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Recreational use of marijuana during pregnancy and negative gestational and fetal outcomes: An experimental study in mice

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ABSTRACT

The prevalence of marijuana use among pregnant women is high. However, the effects on gestation and fetal development are not well known. Epidemiological and experimental studies present conflicting results because of the route of administration, dose, time of exposure, species used, and how *Cannabis* toxicity is tested (prepared extracts, specific components, or by pyrolysis). In this study, we experimentally investigated the effects of maternal inhalation of *Cannabis sativa* smoke representing as nearly as possible real world conditions of human marijuana use. Pregnant mice (n = 20) were exposed (nose-only) daily for 5 min to marijuana smoke (0.2 g of *Cannabis*) from gestational day (GD) 5.5 to GD17.5 or filtered air. Food intake and maternal weight gain were recorded. Ultrasound biomicroscopy was performed on 10.5 and 16.5dpc.On GD18.5, half of the dams were euthanized for the evaluation of term fetus, placenta, and resorptions. Gestation length, parturition, and neonatal outcomes were evaluated in the other half. Five minutes of daily (low dose) exposure during pregnancy resulted in reduced birthweight, and litter size was not altered; however, the number of male pups per litter was higher. Besides, placental wet weight was increased and fetal to placental weight ratio was decreased in male fetuses, showing a sex-specific effect. At the end of gestation, females from the *Cannabis* group presented reduced maternal net body weight gain, despite a slight increase in their daily food intake compared to the control group. In conclusion, our results indicate that smoking marijuana during pregnancy even at low doses can be embryotoxic and fetotoxic.

1. Introduction

Cannabis sativa is popularly known as marijuana and smoking is the commonly used form of this drug. Based on unofficial estimates of drug consumption conducted by the United Nations, it is the most abused drug in the world, with 140 million consumers (UNODC, 2015). Users are young, and exposures occur during their reproductive age (SAMSHA, 2015). Moreover, among pregnant women, it appears more frequently in self-reported questionnaires of drug use during gestation (ACOG, 2015; SAMSHA, 2010). Most of the studies on the toxicity of marijuana use during pregnancy have evaluated the neuro-behavioral effects (Higuera-Matas et al., 2015; Campolongo et al., 2011).

Epidemiological evidence has shown that marijuana impairs the growth trajectory of the fetus, resulting in low birth weight, intrauterine growth retardation (IUGR) and congenital malformation (Hurd et al., 2005; El Marroun et al., 2011; Sherwood et al., 1999; Zuckerman et al., 1989; Fried et al., 1984; Gibson et al., 1983). Maternal health is also negatively affected; marijuana-using mothers present higher prevalence of dysfunctional and precipitous labor, as well as meconium-stained amniotic fluid (Alharbi and El-Guebaly, 2014). Other potential adverse effects of smoking marijuana during pregnancy are lesser known.

The majority of the toxicological knowledge about the effects of *Cannabis sativa* on the reproductive tract and fetal development comes from animal studies. In these studies, exposures are done primarily by gavage of marijuana extract or Δ^9-THC i.p (Abel, 1975). Regardless of the route of administration (inhalation, i.p., p.o., i.v.,

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and i.p), findings of these studies demonstrate increased resorption rate and reduced fetal weight in both mice and rats (Rosenkrantz et al., 1978; Persaud and Ellington, 1967; Kostellow et al., 1978; Harbison and Mantilla-Plata, 1972; Joneja, 1976; Fleischman et al., 1980). None of the studies have reported fetal malformations. Abel et al. (1981) reported that pregnant rats exposed to different doses of *Cannabis* extract (20, 200 mg/kg) throughout gestation presented reductions in weight gain and food consumption. Birth weight was reduced only in those groups exposed during the third week or during the whole gestation. Charlebois and Fried (1980) evaluated the effects of pre-gestational and gestational exposure to *Cannabis* smoke on rats fed low, normal, or high protein diet. They observed that *Cannabis* exposure lengthened the gestation period and increased the occurrence of stillbirths and litter destruction. When exposure was coupled with a high protein diet, these effects were attenuated. Furthermore, evaluation of the outcomes of the groups exposed both before and during gestation suggested a degree of tolerance to the drug effects.

