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Genotoxic evaluations in wistar rats of the hallucinogenic plant extract Ayahuasca

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Abstract

Ayahuasca, a psychoactive infusion, is a sacrament used by indigenous and non-indigenous communities in Brazil and other countries. This beverage has vaunted healing properties;however, its use in a therapeutic context still lacks preclinical data to certify its safety and effectiveness. This study evaluated the genotoxic, mutagenic and cytotoxic potential of ayahuasca in Wistar rats after a single oral dose. Rats of both sexes were randomly distributed into five experimental groups (n=10): negative control that received filtered water, positive control that received doxorubicin and treated groups that received ayahuascaat 1, 5 and 15 times the usual dose taken in human religious rituals.The rats were euthanized 30 hours after dosage. Genotoxicitywas evaluated by flow cytometry, comet assay and micronucleus test. Renal, hepatic and pancreatic functions were evaluated by serumanalysis. Ayahuascashowed low genotoxicity,with an increased frequency of micronuclei only at the highest exposure level, and anon-observed-adverse-effect-level established at 5X the dose, or 1.5 mg/kg bwN,N-dimethyltryptamine a major component of the infusion. No cytotoxic effects were observed in the tested conditions. Furthermore, hepatic, renal and pancreatic functions remained without significant changes for all treated groups.

Keywords: ayahuasca, genotoxicity, cytotoxicity, serum biochemistry, hematological disturbances

Introduction

The empirical use of plants and natural extracts in folk medicine, healing rituals and shamanism is an ancient practice [1].Ayahuasca is a hallucinogenic plant extract with therapeutic and spiritual potential used in shamanicrituals by Amazonian tribes since ancient times.In the 1930s, the use of ayahuasca was incorporated into religious rituals in many ofBrazil's Christiancommunities, mainly *Santo Daime*, *Barquinha* and the *União do Vegetal* (UDV), and now reaches a global scale with representatives in other South American countries, North America, Europe and Asia [2-4]. The use of ayahuascafor religious purposes is granted by Brazilian law, butits commerce and use outside this context arenot allowed[5]. However, the beverage or its constituentscan be found on the internet, as cantour packages to experience ayahuasca in the forest [6].

Ayahuasca is generally produced by the concoction of two Amazonian plants, *Banisteriopsiscaapi,* which contains the β-carbolinesharmine, harmaline and tetrahydro-harmaline, which are inhibitors of monoamine oxidases (MAO), and *Psychotriaviridis*, which contains N, N-dimethyltryptamine (DMT), a serotonin receptor agonist [7]. Thepsychotropic action of ayahuasca is only possible through the synergistic interaction between the main infusion constituents, as the β-carbolines prevent the hydrolysis of DMT in the gastrointestinal tract, allowing its serotoninergic action [8].Beta-carbolineshavealso been shown to present hallucinogenic effects[9]. This neurostimulation may result in various symptoms, including vomiting, diarrhea, increases in blood pressure and heart rate, tremors, transient agitation, drowsiness, behavioral changes and hallucinations [10]. It is believed that the undue association of ayahuasca with other drugs, especially selective serotonin reuptake inhibitors antidepressants, is potentially harmful and may lead to the development of serotonin syndrome [10].

The potential of ayahuasca as a drug of abuse is considered minimal, and its use by members of religious communities showed no deleterious psychosocial effects [11,12]. The therapeutic potential of ayahuascahas been investigated, including its antidepressive effects [13] and treatment for alcohol and cocaine

addiction [12]. The use of ayahuasca to treatcancer has been mainly associated withrelief of psychological symptoms such as anxiety and depression, resulting in a better quality of life, since there are not enough data to indicate antitumor action [14].More recently, the β-carbolineharmine, present in ayahuasca, has been associated with the ability to induce insulin-producing beta cells,increase islet mass and improve glycemic control, in diabetic mice, thus normalizing glycemic levels [15].

