24 h) and chronic (0.05 mg/mL, for 35 days) treatments, showed evidence of gene alterations, mitochondrial dysfunction, and alteration in the transcription pattern of transposable elements of the gypsy superfamily [16].

5. CONCLUSIONS

The alkaline comet assay method was successfully validated in the present study by evaluating the genotoxic potential of the chemicals in *A. aegypti*. It is suggested that the validation of this biomarker of damage to sensitive DNA, either isolated or in association with another cytogenetic method of cytogenotoxicity detection, may reveal differences in sensitivity, in parameters and in data generated from genotoxicity and posterior DNA repair regarding other larvicidal substances. As such, the comet assay can assist in actions for vector control and environmental risk assessment of larvicides.

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