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ENDEREÇO/ADDRESS: ***Biologia Geral e Experimental***, Departamento de Biologia – Universidade Fedral de Sergipe. Av. Mal. Rondon s/n, Jardim Rosa Elze, São Cristóvão, Se; CEP 49100-000; e-mail: jcvilar@bol.com.br *ou* cmorato@bol.com.br.

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CARDIOVASCULAR EFFECTS ON RATS INDUCED BY THE TOTAL ALKALOID FRACTION OF *SIDA CORDIFOLIA*

Márcio Roberto Viana Santos [1]
Murilo Marchioro[1]
Aletéia Lacerda Silveira[2]
José Maria Barbosa Filho [2]
Isac Almeida Medeiros[2]

ABSTRACT

Cardiovascular effects induced by the total alkaloid fraction of *Sida cordifolia* L. (TAF) were investigated in unanesthetized normotensive rats. In these animals, TAF (0.5, 1, 5 and 10 mg/kg, i.v.) induced hypotension, which was attenuated by atropine, L-NAME or hexamethonium at the 10 mg/kg dose, and bradycardia, which was eliminated by atropine at all doses, and attenuated by hexamethonium at 5 and 10 mg/kg, but was unchanged by L-NAME. These results demonstrate that TAF induces hypotension and bradycardia, which appear to be mediated by direct and indirect activation of muscarinic receptors. Nitric oxide also appears to be involved in the hypotensive response.

Keywords: *Sida cordifolia*, total alkaloid fraction, cardiovascular effects, hypotension, rat.

RESUMO

Os efeitos cardiovasculares induzidos pela fração de alcalóides totais da *Sida cordifolia* L. (TAF) foram avaliados em ratos normotensos não-anestesiados. Nestes animais, TAF (0,5; 1; 5 e 10 mg/kg, i.v.) induziu hipotensão, que foi atenuada pela atropina, L-NAME ou hexametônio apenas na dose de 10 mg/kg, e bradicardia que foi abolida pela atropina, em todas as doses, e atenuada pelo hexametônio nas doses de 5 e 10 mg/kg, mas não foi alterada pelo L-NAME. Estes resultados demonstram que TAF induz hipotensão e bradicardia, que parece envolver uma ativação direta e indireta de receptores muscarínicos. Além disso, o óxido nítrico parece também participar do efeito hipotensor induzido pelo TAF.

Palavras-chave: *Sida cordifolia*, fração de alcalóides totais, efeitos cardiovasculares, hipotensão, rato.

INTRODUCTION

The use of medicinal plants for the treatment of human diseases has increased considerably throughout the World. Evaluation of the effects of these plants on organs and systems contributes to the development of the scientific basis for therapeutic applications, and enriches alternatives for the treatment of a growing number of diseases (Elizabetsky, 1986).

Sida cordifolia L. (Malvaceae), a native species of the Brazilian Northeast, is known popularly as "Malva branca". It is used in folk medicine as an antirheumatic and antipyretic (Muzaffer *et al.*, 1991), an antiinflammatory and analgesic (Franzotti *et al.*, 2000), and an antiasthmatic and nasal anticongestant (Ghosh & Dutt, 1930; Mukerji, 1953). Previous studies have demonstrated that vasicine, an alkaloid isolated from this plant (Ghosal *et al.*, 1975), produced hypotension and bradycardia in anaesthetized dogs (Gupta *et al.*, 1977) and unanaesthetized rats (Silveira

[1] Departamento de Fisiologia, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n, Jardim Rosa Elze, São Cristóvão, Se, 49100-000. marcio@infonet.com.br
[2] Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, caixa postal 5009, João Pessoa, PB, 58.051-970.

et al., 2003). In the pharmacological study reported here, we evaluated the effects of the total alkaloid fraction of *Sida cordifolia* L. on the arterial pressure and heart rate of unanesthetized, normotensive rats (*Rattus norvegicus*). We demonstrate that the hydroalcoholic extract of this plant also induces marked hypotension, associated with intense bradycardia, in rats.

MATERIALS AND METHODS

Preparation of the total alkaloid fraction: *Sida cordifolia* leaves (voucher specimen nº. 30171, deposited in the hebarium of the Department of Biology, Universidade Federal de Sergipe, Brazil) were dried, pulverized and immersed in 95% EtOH at room temperature for 72 h. After vacuum concentration, the extract obtained was submitted to conventional acid-base treatment to produce the total alkaloid fraction (TAF). To avoid decomposition, the TAF was dissolved in a cremophor-saline solution (0.1% v/v) at desired concentrations just before each experiment. Cremophor had no effect when tested under controlled conditions (data not shown).

Animals: Male Wistar rats (250-350 g) were used for all experiments. The animals were housed under controlled conditions of temperature (21 ± 1ºC) and lighting (lights on: 06:00-18:00 h), with free access to food and tap water.

Drugs: The drugs used were: heparin sodium salt (Ariston), sodium thiopental (Cristália), atropine sulfate, Nʷ-nitro-*L-arginine* methyl ester (L-NAME), hexamethonium. sodium nitroprusside and cremophor (all from Sigma). All drugs were dissolved freely in saline solution.

Blood pressure measurements: Under sodium thiopental anesthesia (45 mg/kg, i.v.), the lower abdominal aorta and inferior *vena cava* were canulated via the left femoral artery and vein using polyethylene catheters. The catheters were then filled with heparinized saline solution and slid under the skin to emerge between the scapulae. Arterial pressure was measured after 24 h by connecting the arterial catheter to a pre-calibrated pressure transducer (Edwards Lifescience, Irvine, CA, USA) and pressure outputs were recorded by an amplifier-recorder (BioData, Model BD-01, PB, Brazil) connected to a personal computer equipped with an analog-to-digital converter board (BioData, PB, Brazil). Data were collected at a frequency of 200 Hz. For each cardiac cycle, the computer calculated mean arterial pressure (MAP) and pulse interval, referred to here as the heart rate (HR). The venous catheter was inserted for drug administration. Sodium nitroprusside (10 µg/kg) was injected to check the efficacy of catheter insertion.

Experimental protocol: After stabilization of cardiovascular parameters, MAP and HR were recorded before (baseline values) and after randomized i.v. administration of different doses of TAF (0.5, 1, 5 and 10 mg/kg). These administrations were separated by a time interval sufficient to allow full recovery of cardiovascular parameters. A control dose-response curve was then obtained. Dose-response curve were also obtained separately in animals pre-treated with atropine (2 mg/kg; i.v.; 15 min.), a non-selective antagonist of muscarinic receptors (Mitchelson, 1984), L-NAME (20 mg/kg, i.v. 30 min.), a competitive inhibitor of NO-synthase (Moncada & Higgs, 1993) or hexamethonium, a ganglionic blockade (Takahashi & Owyang, 1997).

Statistics: Values are expressed as means ± SEM. The significance of differences among means was tested using one-way ANOVA with Dunnett's post test. All procedures were carried out on Graph Pad Prism™ version 3.02 software.

RESULTS

As expected from control animals, atropine and hexamethonium increased HR significantly, from 350 ± 7 bpm to 485 ± 11 and 416 ± 20 bpm ($p < 0.05$; $n = 6$), respectively. The administration of L-NAME increased MAP and HR from 110 ± 3 mmHg to 136 ± 4 mmHg and from 350 ± 7 bpm to 373 ± 9 bpm ($p < 0.05$; $n = 6$), respectively.

Figure 1 shows original traces of the effect induced by *S. cordifolia* TAF (10 mg/kg, i.v.) in one unanesthetized normotensive rat. In these animals, TAF (0.5, 1, 5 and 10 mg/kg; i.v., randomly) induced transitory hypotension (-1 ± 1; -5 ± 1; -21 ± 4 and -39 ± 3 %) associated with intense bradycardia (-0.7 ± 0.1; -5 ± 0.7; -44 ± 7 and -70 ± 2 %) (Figure 2).

In animals pre-treated with atropine (2 mg/kg), L-NAME (20 mg/kg) or hexamethonium (20 mg/kg), the hypotensive response induced by TAF was only attenuated at the highest dose (10 mg/kg). However, the bradycardic response was eliminated by atropine at all doses, and attenuated by hexamethonium at 5 and 10 mg/kg, although it was not altered by L-NAME (Figure 2).

DISCUSSION

We chose to evaluate the effects of the TAF of *S. cordifolia* on the cardiovascular parameters of unanesthetized rats in order to avoid the possible influence of anesthesia and surgical stress (Smith & Hutchins, 1980; Fluckiger *et al.*, 1985). Baseline MAP and HR values recorded here were similar to those reported in previous studies (Lahlou *et al.*, 2002; Silveira *et al*., 2003; Cunha *et al.*, 2004). Acute administration of TAF induced hypotension associated with bradycardia.

Peterson *et al.* (1984) established that the primary autonomic regulation of sinoatrial node function is carried out by vagal action via stimulation of cardiac muscarinic receptors. Stimulation of these receptors induces intense bradycardia followed by hypotension due to a decrease in cardiac output. It is also well known that activation of endothelial muscarinic receptors induces intense vasodilatation due to release of endothelium-derived relaxing factors (Moncada *et al.*, 1991), mainly NO (Furchgott & Zawadzki, 1980; Moncada *et al.*, 1991). This activation can cause decrease in peripheral vascular resistance and, consequently, hypotension.

