Reproductive Toxicology 77 (2018) 143–153

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Maternal and developmental toxicity of the hallucinogenic plant-based beverage ayahuasca in rats

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ARTICLE INFO

Article history:
Received 15 November 2017
Received in revised form 2 March 2018
Accepted 5 March 2018
Available online 6 March 2018

Keywords:
Ayahuasca
Serotonin syndrome
Developmental toxicity
Banisteriopsis caapi
Psychotria viridis
Teratogenicity
Gestation losses

ABSTRACT

Rats were treated orally with ayahuasca (AYA) on gestation days (GD) 6–20 at doses corresponding to one- (1X) to eight-fold (8X) the average dose taken by a human adult in a religious ritual, and the pregnancy outcome evaluated on GD21. Rats treated with 4X and 8X doses died during the treatment period (44 and 52%), and those that survived showed kidney injury. Rats surviving the 8X dose showed neuronal loss in hippocampal regions and in the raphe nuclei, and those from the 2X dose neuronal loss in CA1. Delayed intrauterine growth, induced embryo deaths and increased occurrence of foetal anomalies were observed at the 8X dose. At non-lethal doses, AYA enhanced embryolethality and the incidence of foetal soft-tissue and skeleton anomalies. This study suggested that AYA is developmentally toxic and that its daily use by pregnant women may pose risks for the conceptus.

1. Introduction

Ayahuasca (AYA) – also known as *hoasca, oasca*, *Daime* and *vegetal* – is a ritualistic psychedelic plant-based beverage traditionally used by Amazonian indigenous populations for spiritual and healing purposes and, more recently, by religious groups, including the *União do Vegetal* (UDV) and *Santo Daime.* This beverage is generally made up of the vine of *Banisteriopsis caapi* (Malpighiaceae) and leaves from *Psychotria viridis* (Rubiaceae) [1]. AYA psychoactive effects are attributed to a synergistic interaction of ß-carboline alkaloids harmine, harmaline and tetrahydroharmine, present in the *B. caapi*, and *N,N*-dimethyltryptamine (DMT) an indole alkaloid found in *P. viridis* [1,2]. ß-carbolines are potent inhibitors of monoamine oxidases (MAO), a group of enzymes that catalyse the oxidative deamination of serotonin, norepinephrine, dopamine and other monoamine neurotransmitters [3]. The hallucinogenic effects of DMT seem to arise mostly from its agonist action on serotonin (5-HT) receptors, primarily the 5-HT_{2A} receptor [4]. ß-carbolines, on the other hand, inhibit MAO-mediated oxidation of DMT in the digestive tract thereby increasing its bioavailability to the brain tissue [5,6].

The religious use of AYA has been regulated in Brazil since 1986 in response to concerns over its safety, while ensuring the freedom of religious practices. According to the Brazilian law, the use of AYA by children during the religious ritual is the parent responsibility, and pregnant women must observe and preserve the well-being and development of the foetus [7], although this use is a matter of heated debate [8,9]. The use of this beverage under the religious context, which occurs normally every 15 days, is also recognized in other South American countries, the United States, and some European countries, including Spain and Italy [10], and more recently in Canada [11].

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https://doi.org/10.1016/j.reprotox.2018.03.002
0890-6238/

Although the commercialization of AYA is prohibited, recreational use of this beverage has increased over the last decades, which has raised concerns regarding its safety in situations other than ceremonial rituals [12]. In particular, the association of AYA with other serotonergic drugs is potentially dangerous and may lead to serotoninergic syndrome [13].

Currently, there is an increasing interest in the therapeutic potential of AYA and its main components, which are thought to be effective in a range of psychiatric illnesses, including depression and drug addiction [14–17]. Studies assessing the toxicological effects of repeated use of AYA are scarce, and toxicological studies conducted with animal models are necessary to assess its safety.

To the best of our knowledge, there is only one previous study on the developmental toxicity of AYA. Oliveira et al. [18] treated pregnant Wistar rats on gestation days 6–20 (GD6-20) with AYA up to a maximum oral dose equivalent to 10-fold the dose usually consumed by a 70 kg adult during a religious ceremony. No signs of AYA-induced maternal toxicity was observed, but AYA increased in a dose-dependent manner the incidence of some foetal skeleton and viscera abnormalities (variations) at dose levels lower than those eliciting overt maternal toxicity.

The botanical origin and proportions of the two plants used in AYA decoctions (*B. caapi* and *P. viridis*) and the preparation procedure, which may vary substantially among the religious and non-religious groups, considerably affects the chemical composition of the beverage [1,2,13,19]. This study in rats evaluated the maternal and developmental toxicity of AYA prepared by an UDV group located in the Federal District of Brazil.

2. Material and methods

2.1. Plant material and preparation of ayahuasca (AYA)

The AYA infusion used in this study was prepared in April 2011 by a "*União do Vegetal*" (UDV) group (*Núcleo Luz do Oriente*) settled in the Federal District of Brazil. *B. caapi* used in the preparation was collected in *Águas Lindas de Goiás* (15°46′17″S; 48°14′56″) and *P.viridis* in *Sobradinho* village (15°75′23″S; 47°72′92″W) Federal District. Voucher specimens of *B.caapi* (ref. Azevedo EP 149880 BRAHMS) and *P.viridis* (ref. Trieto B 149879 BRAHMS) are maintained in the University of Brasilia (UnB) herbarium. Soon after preparation, AYA was frozen and stored at −20 °C for lyophilization (Liotop L101) and use in the experiment. AYA decoction dry matter yield corresponded to 16% (w/v) of the infusion.

The AYA beverage used in this study was the same employed in previous investigations conducted by our research group [20–22]. Measurement of levels of β-carbolines and DMT, and method of analysis were described elsewhere [20,22]. Briefly, harmine, harmaline and DMT concentrations in the AYA decoction were determined by GC–MS/MS, and results were confirmed by LC–MS/MS. Harmine and harmaline analytical standards were from Sigma-Aldrich Co, while DMT was synthesized. The chemical analysis showed that the AYA beverage contained 1.56 mg/mL of harmine, 0.122 mg/mL of harmaline and 0.141 mg/mL of DMT. Tetrahydroharmine was not analysed.

2.2. Animals

Male and nulliparous female *Wistar* rats (90–120 days old and weighing 200–300 g) were from a commercial supplier of laboratory animals (Granja RG, São Paulo, Brazil). Upon arrival at the animal facility of the Faculty of Medicine of the University of Brasilia, rats underwent a 15-day period of acclimation prior to study initiation. All rats were housed in standard polypropylene cages with stainless steel coverlids and pinewood shavings as bedding, and were kept under controlled environmental conditions (12h–12 h, dark-light; 22–25° C; 45–60% humidity). Filtered tap water and a commercial diet for laboratory rats (Labina, Purina®, Brazil) were provided *ad libitum*. Experimental procedures were conducted in accordance with Brazilian animal protection and welfare laws. The research project was approved by the University of Brasilia Ethics Committee on Animal Use (License N° 107766/2010).