Very few pre-clinical studies in animal models have been conducted with ayahuasca, and they are necessary to delineate subsequent safe clinical studies for possible therapeutic use of this beverage. A previous study from our groupshowed that lethality towardfemale Wistar rats can occur at 30 times the human ritual dose (one out of six animals), but the $LD_{50}$was estimated to begreater than 50 timesthe dose; rats treated once at 30times the dose showed increased neuroactivity in brain areas involved in serotoninergic transmission that led to some brain injury[16]. The aim of this study was to investigate the genotoxic and cytotoxic profile of an ayahuasca extract in Wistar rats treated once at 1 to 15 times the ritual dose. Genotoxicity tests are required by regulatory agencies for the approval of new drugs, and to the best of our knowledge, these studies were not previously reported in the scientific literature with ayahuasca.

Materials and Methods

Animals

This study was conducted with 50 nulliparous, healthy Wistar rats of both sexes (25 of each) aged 8-9 weeks.The animals were acquired from Granja RG (São Paulo, Brazil) and allowed to acclimatize for a 15-day period in the Faculty of Health Sciences of the University of Brasilia (UnB) animal house prior to starting the study. The animals were housed in polypropylene cages, kept in a controlled conditions (at 23- 25 C, 45-60% humidity, light/dark 12h/12h cycle), with access to commercial food (Purina®) and filtered water ad libitum. Animals were fasted for 12 h prior to gavage, but with free access to water, and food was re-introduced 4 h after dosing.The animals were weighed before dosage and at termination. The experimental protocol was approved by the Ethics Committee on Animal Use of the UnB Institute of Biological Sciences (UnBDoc 107766/2010).

Ayahuasca infusion

The ayahuasca infusion was provided by a União do Vegetal (UDV) group in the Federal District, Brazil. The doses given to animals were related to the dose taken during a UDVritual (1X), the usual dose,which in this study corresponds to 150 mL for a 70 kg person. The infusion characterization and quantification of the major constituents were described previously [16].A ritual dose of this infusion corresponds to 0.302 mg/kg bw DMT, 3.34 mg/kg bwharmine and 0.261 mg/kg bwharmaline. Appropriately, weighed lyophilized materials were resuspended in 2 mL of filtered water prior to treatment, and administered by oral gavage to the rats.The study include three treated groups that received doses at 1X, 5X, and 15X, the positive control group (CG+) that received intraperitoneal injection of doxorubicin (40 mg/kg bw), and the negative control group (CG-) that received filtered water by gavage. Each group was composed of 10 animals, five males and five females, with a total of 50 animals used in the study.

The animals were euthanized by carbon dioxide (CO2)inhalation 30 hours after receiving the treatment. Five mL of blood was collected from each animal by cardiac puncture and aliquoted into tubes for further bioassays. The femurs were excised, epiphyses cut off and bone marrow was flushed out using a syringewith needle containing 3 ml of fetal calf serum (Difco) and 0.2M EDTA (Invitrogen). The material was collected into a 15mL falcon tube.

Genotoxicity analysis

Comet assay

The comet assay or single cell gel electrophoresis assay (SCGE), originally idealized by Singh [17],was conducted with modifications suggested by Tice et al.[18] and Smith et al. [19].The leukocyte blood fraction was separated by centrifugation, homogenized in low-melting point agarose 0.8% and dropped on slidespreviously coated with normal 1.5% agarose. Slides were placed in a lysis solution for 12 hours (overnight) and electrophoresis was carried out under high alkaline buffer conditions, (pH> 13), 20 min, 20V and 300 mA. After electrophoresis, the slides were soaked in 3 baths of neutralization buffer for 5 min (Tris, pH = 7), with 5 min interval between baths. The slides were allowed to dry, and then fixed in absolute ethanol (5 min), and stained with ethidium bromide (20 g/mL) solution. Analyses were carried out in epifluorescence microscopyusing the software Comet Assay IV-Lite, v4.3 (Axioskop 2, Zeiss). A total of 100 nucleoids per animal were analyzed,classifying damage levels in ranging from 0 (no damage) to 4 (very damaged).DNA damage index was automatically calculated bythe software.