In order to evaluate the role of these receptors in TAF-induced responses, we performed experiments in the presence of atropine, a non-selective antagonist of muscarinic receptors (Mitchelson, 1984). Under these conditions, bradycardia was abolished at all doses, although hypotension was attenuated significantly at 10 mg/kg. This indicates that TAF induces bradycardia either through direct activation of receptors, or indirectly via vagal stimulation and release of acetylcholine into the sinoatrial node. However, hypotension does not appear to be due exclusively to the decrease in cardiac output related to bradycardia, given that it was only attenuated, but not abolished by atropine. This indicates that a decrease in peripheral vascular resistance may also contribute to TAF-induced hypotension.

To verify a possible indirect effect of TAF via the vagal nerve, we performed experiments with hexamethonium, a ganglionic blocker (Takahashi & Owyang, 1997). This drug attenuated bradycardia and hypotension significantly, but did not eliminate them altogether. This indicates that the bradycardic response is mediated by two distinct pathways, indirect and direct activation of cardiac muscarinic receptors.

Finally, we investigated the role of nitric oxide. In this condition, L-NAME, a competitive inhibitor of NO-synthase (Moncada & Higgs, 1993), changed the hypotensive response only at the 10 mg/kg dose, suggesting that NO is at least partly involved in the hypotensive effect.

These results agree with those of Silveira *et al.*

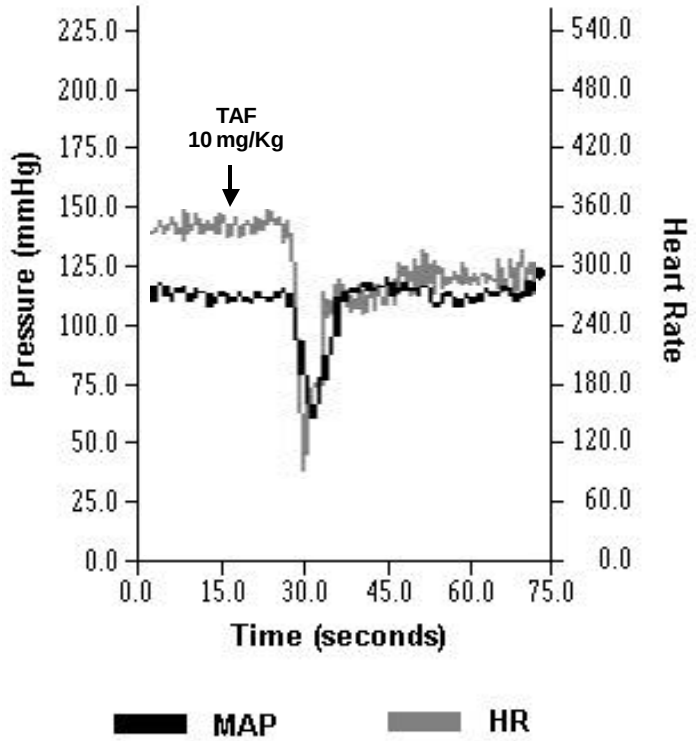

Figure 1. Original traces showing the effect of TAF (10 mg/kg; i.v.) on MAP and HR in one non-anaesthetized normotensive rat. The arrow indicates point of TAF administration.

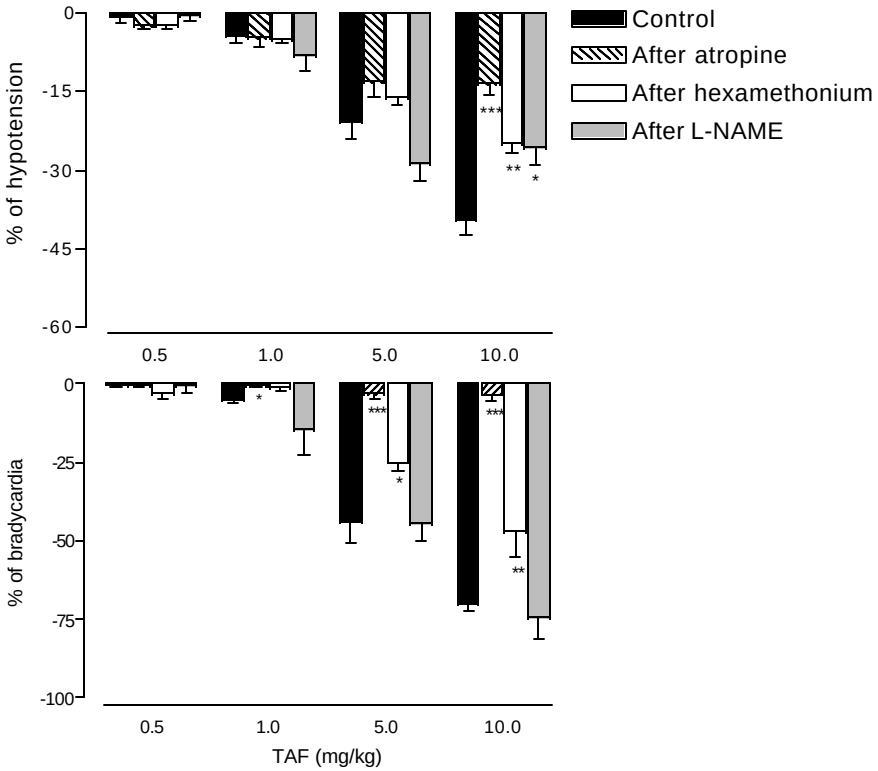

Figure 2. Hypotensive and bradycardic response induced by TAF (0.5, 1, 5 and 10 mg/Kg; i.v.) in non-anaesthetized normotensive rats before (Control) and after pre-treatment with atropine (2 mg/kg), hexamethonium (20 mg/kg) or L-NAME (20 mg/kg). Values are expressed as mean ± SEM of six experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs Control.

(2003), who demonstrated that vasicine, an alkaloid isolated from *S. cordifolia* (Ghosal *et al*., 1975), also produced marked hypotension associated with bradycardia in unanesthetized rats. The effects recorded in the present study may thus be due to the presence of vasicine in the TAF, although further data would be required to confirm this.

Overall. our results demonstrate that the TAF of *S. cordifolia* induces hypotension and bradycardia, apparently mediated by direct and indirect activation of muscarinic receptors. Nitric oxide also appears to be involved in the hypotensive response.

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ANTINOCICEPTIVE ACTIVITY OF THE AQUEOUS EXTRACT OF *BAUHINIA CHEILANTHA* (BONG.) STEUD. (LEGUMINOSAE: CAESALPINIOIDEAE)

Adriana M. O. Silva[1]
Flavia Teixeira-Silva[1]
Rogéria de Souza Nunes[1]
Rosilene Moretti Marçal[1]
Sócrates Cabral de Holanda Cavalcanti[1]
Ângelo Roberto Antoniolli[1]

ABSTRACT

Bauhinia cheilantha (Leguminosae: Caesalpinioideae) is a common plant of the Brasilian Caatinga widely used in folk medicine as an analgesic. In order to verify this effect pharmacologically, the antinociceptive activity of the plant was evaluated through the administration of its aqueous extract in mice. The extract was administered orally (400 mg/kg) 60 minutes before a writhing test, and was found to reduce nociception by 54.4%. The effects of formalin (1%) were also reduced by the extract at all doses. Naloxone (5 mg/kg, i.p.) and caffeine (10 mg/kg, i.p) reverted the effect of the extract. In a hot plate test, the extract (100mg, 200mg and 400 mg/kg) increased latency time by 39.8%, 30.7% and 32.8%, respectively. There was no acute toxicity in doses up to 3g/kg. The aqueous extract of the *B. cheilantha* bark revealed antinociceptive activity in all the models tested, effects that are possibly associated with the opioid and adenosine systems.

Keywords: *Bauhinia cheilanta*, antinociceptive, aqueous extract.

RESUMO

Bauhinia cheilantha (Leguminosae-Caesalpinioideae) é uma planta comum da caatinga amplamente utilizada na medicina popular como analgésica. Para verificar farmacologicamente este efeito, a atividade antinociceptiva desta planta foi verificada através do extrato aquoso da entre-casca, administrado em camundongos. O extrato (400 mg/kg), administrado por via oral 60 minutos antes do teste reduziu a nocicepção em 54,4%. Os efeitos da formalina (1%) também foram reduzidos pelo extrato em todas as doses. Naloxona (5 mg/kg, i.p.) e cafeína (10 mg/kg, i.p) também reverteram este efeito. No teste da placa quente o extrato de *B. cheilantha* nas doses 100mg, 200mg e 400 mg/kg, aumentou o tempo de latência em 39.8%, 30.7% e 32.8%, respectivamente. Não houve toxicidade aguda até a dose de 3g/Kg. O extrato aquoso da entre-casca de *B. cheilantha* mostrou atividade antinociceptiva nos modelos testados, efeito possivelmente associado com os sistemas opióide e adenosina.

Palavras-chave: *Bauhinia cheilanta*, antinociceptivo, extrato aquoso.

INTRODUCTION

Many plants of the Brasilian flora are used in local folk medicine, but very few have been pharmacologicaly validated. Among the unstudied majority is the legume *Bauhinia cheilantha* (Bong.) Steud., a small tree (average height 5 m), which is relatively common in the semi-arid Brasilian Caatinga. Known locally as *mororó*, the extract of its bark is used as an analgesic, but there are no pharmacological data on its properties.