2.3. Mating procedure

For mating, three females were placed into the cage of one male for 3 h at the end of photoperiod dark phase (6:00-9:00 a.m.). Copulation was confirmed by the presence of spermatozoa and oestrous cycle epithelial corneal cells in the vaginal smear. The day on which copulation occurred was designated day 0 of pregnancy (GD0). Sperm-positive rats were allocated at random to control and AYA-treated groups.

2.4. Treatment

As required, weighed lyophilized AYA was resuspended in 2 mL of filtered water and administered by gavage to rats. From GD6–20, rats were treated with single daily doses of AYA corresponding to one-human equivalent dose (1X), two- (2X), four- (4X) and eight-fold (8X) the typical dose (adjusted to body weight) taken by a human adult during a UDV religious ritual (150 mL by a 70 kg adult). Control rats received 2 mL of the vehicle (filtered water) only. One-human equivalent dose (1X) of this infusion corresponds to a dry matter content of 343 mg/kg bw/day, and 0.30 mg/kg bw/day DMT, 3.34 mg/kg bw/day harmine and 0.26 mg/kg bw/day harmaline (Table S in Supplementary material).

Twenty-five pregnant females were treated per dose group. Cages were inspected daily for deaths, clinical signs of toxicity and behavioural abnormalities. Body weight and total amount of food consumed were measured every third day.

2.5. Caesarean section

Rats were euthanized by CO_2 inhalation on GD21. After maternal death, the gravid uterus was quickly removed, and weighed with its contents. Ovaries were excised, cleared of adhering fat, and the number of corpora lutea graviditatis was counted. The numbers of dead and living foetuses and resorptions were recorded as well. The method of Salewski [23] was employed to reveal sites of completely resorbed implantations. Maternal liver, spleen, kidney, stomach, heart and brain were removed, weighed and fixed in formalin solution. Placentas and living foetuses were weighed, crown-rump length was measured and the sex determined. All foetuses were examined for externally visible abnormalities under a stereomicroscope. About 52–57% of foetuses from each litter, selected at random, were fixed in a 5% acetic acid, 2% formaldehyde (37%), 72% ethanol and 21% water solution and further evaluated for visceral abnormalities using a micro-sectioning technique adapted from Sterz [24]. The remaining foetuses were fixed in acetone, macerated in KOH, cleared with a glycerin–KOH solution, and stained with Alizarin Red S for skeleton evaluation [25]. Soft-tissue and skeleton abnormalities were identified and recorded using the internationally harmonized terminology glossary for developmental anomalies in laboratory animals – version 2 [26], and classified according to the scheme agreed upon by experts in a series of international workshops held in Berlin [27,28].

2.6. Histological processing of maternal organs

After fixation in formalin solution, spleen, liver and kidneys were embebbed in paraffin blocks, sectioned (5 μm sections) using

a Leica® microtome and mounted on slides, which were subsequently stained with eosin-haematoxylin. Histological images were acquired and evaluated using a ScanScope CS histological scanner (*Aperio ePathology Solutions*, CA, USA) at x400 magnification (resolution 0.25 μ/pixel) using the ImageScope (*Aperio Software program*, CA, USA). Histological evaluation considered the following tissue alterations: cytoplasmic membrane structure (disruption); loss of cytoplasmic organelles; nuclei piknosis; vascular congestion; inflammatory infiltration; oedema and areas indicative of reversible (hydropic and fat) or irreversible lesion (necrosis and apoptosis). Triplicate slides of each organ were analysed and the resulting data evaluated for the presence or absence of tissue alteration.

For neurotoxic evaluation, brains from 4 randomly selected animals per group were cut into 50 μm slices with a vibrating microtome (KD 400) and stained using Nissl, a well-established method for brain cell density. Viable neurons were counted in the following regions: the hippocampal formation (areas of *Cornu Ammonis* CA1, CA2, CA3 and Dentate Gyrus, DG); amygdaloidal complex (Posterior Basolateral Nucleus, PBN) and the Dorsal Raphe Nuclei (DRN). The degree of neuronal loss was quantified under a Leica DM 2000 microscope with subsequent analysis by Application Suite software (LAS) Core V4.1. Neuron counts in the right and left hippocampus were performed in a perimeter of 500 μm to the tip area of the dentate gyrus, 1000 μm for the CA1, CA3, amygdaloidal complex and raphe nuclei areas and 900 μm for the CA2 area [29]. One slide containing two brain slices of each animal was analyzed. All slides were randomly coded to blind the evaluator.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey ad hoc test or, alternatively, by Kruskal-Wallis test [30] and Dunn's multi-comparison test [31], or Mann-Whitney *U* test [32], whenever the data did not fit a normal distribution. Values were reported as means ± standard error (SEM), or the median and range (minimum- maximum value). Proportions were analysed by the chi-square test or by the Fisher exact test. In any case, a difference was significant when $p < 0.05$. Statistical calculations were performed using IBM SPSS Statistics for Windows, Version 19 (IBM Corp., released 2010), or GraphPad Prism version 5 (GraphPad Software, Inc., released 2007).

3. Results

3.1. Maternal toxicity

All vehicle-control rats and all rats treated with the two lowest doses of AYA survived to the scheduled euthanasia on GD21. Several AYA treatment-related deaths, however, occurred in the two highest dose groups. As illustrated in Fig. 1, only 56% and 48% of females treated with 4X and 8X, respectively, survived to GD 21. The survival curves for controls and lowest dose groups (1X and 2X) groups differ ($p < 0.05$) from those obtained for the highest dose groups (Fig. 1). As expected, the period of treatment (number of daily doses) prior to death was shorter for rats exposed to the highest dose (8X) compared to the second highest dose (4X). The median number (minimum–maximum values) of treatment days before death was 4 (2–12) days for the 8X group, and 8 (4–13) days for the 4X group ($p < 0.05$; one-tailed Mann-Whitney *U* test). In all cases, clinical signs of toxicity such as piloerection, vocalization, chromodacryorrhea, tremors, hind limb hyperextension, rigidity, cyanosis, lethargy and/or seizures preceded the death. Piloerection, tremors, lethargy and vocalization (within a few minutes after AYA administration only) were also observed in treated rats that survived to GD21, particularly among those that received the highest doses.