In vivo micronucleus test

The in vivomicronucleus test was performed with the rat femoral bone marrow, according to the OECD 474/1997[20]. Theinitial erythrocyteseparation was conducted in a10 µm pore cellulose membrane, followed by discontinuous Percoll® gradientcentrifugation for theremoval of residual granules[21]. The erythrocytes were fixed for 10 minutes with methanol, and stained with Giemsa. The frequency of immature, micronucleated polychromatic erythrocytes (PCE) in a sample of 2000 polychromatic erythrocytes (PCE) per animal wascounted [22].Mature, normochromatic erythrocytes (NCE) were also

counted, and possible cytotoxic effect was calculated by the ratio of PCE with a sample of 200 erythrocytes per animal and the total cells (PCE + NCE), expressed as % PCE.

DNA fragmentation analysis by flow cytometry

One hundredµL of the bone marrow sample collected in SFB wasfixed in 500 µL of Karnovsky's solution and kept at 4 C. The collected cells were fixed in ethanol 70% at 4oC and stored until the procedure was carried out. To analyze DNA fragmentation the sample was washed in phosphate buffer saline (PBS) and incubated in 50 µg/ml RNAse A for 30 min. at 37 C. The staining was carried out with50 mg/mL propidium iodidefor 30 min at room temperature. Ten thousand events per individual were quantified in a CyFlow® flow cytometer (Partec, Germany) and the analysis performed by WindowsTMFlowMax ® software.

Hematologic and biochemical evaluation

A 0.5 mL aliquot of whole blood collected in 0.2 M EDTA from each animal was analyzed in an automated Sysmex Poch100iV Diff ™ hematology analyzer calibrated for rats. Hemoglobin (HGB), total hematocrit (HCT), erythrocyte (RBC), white blood cell count (WBC), platelet parameters (PLT), corpuscular volumes and their rates of change were analyzed. Serum biochemical analysis of hepatic function (AST, ALT, total, direct and indirect bilirubin), renal (creatinine and urea), pancreatic (amylase and lipase) and metabolism of triglycerides, were kindly provided by the Sabin Laboratory, Brasilia, DF, Brazil.

Statistical analyses

Statistical comparison between control and treated groups was performed using the Shapiro-Wilk normality test for decision making (parametric or non-parametric test). For samples with normal distribution One-Way ANOVA was used with post hoc Bonferroni test. For samples with non-normal distribution the Mann-Whitney test was used (p 0.05), nonparametric test.

Results

None of the animals treated with the doses tested died or had any adverse clinical signs over the length of the study. No macroscopic alterations and no difference in relative organ weights (liver, kidney, spleen and brain) were observed in the CG- and treated groups (data not shown).

Genotoxicity

Comet Assay

Table 1 shows the DNA damage index estimated by the comet assay for controls and ayahuascatreated groups. No significant differences in damage index between the treated groups and CG- were observed.The CG+ group treated with doxorubicin showed a significantlyhigher DNA damage index when compared to all the other groups, as expected for this drug.

Table 1. DNA damage index of controls (CG- and doxorubicin CG+) and ayahuasca treated (1X, 5X and 15X the ritual dose) groups, estimated by the comet assay. Values are the mean ± standard error; 5 animals per sex per group

CG-	155.6 ± 24.05	181.4 ± 31.76	168.5 ± 19.27
Doxorubicin	258.4 ± 18.58*	238.8 ± 8.75*	248.6 ± 10.22 *
1X	122.6 ± 33.16	189.8 ± 22.01	156.2 ± 21.85
5X	156.2 ± 22.81	145.4 ± 32.60	150.8 ± 18.84
15X	197.2 ± 15.10	147.2 ± 23.74	172.2 ± 15.66

* indicates statistical difference with all the other groups (p 0.05)

Micronucleus test

The frequencies of micronucleated polychromatic erythrocytes (PCE) are shown in Table 2, and were very low for all groups, except the doxorubicin CG+, as is expected for a genotoxic drug.Groups treated at smaller doses (1X and 5X) showed no significant differences compared to CG-; however, the frequency for the 15Xwas significantly higher than the negative control. There were no significant differences between the sexes.