THC and its metabolites are able to cross the placental barrier and reach the fetus (Hutchings et al., 1989; Jakubovic et al., 1973). The endocannabinoid (eCB) system has an important role in reproduction, from the earliest stages of ontogenic development to parturition, including fertilization, embryo implantation, and placentation (Sun and Dey, 2012). The endocannabinoid system is present in different organs where it plays multiple physiological roles. It is composed of the cannabinoid receptors, CB1 and CB2, which are G protein-coupled receptors that are differentially distributed in the organs (Park et al., 2003; Das et al., 1995; Galiegue et al., 1995), and endogenous molecules (endocannabinoids) derived from arachidonic acid: anandamide (*N*-arachidonylethanolamine—AEA) and 2-arachidonoylglycerol (2-AG). Marijuana's Δ^9-THC can also bind to CB receptors and activate multiple intracellular signal transduction pathways.

Studies in humans have many confounding factors (e.g., lifestyle, socioeconomic and nutritional status, age, and tobacco use) that make it difficult for interpretation and establishment of a causal relationship between smoking marijuana and poor gestational outcomes. Furthermore, toxicological studies conducted in animals use intraperitoneal injections or oral gavage of Δ^9-THC to perform the exposures, which exclude the interaction of compounds present in the smoke that could also contribute to pregnancy disorders, and the doses used are far beyond the dose commonly experienced by humans (Abel, 1975). Most of the published reviews have acknowledged that there are several uncertainties on the effects of maternal marijuana use on gestational and fetal outcomes (Volkow et al., 2014). There is lack of information on biological mechanisms, whether fetal developmental disruptions occur indirectly (maternally mediated), directly, or as a combination of both, and alterations in placental function, changes in hormonal balance, on sex-specific effects, effects on organogenesis of the kidney, lungs, spleen, and thymus.

These aspects and the spreading legalization of recreational use of *Cannabis sativa* point out that there is an urgent need of further toxicological studies to better recognize the effects and elucidate the mechanism involved in this association. In the present study, we developed an experimental murine model to study the effects of recreational use of marijuana during pregnancy to mimic human "real world" exposures in terms of dose and use to evaluate the effects on gestational and fetal outcomes.

2. Material and methods

2.1. Animals

This study was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo (Protocol no. 5910070714). We conducted the experiments in agreement with the National and Institutional Guidelines for Animal Welfare. All animals were treated humanely with due consideration being given to the alleviation of distress and discomfort. Two groups of Balb/C mice (inbred strain) were used in this study: 20 females aged 60 days and 3 males aged 80 days, with proven fertility from our University's Animal Facility. We raised and maintained the animals in a ventilated cage system with food and water *ad libitum*. Temperature ($21 \pm 2\,^{\circ}C$) and light (12/12-h light/dark cycle) were controlled.

2.2. Drug

Marijuana (containing 0.3% Δ^9-THC) used in this study was donated for scientific purposes by the Núcleo de Perícias Médica Legais—Instituto de Criminalística de Marília legally authorized by the 3° Vara Criminal da Comarca de Marília, São Paulo, Brazil. The drug comes from a drug bust conducted by the local police.

2.3. Exposure system

The exposure system (Fig. 1) is composed of a pump that blows air through a HEPA filter into a pulse dumper. The airflow is split into two directions that pass across the valves that control the flow. One flow goes to the smoking chamber and the other directly to the mixture chamber. The smoking chamber is a 1-L sealed box with an aperture for the airflow, creating a positive pressure that forces the airflow to pass through the cigarette, and consequently the smoke flows to the mixture chamber. The mixture chamber is a 1-L sealed box with a bulkhead to promote the mixing process. Air-smoke flow was controlled at 1.2 L/min. The mixed smoke-air goes to the manifold where mice are arranged in individual tube-type holders (n = 8). The prepared marijuana cigarette lasts for 5 min of exposure in this system. An identical system was built to conduct exposures to the control group.