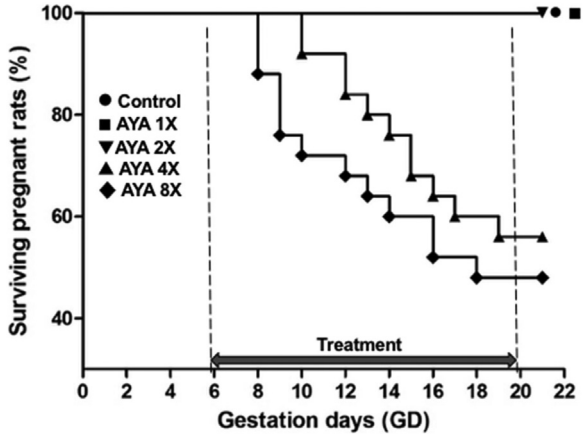

Fig. 1. Survival over time (Kaplan-Meier plot) of rats treated with ayahuasca infusion (AYA) during pregnancy. Wistar rats (N = 25 per group) were treated by gavage with daily doses of AYA (control, 1X, 2X, 4X and 8X) from gestation day (GD) 6–20 and euthanized on GD21. No death was noted in the control group and in the groups of rats treated with the two lowest doses of AYA (1X and 2X).

As shown in Table 1, AYA reduced food consumption over the whole treatment period at all tested dose levels, except the 2X dose. This decrease was significant at the highest dose (8X) during the whole treatment period. AYA did not decrease significantly the mean maternal body weight gain (after subtracting gravid uterus weight) compared to the control group weight gain at any dose level. Nonetheless, the maternal weight gain in the group treated with the second highest dose (4X) was lower than the weight gain noted in the group treated with the highest dose tested (8X).

Common macroscopic findings of the post-mortem examination (GD21) were dilated stomachs (at least one rat per treatment group), and dilated intestines (3–10 rats per dose group). These visceral abnormalities were found in AYA-treated rats only, yet their occurrence was not clearly dose-related. Spots of fat tissue were noted on the liver of rats from three AYA-treated groups (2X, 4X and 8X). No other relevant macroscopic pathology finding was noted at the necropsy of rats that survived to the scheduled euthanasia (GD21).

Except for an increase in stomach weight finding (consistent with a dilated organ noted at the necropsy) that was unrelated to the dose level, no AYA-induced change of organ weight was found (Table 2). The most striking finding of histopathological examination was a dose-dependent increase in the incidence of hydropic cell degeneration (renal tubule cells swelling) in the kidneys of rats treated with the two highest doses of AYA (Table 3 and Fig. 2). Hydropic cell changes are reversible and early signs of injury that, upon continued exposure to a nephrotoxic agent, may further evolve to an irreversible tubular cell injury (*e.g.* necrosis) [33]. A non-significant trend to an increase in the occurrence of signs of liver and spleen tissue injury at the highest doses was also noted (Table 3).

To investigate the effect of AYA in the brain tissue, data from both hemispheres were combined, as there were no significant differences between the neuronal counts of the left and right brain hemispheres of the same animal (data not shown). The neuron density results (cells/μm^2) in these areas, for both control and AYA-treated groups, are shown in Table 4. A significant decrease in the number of viable neurons compared to controls was observed in the CA1 hippocampal layer of the 2X, 4X and 8X groups, and in the CA2 and CA3 layers of the 8X group. The neuronal loss observed in the hippocampus regions of animals from the highest dose group is shown in Fig. 3. In addition, there was also a significant decrease in

Table 1
Effects of ayahuasca (AYA) infusion administered by gavage to Wistar rats on gestation days (GD) 6–21 on maternal weight gain and food consumption. X: human equivalent dose.

Treatment	AYA oral dose (dry matter: g/kg bw/day)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Treated pregnant rats	25	25	25	25	25
Rats surviving to GD21	25	25	25	14	12
Maternal body weight (g)					
GD0	258.9 ± 2.7	254.6 ± 2.9	258.4 ± 2.5	252.1 ± 3.2	262.2 ± 3.7
GD21	344.4 ± 4.4	332.8 ± 6.7	324.4 ± 5.4	320.3 ± 7.2	331.3 ± 7.1
Food consumption[#] (g)					
GD6–9	61.4 ± 2.7	43.0 ± 1.4*	57.7 ± 2.1	38.6 ± 3.0*	40.6 ± 3.0*
GD9–12	62.8 ± 2.4	45.8 ± 2.5*	64.4 ± 2.0	41.0 ± 4.9*	40.9 ± 3.4*
GD12–15	62.0 ± 2.3	46.6 ± 2.8*	66.0 ± 2.7	48.2 ± 3.9*	48.7 ± 3.5*
GD15–18	61.3 ± 2.0	54.3 ± 2.2	61.7 ± 2.1	55.4 ± 3.9	46.0 ± 2.8*
GD18–21	62.8 ± 2.5	56.7 ± 2.7	59.4 ± 2.4	54.4 ± 3.3	48.2 ± 4.4*
Maternal weight gain (Δg)					
GD 0–21	85.5 ± 4.6	78.2 ± 5.6	66.0 ± 4.9	68.2 ± 6.0	69.0 ± 7.2
GD 6–21	73.8 ± 4.4	64.6 ± 5.4	49.4 ± 4.7*	55.7 ± 5.6	50.2 ± 7.8
Gravid uterus weight (g)	63.3 ± 3.3	51.1 ± 4.0	45.8 ± 3.9*	54.6 ± 4.4	31.8 ± 6.2*
Maternal weight gain minus uterus weight (Δg)	22.2 ± 4.6	27.0 ± 3.6	20.2 ± 3.1	13.6 ± 2.9	37.2 ± 4.3

Data are shown as mean ± SEM.
* Significantly different from control values, $p < 0.05$.
# Total amount of food consumed per rat in 3 days.

Table 2
Effects of rat exposure to ayahuasca (AYA) beverage during gestation (GD6-20) on the weight of maternal organs measured on GD21. X: human equivalent dose.

Treatment	AYA oral dose (dry matter: g/kg bw/day)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Pregnant rats (N)	25	25	25	14	12
Organ weight (g)					
Spleen	0.86 ± 0.03	0.81 ± 0.03	0.86 ± 0.03	0.79 ± 0.04	0.90 ± 0.05
Brain	2.07 ± 0.04	1.99 ± 0.03	2.00 ± 0.02	1.97 ± 0.03	2.08 ± 0.03
Heart	0.98 ± 0.02	0.94 ± 0.02	1.01 ± 0.03	0.89 ± 0.02	1.05 ± 0.06
Stomach	4.33 ± 0.34	6.82 ± 0.63*	6.58 ± 0.50*	4.19 ± 0.44	6.88 ± 1.15
Liver	13.87 ± 0.42	13.50 ± 0.36	13.62 ± 0.27	13.09 ± 0.44	13.71 ± 0.44
Kidneys[+]	0.98 ± 0.02	0.96 ± 0.02	0.97 ± 0.02	0.93 ± 0.03	1.02 ± 0.03

Data are shown as means ± SEM and analysed by ANOVA and Tukey ad hoc test.
+ Average weight of left and right kidneys.
* Significantly different from control values, $p < 0.05$.

Table 3
Histopathology findings in rat organs examined at C-section (GD21) following oral exposure to ayahuasca (AYA) beverage during on gestation days 6–20. X: human equivalent dose.