A variety of pharmacological properties have been reported for species of the genus *Bauhinia.* For example, the seeds of *Bauhinia monandra* affect haemagglutination positively, although this effect is eliminated by autoclaving (Abreu et al, 1990; Penate et

[1] Departamento de Fisiologia, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n, Jardim Rosa Elze, São Cristóvão, Se, 49100-000. rosilenemoretti@ufs.br

al, 1988), and the leaves of *Bauhinia tarapotensis* have antioxidant properties (Braca et al., 2001). Other species of the genus *Bauhinia* have been reported to have properties related to the treatment of malaria and hypoglycemia, and the regulation of thyroid hormones (Kittakoop et al., 2000; Lemus et al., 1999; Pepato et al., 2002; Panda & Kar, 1999).

Several chemical compounds have been purified from different species of *Bauhinia*, including lectins, racemosol, and demethylracemosol. (Silva & Filho, 2002; Allen et al., 1980, Kittakoop et al., 2000). The medicinal uses of *B. cheilantha* have been reported in the ethnobotanical literature (eg. Andrade-Lima, 1988), but there have been no pharmacological studies of the analgeic properties of this plant, a lack of information which motivated the present study.

MATERIAL AND METHODS

Plant material: Bark of *B. cheilantha* was collected during the wet season in the village of Santa Rosa do Ermírio, Sergipe (09°45'S, 37°40'W). The species was identified by the biologist Gilvane V. Souza, and the voucher specimen (number 007490) is deposited at the herbarium of the Departamento de Biologia, Universidade Federal de Sergipe, Brasil.

Preparation of aqueous extract (AE): The bark was dried at 40°C in a forced air oven (Marconi MA 037) for 48 h and triturated in a mill to obtain a powder. This powder was added to distilled water (1:5 w/v) at 75°C and infused for 30 min in order to constitute the aqueous extract. After infusion, the AE was filtered and freeze dried in a VirTis bench top freeze dryer, yielding a brown powder (8%), which was diluted and used in the pharmacological tests. For the experiments, the extract was reconstituted in water at three different concentrations – 100 mg/mL (AE100), 200 mg/mL (AE200) and 400 mg/mL (AE400), which were administered orally to the mice 60 minutes before each experiment.

Animals: Male and female Swiss mice (20-35 g) were used as test animals. The animals were maintained in plastic cages, with food and water *ad libitum*, but were fasted for 12 hours prior to the oral administration of test substances. All experiments were performed in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals.

Preparation of drugs: Drugs used in the experiments were diluted to an injection volume of 0.1 mL/10g of animal weight, except when defined in the text. Acetic acid 0.6% (Merck), morphine hydrochloride (Sigma), formalin 1% (Baker), naloxone hydrochloride (Sigma), and caffeine (Sigma) were all diluted in water. Indomethacin (Sigma) was diluted in water/0.1 N NaOH (pH = 8). Indomethacin, morphine, naloxone and caffeine were injected 15 minutes before tests.

Acute toxicity: Acute toxicity of the plant was verified in three groups of mice (n = 5/ne group (n = 5) received distilled water; the others received the aqueous extract in increasing doses (1, 2, and 3 g/kg; p.o.). The mortality was observed during 48 hours.

Pharmacological tests

Antinociceptive activity was verified using the writhing test induced by acetic acid 0.6% (Koster et al. 1959), the formalin 1% test (Dubuisson et al., 1977; Hunskaar, 1986) and the hot plate test (Eddy & Leimbach, 1953).

Writhing test: Six groups of mice (n = 9) were tested. All animals received acetic acid 0.6% (0.1 mL/ 10g; i.p.), and AE100, AE200 and AE400 was administered p.o. to three groups 1 h before the nociceptive agent. One control group received distilled water instead of the aqueous extract. Morphine (2.5 mg/kg, i.p.) and indomethacin (10 mg/kg, i.p.) were used as standards in the remaining groups. Ten minutes after the acid was administrated, the number of writhes was

recorded during a period of 20 min.

Hot plate test: The experiment started 60 min (time zero) after p.o. administration of AE100, AE200 and AE400. The mice (n = 9) were placed on an aluminum plate heated to 55±0.5ºC and the time elapsed to the moment when they licked their hind paws was recorded (hereafter referred to as latency). This procedure was repeated at times 0, 15, 30 and 60 min. In order to avoid damage to the paws of the animals, time standing on the plate was limited to 30 seconds. Morphine (5 mg/ kg, i.p.) was used as the reference drug.

Formalin test: In a group of nine mice, AE100, AE200 and AE400 was administered p.o., and after 60 min., 0.02 mL of 1% formalin was administered to the subplantar of the left posterior paw. The time that each mouse spent licking its paw was recorded. Control animals received distilled water, p.o. Morphine was used as a standard test drug (7.5 mg/kg, i.p.). The reaction to pain was measured 0-5 min (1st phase) and 20-25 min (2nd phase) after administration of the stimulus. In order to confirm the possible participation of the opioid system, naloxone (5 mg/kg; i.p.) was administered with AE100 to a second group (n = 8). Morphine combined with naloxone (7.5 mg/kg, i.p. and 5 mg/kg; i.p., respectively) was administered to a third group 15 minutes prior to the injection of formalin. We also administered caffeine (10 mg/kg, i.p.) associated with AE100 to verify the influence of the adenosine system on the effect of the aqueous extract.

Statistical analysis: Results of the hot plate test were analysed using two-factor parametric ANOVA, in which the variable was the latency and the two factors being simultaneously verified were the time interval of the four experiments and the drugs (extract and morphine dosage, and the control). The Tukey test for multiple comparisons of the means was applied to all F values with $p<0.05$. The results of the writhing and formalin tests were analysed using the Kruskal-Wallis non-parametric one-way ANOVA (samples do not come from normal population and the variances are heterogenous), with the Nemenyi test for multiple comparisons of the medians (Zar, 1996).

Percentage inhibition = (1-Vt/Vc) x 100, where Vt and Vc represent the number of writhes or time spent licking the paw for the treated and control groups, respectively. The percentage increase in latency = (1-(Xc/Xt)x 100), where Xc and Xt are the average latency for control and treated groups, respectively.

RESULTS AND DISCUSSION

The aqueous extract of *B. cheilantha* reduced significantly the frequency of writhing induced by acetic acid (Kruskal-Wallis: $F_{0,05;5;47} = 32.4$; $p<0.0005$), with the number of events reduced by up to 54.4%, in comparison with the control, at 400 mg/kg (Nemenyi: $q_{0,05;\infty;6} = 5.1$; $p<0.05$, Table 1). Positive results for the writhing test do not necessarily means that the extract has an analgesic effect, however, as this test is sensitive not only to analgesics, but also to substances such as muscle relaxants, antihistamines, monoamine oxidase inhibitors, adrenergic blockers, and neuroleptics (Chernov *et al*., 1967; Hendershot & Forsaith, 1959; Loux *et al*., 1978; Pearl *et al.*, 1968). The formalin and hot plate tests were employed in order to confirm the analgesic properties of the extract.

The hot plate model involves a supraspinal response to thermal stimuli. Drugs with supraspinal action affect this response, especially at temperatures of 55ºC or more (Ankier, 1974; Magalini *et al.*, 1979). The results indicate no statistical difference in mean latency among the experiments at time zero, 15, 30 and 60 min (ANOVA: $F_{0.05(1)3,140} = 0.956$; $p>0,05$; Table 2) and no interaction of the experiments with drugs (including control) affecting latency means ($F_{0.05(1)12,140} = 0.735$, $p>0.05$). These results indicate homogeneity in the mean latency of control, morphine, and extract groups in all four experiments, but the most important general finding was the significant differences among drugs (including control), independently of time (ANOVA: $F_{0.05(1)4,140} = 27.8$; $p< 0.0005$).

The accentuated significance of this result may be due primarily to the morphine effect, but we were particularly interested in verifying the contribution of the extract to the significance of the variance and which dose affected the result. As no significant differences were found among experiments, the grand mean of each extract dose was compared to the grand mean of the control. The results indicate that all doses of the extract were significantly different from control, with an increase of 39.8% (AE100), 30.7% (AE200) and 32.8% (AE400) in latency of the response to the administration of the extract. (Tukey: AE100, AE200, and AE400 vs. control group, respectively: $q_{0.05,140,5} = 6.74$; $p<0.05$; $q_{0.05,140,5} = 4.51$; $p<0.05$; $q_{0.05,140,5} = 4.9$; $p<0.05$; Table 2). This response suggests that, like morphine, the aqueous extract interferes with the central pain induced mechanism, reducing its nociceptive response to thermal stimuli.

The aqueous extract of *B. cheilantha* altered the paw licking time significantly in both phases of the test (Kruskal-Wallis: $F_{0.05(1)4;39} = 15.3$; $p<0.0005$; $F_{0.05(1)4;39} = 13.7$; $p<0.0005$; respectively). In the first phase, AE100 reduced paw licking time significantly (Nemenyi: $q_{0.05,\infty,5} = 4.1$; $p<0.05$). In the second phase, all doses inhibited the formalin effect (Nemenyi: $q_{0.05,\infty,5,} = 5.3$; $p<0.05$; $q_{0.05,\infty,5} = 4.2$; $p<0.05$; $q_{0.05,\infty,5} = 4.2$; $p<0.05$). Table 3 shows the percentage inhibition of the pain reaction produced by morphine and the plant's aqueous extract.