2.3.1. Marijuana cigarettes

Marijuana cigarettes were prepared by grinding 200 mg of *Cannabis sativa* and manually filling commercially available blank cigarette tubes. This allowed us to prepared standardized cigarettes.

2.4. Exposure protocol

Twenty healthy female mice were randomly distributed in the *Cannabis* or control group (n = 10 mice per group). During 7 consecutive days, all females were trained to be familiar with the experimental procedures and researchers.

After the training period, the females were housed with males (2:1), and the presence of a vaginal plug or sperm in the vaginal lavage was considered as the evidence of mating and the 0.5 gestational day (GD) was determined. Pregnant mice were exposed daily for 5 min to marijuana smoke or filtered air from GD 5.5–17.5 (Fig. 2). The exposure time was similar to that described by Lichtman et al. (2001), with some modifications.

2.5. Characterization of the exposure

Maternal exposure was characterized by the presence of Δ^9-THC metabolites (THC–COOH) in urine samples. Urine was collected every 24 h after exposure, according to the protocol of Khosho et al. (1985).

2.5.1. Chemicals

The following chemicals reagents were used: sodium hydroxide (Merck—Darmstadt, Germany UK), acetic acid (Merck—Darmstadt,

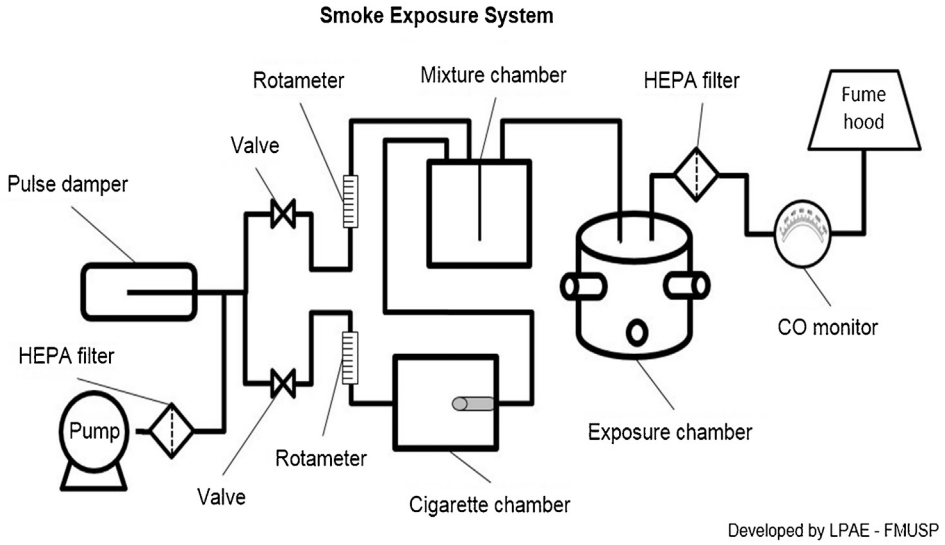

Fig. 1. Schematic representation of the smoke exposure system.

Germany UK), hexane (Sigma–Aldrich Sigma–Aldrich—St. Louis, MO, USA), ethyl acetate (LabSynth São Paulo, Brazil, BR), BTSFA in 1% TMCS (Cerilliant/Sigma–Aldrich—St. Louis, MO, USA) and standards: THC, CBD, CBN, THC-COOH at concentration 1 mg/mL and D_3 THC 1 μg/mL (Cerilliant/Sigma–Aldrich—St. Louis, MO, USA).

2.5.2. Sample preparation for GC–MS analysis

Urine samples (200 μL) were hydrolyzed with NaOH. After the hydrolytic step, the solution was made acidic and then extracted by mixing hexane/ethyl acetate (9/1). The organic layer was collected and dried under nitrogen stream. The extract was derivatized by adding 50 μL of BSTFA with 1% TMCS and incubated at 80 °C for 30 min. One microliter of the final solution was then injected into the gas chromatography column. As an internal standard, 50 μL of D_3 THC (1 μg/mL) was used.