Treatment	AYA oral dose (dry matter: g/kg bw/day)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Rats examined on GD21 (N)	25	25	25	14	11
Organs with abnormalities: N (%)					
Spleen					
Congestion	1(4.0)	2(8.0)	2(8.0)	2(14.3)	3(25.0)
Hemorrhage	1(4.0)	2(8.0)	1(4.0)	2(14.3)	3(25.0)
Necrosis	0(0)	0(0)	0(0)	1(7.1)	1(8.3)
Liver					
Congestion	1(4.0)	2(8.0)	3(12.0)	2(14.3)	3(25.0)
Hyaline cylinder	0(0)	0(0)	1(4.0)	0(0)	1(8.3)
Hydropic degeneration	3(12.0)	3(12.0)	2(12.0)	2(14.3)	3(25.0)
Steatosis	1(4.0)	2(8.0)	3(12.0)	2(14.3)	3(25.0)
Inflammatory infiltrate	0(0)	0(0)	1(4.0)	2(14.3)	2(16.7)
Necrosis	0(0)	0(0)	1(4.0)	2(14.3)	2(16.7)
Kidneys					
Hyaline cylinder	0(0)	0(0)	1(4.0)	1(7.1)	1(8.3)
Congestion	2(8.0)	2(8.0)	2(8.0)	3(21.4)	1(8.3)
Hydropic degeneration	2(8.0)	3(12.0)	4(16.0)	6(42.9)*	6(50.0)*
Steatosis	0(0)	0(0)	0(0)	0(0)	1(8.3)
Inflammatory infiltrate	0(0)	0(0)	0(0)	1(7.1)	2(16.7)
Necrosis	0(0)	0(0)	0(0)	1(7.1)	1(8.3)

Data were analysed by the chi-square test.
* Significantly different from control values, $p < 0.05$.

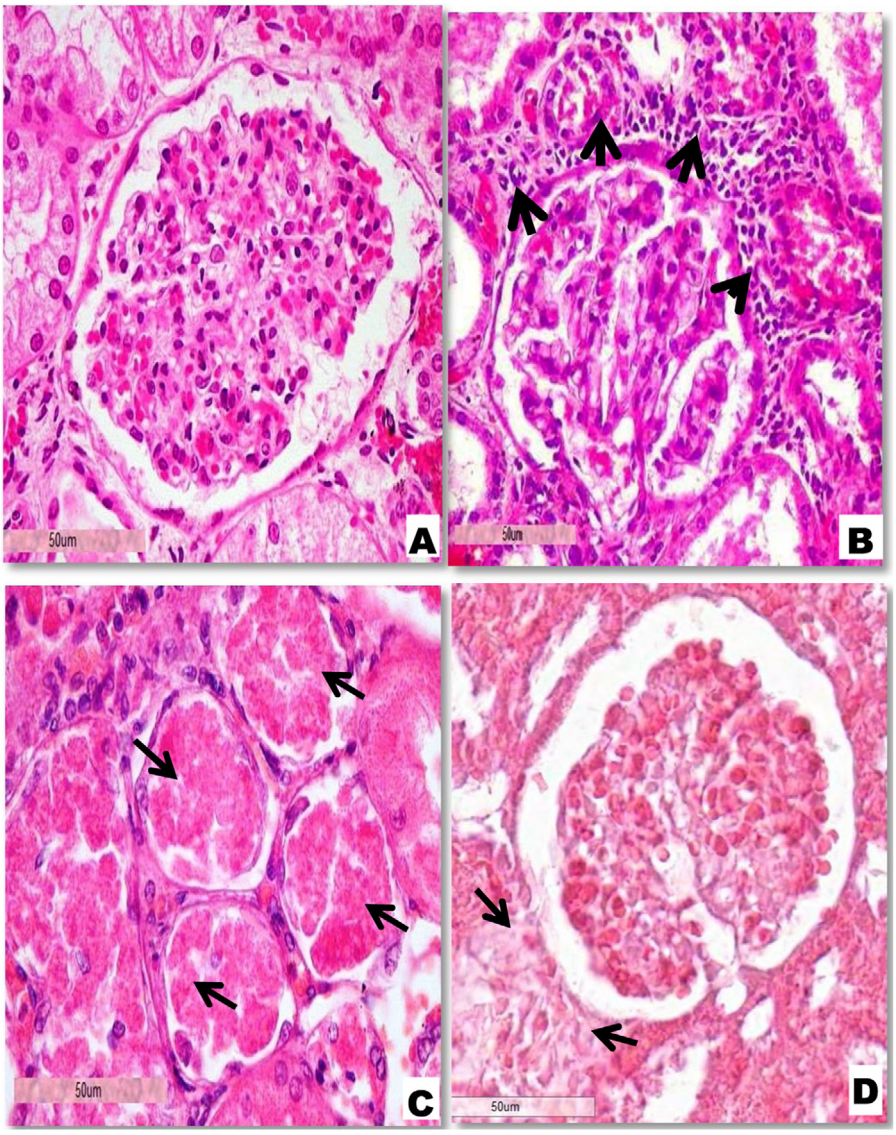

Fig. 2. Histopathological findings in kidneys of pregnant Wister rats on GD21. (A) Photomicrograph from a kidney section of a control group rat; (B-D) of rats treated orally with ayahuasca (AYA) at doses corresponding to eight-fold (8X) the average human dose taken in religious rituals, showing (B) inflammatory infiltrate, (C) necrosis and (D) hydropic cell degeneration. H&E staining, ×400.

Table 4
Neuron density (cells x 1000/μm^2) in specific brain regions of female *Wistar* rats exposed to ayahuasca (AYA) for 15 days (n = 4 animals for all groups). X: human equivalent dose. Results are presented as mean ± SEM and (%) relative to the control group (0), which was considered as 100% viable neurons.

	AYA oral dose (dry matter: g/kg bw/day)				
Region	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
CA1	5.6 ± 0.4	4.8 ± 0.4 (85.7)	3.9 ± 0.2* (69.6)	3.7 ± 0.2* (66.1)	3.3 ± 0.3* (58.9)
CA2	10.9 ± 1.1	9.4 ± 0.7 (86.2)	7.6 ± 0.3 (69.7)	7.2 ± 0.6 (66.0)	7.1 ± 1.3*(65.1)
CA3	7.1 ± 0.5	7.0 ± 0.6 (98.6)	5.5 ± 0.3 (77.5)	5.3 ± 0.5 (74.6)	5.0 ± 0.3*(70.4)
GD	17.8 ± 1.6	16.7 ± 1.1 (93.8)	16.4 ± 0.8 (92.1)	16.1 ± 0.1 (90.4)	16.0 ± 0.9 (89.9)
BA	5.6 ± 0.4	5.4 ± 0.3 (96.4)	5.3 ± 0.4 (94.6)	4.7 ± 0.1 (83.9)	4.6 ± 0.1 (82.1)
DRN	6.9 ± 0.7	6.8 ± 0.5 (98.6)	5.9 ± 0.4 (85.5)	5.2 ± 0.2 (75.4)	5.0 ± 0.2*(72.5)

CA1, CA2 and CA3: hippocampus areas; DG: dentate gyrus; BA: basolateral amygdala; DRN = dorsal raphe nucleus.
* Significantly different from control values, $p < 0.05$.

neural density in the DRN compared to the controls in the 8X group (Table 4).