Naloxone, a non-selective opioid receptor antagonist, was used to elucidate the possible mechanisms of the *B. cheilantha* extract (Magalini *et al.*, 1979). In the first phase, Kruskal-Wallis revealed no significant differences among the following groups: control, morphine plus naloxone, and AE100 plus naloxone (Kruskal-Wallis: $F_{0.05(1)2;20} = 2.2$; $p>0.10$, Table 4), which suggests that the effect of the aqueous extract may involve an opioid system. However, when comparing the control, AE100, and AE100 plus caffeine groups, significant differences were found in both phases of the test (Kruskal-Wallis: $F_{0.05(1)2,20} = 13.2$;

Table 1. Effects of control, AE100, AE200, and AE400, indomethacin (10 mg/kg), and morphine (2.5 mg/kg) on the frequency of writhing induced by acetic acid 0.6%.

Group	Dose (mg/kg)	Median	%
Control	-	27	-
Morphine	2.5	3*	69.2
Indomethacin	10	2*	79.8
AE	100	18	17.2
AE	200	14	41.9
AE	400	6*	54.4

n = 9
* $p<0.05$ compared to control, Nemenyi test after Kruskal-Wallis.
% = percentage of writhes inhibition scores.

Table 2. Effects of *B. cheilantha* aqueous extract (AE) and morphine on latency time in the hot plate test.

Groups	Dose (mg/kg)	Latency time (Mean±SEM)				Grand Mean	%
		0 min	15 min	30 min	60 min		
Control	-	13.11±1.02	13.25±1.29	9.55±1.15	8.73±1.33	11.16	-
Morphine	5	29.09±0.91	25.66±2.17	27.52±1.26	25.59±1.83	26.96*	58.6
AE	100	18.45±2.22	14.93±1.67	19.83±2.88	20.92±2.66	18.53*	39.8
AE	200	15.78±1.56	14.71±2.51	17.5±2.70	16.4±2.57	16.1*	30.7
AE	400	18.45±1.92	14.87±1.56	16.38±2.3	16.75±2.31	16.61*	32.8

n = 9 for each group at each time interval.
* $p<0,05$, compared to control, Tukey test after two way ANOVA.
%, percentage of latency time increase.
SEM, standard error of the mean.

$p<0.0005$; $F_{0.05(1)2,20} = 82.7$; $p<0.0005$, respectively). In the first phase, caffeine reverted the AE effect (Nemenyi: $q_{0.05\ \infty;5} = 1.8$; $p>0.05$; Table 5), which indicates participation of the adenosine system in the analgesic effect. Adenosine may interact with four subtypes of receptors (A_1, A_{2a}, A_{2b} and A_3), but, as caffeine is a non-selective antagonist, further studies will be necessary to determine how these subtypes are involved in the response.

In order to evaluate the acute toxicity of *B. cheilantha*, the mice were submmited to increasing doses of the extract. No deaths ocurred, even at the highest dose (3 mg/kg), indicating low toxicity, as reported by Lorke (1993).

Table 3. Effect of *B. cheilantha* aqueous extract (AE, 100, 200, and 400 mg/kg) and morphine (7.5 mg/kg) in the 1% formalin test.

Groups	Dose (mg/kg)	1st phase		2nd phase	
		Median	%	Median	%
Control	-	35	-	21	-
Morphine	7.5	10*	77.4	0*	64.0
AE	100	20*	53.9	0*	57.4
AE	200	24	17.9	0*	46.1
AE	400	29	-3.7	0*	46.2

n = 9 for each group at each time interval
* $p<0.05$ compared to control, Nemenyi test after Kruskal-Wallis.
% = inhibition percentage of pain reaction scores.

Table 4. Effect of naloxone (5 mg/kg, i.p.) on morphine (10 mg/kg, p.o.) and AE100 in the formalin test.

Groups	1st phase		2nd phase	
	Median	%	Median	%
Control	54.5	-	29.5	-
Morphine + Naloxone	55.5	-10.9	18	21.2
AE100 + Naloxone	47.5	38.2	0*	73.3

n = 8 for each group at each tim e interval.
*$p<0.05$ compared to control, Nemenyi test after Kruskal-Wallis .
% = inhibition percentage of pain reaction scores

Table 5. Effects of caffeine on AE100 in the formalin test.

Groups	1st phase		2nd phase	
	Median	%	Median	%
Control	53.5	-	27.0	-
AE100	23.5*	68.7	0.0*	56.1
AE100 + Caffeine	37.0	25.1	0.0*	61.0

n = 8 for each group at each time interval.
*$p<0.05$ compared to control, Nemenyi test after Kruskal-Wallis.
% = inhibition percentage of pain reaction scores.

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ELECTROPHYSIOLOGICAL EFFECTS OF SODIUM THIOPENTAL ON THE RIGHT ATRIUM OF THE RABBIT (*ORYCTOLAGUS CUNICULUS*)

Eduardo Antônio Conde-Garcia[1]
Marilene de Paula-Carvalho[1]
Antonio Edilson Nascimento[1]
Vanda Rodrigues de Oliveira[1]
Marcli Costa da Silveira Libório[1]

ABSTRACT

This study describes the effects of sodium thiopental (40 mg/l) on the rabbit atrium. The electrical endocardial signals and the intracellular action potentials were recorded. The results revealed a reduction in the atrial impulse velocity (from 75 ± 3 cm/sec to 63 ± 7 cm/sec), disorganization of the propagated electrical front wave, reduction of the spontaneous atrial pacemaker rate, depolarization of atrial cells (quiescent: 3.46 ± 1.2 mV; under electrical stimulation: 3.1 ± 0.5 mV), and an increase of the atrial cellular refractory period (from 52 ± 5 msec to 117 ± 8 msec). Atropine sulfate (1 mg/l) did not prevent or abolished the bradicardia produced by the sodium thiopental.

Keywords: Electrocardiophisiology, action potentials, atrial cells, sodium thiopental.

RESUMO

O estudo descreve os efeitos do tiopental sódico (40 mg/l) sobre átrios de coelho. Foram registrados os sinais elétricos endocárdicos e os potenciais de ação intracelulares. Os resultados mostraram redução da velocidade de propagação do impulso atrial (75 ± 3 cm/sec a 63 ± 7 cm/sec), desorganização da frente de onda propagada, redução da freqüência espontânea do marcapasso atrial, despolarização de células atriais mantidas em repouso (3.46 ± 1.2 mV) ou sob estimulação elétrica (3.1 ± 0.5 mV), e aumento do período refratário das células atriais (52 ± 5 msec a 117 ± 8 msec). O sulfato de atropina (1 mg/l) não preveniu ou aboliu a bradicardia produzida pelo tiopental sódico.

Palavras-chave: eletrocardiofisiologia, potencial de ação, células atriais, tiopental sódico.

INTRODUCTION

Sodium thiopental (NaTHIO) is a well-known barbiturate. Its rapid hypnotic properties derive from its effective liposolubility, a property due to the presence of a sulfur atom substituting an oxygen atom in the ureic residue of the barbiturate ring. At blood concentrations of up to 16 mg/ml, conscience is lost quickly (Evers & Crowder, 2001). Patients of normal body weight who receive small doses of NaTHIO normally wake up around 5 to 10 minutes later. Rapid recovery occurs as a consequence of the redistribution of NaTHIO among different tissues and organs, mainly those with high blood flow demands. In plasma, 85% of NaTHIO is bound to albumin molecules and, as a consequence, patients with severe hypoalbuminemia may need less NaTHIO to lose consciousness during anesthetic procedures.

Becker & Tonnesen (1978) reported some cardiovascular effects produced by NaTHIO in patients anesthetized exclusively by this drug. These effects included an increase in heart rate during the induction phase, a decrease in systolic blood pressure, and an increase of the ventricular pre-ejection period.

Since Beattie *et al.* (1930) showed that chloroform facilitates the appearance of ventricular fibrillation, the

[1] Departamento de Fisiologia, Laboratório de Biofísica do Coração. Universidade Federal de Sergipe, Av. Marechal Rondon, s/n, Jardim Rosa Elze, São Cristóvão, Se, 49100-000. egarcia@ufs.br

anesthetic side-effects have been investigated exhaustively. However, Price & Ohnishi (1980) have emphasized the need for a better understanding of the effects of NaTHIO on the conduction of electrical impulses and myocardial excitability.

This paper describes the electrophysiological effects promoted by this thiobarbiturate. The following parameters were studied: a) conduction velocity of the atrial electrical impulse, b) organization of the wave front of the propagated electrical impulse, c) spontaneous pacemaker rate, d) cellular resting potential in quiescent and electrically stimulated myocardial fibers, e) morphology of propagated action potentials, and f) electrical refractory period of the atrial cells. These parameters are especially important given the use of NaTHIO in animal research as a hypnotic drug or as an auxiliary to prevent ischemic/reperfusion-induced cardiac arrhythmias (Ruigrok *et* al., 1985; Schultz *et* al., 1997; Conradie & Coetzee, 1999; Kato & Foëx, 2002).

MATERIAL AND METHODS

The present study was carried out on adult rabbits (1.5-2.0 kg) of both sexes. Animals were sacrificed by a blow applied to the base of the skull. Their hearts were removed immediately and immersed in a modified Tyrode solution (in mM: NaCl 137, KCl 5.0, $MgCl_2$ 0.5, $NaHCO_3$ 12, $CaCl_2$ 1.8, Glucose 6.0, NaH_2PO_4 1.8). The right atrium was separated and mounted in a chamber with its endocardial surface facing upwards, superfused in Tyrode at 34.0±0.5°C (UNITEMP, model 111, FANEM, Cumbica, Guarulhos, Sao Paulo, SP), aerated and buffered with carbogen mixture (95% oxygen plus 5% carbon dioxide, <1% error). The test solution was prepared by adding NaTHIO (Thionembutal, Abbott, Laboratórios do Brasil, Ltda., São Paulo, SP) to the Tyrode.