Table 2. Frequencies of micronucleated polychromatic erythrocytes (PCE) of controls (CG- and doxorubicin CG+) and ayahuasca treated (1X, 5X and 15X the ritual dose) groups, estimated by the micronucleus test. Values are the mean ± standard error; 5 animals per sex per group

	Female	Male
CG-	1.8 ± 0.8[a]	1.6 ±0.81[a]
Doxorubicin	27.2 ± 1.39 *	28 ±1.58 *
1X	1.8 ±0.58	2 ±0.83
5X	2.2 ± 0.8	1.8 ±0.66
15X	3.6 ±0.51[b]	4.2 ±0.73[b]

* Indicates statistical difference with all the other groups; different letters indicate statistically significant differences between groups (p 0.05)

The cytotoxicity, measured as the % PCE related to the total cells, isshown in Fig. 1.Only the doxorubicin CG+showed a significant decrease when compared withall other groups.

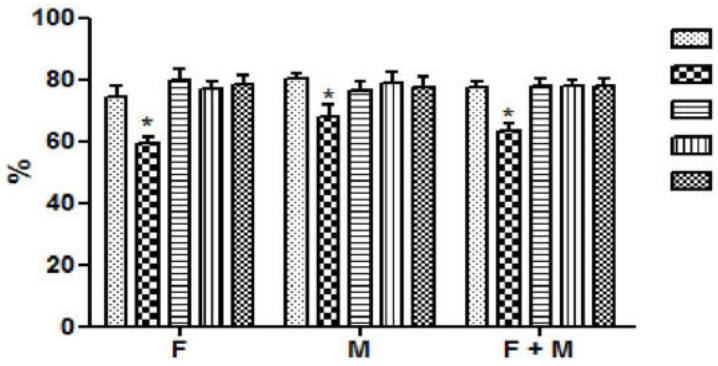

Figure1. Cytotoxicity (% PCE related to the total cells) in the micronucleus test. F = female; M = male. * Indicates statistical difference with the other groups.p 0.05; 5 animals per sex per group.

DNA fragmentation by Flow Cytometry

The results of the analysis of DNA fragmentation by flow cytometry are shown in Fig.2. Females from the group treated at 1X showed a significantly higher percentage of fragmentation when compared to the CG-, a difference that was also detected when both sexes were considered together. Again, this response was as expected, with higher % of fragmentation than all the other groups.

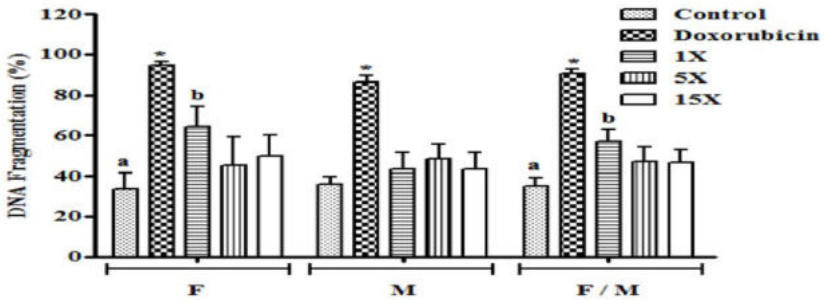

Figure 2. DNA fragmentation by flow cytometry. F = female; M = male. *Indicates statistical difference with the other groups; different letters indicate statistically significant differences between groups.p 0.05; 5 animals per sex per group

Hematologicaland serum biochemical analysis

There were no significant differences between the ayahuascatreated groups and the negative control group in hematological parameters analyzed in both males and females (data not shown).

