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Genotoxicity assessment of the antimalarial compound artesunate in somatic cells of mice

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ABSTRACT

Artesunate is a derivate of artemisinin that is both an antimalarial agent and acts cytotoxically on tumor cells. Despite its therapeutic use, its *in vivo* genotoxic potential has still not been evaluated. This study, therefore, was an investigation into the effects of a single oral administration of artesunate with an *in vivo* comet assay that analyzed leukocytes from peripheral blood and liver cells, and a micronucleus (MN) assay of bone marrow cells from male Swiss mice. The artesunate was administered by oral gavage at doses of 5, 50 and 100 mg/kg. Cytotoxicity was assessed by scoring 200 consecutive polychromatic (PCE) and normochromatic (NCE) erythrocytes (PCE/NCE ratio). The results demonstrate that artesunate induced significant DNA damage only in liver cells and that high doses of artesunate caused an increase in the mean number of micronucleated polychromatic erythrocytes (MNPCE). Under our experimental conditions, artesunate showed weak genotoxic effects at low doses and clastogenic effects at high doses. The PCE/NCE ratio indicated no cytotoxicity. The data obtained suggest caution about either continuous or high-dose use of artesunate by humans.

1. Introduction

Malaria is the most devastating endemic tropical disease in the world, with between 300 and 500 million clinical cases and almost three million deaths every year. Most of these deaths are caused by infection with *Plasmodium falciparum*, the most harmful species of malaria parasite (WHO, 2001). The main active principle of the *Artemisia annua* plant is known as artemisinin (Klayman, 1985). Artemisinin and its derivatives, such as artesunate, stand out among the chemotherapy medicine available for the treatment and chemoprophylaxis of malaria because this anti-malarial medicine presents no clinically relevant resistance to plasmodium (Meshnick et al., 1996; Dhingra et al., 2000; Olliaro, 2001). Artemisinin has been chemically modified to produce derivatives such as: sodium artesunate, artemether, arteether, dihydroartemisinin, and artelinic acid. These compounds have been formulated for oral, rectal, and parenteral administration. Combinations of these medicines have presented fast antimalarial activity and an absence of clinically important resistance (Woodrow et al., 2005). Artesunate is the most versatile of these derivatives, because it is easily soluble in water, which has facilitated the development of oral and rectal formulas, and thus is often used both in monotherapy and combined with other drugs (WHO, 2001; Angus et al., 2002).

The combination of oral artesunate and mefloquine is commonly used to treat malaria in Southeast Asia. The addition of artesunate accelerates the therapeutic response, reduces recrudescence rates and transmissibility, prevents the occurrence of resistance, increases patient compliance, and also provides a highly effective treatment for hyperparasitemic patients and recrudescent infections (Nosten et al., 1994, 2000; Luxemburguer et al., 1995; Price et al., 1995).

The combination of artesunate and amodiaquine is an ACT (artemisinin-based combination therapy) recommended by the World Health Organization (WHO) for use in malaria control programs, and is a first-line treatment for African children with non-complicated malaria (WHO, 2004).

Considering the use of artesunate in the treatment of malaria and the inexistence of *in vivo* mammal cell studies investigating its genetic toxicity, the main objective of this study was to evaluate the genotoxic/mutagenic potential of artesunate in the peripheral blood cells, liver and bone marrow of male albino (*Mus musculus*) *Swiss* mice, using the comet assay (SCGE) and the micronucleus test.

2. Materials and methods

2.1. Artesunate

Dihydroartemisinin was prepared from artemisinin using a routine procedure (Brossi et al., 1988). The acid chloride RCOCl was prepared from the corresponding carboxylic acids by heating them with thionyl chloride at 50–60 °C for 2–3 h and

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reacting them with dihydroartemisinin in the presence of triethylamine in dry dichloromethane at 0 °C for 2 h to furnish ester derivatives in 49–58% yields. Artesunate was prepared from dihydroartemisinin (DHA) by reacting it with succinic acid anhydride in basic medium. Pyridine was used as a solvent, sodium bicarbonate as a base and DMAP (*N*,*N*-dimethylaminopyridine) and triethylamine in 1,2-dichloroethane were used as catalysts, with yields of up to 100%. The mixture was stirred up to 9 h to obtain artesunate in quantitative yield. The product was further re-crystallized from dichloromethane. Alpha-Artesunate was exclusively formed (m.p. 135–137 °C).