Surface records were performed with the aid of a Teflon®-coated silver wire electrode (150 mm) inserted into a hypodermic stainless steel needle (length:100 mm, outer diameter: 1 mm) used as a grounded pole. The electrode was then mounted on a mechanical micromanipulator to allow it to be displaced smoothly (X-Y axes) along the endocardial surface. Electrical signals captured by this roving electrode were amplified differentially, monitored, and photographed by an oscilloscope camera (Differential Amplifier 5A22N, C-50 Oscilloscope Camera, D44 Dual Beam Oscilloscope, TEKTRONIX, Inc. Beaverton, Oregon, USA).

Intracellular readings were taken with 3M KCl-filled glass microelectrodes (DC resistance equal to 40 MW, tip diameter about 0.1mm: Oliveira-Castro & Machado, 1969). Microelectrode signals were sent to a high input impedance amplifier (M701, W-P Instruments, Inc., New Haven, Connecticut, USA) and then to a plug-in oscilloscope amplifier (5A48 Dual Trace Amplifier, TEKTRONIX, Inc. Beaverton, Oregon, USA). Following the experimental protocol, the atrium was stimulated electrically using ungrounded electrical current pulses (DS2 Isolator Unit, D4030 Pulse Programmer, DIGITIMER Limited, Welwyn Garden City, Hertfordshire, England). The stimuli were delivered through a pair of stainless steal electrodes placed at the right atrial appendage.

To evaluate the effect of thiobarbiturate on atrial impulse velocity, the atrial pacemaker frequency was set at a rate 20% higher than the spontaneous one. The conduction velocity was measured through previously selected pathways with uniform conduction of electrical signals. They were placed 4 to 6 mm from the stimulus electrodes to avoid interference of the stimulation field. Atrial impulse speed was estimated by displacing the surface electrode at constant steps (0.5 or 0.6 mm) and by simultaneous measurement of the time elapsed before the wave reached the electrode. It was possible to estimate the velocity of the atrial impulse by plotting each displacement against its corresponding time delay, based on the steepness of the regression line used to fit the experimental data.

To evaluate the effects of the barbiturate on atrial pacemaker activity, the electrical impulses recorded by

the surface electrode were counted, and the spontaneous rate was determined in detail (D4030 Pulse Programmer, DIGITIMER Limited, Welwyn Garden City, Hertfordshire, England; 1830 Interval Generator, 1832 Preset Control, 1831 Pulse Control Module, W-P Instruments, Inc. New Haven, Connecticut, USA).

To study the effects of NaTHIO on cellular resting potential, special care was taken with regard to the grounding system of the organ bath. For this, the silver electrode was covered with chloride, according to the technique described by Geddes (1972), in order to minimize junction potentials. This electrode, connected to the ground, was immersed in 3M KCl, with an agar/3M KCl bridge connecting it to the organ bath. The aim was to obtain electrical stability, and no electrical drift was recorded in the organ bath when the Tyrode voltage was monitored during 3 hours.

Statistical Analysis: Results are presented as means ± standard deviation. Student's t-test was used to analyze differences between means.

RESULTS

1. Effects of sodium thiopental on the propagated atrial electrical wave

Figure 1 shows two sets of electrical waves recorded from the atrial endocardium by moving the surface electrode at regular steps. The experiments were carried out on a paced right atrium (2 Hz). In the control (Figure 1A), the interval between successive waves was highly regular (electrode displacement step = 0.6 mm), indicating uniform propagation. Conduction velocity was calculated at 73 cm/sec ($r^2 = 0.9992$). In the experimental procedure (Figure 1B), waves were recorded in the presence of NaTHIO (40 mg/l). The surface electrode was displaced at steps of 0.5 mm and the impulse velocity of the atrium decreased to 56 cm/sec ($r^2 = 0.9954$, $p < 0.001$), 22% lower than the control value. Wave morphology was also less regular, and in several cases, irregularities indicate loose organization of atrial impulses. Figure 1C presents the regression lines for control and NaTHIO data points. Similar results were obtained for six other atria in which NaTHIO (40 mg/l) decreased the impulse velocity from 75 ± 3 cm/sec to 63 ± 7 cm/sec (n = 18 trials, $p < 0.001$). This effect had not disappeared completely 30 minutes after removal of the barbiturate from the organ bath.

2. Effects of sodium thiopental on the atrial pacemaker rate

At 60 mg/l, NaTHIO reduced the atrial pacemaker rate 16 to 47% (Figure 2). This negative chronotropic effect was eliminated partially or completely during the washout (n = 13 atria, 25 trials, $p < 0.001$).

At a doseage of 40 mg/l, NaTHIO decreased the spontaneous atrial rate progressively, and complete asystole was observed in several experiments (see Figure 3). Initial control rate was 158 bpm, but during NaTHIO (40 mg/l), it decreased progressively, sometimes to zero (interruptions in the fitting line). This effect was not altered by the application of atropine sulfate (1 mg/l, Sigma Chemical Co., St. Louis, MO, USA) 20 minutes before NaTHIO. However, removal of NaTHIO from the perfusion solution resulted in a return to the control rate. Similar results were obtained for three other atria (n = 6 trials). In some experiments, NaTHIO (40-60 mg/l) induced the appearance of isolated extrasystoles or even activated rapid ectopic foci (results not shown).

3. Effects of sodium thiopental on the resting potential of the myocardium

Figure 4 shows an intracellular record obtained from a quiescent atrial cell (resting potential = 81 mV). Downward pointing arrows indicate the moment when NaTHIO (40 mg/l) was added to the bath and upward arrows indicate when the barbiturate was removed from. NaTHIO promoted depolarization (3.46 ± 1.2 mV) when present in the solution bath. The depolarizing effect was not reverted completely by the washout. Similar effects were observed in five other atria (3.06 ± 1.4, n = 18 trials, $p < 0.001$).

NaTHIO (40 mg/l) also promoted depolarization in electrically stimulated atrial tissue (1.2 Hz). Figure 5

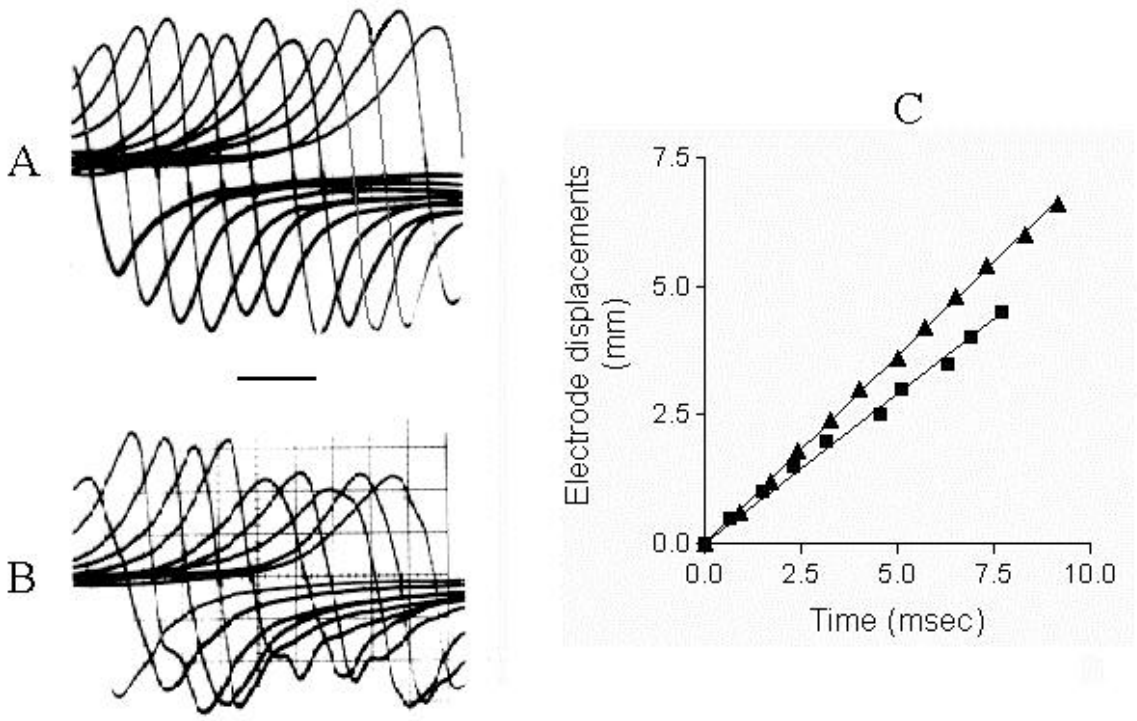

Figure 1. Effect of NaTHIO (40mg/l) on the rabbit atrial conduction velocity. Superimposed electrical waves recorded on the endocadial surface by displacing a surface roving electrode at constant steps. A: control (step=0.6mm); B: test solution (Tyrode + NaTHIO 40mg/l, step=0.5mm); Atrial impulse velocities were determined (C) by the steepness of regression lines. NaTHIO reduced the impulse velocity about 23 percent from 73cm/sec control (triangles, r^2=0.99) to 56cm/sec (squares, r^2=0.99, $p<0.001$). The experiment was carried out on paced atrium (2Hz, 34±0.1°C; Horizontal bar: 2msec).