2.5.3. GC–MS analysis

Analyses were performed on a 7890A-5975C GC–MS system (Agilent Technologies) with an HP-5 MS equipped with a capillary column (30 m × 0.25 mm × 0.25 μm) and using a splitless injection. The injection temperature was 280 °C. The carrier gas was helium at a flow rate of 1 mL/min and the injection volume was 1 μL. The column temperature was maintained at 170 °C for 0.5 min and then increased to 200 °C at a rate of 35 °C/min. The temperature was subsequently increased to 290 °C at a rate of 20 °C every 2 min. The temperature of the transfer line was 280 °C. Temperatures of the ion source and quadrupole were maintained at 280 °C and 200 °C, respectively. Selective ion monitoring mode (SIM) was used with a dwell time of 50 ms. Retention times were 5.90 and 6.66 min for THC-d3 and THC–COOH, respectively. The following qualitative ions (*m*/*z*) were analyzed: THC-d3, 306, 374, 389; THC, 303, 315, 371, 386; and THC–COOH, 371, 372, 473, 488.

2.6. Gestational outcomes

Dams were weighed daily during the exposure period, and food intake was recorded. On GD 18.5, half of the dams were euthanized by i.p. injection of sodium pentobarbital (200 mg/kg body weight), the abdominal wall was immediately opened, and the uterus was

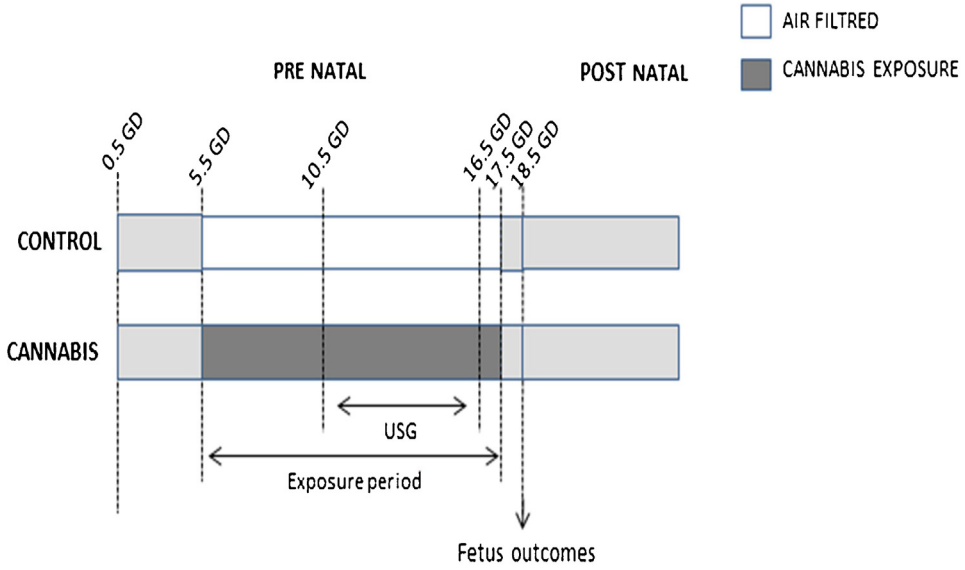

Fig. 2. Schematic illustration of the exposure protocol and the ultrasonography examination timetable.

examined carefully. The fetuses and placentas were removed, checked for malformations, and weighed. The other half of pregnant mice delivered naturally, and they were continuously monitored in order to verify whether the offspring born were dead or alive.