Serum biochemical analyses to investigate liver (alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin), renal (urea and creatinine) and pancreatic function (serum amylase and lipase) are shown in Table 3.Liver function was altered bydoxorubicin, showing a significant increase in TGP and TGOcompared with the CG-. On average, treatment with ayahuasca at the tested doses produced no significant change in liver, kidney or pancreatic function compared with CG-,although there was an increase in urea and creatinine observed in the 15X group. The doxorubicin CG+showed significantly higher triglyceride concentrations when compared to CG-.The serum concentration of triglycerides at the 15X dose was significantly lower than all other groups.

Table 3. Analysis of serum biochemistry of controls (CG- and doxorubicin CG+) and ayahuasca treated (1X, 5X and 15X the ritual dose) groups. Values are the mean ± standard error; 10 animals per group, male and females.

	CG-	Doxorubicin	1X	5X	15X
AST	111.4±11.6	209.2±37.1*	91.7±10.0	98.3±11.4	113.2±12.4
ALT	36.7±2.6	52.7±4.3 *	37.8±2.8	36.3± 3.5	36±5.2
Total bilirubin	45.0±2.2	51.2±3.2	43±6.1	47.6±2.4	47.2±2.7
Direct bilirubin	33.5±2.8	33.7±2.9	35.8±2.3	31± 1.7	34.55±2.7
Indir. bilirubin	11.5±1.6	17.5±2.6	7.25±5.1	16.6±2.5	12.6±1.8
Urea	39.38±3	40.38±2.5	44.6±3.2	39.6±2.4	110.3±42.4
Creatinin	24.6±0.9	27.7±1.5	25.6±1.73	25.5±1.2	104.8±50.7
Amylase	1524±149.0	1527±113.4	1699±81.	1619±61.	2781±1175
Lipase	19.5±0.4	17.2±2.0	17.5±1.4	18.2±0.9	18.3±0.8
Triglycerides	57.6±4.1[a]	88.6± 10.9[b]	79.4±9.5	73.4±5.6	52.5±5.1*

Discussion

Beverages consumed as infusions, such as tea and coffee, are common worldwide. Infusion or concoction processes can either inactivate antimutagenicor activate promutageniccompounds [23]. In general, plant extracts may containcompounds with both properties, hence the importanceof carrying outevaluations such as the present one to protect human populations. The preparation of ayahuascainfusionvariesconsiderably among religious groups, mainly regarding the proportion of the plants used, the plant cultivars and the duration of the concoction[24-25], which can last around 3 to 4 hours.

In this study, a single oral dose of ayahuasca was used to treat animals of both genders at 1X, 5X and 15X times the ritualistic doseused by theUDV, corresponding to 0.3, 1.5 and 4.5 mg/kg bw DMT, respectively. No adverse clinical signs were observed in the animals throughout the study. There were no statistically significant differences in the body weight and relative organ weights among the treatedgroupsand negative control. No major macroscopic alterations were observed inany organ from treated animals.

To the best of our knowledge, this is the first in vivostudy of the genotoxicity and cytotoxicity of ayahuasca. Previousin vitro studies conducted with -carbolines,one of the main constituents of the infusion, reported conflicting results. In a review paper, Meester et al. [26] concluded that harman and norharman do not behave as true mutagens, but may modify and increase genotoxic and toxic properties of other compounds. Boeira et al. [27] found that harman and harminewere genotoxic in V79 cells (Chinese hamster fibroblasts), probably due to their ability to induce DNA strand breaks.In yeast, harmalinewas shown to induce DNAlesions during

the exponential growth phase, when cells are more sensitive[28]. However, Picadaet al. [29] showed no genotoxic effects of harmine in fungi, bacteria and cell culture. Furthermore, -carbolines, including harmine and harmaline, were shown to have antigenotoxicand antimutageniceffectsin yeast and mammalian cells, respectively, probably due to the antioxidative hydroxyl radical-scavenging property of the molecules [30]. Chen et al. [31] showed that harmine derivatives have antitumor activity, but also cause acute neurotoxicity, mainly through apoptosis.