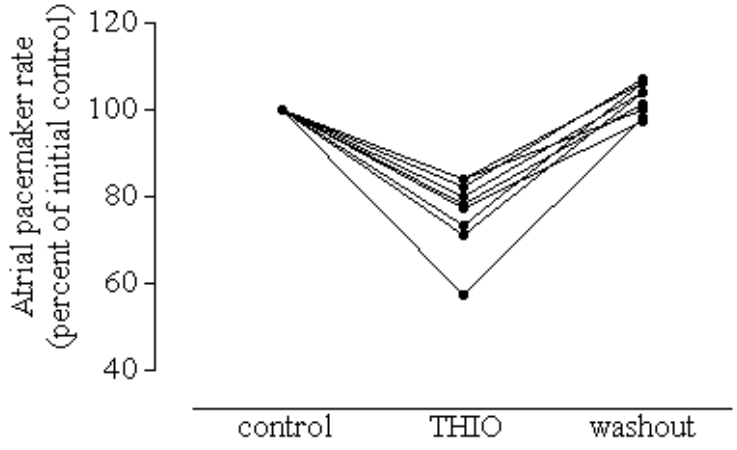

Figure 2. Negative chronotropic effect produced by NaTHIO (40mg/l) on the rabbit atrial pacemaker (n=13 atria). This effect ranged from 16 to 47 percent of the control rate. Experiments were performed on spontaneous beating atria (34±0.1°C, $p<0.001$).

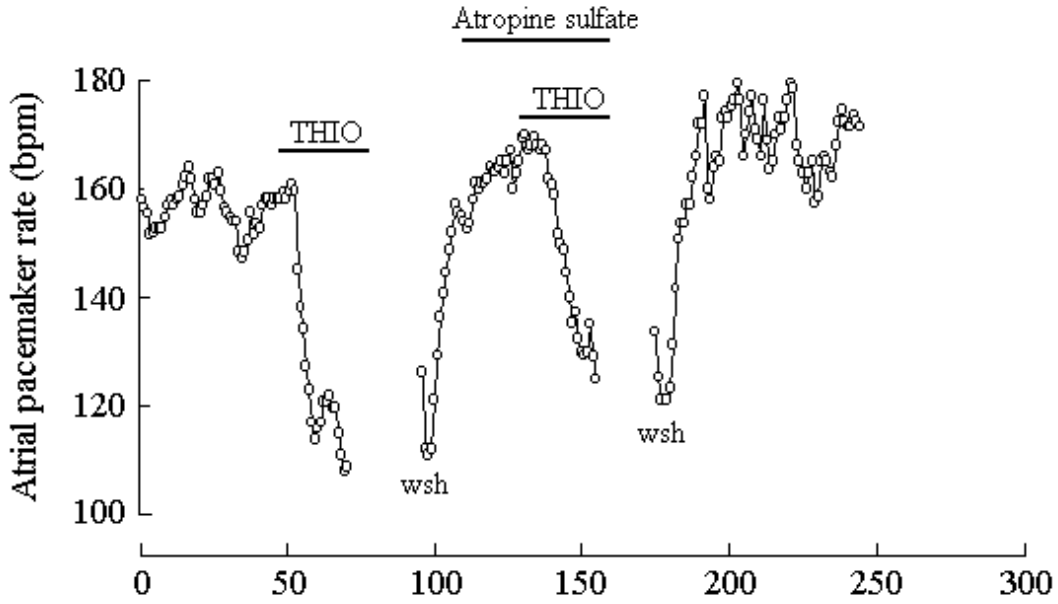

Figure 3. NaTHIO (40mg/l) induces asystole (interruption of the fitting line) in the rabbit right atrium. The asystole was observed in 4 of 13 rabbit atria assayed but bradycardia was present in all of them. This negative chronotropic effect could not be prevented by atropine sulfate (1mg/l) applied 20 minutes before and during the barbiturate action (2Hz, 34±0.1°C).

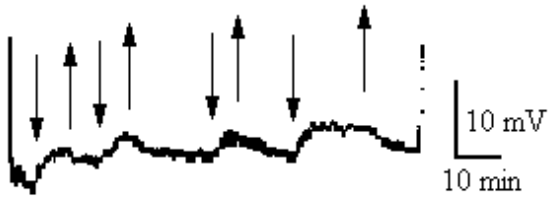

Figure 4. Effect of NaTHIO (40mg/l) on the quiescent rabbit atrial cell (initial resting potential=81mV). Downward and upward arrows mark when NaTHIO was added or removed, respectively, from the organ bath. Note that it produced small depolarizations (3.46±1.2mV). This effect was not completely abolished during washout (34±0.1°C).

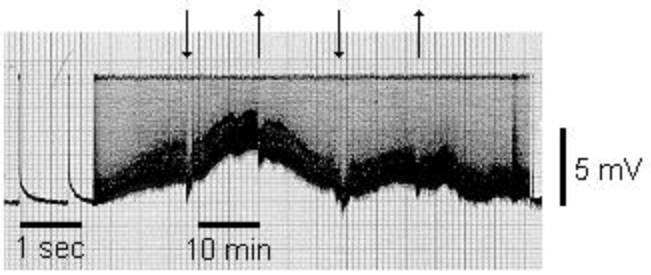

Figure 5. Effect of NaTHIO (40mg/l) on the resting potential of a rabbit atrial paced cell (1.2Hz). Thiobarbiturate depolarized the cell (downward arrows). Similar result was seen in other 5 atria (3.1±0.5mV, 34±0.1°C). Upward arrows stand for the washout.

shows intracellular records obtained from a stimulated atrium. The amplitude of action potentials is truncated due to the high gain needed for monitoring resting potential. Up and down arrows indicate, respectively, when NaTHIO (40 mg/l) was added to or removed from the bath. Note that NaTHIO induced depolarization. Similar results were obtained from five other atria (n = 12 trials) in which NaTHIO depolarized atrial cells 3.1 ± 0.5 mV ($p < 0.001$). Nevertheless, in contrast to the quiescent myocardium, recovery of control resting potential was faster in the paced atrium, and residual depolarization did not occur.

4. Effects of sodium thiopental on the morphology of the propagated action potential in atrial tissue

Figure 6 shows superimposed traces of action potentials that were randomly obtained from atrial cells located in a previously selected myocardium area (Zoom Stereo Microscope, model SZ-III, Olympus Optical Co., Ltd., Tokyo, Japan, ocular with embedded reticle). Control action potentials are seen in the Figure 6A, and Figure 6B presents action potentials recorded when NaTHIO (20 mg/l) was added to the external medium. Arrows indicate the maximum amplitude of the fast component of the myocardial action potentials. In the presence of NaTHIO, the amplitude of the fast component was reduced and was exceeded by that of the slow component (Paes de Carvalho *et al.*, 1966, 1969).

5. Effects of sodium thiopental on the cellular refractory period

Figure 7 shows action potentials obtained from an atrial cell. The upper panel presents the control, and the lower panel depicts the effect of NaTHIO (40 mg/l) on the cellular refractory period. Extrasystolic stimuli, applied at different coupling intervals (between normal and premature stimuli), permitted measurement of the cellular refractory period. In the control, the refractory period was 73 msec, but it increased to 127 msec when NaTHIO was added to the bath. Similar results were

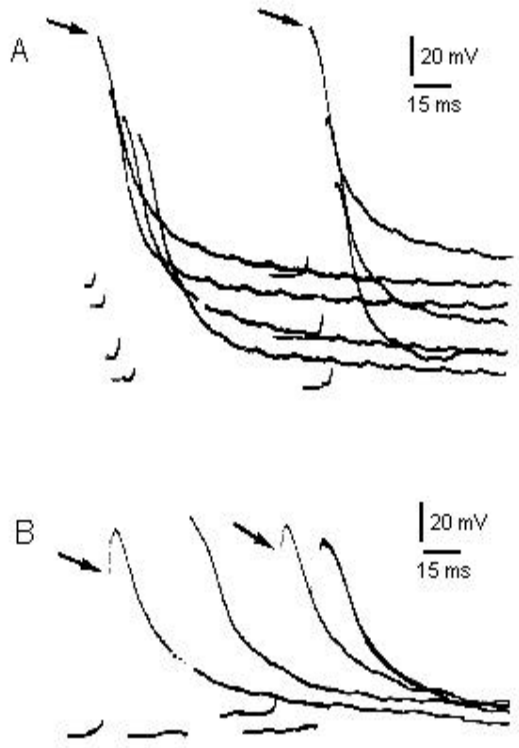

Figure 6. Superimposed traces of propagated action potentials obtained in different cell of the atrial endocardial suface. A: six action potentials recorded on control solution. All of them showed a well-developed fast component (depolarization phase). B: four action potential were recorded in the same atrial area, but after adding NaTHIO (40mg/l). Under the barbiturate action, several action potentials showed depressed fast components (arrows), suggesting a partial inhibition of the sodium current (34±0.1°C, stimuli: 30mV, 1ms, 2Hz).