In summary, several clinical signs of toxicity, and a dose-related increase in mortality indicated that the two highest doses of AYA tested in this study were maternally toxic. A higher incidence of signs of reversible kidney injury in rats treated with the two highest doses that survived to GD21 corroborates this conclusion. In addition, decreased neural density was observed in the hippocampus and DRC, mainly in the highest treated dose, but also in the CA1 hippocampus region at the three highest doses.

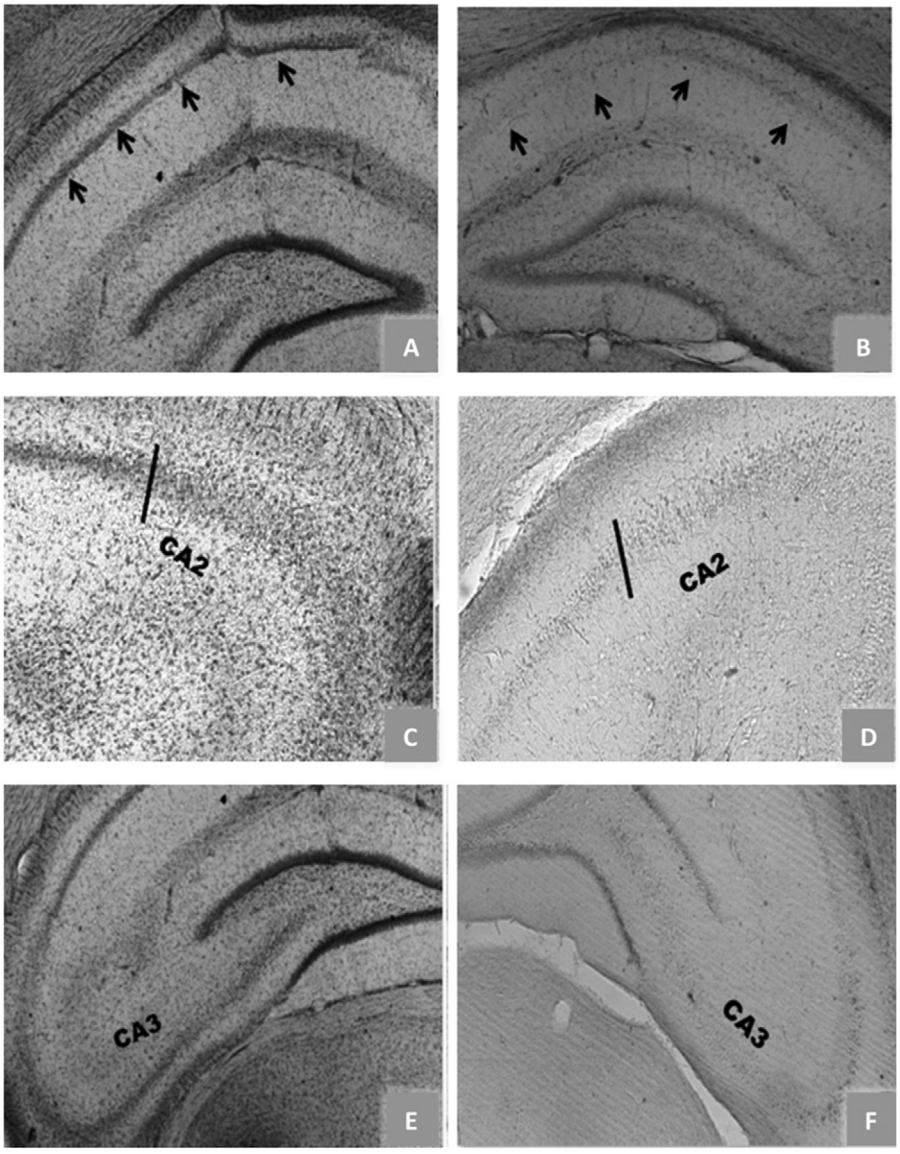

Fig. 3. Photomicrography of the CA1 (arrows), CA2, and CA3 regions of animal representative of the control group (A, C, E) and AYA-treated group at 8X dose (B, D, F). 4× magnification. Nissl coloration. Lower neuron density compared to controls was observed in the CA1, CA2 and CA3 areas of female AYA treated rats.

3.2. Developmental toxicity

As shown in Table 5, control and AYA-treated groups had comparable numbers of *corpora lutea graviditatis* per dam. The number of implantation sites per dam was not altered by treatment from GD6 onwards either. Therefore, since implantation in the rat takes place on GD6–8 [34] one may conclude that AYA did not impair ongoing implantation nor did it induce very early post-implantation losses, that might remain undetected by Salewski's technique. Nevertheless, at nearly all doses of AYA, the number of resorptions per litter was higher than that in the vehicle-control group, a difference that was significant at the 8X dose group (Table 5). The significant decline in the number of live foetuses per litter (litter size) in the 2X and 8X groups is consistent with the interpretation that AYA did increased the incidence of post-implantation losses. AYA-induced enhancement of post-implantation losses was mostly due to an increase in the number of early resorptions, or embryo deaths occurring within a short period (few days) after implantation. A non-significant 5–17% decrease in foetal body weight compared to control foetus weight was noted in all AYA treated groups (Table 5).

External examination did not reveal any gross structural abnormality in foetuses from control and AYA-treated groups. Treatment with AYA increased the incidence of soft-tissue abnormalities such as dilated brain lateral and third ventricles (Table 6), which are "gray-zone" anormalies, i.e, it is unclear whether it is permanent (*i.e.*, persists after birth), or it adversely affects the survival or health [28]. The treatment also increased the incidence of malpositioned organs (kidneys, testes, ovaries, and uterus), and of livers exhibiting abnormal shape and with one additional lobe (Table 6), which are considered of low severity and classifiable as variations [28]. The occurrence of some of these visceral abnormalities (*e.g.*, dilated cerebral ventricles and malpositioned testes and ovaries) was enhanced even at the lowest dose tested (1X). Absence of ovaries (a malformation) was recorded in two foetuses from two dams treated with the lowest dose of AYA tested (1X).

Table 7 shows the incidence of skeleton abnormalities in control and AYA-exposed foetuses. An increased incidence of incompletely ossified skull bones was the most frequent skeletal abnormality in foetuses from dams treated with AYA on GD6–20. The increase in the occurrence of incompletely ossified nasal,

Table 5
Caesarean section data (GD21) of rats treated orally with ayahuasca (AYA) on gestation (GD) days 6–20. X: human equivalent dose.

