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Biomarkers of genotoxicity and genomic instability in a non-human primate, *Cebus libidinosus* (*Cebidae*, *Platyrrhini*), exposed to nitroimidazole derivatives

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ABSTRACT

The genotoxicity of two nitroimidazole derivatives, ornidazole (ONZ) and metronidazole (MTZ) in the peripheral blood lymphocytes of *Cebus libidinosus* (CLI) (Primates, Cebidae) was assessed. Endpoints measured included sister chromatid exchange (SCE) frequency, cell proliferation kinetics (CPK), replication index (RI), mitotic index (MI), and damage incidence in or near CLI heterochromatin regions. MI and SCE values following ONZ or MTZ treatments were significantly different ($p < 0.001$) from control. SCE frequency per chromosome was not proportional to chromosome length. The chromosomes most affected for SCE were 1, 2, 4, 6, 11–13, 17, and 18, many of which possess interstitial or terminal heterochromatin. In the CLI genome, chromosomes 11 and 17 showed higher susceptibility to damage RI was the only biomarker that did not show statistically significant differences between control and treated cultures. *C. libidinosus* bands 11q1.4 and 11q1.5 may be hot-spots in the context of nitroimidazole exposure.

1. Introduction

Non-human primates have been used in genetic studies due to their phylogenetic proximity and physiological similarity to humans. Non-human primates are susceptible to many human diseases, such as osteoporosis, arteriosclerosis, autoimmune diseases, and cancer [1]. As determined by chromosome painting with human probes, the karyotype of *Cebus* (neotropical primates, *Cebidae*) is closely related to that of humans [2–5]. This primate is a valuable experimental model for human metabolic, hormonal, and neurophysiological processes [6–9]. *Cebus libidinosus* (CLI) is also an interesting experimental model for genotoxicity monitoring [10–12]. Genotoxic biomarkers represent early biological effects which may be quantified through various endpoints. The most extensively used biomarkers are sister chromatid exchanges (SCEs), micronuclei (MN), chromosomal aberrations (CA), mitotic index (MI), cell proliferation kinetics (CPK), and DNA double strand breaks (DSBs).

Several experimental designs have been developed for analysis of the genotoxic effects of imidazole compounds, with special interest attached to the 5-nitroimidazoles [13–16], which can be used as food preservatives and as human therapeutic products—antibiotics, antimycotics, and antifungals [17,18]. Animal reproduction centres and zoos use nitroimidazoles in veterinary applications. The neotropical primates *Saimiri*, *Alouatta*, *Callitrhix*, and *Cebus* are among the animals frequently treated with nitroimidazole antiparasitic agents [19].

Metronidazole (1-hydroxyethyl-2-methyl-5-nitroimidazole, MTZ), has been regarded as the "gold standard" agent, against which all other antibiotics with anaerobic antimicrobial activity should be compared. MTZ is one of the ten drugs most commonly used during pregnancy, and it appears in the "essential drug list" of the World Health Organization. MTZ is also a key component of combination therapies used to eradicate *Helicobacter pylori*, the microaerophilic bacterium which chronically infects the stomachs of more than half of the world' population, causing peptic ulcer with an early risk for gastric cancer [20]. Ornidazole, 1-(3-chloro-2-hydroxy)-propyl-2-methyl-5-nitroimidazole, ONZ) has similar antimicrobial effects and is also used in treatment of protozoal infections and in treatment and prophylaxis of anaerobic

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bacterial infections. ONZ is well absorbed and distributed after oral administration, and has a longer half-life than MTZ [21].

Conflicting results have been reported in relation to the genotoxic activity of imidazole derivatives [22–24]. Non-mutagenic, teratogenic and carcinogenic effects have been reported [25–29]. Physical and chemical agents may differentially affect susceptible regions of the genome (e.g., heterochromatin vs. euchromatin), and this may be the case for 5-nitroimidazoles [30,31]. In this report, we present the results of an *in vitro* genotoxicity study of MTZ and ONZ, in the peripheral blood lymphocytes of *Cebus libidinosus,* a non-human primate. We determined the sister chromatid exchange (SCE) frequencies, cell proliferation kinetics (CPK), replication index (RI), and mitotic index (MI), and we analyzed the incidence of damage either in or near heterochromatin regions, by applying the C-bands and sequential G-bands SCE techniques.

2. Materials and methods

2.1. Samples

All cytogenetic studies were performed in peripheral blood lymphocytes (PBL) from six *Cebus libidinosus* (CLI) individuals (three males and three females) kept in captivity for almost 2 years at the Dirección Provincial de Fauna de Corrientes and at the EBCo – CONICET (Estación Biológica Corrientes—Consejo Nacional de Investigaciones Científicas) (Argentina). Their diet and general health state were controlled by the veterinary team. All the animals were free of parasitic diseases during the last 6 months before the peripheral blood samples were taken. The animals had no contact with humans other than their keepers. All blood samples were obtained by venipuncture using a heparinized sterile syringe (Heparin, Abbot).

2.2. Drugs

MTZ (Flagyl®, Roche) and ONZ (Tiberal®, Roche) were used at a concentration = 10 μg/ml, comparable to the plasma levels of these drugs in human therapeutics [32].