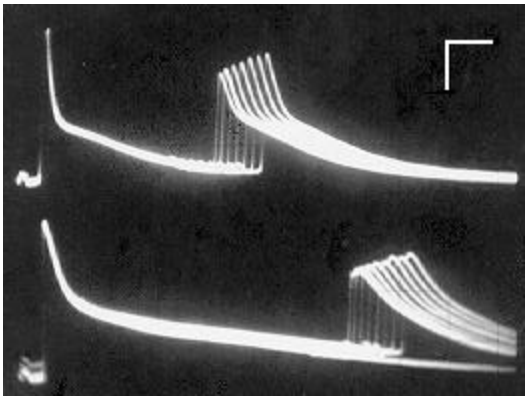

Figure 7. Effect of NaTHIO (40mg/l) on the cellular refractory period determined by applying extrasystolic stimuli with different coupling interval. Upper panel: control (refractory period equal to 73msec); lower panel: test with 40mg/l of NaTHIO (refractory period equal to 127msec). Experiment carried out at 34±0.1°C. Calibration bars: 20mV (vertical), 20msec (horizontal).

obtained in fifteen other cells (n = 4 atria; control : 52 ± 5 msec; test: 117 ± 8 msec; $p < 0.001$).

DISCUSSION

In spite of the development of new hypnotic agents, NaTHIO is still employed as an anesthetic in many experimental procedures with animals. This paper contributes to the understanding of its effects on the mammalian myocardium. The results showed that NaTHIO promotes arrhythmogenic effects similar to electrical wave front fragmentation, reduction of the fast component of action potential amplitude, and cellular depolarization. On the other hand, the increase of the refractory period may have an anti-arrhythmogenic effect associated with NaTHIO.

Our results show that NaTHIO promotes electrical changes in the myocardium that can promote and sustain cardiac arrhythmias. This is because it reduces the myocardium impulse velocity and simultaneously disorganizes the propagated wave front of the electrical impulse. This could facilitate the establishment of a chaotic state in electrical wave propagation. It is important to note that the irregular morphology observed in the surface records during NaTHIO action (Figure 1B) represents a form of wave front fragmentation that is a consequence of micro-accelerations and micro-deaccelerations of the propagated impulse. This facilitates re-entry mechanisms in the myocardium tissue, leading to the appearance of cardiac arrhythmia. The thiobarbiturate also decreased impulse propagation velocity, probably due to the decrease of the fast sodium currents that are responsible for the depolarization phase of propagated action potentials. This effect became clear because NaTHIO reduced clearly the fast component of the myocardial action potential (Figure 6B). Similar sodium current inhibition has been recorded for sodium pentobarbital (Wartenberg *et al*., 2001) – a close NaTHIO analogue.

Becker & Tonnesen (1978) observed a cardiac rate increase during the sleep induction phase in patients anesthetized with NaTHIO. However, in the isolated rabbit right atrium, this barbiturate promoted bradycardia, sometimes followed by an asystole. The tachycardic effect related by these authors could be explained by the depression of myocardial contractility, given that NaTHIO is known to reduce inward calcium flow in myocardial cells (Komai & Rusy, 1991, 1994a, 1994b; Park & Lynch, 1992; Housmans *et al.*, 1995; Bettens *et* al., 1996; Descorps *et al.*, 2001). This entry of calcium ions is important for the promotion of calcium-induced calcium release in the myocardial cells (Sitsapesan & Williams, 1994; Bassani *et* al., 1995; López-López *et* al., 1995; Sipido *et* al., 1998; Wier & Balke, 1999; Shannon *et* al., 2000; Bers, 2002) and initiatiation of the contractile process. Depression of myocardial contractility would lead to a decrease in arterial blood pressure, triggering a reflex response from the aortic pressure baroreceptors. Under such conditions, the sympathetic tonus of the heart would be enhanced and the cardiac rate increased. The depressor effect of NaTHIO on the atrial pacemaker cells does not seem to be mediated by release of acetylcholine from the parasympathetic nervous endings, given that the muscarinic blockade with atropine sulfate did not have any effect.

Our data suggest that NaTHIO modifies myocardial performance by acting on the ionic cellular currents responsible for electrogenesis in the cardiac tissue. To study this, resting potentials from quiescent and non-quiescent myocardial cells were measured (Figs. 4 and 5). In both cases, thiopental depolarized the myocardium. It is known that the resting potential is maintained by a complex balance between depolarizing currents, which are mainly carried by sodium and calcium ions, and hyperpolarizing currents, carried by potassium ions. The depolarizing effect of thiopental should thus be due either to an increase of the inward sodium-calcium current or to a decrease of the ouward potassium current. The depressor effect of NaTHIO on potassium channels was described recently (Pancrazio *et al.*, 1993; Carnes *et al.*, 1997; Martynyuk

et al., 1999). In fact, NaTHIO, as demonstrated elegantly by Heath & Terrar (1996), is a selective blocker of the K_s potassium channels (a subtype of the delayed potassium channel that is not sensitive to sotalol, a beta-adrenergic agonist). However, this channel is not involved in diastolic depolarization and thus does not appear to be related to the chronotropic effects of thiopental. It remains to be understood whether NaTHIO also enhances the inward rectifier conductance of the potassium channel (K_1) or reduces the slow inward sodium and calcium currents during the pacemaker action potential foot.

The increase in the refractory period observed during the action of NaTHIO is probably dependent on the blockade of the K_s channels. It is now well known that substances which reduce potassium conductance, such as amiodarone, 4-aminopyridine, dronedarone, tetraethylamonium, etc., also increase the tissue refractory period (Raatikainen *et* al., 2000; Workman *et* al., 2000; Li *et* al., 2001; Sun *et al*., 2002). These drugs produce long-lasting action potentials and lead to an increase of the effective tissue refractory period, and thus inhibit the re-entry circuits of myocardial tissues. Because thiopental is also able to prolong the action potential, it could act as an anti-arrhythmogenic agent in stressed myocardia.

In addition to its effects on the ionic currents carried by calcium and potassium, NaTHIO reduces the fast sodium inward current. This effect does not seem to be related to cellular depolarization because the changes in the membrane resting potential are of small magnitude (3 - 5 mV). Our results indicate that NaTHIO interferes, to a certain extent, with the fast sodium channels, contributing to a reduction in the atrial electrical wave velocity. Such effects lead to a decrease in the myocardial safety factor, which depends on the amplitude of the propagated action potential. This facilitates the appearance of impulse conduction blocks. If such effects predominate during the action of NaTHIO, the myocardium will become more vulnerable to the appearance of arrhythmias. However, cardiac behavior will ultimately depend on the balance of the pro- and anti-arrhythmogenic effects that are related to NaTHIO.

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MUTAGENIC EFFECTS OF TOXIC RESIDUES ON THE MICRONUCLEI OF FRESH WATER TELEOSTEI

Mithitaka Soma[1,2]
Luiza Ishikawa-Ferreira[1]
Francisco Borba Ribeiro Neto[3]

RESUMO

Neste estudo foi aplicado o teste de micronúcleo em teleósteos que habitam o Reservatório de Americana, rio Piracicaba, São Paulo, para verificar o grau com que os resíduos agrotóxicos presentes naquele sistema aquático estão afetando o material genético dos peixes que ali vivem e se reproduzem. O teste foi também aplicado num grupo controle constituído por teleósteos coletados em ambientes não poluídos localizados nos rios Atibaia e Atibainha, tributários do rio Piracicaba. Os resultados mostraram que a freqüência de micronúcleos nos eritrócitos, quando comparado com o controle, foi significantemente maior nos peixes do reservatório, indicando que as suas águas estão contaminadas com resíduos tóxicos num grau capaz de causar mutagênese nos peixes.

Palavras-chave: teleósteos, poluição aquática, micronúcleos, mutagênese.

ABSTRACT

In this study, the micronucleus test was applied to teleostei inhabiting the Americana reservoir on the Piracicaba river in São Paulo, in order to evaluate the effects of the toxic agricultural residues present in the reservoir on the genetic make-up of the fish that live and reproduce there. The test was also applied to a control group of teleostei collected in non-polluted environments in the Atibaia and Atibainha rivers, tributaries of the Piracicaba. The results showed that the frequency of micronuclei in the erythrocytes was significantly greater in fish from the reservoir in comparison with those from the control group. This indicates that the waters of the reservoir are contaminated with toxic residues in amounts capable of causing mutagenesis in its fish.

Keywords: teleostei, aquatic pollution, micronucleus, mutagenesis.

INTRODUCTION

When organisms are exposed to mutagenic agents, they may suffer DNA lesions which increase the risk of the development of tumors, if not repaired by its defense systems. Genotoxic agents include the phenol compounds (Stich, 1991), N-nitrosamine (Ashby *et al.*, 1991), sulphur-35 (Monakhov, 1991), and the ionizing radiation procedure (Schimid & Bauchinger, 1980; Natarajan *et al.*; 1986; Jong *et al.*, 1988; Eisele *et al.*, 1991; Doloy *et al.*, 1991). The indiscriminate use of agro-toxins is of special concern, because toxic compounds, such as heavy metals, accumulate in the soil and from there, are washed into rivers and water tables, with serious consequences for the species at the top of the food-chain.

The development of techniques which permit the precocious identification of the presence of mutagenic agents is of great importance for the identification of environments that have been polluted. Cytogenetic analysis is one of the most common of these techniques, which consists of preparing cell cultures of exposed individuals, or analyzing the cells of the hematopoietic system directly. Clastogenic

[1] Faculdade de Ciências Biológicas, Pontifícia Universidade Católica de Campinas, São Paulo.
[2] Departamento de Herpetologia, Instituto Butantan, São Paulo.
[3] Núcleo de Fé e Cultura da PUC de São Paulo.

agents rupture the chromosomes and micronuclei, while aneugenic agents interfere with the mitotic spindle apparatus through numeric alterations to the chromosomes. These alterations are due to the interruption of chromosome migration to the poles, which originates micronuclei.