Examination of the gravid uterus allowed us to count the number of implantation sites and identify resorptions. For this, the fetuses and placentas were carefully dissected and weighed. The sex of the fetuses was recorded, and the number of corpora lutea in the ovaries was counted under a stereomicroscope. Half of this material was fixed in 4% paraformaldehyde in PBS and the other half was frozen (−80 °C) for further analyses. In addition, the following parameters were calculated (US EPA, 1996):

$$\text{Pre-implantation loss index} = [(\text{Ncl} - \text{Nis})/\text{Ncl}] \times 100]$$

$$\text{Post-implantation loss index} = [(\text{Nis} - \text{Nftp})/\text{Nis}] \times 100]$$

$$\text{Implantation index} = [\text{Nimp} / \text{Ncl}] \times 100$$

where Ncl represents the number of corpora lutea, Nis is the number of implantation sites, Nimp represents the number of implants, and Nftp represents the number of full-term pups.

2.7. Fetal development

Fetal development was evaluated during pregnancy using ultrasound biomicroscopy (Mu and Adamson, 2006). On GD 10.5 and 16.5, morphological evaluation of all pregnant mice was performed, with two fetuses per dam, where the crown-rump length (CRL), biparietal diameter, abdominal anteroposterior diameter, abdominal transverse diameter, placental diameter, and placental thickness were assessed. The analyses were performed with the aid of a high-frequency ultrasound imaging system (Vevo 2100, Visual Sonics, Toronto Canada) with a 40-MHz transducer by MMV who was blind to the group. The limited time for the animal's isoflurane sedation and examination duration was set at 30 min. Furthermore, a random sample of the fixed fetuses from each group was selected and the organs were dissected and weighed to verify the fetal development. Fetal-to-placenta weight ratio was calculated as an indicator of fetal–placental dysfunctions. This measure is expected to increase as pregnancy progresses; if abnormally low or high, it could be indicative of poor fetal outcomes.

2.8. Statistical analyses

Statistical Package for Social Sciences® (SPSS) version 17.0 was used for all statistical analyses. Data are presented as means and standard deviations. For comparison of reproductive parameters between the groups, Student's *t*-test or the Mann–Whitney test for independent samples was used. Chi-squared test was used for comparison of reproductive indexes between the groups. For some parameters, in order to monitor the variability within a group, the coefficient of variation for each variable (CV = standard deviation/mean) was calculated. Statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Characterization of the exposure

It was possible to detect, identify, and confirm the presence of THC-COOH, the principal biomarker of *Cannabis* exposure, in all the 20 urine samples. The detection and identification were performed by confirmation of *m*/*z* THC-d3, 306, 374, 389; THC, 303, 315, 371, and 386 —COOH THC, 371, 372, 473, 488, besides the peak intensities.

3.2. Gestational outcomes

Table 1 shows the results of gestational outcomes. There was no difference in the number pups per litter and death of fetus between the groups. The *Cannabis*-exposed group presented more male pups when compared to control group ($p = 0.04$). We did not observe any significant difference in the implantation index and pre- and post-implantation loss indexes between the groups. However, post-implantation loss index was almost two times higher in the *Cannabis*-exposed group.

Maternal total body weight gain during pregnancy was not different between the groups; however, when the pregnant uterus weight and the initial maternal weight were excluded, there was a significant difference that is known as the net maternal weight gain ($p = 0.03$). In the first week of exposure (from GD 5.5–12.5), pregnant females of the *Cannabis* group presented a small reduction in food consumption (weekly or daily food intake) compared to control group, but this difference failed to attain significance. On the second week (from GD 13–18.5), this scenario was reversed. The *Cannabis*-exposed dams consumed, weekly or daily, more food compared to control dams, but again the differences were not statistically significant (Fig. 3).

3.3. Fetal development

3.3.1. Ultrasound evaluation

No difference was found in the intrauterine fetal growth and placental development in the period between GD 10.5 and 16.5 when assessed by ultrasound biomicroscopy. Skeletal or visceral malformations were not seen in the offspring of both groups during morphological ultrasound examination (Fig. 4).

Table 1
Mean standard deviation (SD) values and the coefficient of variation of gestational outcomes.