In this study, the comet assay of circulating leukocytes did not show a significant difference in the DNA damage index in ayahuasca treated animals compared to negative control. However, flow cytometric analysis of bone marrow cells showed higher DNA fragmentation only in females treated at 1X the ritual dose, but not at higher doses, indicating that this effect was not dose dependent, and may be a random effect. Comet assay and flow cytometry analysis are complementary: while the latterprovides a quantitative measurement of DNA damage, the comet assay indicates the severity of the damage. Additionally, the comet assay performed in the peripheral blood evaluates hematopoietic cells at the final developmental stage, and provides information about the effect of a substanceon non-dividing cells, while the flow cytometry in the bone marrow evaluated actively dividing cells.

Micronucleus test showed a slight butsignificant increase in the PCEfrequency at the highest ayahuasca dose, although much lower that what was observed with the doxorubicin positive control group. Snyder [32]showedthat the presence of the N-dimethyl group in the molecule is related to clastogenic action, probably due to its ability to intercalatethe DNA through non-covalent binding, or by inhibition of topoisomerase. Although DMT has anN-dimethyl group,thestudy using pure DMT produced no genotoxic effects [32].Other in vivostudies showedthe absence of clastogenic effects of -carbolines.Picadaet al. [29]showed that harmine (46 mg/kg bw) and harman (30 mg/kg bw) injected intraperitoneally in micedid not induce micronucleus formation nor changes in chromosomal structure. The harmine dose was similar to the highest oral dose used in our study (15X, 50 mg/kg bwharmine).

A lower proportion of immature polychromatic erythrocytes over the total number of erythrocytes may indicate the cytotoxicity of a given compound due to inhibition of cell proliferation. In this study, no significant differences were found in this parameter between the ayahuasca treated groups when compared to the negative control. Hence, we can conclude that ayahuasca had no effect on bone marrow cell proliferation, indicating no cytotoxicity of this infusion at the tested doses.

Studies with yeast showed that the cytotoxic and mutagenic effectof harmine is only detected during the fungus'exponential growth phase and is dependent of the alkali medium concentration [28]. In vitro studies with human cells conducted by Jimenez et al.[33] indicated that harmine and harmaline were not capable of inducing events related to double strand breaks, although they produced necrosis and apoptosis at higher concentrations.

In this study, we found no significant differences in hematological parameters between the ayahuascatreated groups and the CG-, indicating no effect on maturation of red and white blood cells. Riba et al. [34]found no clinically relevant alterations in the hematological or biochemical parameters of six human male volunteerswhoingested anayahuasca infusion that contained from 0.5 to 1 mg/kg bw DMT. These doses are between the 1X and 5X doses used in the treated rats in our study (0.3 and 1.5 mg/kg bw DMT).

AST, ALT and bilirubin results indicated that ayahuasca did not alter theliver function of the treated animals. Doxorubicin did alter liver function, as shownby the significant increase in AST and ALT in the CG+. The pancreatic function reflected by serum amylase and lipase was also not affected by the ayahuasca treatment at the two lower dose levels, although an increase in amylase concentration in the 15X group was observed. This result maysuggest a possible biological effect of carbohydrates associated with low concentrations of circulating triglycerides, which was observed only in this group.

In conclusion, the data presented suggest that a single ayahuasca treatment at 1X and 5X the usual ritual dose showed an absence of genotoxicity in Wistar ratsthrough micronucleus test. A higher PCE frequencywas observed at the 15X dose,and a non-observed-adverse-effect-level (NOAEL) can be established at 5X dose, or 1.5 mg/kg bw DMT. Higher DNA fragmentation was observed only at the lowest dose, but was considered a random effect, not related to the treatment.Cytotoxicity was not observed in any treated group, and nor were any major hematological and biochemical alterations that would affect physiological functions. Further studies should be carried out with chronicallyexposed animals to complete the genotoxic evaluation of ayahuascaunder the investigationof itstherapeuticpotential.

Author Contribution

Melo Jr and Souza-Filho, carried out experiments, Pic-Taylor A, Supervisor of Melo Jr and responsible for the research project, Grisolia CK study design and laboratory support, Caldas ED analytical chemistry support and statistical analysis.

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