Treatment	AYA oral dose (dry matter: g/kg bw/day)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Pregnant rats on GD21	25	25	25	14	12
Corpora lutea graviditatis (N)	11.7 ± 0.4	12.0 ± 0.3	12.5 ± 0.46	10.6 ± 0.6	13.2 ± 1.2
Implantation sites (N)	9.8 ± 0.4	8.8 ± 0.6	8.8 ± 0.6	9.3 ± 0.6	8.6 ± 0.9
Resorptions (N)	0.6 ± 0.2	1.4 ± 0.3	2.1 ± 0.4	1.4 ± 0.3	3.9 ± 1.3*
Early (N)	0.2 ± 0.1	0.9 ± 0.2	1.6 ± 3.5*	1.1 ± 0.3	1.8 ± 0.7*
Intermediate (N)	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	1.1 ± 0.8
Late (N)	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1
Loss of the whole litter (N)	0	0	0	0	3#
Dead foetuses (N)	1	0	0	2	3
Litter size (N)	9.2 ± 0.5	7.4 ± 0.6	6.5 ± 0.6*	7.7 ± 0.7	5.9 ± 0.9*
Sex ratio (M/F)	1.0 (0.0–3.5)	0.8 (0.0–2.0)	1.5 (0.3–4.0)	0.5 (0.3–1.5)	0.75 (0.0–1.5)
Placenta weight (g)	0.56 ± 0.01	0.58 ± 0.03	0.61 ± 0.01	0.56 ± 0.01	0.59 ± 0.07
Foetal body weight (g)	4.8 ± 0.2	4.3 ± 0.2	4.5 ± 0.1	4.5 ± 0.2	4.0 ± 0.3
Crown rump length (mm)	39.1 ± 0.6	39.3 ± 0.8	39.5 ± 0.5	39.0 ± 0.7	37.7 ± 1.5

Sex ratio is shown as the median and range (minimum-maximum value) and analysed by non-parametric tests (Kruskal-Wallis test and Dunn's multiple comparison test). All other data are means ± SEM that were compared by ANOVA and Tukey post hoc test.
* Significantly different from control values, $p < 0.05$.
# 2 dams with resorption of the whole litter, 1 dam with 3 dead foetuses (runts).

Table 6
Occurrence of soft-tissue abnormalities in the offspring of rats treated orally with ayahuasca (AYA) on gestation days (GD) 6–20. Foetuses were removed on GD 21. X: human equivalent dose.

Treatment	AYA oral dose (dry matter: g/kg bw/day)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Foetuses (litters) examined, N	95 (25)	87 (25)	69 (25)	54 (14)	29 (9)
Foetuses (litters) with anomalies (%), in:					
Brain					
Lateral and third ventricles (dilated)	0 (0)	8 (16.0)	1.4 (4.0)	1.8 (7.1)	3.4 (11.1)
Liver					
Misshapen	12.6 (32.0))	49.4* (52.0)	52.2*(56.0)	64.8* (78.5)*	55.2*(77.6)*
Additional lobe	4.2 (16.0)	11.5 (20.0)	10.1 (16.0)	7.4 (21.4)	6.9 (11.1)
Kidneys					
Malpositioned	0 (0)	1.1 (4.0)	0 (0)	9.2* (28.5)*	0(0)
Ureter					
Misshapen	0 (0)	0 (0)	5.8*(16.0)	16.7* (35.7)*	13.8* (22.2)
Testes					
Malpositioned	2.1(4.0)	10.3*(28.0)*	10.1* (28.0)*	3.7 (14.3)	10.3* (33.3)*
Ovaries					
Malpositioned	0 (0)	11.5* (28.0)*	4.3 (8.0)	1.8 (7.1)	3.4 (11.1)
Absent	0 (0)	2.3 (8.0)	0 (0)	0 (0)	0 (0)
Misshapen	2.1 (4.0)	1.1 (4.0)	2.8 (8.0)	0 (0)	0 (0)
Uterus					
Misshapen	0(0)	1.1 (4.0)	1.4 (4.0)	5.5* (21.4)*	3.4 (11.1)
Malpositioned	0 (0)	2.3 (8.0)	0 (0)	5.5* (21.4)*	0 (0)

Data were analysed by the Fisher exact test.
* Significantly different from control values, $p < 0.05$.

frontal, parietal, interparietal, supraoccipital and squamosal bones did not indicate a definitive dose response relationship, and in most cases was detected at the lowest dose of AYA tested (1X) (Table 7). Enhanced incidences of incompletely ossified and unossified sternebra, supernumerary lumbar ribs, and zygomatic bone fused with maxilla were observed in foetuses from AYA-treated dams as well.

In summary, treatment with AYA on GD6–20 impaired prenatal development of rats. At the highest of dose tested (8X), AYA was lethal to the embryo/foetus, delayed foetal growth, and increased the incidence of structural abnormalities (mostly variations). Findings indicative of developmental toxicity, including an increased occurrence of post-implantation gestation losses and foetal soft-tissue and skeleton abnormalities (mostly variations), were also noted at 2X dose level, which did not cause overt signs of maternal toxicity. Although not having reached statistical significance, a 17% decrease in foetal body weight (noted at the 8X dose group) is of potential biological significance and cannot be overlooked. Moreover, the increased incidence of incompletely ossified bones in the foetuses of AYA-treated groups is also consistent with the hypothesis that the treatment may have caused a delay in prenatal growth

4. Discussion

The AYA infusion tested in this study proved to be highly toxic to pregnant rats. The highest doses tested (4X and 8X), killed 42 and 58% of treated rats, respectively, and induced discernible histopathologic changes in the kidneys (hydropic cell degeneration) of nearly half of the rats that survived to scheduled euthanasia (GD21). Hydropic degeneration may be a reversible change, or an early indication of necrosis, an irreversible damage. At any rate, it is a nonspecific renal tissue change arising from a perturbation of cell function associated to repeated administration of doses of AYA as high as 4-fold and 8-fold the equivalent human dose. It is unclear, however, whether this potentially reversible nephrotoxic damage contributed to AYA-induced deaths. Our previous study showed that male rats exposed to repeated AYA doses also died at

Table 7
Occurrence of skeleton abnormalities in the offspring of rats treated orally with ayahuasca (AYA) on gestation days (GD) 6–20. Foetuses were removed on GD21.