Parameter	Group	N	Mean	SD	CV	p
Maternal Initial weight (g)	Cannabis	10	22.5	1.5	7%	ns
	Control	10	21.2	1.4	7%	
Maternal final weight (g)	Cannabis	10	36.3	3.3	9%	ns
	Control	10	38.3	3.9	10%	
Net maternal body weight gain (g)	Cannabis	5	3.9	1.8	46%	0.03
	Control	5	8.1	1.3	17%	
Uterine weight (g)	Cannabis	5	10.8	1.4	13%	ns
	Control	5	11.7	3.0	25%	
Number pups per litter	Cannabis	10	7.5	1.3	17%	ns
	Control	10	7.3	3.0	41%	
Number male pups per litter	Cannabis	10	4.2	1.7	41%	0.04
	Control	10	2.7	1.3	50%	
Number female pups per litter	Cannabis	10	2.7	1.0	38%	ns
	Control	10	3.3	1.7	52%	
Number dead fetuses	Cannabis	10	0.7	1.6	234%	Ns
	Control	10	0.1	0.3	316%	
Implantation index	Cannabis	5	79.9	16.8	21%	Ns
	Control	5	80.9	12.1	15%	
Number Total Resorption	Cannabis	5	2.0	1.0	50%	Ns
	Control	5	1.2	0.8	70%	
Number Late Resorption	Cannabis	5	0.2	0.4	224%	Ns
	Control	5	0.0	0.0		
Pre implantation loss Index	Cannabis	5	20.1	16.8	84%	Ns
	Control	5	19.1	12.1	63%	
Post implantation loss index	Cannabis	5	27.4	15.8	58%	Ns
	Control	5	14.8	9.2	62%	
Number of Corpus Luteum	Cannabis	5	11.6	2.7	23%	Ns
	Control	5	12.0	1.4	12%	

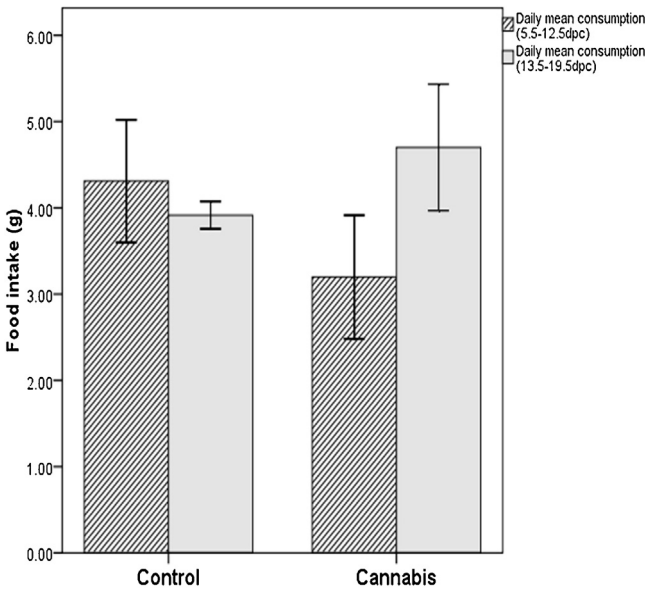

Fig. 3. Graphic representation of the daily food intake (g) of the pregnant mice of the control and Cannabis exposed groups in the first (from 5.5 to 12.5 GD) and second week (from 13.5 to 18.5 GD) of exposure.

3.3.2. Fetal body and organ weights

Fetal weight was significantly lower in pups delivered from pregnant mice exposed to *Cannabis* smoke when compared to the control group ($p = 0.02$); a mean reduction of 9.9% in birth weight was observed. When analyzed by litter, mean fetal weight per litter was significantly different ($p = 0.03$).

When analyzed by sex, males from the *Cannabis* group weighed 14.3% less (0.15 g) than male pups from control group ($p = 0.001$). In relation to female pups, we did not observe differences in birth weight (Table 2). Percentiles of male and female fetal weight are depicted in Table 3. Evaluation of weight of the fetal organs revealed significant decrements in the weights of the lungs ($p = 0.001$), brain ($p = 0.002$), thymus ($p = 0.033$), and liver ($p = 0.008$) of neonates from the *Cannabis* group (Fig. 5).