2.3. Cultures

Duplicate cultures from *Cebus libidinosus* peripheral blood were prepared according to [33], with slight modifications. Briefly, an aliquot (0.5 ml) of each of the blood samples was placed in a sterile flask containing 7 ml RPMI medium supplemented with 1.5 ml fetal bovine serum (Gibco), 0.1 ml phytohemagglutinin (Gibco) and 32 μM bromodeoxyuridine (Sigma). MTZ and ONZ were added at the start of the cultures, which were then incubated for 72 h at 37 °C. Control cultures were prepared with physiological solution, which was also the vehicle used to prepare the drug solutions. Two hours before harvesting, colcemid (0.2 ml, 10 μg/ml; Sigma) was added to each culture flask. For harvesting, cells were centrifuged at approximately 1000 rpm for 10 min. The supernatant was removed and pre-heated (37 °C) 0.075 M KCl hypotonic solution, 5 ml, was added. Cells were re-suspended and incubated at 37 °C for 45 min. The supernatant was removed by centrifugation, and fixative (methanol: glacial acetic acid, 3:1; 5 ml) was added. The fixative was removed and the procedure repeated twice. To prepare the slides, five drops of the fixed cell suspension were placed on clean glass slides and air-dried. Cells were stained following a modified fluorescence plus Giemsa (FPG) technique [34]. Slides were stained for 20 min in 0.05% (w/v) Hoechst 33258 solution, rinsed with tap water, placed under a near-UV lamp for 90 min, covered with Sorensen's buffer, pH 6.8, and stained with 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min.

2.4. Mitotic index (MI)

The MI was estimated as the proportion of mitotic cells in 2000 cells counted for each culture (control and exposed to nitroimidazole derivatives) in Giemsa-stained slides in the six samples [35].

2.5. Cell proliferation Kinetics (CPK)

The proportion of first (M_1), second (M_2) and third (M_3) division cells was scored in 100 consecutive metaphases from each duplicate 72 h culture, for each experimental point. The cells were scored considering first mitosis (both chromatids dark-stained), second mitosis (one chromatid of each chromosome dark-stained) and third and/or subsequent mitosis (a proportion of chromosomes with both chromatids light-stained). The replication index (RI) was calculated as: $RI = ((M_1 \times 1) + (M_2 \times 2) + (M_3 \times 3))/100$ [36].

2.6. Sister chromatid exchange (SCE)

The frequency of sister chromatid exchange (SCE) was counted in 50 harlequin-stained metaphases, all with 54 centromeres in CLI metaphases, per culture, concentration, and animal.

For SCE frequency analysis, cells undergoing the second division (one chromatid of each chromosome dark-stained) were considered. The results are expressed as the frequency of SCE per metaphase [36].

2.7. Sequential G-banding

A new protocol to obtain sequential G-banding/SCE was verified and established with CLI metaphases, following Ref. [30]. G-bands were obtained using Wright stain, as follows: After one week at room temperature in dark containers (or, alternatively, after 5 h at 37 °C) slides were pretreated for 2.5 min in 2xSSC at 65 °C, washed with distilled water, and covered with a 3:1 Wright stain: Sorensen buffer in the dark for 1.5 min. Slides were quickly washed with abundant tap water and left in the dark until photographed. After recording all the available metaphases, slides were de-stained with a dehydration/hydration procedure using ethanol at 10%, 30%, 70%, 30%, 10% (3 min each), and finally washed for 10 min in distilled water. The fluorescence-plus-Giemsa (FPG) technique was then applied, following Ref. [34], adapted to specific laboratory conditions.

The numbers of SCEs were counted in whole chromosomes and identified at a sub-chromosomal level, using the sequential G-band (400 band resolution)/SCE technique.

2.8. Recording of images

G-banded slides were photographed at 1000X with a Leica photomicroscope, using a Pixera® viewfinder, recording the landmarks of each metaphase. After FPG, slides were scanned and metaphases showing SCE were re-photographed at the same scale at which they had been previously recorded. Each pair of images for CLI metaphases was then processed using Microsoft Photoshop® 5.0 and printed on high-quality paper (Epson®).

2.9. Statistical considerations

The analysis was performed considering two complementary views: (a) a "macro-cytogenetic" perspective, in which each chromosome was considered as an independent unit and the SCEs observed in each chromosome were compared with the expected SCE distribution, according to the relative size of each unit, along with the expected comparisons between treated cultures and controls; and (b) a "micro-cytogenetic" perspective, considering G-bands as units, and counting the value of the frequency of the SCE in each band with or without the addition of each nitroimidazole to the CLI cultures. In all cases, a chi-squared test was used to compare expected/observed frequencies of SCE, using Statistica v 5.0 [37], in which the expected frequencies were those of the control cultures.

Statistical analysis for biomarkers (Replication Index, Mitotic Index and general Sister Chromatid Exchanges) was performed using one-way analysis of variance (ANOVA). A statistically significant difference was set at $p < 0.05$.

3. Results

3.1. Cebus libidinosus karyotype

The karyotype of *Cebus libidinosus* presented 52 autosomes and a sexual pattern XY in the six animals analyzed [38].

3.2. Biomarkers and genotoxic nitroimidazole effects in CLI metaphases

A genotoxic effect biomarker is an early alteration that can be quantified as resulting from the action of a given agent. The biomarkers used were mitotic index (MI), replication index (RI) and sister chromatid exchanges (SCEs) whose expression reflects possible alterations of the cell cycle or genetic damaging events at the chromosomal level.

The MI and SCE/cell values of the ONZ- and MTZ-treated cultures were significantly different from those of the control. The RI was the only biomarker that did not show statistically significant differences. Table 1 shows the averages obtained for the biomarkers used.

The MI values for ONZ and MTZ were similar ($F = 9.46$, $p < 0.001$). When the SCE frequency was evaluated, a statistically significant