Treatment	AYA oral dose (dry matter: g/kg bw/d)				
	0 (0)	1X (0.34)	2X (0.68)	4X (1.37)	8X (2.74)
Foetuses (litters) examined, N	135 (25)	98 (25)	93 (25)	55 (14)	40 (9)
Foetuses (litters) with anomalies (%) in:					
Skull					
Nasal bone					
(incomplete ossification)	0(0)	6.1*(20.0)	3.2(8.0)	0(0)	25.0*(55.5)*
Frontal bone					
(incomplete ossification)	3.7(20.0)	22.4*(52.0)*	12.9*(20.0)	14.5*(35.7)	20.0*(44.4)
Parietal bone					
(incomplete ossification)	3.7(20.0)	19.4*(52.0)*	8.6(16.0)	1.8(7.1)	17.5*(33.3)
Interparietal bone					
(incomplete ossification)	3.7(20.0)	22.4*(52.0)*	12.9*(20.0)	14.5*(35.7)	20.0(44.4)
(misshapen)	1.4(8.0)	2.0(8.0)	0(0)	0(0)	0(0)
Supraoccipital bone					
(incomplete ossification)	7.4(36.0)	11.2(44.0)	10.7(24.0)	5.4(21.4)	15.0(33.3)
(hole)	0.7(4.0)	1.0(4.0)	12.9*(40.0)*	7.2*(28.5)*	0(0)
(misshapen)	13.3(72.0)	33.6*(72.0)	39.7*(72.0)	32.7*(64.2)	27.5(66.6)
(unossified)	0(0)	2.0(8.0)	0(0)	0(0)	0(0)
Squamosal bone					
(incomplete ossification)	11.1(52.0)	33.3*(52.0)	45.1*(68.0)	25.4(71.4)	40.0*(55.5)
(misshapen)	0(0)	19.3*(44.0)*	1.0(4.0)	0(0)	42.5*(33.3)*
Zygomatic bone					
(incomplete ossification)	0(0)	4.0*(16.0)	0(0)	0(0)	5.0(22.2)
(misshapen)	0(0)	0(0)	0(0)	0(0)	7.5*(33.3)*
(unossified)	0(0)	0(0)	0(0)	1.8(7.1)	0(0)
(fused with maxilla)	0(0)	12.2*(44.0)*	13.9*(44.0)*	0(0)	2.5(11.1)
(fused with squamosal)	0(0)	1.0(4.0)	0(0)	5.4(14.2)	0(0)
Sternum, sternebra					
(incomplete ossification)	0(0)	2.0(8.0)	26.8*(36.0)*	0(0)	0(0)
(unossified)	4.4(24.0)	31.6*(68.0)*	39.7*(92.0)*	25.4*(50.0)	42.5*(55.5)
Vertebral column					
Vert cent. (split)	2.2(12.0)	3.1(12.0)	0(0)	0(0)	12.5*(33.3)
Vert cent (misshapen)	0(0)	3.1(12.0)	1.0(4.0)	0(0)	30.0*(55.5)*
Vert cent (unossified)	0(0)	0(0)	0(0)	0(0)	5.0(22.2)
Ribs, lumbar					
(rudimentary)	17.0(64.0)	20.4(52.0)	24.7(52.0)	9.1(35.7)	25.0(55.5)
(supernumerary)	0(0)	8.2*(20.0)	4.3*(12.0)	0(0)	10.0*(33.3)*

Data were analysed by the Fisher exact test.
* Significantly different from control values, $p < 0.05$.

4 and 8X the ritual human equivalent dose, but when the intake occurred every two days, the animals survived until termination, after 72 days of exposure [22]. The lethal acute (single) dose for female rats of the same AYA infusion was higher than 50X the ritual human equivalent dose [20].

The most plausible hypothesis is that AYA-caused deaths resulted from its neurotoxic effects, which could not be reversed in 24 h. As aforementioned, prior to death AYA-treated rats displayed neurotoxic symptoms such as tremors, rigidity, hyperextension of hind limbs and seizures. At least three of these symptoms (hyperextension or abduction of hind limbs, tremors and rigidity) are typical symptoms of serotonin syndrome in rodents [35,36].

In humans, serotonin syndrome is a potentially life-threatening event that involves the overstimulation of $5HT_{A1}$ and $5HT_{A2}$ receptors in the peripheral and central nervous system. Overexposure to serotonergic drugs and or MAO inhibitors, and a combination of MAO inhibitors and 5HT selective serotonin reuptake inhibitors (SSRIs) may trigger a serotonin syndrome [37]. The fact that DMT exerts an agonist action on $5HT_{A2}$ (and possibly also on $5HT_{2C}$) receptors [38], and that harmine and harmaline are potent MAO inhibitors is consistent with the hypothesis that high doses of AYA containing these alkaloids precipitated a severe serotonin syndrome after repeated exposure that evolved to convulsions and death. Castro-Neto et al. [39] reported increased levels of serotonin in the hippocampus of rats treated once with AYA, together with serotonin, noradrenaline, and dopamine in the amygdala.

A significant decrease in the number of viable neurons was observed in the hippocampus and DRN brain regions of female rats that survived the AYA treatment at the highest dose, which led to a breakdown of neuronal layer architecture at the highest dose tested. An acute dose study conducted by our research group showed that a single high AYA dose (30X) significantly activates neurons of the CA1, CA2 and CA3 regions of the hippocampal formation, amygdaloidal complex and DRN [20]. The authors also reported positive cell staining with Fluoro-Jade B in the DRN and amygdaloidal complex areas, suggesting that hyperactivation may result in cell injury. However, in this single dose study, no permanent damage or change in brain histology and cell number was observed.

Study findings indicated that 8X, an AYA dose overtly toxic to the mother, increased embryolethality, retarded embryo-foetal growth and increased the incidence of several soft-tissue and skeleton anomalies generally classified as variations. The incidence of embryo deaths and foetal soft-tissue and skeleton structural abnormalities was also enhanced at non-maternally toxic doses. A higher occurrence of embryo deaths (mostly early resorptions) and foetal anomalies at 1X and 2X doses, which did not cause toxicity signs other than a loss of neurons in CA1 brain area (2X), suggested that the developing conceptus was more susceptible than the maternal organism to harmful effects of AYA.

Although proving to be a developmental toxicant, AYA did not increase the frequency of malformations at any dose level. The intramembranous ossification of rat skull flat bones takes place near term [40] and a higher proportion of incompletely ossified skull bones observed on GD21 can be interpreted as a sign of delayed ossification (a change potentially reversible after birth),

or a variation rather than a permanent structural change (or malformation). Skeletal abnormalities, such as rudimentary (extra) lumbar ribs and misshapen supraoccipital bone, showed a high incidence in control foetuses and are also classifiable as variations. Fused bones are generally considered malformations, and "zygomatic bone fused with maxilla" has been classified as malformation by many toxicologists [27], while others think that this foetal skeleton observation is a variation [41]. Nonetheless, the fusion of zygomatic bone with maxilla normally occurs later during postnatal growth and consequences of an anticipated fusion are not clear. Along the same line, most soft tissue abnormalities, the occurrence of which increased in AYA-exposed foetuses, are considered variations rather than malformations [28]. In sum, although being selectively toxic and lethal to developing embryos, AYA did not cause a clear teratogenic effect in rats.