3.3.3. Placenta

Exposure to *Cannabis* smoke during pregnancy affected the placental weight (Table 2).When all pups were analyzed together, there was a significant increase in placental weight ($p = 0.04$). Fetal-to-placental weight ratio (F/P) was significantly decreased ($p = 0.009$). When analyzed by gender differences between the groups, placental weight and F/P were significant only for male pups.

4. Discussion

In this study, marijuana smoke inhalation negatively affected the gestational and fetal outcomes in the Balb/C mice model of

Table 2
Mean, standard deviation (SD) values of fetal and placental weight by groups and separated by gender.

Parameter	Group	N	Mean	SD	CV	p
Birth weight per litter (g)	Control	5	1.02	0.16	16%	Ns
	Cannabis	5	0.9	0.018	2%	
Birth weight (g)	Control	37	1.01	0.15	15%	0.02
	Cannabis	36	0.91	0.09	10%	
Litter weight (g)	Control	5	7.44	2.2	30%	Ns
	Cannabis	5	6.54	1.3	20%	
Placental weight (g)	Control	37	0.10	0.03	30%	0.04
	Cannabis	36	0.15	0.04	27%	
Fetal/Placental weight ratio	Control	37	11.03	2.39	22%	0.009
	Cannabis	36	6.59	1.48	22%	
MALE PUPS						
Body weight (g)	Control	18	1.04	0.15	14%	0.001
	Cannabis	24	0.89	0.11	12%	
Placental weight (g)	Control	18	0.10	0.02	20%	0.03
	Cannabis	24	0.15	0.04	27%	
Fetal/Placental weight ratio	Control	18	10.90	2.47	23%	0.004
	Cannabis	24	6.33	1.76	28%	
FEMALE PUPS						
Body weight (g)	Control	19	0.96	0.15	16%	Ns
	Cannabis	12	0.94	0.06	6%	
Placental weight (g)	Control	19	0.10	0.04	40%	Ns
	Cannabis	12	0.14	0.02	14%	
Fetal/Placental weight ratio	Control	19	11.29	2.72	24%	Ns
	Cannabis	12	7.10	0.68	10%	