2.2. Chemicals

Doxorubicin (DXR, Oncodox®, Meizler) was used as the DNA damaging agent in the comet and micronucleus assays, and was prepared by dissolving in sterile water. The other main chemicals were obtained from the following suppliers: normal melting point (NMP) agarose (Cat. No. 15510–019: Invitrogen) low melting point (LMP) agarose (Cat. No. 15517–014: Invitrogen), sodium salt *N*-lauroyl sarcosine (L-5125: Sigma) and ethylenediaminetetraacetic acid (EDTA) (Merck).

2.3. Animals and dosing

Experiments were carried out on 12-week old male Swiss albino mice (*Mus musculus*), weighing 25–30 g. The animals were acquired from the animal house of the Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil and kept in polyethylene boxes ($n = 6$) in a climate-controlled environment (25 ± 4 °C, 55 ± 5% humidity) with a 12 h light/dark cycle (7 a.m.–7 p.m.). Food (NUVILAB CR1 – NUVITAL) and water were available *ad libitum*. The mice were divided into experimental groups of six animals/box. Artesunate was diluted in water and administered in a single dose of 0.5 mL by gavage at concentrations of 5, 50 and 100 mg/kg body weight. These concentrations were based on our acute toxicity studies in mice, as well as on studies of its antimalarial effects (Angus et al., 2002). The negative control group received distilled water. The positive control group received an intraperitoneal injection of Doxorubicin 80 mg/kg. The animals used in this study were sacrificed by cervical dislocation. The experimental design is presented in Table 1. The Animal Bioethics Committee of FAMEMA, Marília, Brazil, approved this study on July 30, 2009 (protocol number 140/09), in accordance with federal government legislation on animal care.

2.4. comet assay

The comet assay (SCGE) was carried out according to the method described by Speit and Hartmann (1999), which is based on the original work of Singh et al. (1988), and the guidelines discussed by Tice et al. (2000), as well as additional modifications. Twenty-four hours after the treatment, peripheral blood leukocytes and liver cells from six Swiss mice from each group were sampled. Liver samples were washed in saline solution, in an ice bath. A small portion was transferred to a Petri dish containing 1 mL of Hank's solution (pH 7.5) and then homogenized gently with small pinches. A 10-μL aliquot of cells from each animal was mixed with 120 μL of 0.5% low melting point agarose at 37 °C, and immediately spread on microscope slides pre-coated with 1.5% normal melting point agarose. Coverslips were added and the slides were allowed to gel at 4 °C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL of distilled water and 1% sodium lauryl sarcosine), plus 1 mL of Triton X-100 (Merck) and 10 mL of dimethylsulfoxid (Merck). The slides, which were protected from light, were kept at 4 °C for 1 h and then placed in the gel box, positioned at the anode end, and immersed in a high pH (>13) electrophoresis buffer (300 mM NaOH per 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4 °C for 20 min prior to electrophoresis in order to allow the DNA to unwind. The electrophoresis run was carried out in an ice bath (4 °C) for 20 min at 300 mA and 25 V (0.722 V cm^{-1}). The slides were then submerged in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethyl alcohol for 10 min. The slides were dried and stored overnight or longer before staining. For the staining process, each slide was briefly rinsed in distilled water, covered with 30 μL of 1× ethidium bromide staining solution prepared from a 10× stock (200 μg/ml) and covered with a coverslip. The material was evaluated immediately at 400× magnification, using a fluorescence microscope (Olympus BX 50) with a 515–560 nm excitation filter and a 590 nm barrier filter.