Since AYA has a serotonergic mode of action, causes a serotonergic syndrome and has putative anti-depressive effects [20], it is fair to think that AYA could share some of the effects of SSRIs on prenatal development. Indeed, epidemiology investigations showed that the use of SSRIs during pregnancy may harm the unborn child leading to low birth weight, increased neonatal care and admission to the Intensive Care Unit, preterm births, and foetal/neonatal death [42,43]. Although a possible association between AYA *in utero* exposure with cardiac defects was observed in study, retrospective reviews and meta-analyses failed to detect a consistent increase in the risk of congenital anomalies in babies prenatally exposed to SSRIs [44,45]. Studies in rodents suggested that *in utero* exposure to SSRIs adversely affects motor development and increase immobility in the forced swimming [46,47]. It was also suggested that developmental exposure (including prenatal exposure) to SSRIs (*e.g.* fluoxetine) may alter epigenetic programming in rodent brain causing long term behavioural modifications [48]. Although SSRIs and AYA have distinct modes of action (SSRIs act on 5HT transporters, while AYA inhibits MAO and has agonistic action on $5HT_{2A}$ receptors), it is plausible to hypothesize that, like SSRIs, AYA-mediated overstimulation of the embryo-foetal 5HT system may adversely affect neurological and behavioural development. Only a few studies in humans, however, investigated the effects of prenatal exposure to SSRIs on cognitive functions and the risk of psychiatric illnesses [47]. The postnatal neurological and behavioural development of the offspring exposed to AYA during pregnancy (to doses that cause no overt maternal toxicity, *e.g.* equal to and or lower than 2X) deserves to be further investigated.

The AYA beverage tested in this study was a more potent maternal and developmental toxicant than the infusion used by Oliveira et al. [18]. These authors found no indication of marked maternal toxicity even at the highest dose tested (10X a typical ritual dose corresponding to 100 mL taken by a 70 kg bw adult). Except for a decline in pregnancy weight gain during the first days of AYA administration (GD6–9 and 9–12), Oliveira et al. found no significant maternal weight gain deficit. Moreover, they did not observe maternal deaths, nor did they find an embryolethal effect in doses up to 10X, whereas this study found many maternal deaths at the two highest doses (4X and 8X) and embryo deaths at all doses of AYA. Along the same line, Oliveira et al. [18] reported that treatment caused no clinical sign of toxicity at selected doses, whereas we noted symptoms compatible with a serotonin syndrome, particularly at doses equal to and higher than 4X.

The reason why the two infusions differed from each other regarding both maternal and developmental toxicities is not entirely clear. Plausible explanations are distinct concentrations of the major plant alkaloids (β-carbolines and DMT) and or the presence of other still unknown toxicants in the AYA beverages. Differences in the AYA chemical composition are expected and due to the intrinsic factors of the plants used in the preparation (environmental factors and cultivation), their proportion in the infusion and preparation time (boiling, decoction and concentration), which are particular to each AYA religious community. The AYA used in the present study was prepared by a UDV group using plants collected in the Federal District, in Midwestern of Brazil, while the one used by Oliveira et al. [18] was prepared by another religious group, using plants collected in the state of São Paulo, located in the Southern East of the country. The chemical profiles of the AYA infusion used in the present study and in Oliveira et al. [17] are shown in Supplementary material. The infusion used by Oliveira et al.'s study contains higher concentrations of DMT and harmaline (corresponding to 6.1 and 8.9 mg/kg bw/day, respectively, at 10X dose) than the levels at the 8X dose used in the present study (2.4 and 2.1 mg/kg bw/day, respectively), but lower harmine levels (19.5 mg/kg bw/day against 26.7 mg/kg bw/day in our study). It is possible that the differences in the concentrations of MAO inhibitors and DMT and the interaction between them made the AYA infusion tested in this study an inducer of serotonin syndrome stronger than that evaluated by Oliveira et al.

Gestational losses observed in this study were predominantly due to early resorptions, that is, prenatal losses corresponded to embryo deaths that occurred a few days after implantation, within the first days of treatment with repeated doses of AYA at 2X or higher. It is unclear whether AYA embryolethal effect would occur at similar doses after a single administration or when the time interval between two consecutive doses is longer than one day, which is the context of the religious use of this beverage. In most religious groups, the rituals occur every 15 days, with extra sessions in some festive occasions, and pregnant women are advised to take lower portions [9,10]. Various studies have shown the safety of AYA use under the religious context [49–51]. A study conducted with adolescent users indicated no significant difference with the control group (non-users) on neuropsychological measures, including increased attention, visual search, verbal and visual abilities, memory, and mental flexibility [50]. Furthermore, compared to controls, considerable lower frequencies of positive scoring for anxiety, body dismorphism, and attentional problems were detected among ayahuasca-using adolescents [51].

This study has some limitations that deserves to be discussed. The first is the lack of THH analysis in the AYA infusion, which hampered a comparison with the levels found in the infusion used by Oliveira et al. The other limitations are inherent to the metabolic, physiological and pharmacokinetic differences between rats and humans, which may limit the extrapolations of the study findings between the two species. For example, a very common symptom observed in humans after AYA consumption is vomiting [12], a symptom that is not observed in rats. Hence, the actual AYA dose that is absorbed by humans after ingestion may be lower than what is available for absorption by the rat.

5. Conclusions

Results presented in this study showed that repeated intake of AYA beverage prepared by an UDV group (Federal District, Brazil) was lethal to pregnant rats at doses equal to or higher than 4-fold the single human dose (4X) taken by an adult during the UDV religious ceremony. A non-observed-adverse-effect-level (NOAEL) for maternal toxicity was set at 1X the human dose, corresponding to 343 mg/kg bw/day of AYA powder (or 3.34 mg of harmine, 0.26 mg of harmaline and 0.30 mg of DMT per kg bw/day). This NOAEL was related to the neurotoxic effects seen at the 2X dose (decreased viable neurons in the CA1 hippocampal layer). Since the lowest dose of AYA tested caused only a small increase in the incidence of skeleton and soft-tissue variations (findings of minor toxicological significance), this dose level (1X) was also determined as the study NOAEL for developmental toxicity.

Although the data presented has limited relevance for the human religious context, where the exposure occurs normally every 15 days, this study suggested that the repeated daily intake of AYA by pregnant women at doses higher than one-human ritual equivalent dose may pose risks for the conceptus. Furthermore, women of child-bearing-age should be cautioned over the use of AYA for recreational purposes.

Funding

This work received the financial support of the Federal District Research Foundation (FAP-DF; Grant 193000358/2010). LG Motta and JA Morais were granted Master's scholarships by the REUNI Program of the University of Brasilia.

Conflict of interest

None.

Acknowledgment

We thank the UDV group *Núcleo Luz do Oriente* for providing the ayahuasca for the conduction of this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.reprotox.2018.03.002.

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