Micronuclei are formed during mitotic cell division, in the transition from metaphase to anaphase, when chromosomes and acentric fragments not attached to the spindle apparatus, or delayed during the mitosis to the poles, remain in the cytoplasm in the form of small nuclei. These micronuclei are useful for biomonitoring, because they indicate exposure to mutagenic agents. The toxic effects of substances, such as glyphosate, dichlowos, surfactants, mixtures of chemical compounds, and heavy metals like cadmium and copper, have been verified in aquatic organisms through the presence micronuclei (McKlim *et al.* 1970; Folmar *et al*. 1979; Ariyoshi et al. 1990; Horsberg & Hoy, 1990; Ensenbach & Nagel, 1995; Gardner & Yevich, 1970; Tort & Torres, 1988).

In recent decades, the increasing use of chemical products has caused an accumulation of residues in the environment, which have serious consequences in aquatic habitats (Blaxhall, 1972; Pritchard, 1993). The aim of the present study is to evaluate the effects of the pollution of the waters of the Americana reservoir in the municipality of Americana, São Paulo, through analysis of the frequency of micronuclei in the erythrocytes of the peripheral circulation in local fish.

MATERIALS AND METHODS

The focus of this pilot study is to verify the number of micronuclei in the erythrocytes of fish, based on the assumption that all individuals inhabiting an environment polluted with toxic residues will have more micronuclei in comparison with those from unpolluted habitat. For this, two groups of fish (control and experimental) were collected within the same region of the State of São Paulo. Five species were collected in both groups: *Astyanax faciatus* and *Serrasalmus spilopleura* (Characidae), *Hoplias malabaricus* (Erythrinidae), *Pimelodella gracilis* (Pimelodidae) and *Geophagus brasiliensis* (Cichlidae)

Control group specimens were collected at two sites on the Atibainha river, near Piracaia, and one on the Atibaia river, upriver from Bom Jesus dos Perdões. These sites were chosen because of the general lack of pollution in the area. The experimental group was collected at three sites in the Americana reservoir, on the Piracicaba river, in the municipality of Americana (22º44'S, 47º14'). The reservoir is formed by tributaries of the Piracicaba river, which pass through a number of agricultural areas, characterized by high levels of pollution. The reservoir suffers considerable local impact, from the dumping of urban and industrial sewage by the city of Campinas, to increasing agricultural activity, which produces toxic residues that drain directly into the aquatic environment. All specimens were collected with fishnets and hooks, in order to obtain living individuals in good condition. Samples were collected during the dry season (March to August) and the rainy season (September to February).

Blood samples were collected from the tail vein or directly from the heart, using a hypodermic syringe and needles previously heparinized. Slides were prepared with the blood smears and left to dry outdoors. They were then fixed with methanol PA during 3 minutes, and stored in boxes to be taken to the Cellular Biology Laboratory of the Pontifical Catholic University of Campinas, where they were dyed with Schiff reagent for analysis. Dyeing, by the Feulgen technique, consisted of hydrolyzing slides in HCl 1N immersed in water at 60ºC during 8 min. Slides were then immediately immersed in iced H_2O at approximately 4ºC, to halt hydrolysis. Slides were then washed in running tap water for 10 minutes, and quickly rinsed in distilled water. After being dried well, the slides were dipped in Schiff reagent in the dark for one hour at room temperature, and then mounted with Permount and cover slips, and left to dry until the following day. The cells were analyzed under binocular microscopes.

From each species of both groups (control and experimental) we took 10 samples in order to verify the amount of micronuclei, 5 samples in each dry and rainy season. In each sample we analyzed 3,000 erythrocyte cells, establishing in this way the number of micronuclei per species in the two seasons for both groups (Table 1). The frequency of micronuclei was determined according to erythrocytes of the peripheral circulation, following the technique proposed by Schimid (1975), adapted to fish, observing the criteria proposed by Titenko-Holand *et al.* (1997). In order to evaluate the genotixic effect, we only considered positive (mutagenic) the following associated conditions: occurrence of at least 3 micronuclei for every 1000 cells, micronuclei coloration identical to that of the nuclei and smaller structures, or equal to 1/3 of the main nucleus and clearly separated in the cytoplasm (Grassi et al., 2002). The number of micronuclei was grouped for all species, because the question is focused primarily on the variation of these micronuclei between seasons and polluted-unpolluted areas. The results were analyzed through a two-way Anova (pollution and precipitation as the two factors) followed by the Tukey test, with a 5% significance level

RESULTS

Table 2 shows the results of the analysis of variance. As expected, the mean number of micronuclei was significantly greater in the experimental group (Americana reservoir) in comparison with the control group ($F_{0.05(1)1,56} = 889.24$, $p<0.01$). The mean number of micronuclei was also significantly larger in the dry season in comparison with the wet ($F_{0.05(1)1,56} = 20.01$, $p<0.001$), without interaction between factors ($F_{0.05(1)1,56} = 3.11$, $p>0.05$), which reflects that the difference among levels of one factor is constant at all levels of the other. These results indicate clearly that the reservoir is very polluted with genotoxic material, either clastogenic or aneugenic or both, and that the dry season was the period of highest toxicity in both environments. Table 3 shows the significance of the multiple comparisons between the means of the micronuclei frequencies from both environments and between seasons.

DISCUSSION

Mutagenetic studies based on the observation of micronuclei are both practical and reliable, due to the fact that all clastogenic and aneugenic agents cause alterations in chromosome numbers or morphology. Such alterations may be detected with greater precision through karyotype analysis, which pinpoints the smallest alterations, such as gaps, which cannot be identified using the micronucleus technique. The latter method is nevertheless favored by its simplicity, dependability, and speed, not to mention the fact that it does not cause suffering to the animals. As similar

Table 1. Frequency distribution of micronuclei between the factors pollution and precipitation for statistics: Dry Control x Dry Experimental, I, II, III grouped and Wet Control x Wet Experimental, I, II, III grouped.

Season	Species	Control Group (Unpolluted)			Experimental Group (Polluted)		
		I	II	III	I	II	III
Dry	*A.faciatus*	6	7	7	18	23	26
	H.malabaricus	5	5	6	17	21	23
	P.gracilis	6	8	7	21	23	23
	G.brasiliensis	6	6	8	20	24	26
	S.spilopleura	5	7	7	19	21	24
Wet	*A.faciatus*	4	4	6	16	17	21
	H.malabaricus	3	5	5	16	17	20
	P.gracilis	3	6	6	18	20	21
	G.brasiliensis	5	5	6	17	19	23
	S.spilopleura	5	6	7	17	19	22

I, II, III – collection sites

conclusions can be drawn from micronucleus testing, it is clearly preferable over karyotyping.

Our analysis shows clearly that the Americana reservoir is heavily polluted and that its fish are contaminated, endangering their populations and the health of the humans that eat them. Despite the greater leaching of toxins by rainwater during the wet season, fewer micronuclei were recorded during this season, as reported by Grassi (2002). A possible explanation for this is that the reduced volume of standing water in the dry season reduces flow and increases the precipitation of toxins in the bottom sediments, resulting in greater exposure for the fish. In the subsequent rainy period, the renewed flow of water dilutes and removes the accumulated toxins.

It is pertinent to ask why some cells have more than one micronucleus. Do these cells receive micronuclei from the previous cell division, or does each new nucleus form its own micronuclei, which migrate to the same cell? Another possibility is that several acentric fragments get together randomly to form a macronucleus. Both clastogenic and aneugenic agents may provoke the formation of a single micronucleus, but they may sometimes form two or more. This is normally rare, however, under low concentrations of mutagenic agents. Soma (2000) showed that cells receiving higher doses of X radiation tended to have more micronuclei.

The mutagenic agents may be natural or artificial. The former originate from natural sources, such as radiation from the chemical elements that form the soil. Artificial agents are synthetic chemicals, or those derived from synthetic reactions. Each year, the number of synthetic compounds increases, due to industrialization and the technological development of manufacturing, transportation, and agriculture, part of which ends up in the environment. In fish, as well as other vertebrates, hematopoietic tissues respond rapidly to genotoxic agents, within four to five days of exposure. In fish, the hematopoietic organ is the cephalic kidney, in contrast with mammals, in which it is the bone marrow. These organs respond quickly because they are subject to a constant process of intensive cell division, making their cells vulnerable to mutation and the formation of micronuclei.

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Table 2. Analysis of Variance summary between the distribution of micronuclei of the treatments (polluted and unpolluted groups) and blocks (dry and wet seasons).

Source of Variation	Degrees of freedom	Sum of square	Mean square	F
Between Treatments A	1	3226.67	3226.67	889.24***
Between Blocks B	1	72.6	72.6	20.01***
Interaction A x B	1	11.27	11.27	3.11 ns
Error	56	203.2	3.63	

*** $p<0.001$; ns, no significant at 5% level

Table 3. Tukey multiple comparisons of the means of micronuclei according to environment and seasons.

Samples	means		q
Control (Co) x Reservoir (Re)	5.74 (Co)	20.4 (Re)	41.89***
Wet (We) x Dry (Dr)	11.97 (We)	14.17 (Dr)	6.28***

*** $p<0.001$

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