The extent and distribution of DNA damage indicated by the SCGE assay was evaluated by examining at least 100 randomly selected and non-overlapping cells (50 cells per coded slide) per animal in a blind analysis (six mice per group). These cells were scored visually, according to tail size, into the following four classes: class 0 – no tail; class 1 – tail shorter than the diameter of the head (nucleus); class 2 – tail length 1–2 times the diameter of the head; and class 3 – tail length more than twice the diameter of the head. Comets with no heads, with nearly all of the DNA in the tail or with a very wide tail were excluded from the evaluation because they probably represented dead cells (Hartmann and Speit, 1997). The total score for 100 comets, which ranged from 0 (all undamaged) to 300 (all maximally damaged), was obtained by multiplying the number of cells in each class by the damage class.

2.5. Micronucleus assay

The assay was carried out following standard protocols, as recommended by Schmid (1975) and Krishna and Hayashi (2000). The same six mice per group used in the comet assay were used in the micronucleus test. Animals were sacrificed 24 h after the treatment. The bone marrow from both femurs was flushed out using 2 mL of saline solution (NaCl 0.9%) and centrifuged for 7 min. The supernatant was discarded and smears were made. The slides were coded for a "blind" analysis, fixed with methanol and stained with Giemsa solution. For the micronucleated cell analysis, 2000 polychromatic erythrocytes (PCE) per animal were scored to determine the mutagenic effects of artesunate. To detect possible cytotoxic effects, the PCE/NCE (polychromatic/normochromatic erythrocyte) ratio from 200 erythrocytes/animal was calculated (Gollapudi and McFadden, 1995). Coded slides were scored with a light microscope at 1000× magnification. The mean number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability (standard deviation) based on intragroup differences among animals.

2.6. Statistical analysis

After verifying for normal distribution, the data obtained from micronucleus and SCGE assays were submitted to one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparison test (Sokal and Rohlf, 1995) with the GraphPad Instat® software (version 3.01). The results were considered statistically significant at $P < 0.05$.

3. Results

The comet assay results are shown in Tables 1 and 2, where the animals results for the different doses of artesunate and DXR-positive control are compared with the negative control (water). As expected, when the positive control was compared with the negative control, we found that DXR induced a significant increase ($P < 0.001$ or greater) in comet assay DNA migration for leukocyte and liver cells (Tables 1 and 2). We found a significant increase in DNA migration only at a 5 and 50 mg/kg dose in liver cells. There was no statistical difference in DNA migration between the three tested doses of artesunate. At the dosage that induced a significant increase in DNA damage in liver cells cells, most of the damage was minor (class 1), with only a very few cells showing a large amount of damage (classes 2 and 3).

Table 3 shows the micronucleus test results obtained for male Swiss mice treated with artesunate: the mean number of micronucleated polychromatic erythrocytes (MNPCE) in untreated controls and treated animals. As expected, the positive mutagen DXR induced a statistically significant increase in MNPCE. The clastogenicity test revealed an increase in the mean number of MNPCE at all tested doses, being statistically significant at the two highest doses. The observed clastogenicity was dose-related. The tested substance no caused a statistically significant decrease in PCE/NCE ratios. These data suggest that artesunate had a weak clastogenic effect on the bone marrow cells of mice without cytotoxic effects.

4. Discussion

Before the approval of new pharmaceutical products, either natural or synthetic, both their therapeutic and their toxicological effects must be evaluated, so that only substances free from potential mutagenic effects are introduced (Rodeiro et al., 2006). Several studies have suggested that mutagenic agents produce carcinogenic effects (Chandra et al., 2006). The combined use of the comet Assay and micronucleus test in a single study has yielded good results. Vasquez (2010), using the damage-inducing agents Mitomycin C and Cyclophosphamide, tested both techniques concomitantly on different tissues of rats and mice, and thus reduced the number of animals used without compromising the efficacy of the test.

Through the comet assay, it is possible to quantify and distinguish different DNA damage levels, seeing that the evaluation of scores for each experimental group is highly important. In this