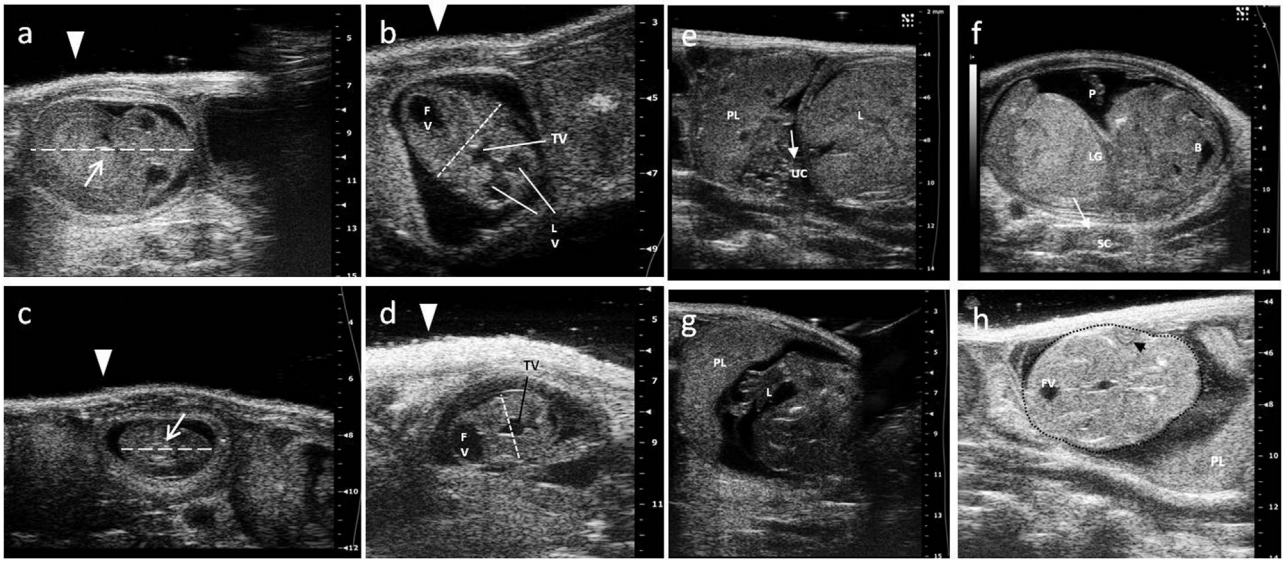

Fig. 4. Morphologic examination of fetuses from Cannabis group. Ultrasound images of fetuses at 10.5 dpc (a–d) and 16.5 dpc (e–h). (a,c) Sagittal view of the fetus: CR length detached line and arrow indicates the heart. (b,d) Horizontal section of the head: TV, third ventricles; LV, left ventricles; FV, forth ventricle. (e) Cross section of the placenta (PL) and fetus at the liver (L) and umbilical cord (UC) topographic level. (f) Cross section of the fetus showing the anterior paw (P), lung (LG) and spinal cord (SC). (g) Cross section of the placenta (PL) and the fetus showing the anterior limb (L). (h) Horizontal section of the term fetal head (dotted line), skull parts are represented by bright white lines, eye (arrow head).

Table 3
Percentiles of male and female birth weight of Cannabis and control groups.

Parameter	Group	Percentiles						
		5	10	25	50	75	90	95
Female Birth weight (g)	Control	0.67	0.69	0.91	0.96	1.00	1.17	
	Cannabis	0.83	0.83	0.90	0.95	0.99	1.00	
Male Birth weight (g)	Control	0.82	0.82	0.94	1.03	1.15	1.24	
	Cannabis	0.68	0.72	0.84	0.92	0.96	1.03	1.07

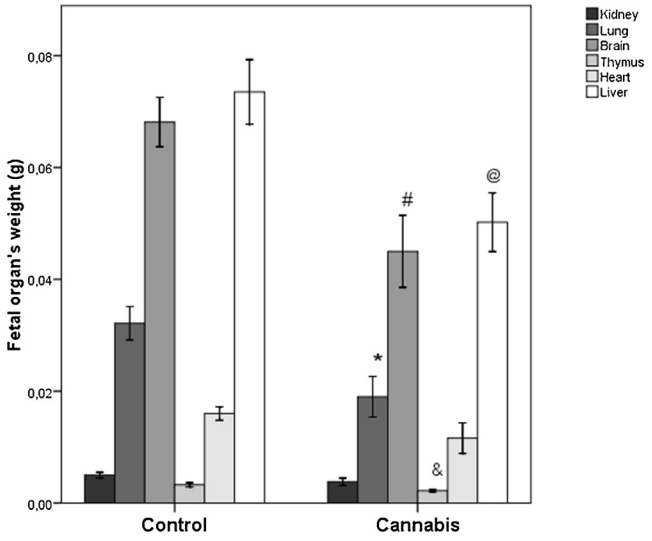

Fig. 5. Mean kidney, lung, brain, thymus, heart and liver weights (g) in Cannabis and control groups.

exposure. Five minutes of daily (low dose) exposure during pregnancy resulted in reduced birth weight, and litter size was not altered; however, the number of male pups per litter was higher. Besides, placental wet weight was increased and fetal- to-placental weight ratio was decreased in male fetuses, showing a sex-specific effect. Although not significant, the difference in the post-implantation loss index was two times higher in marijuana-smoking female mice. Exposure procedures and animal handling and manipulation did not affect maternal food intake in both the control and *Cannabis* groups. At the end of gestation, females from the *Cannabis* group presented reduced maternal net body weight gain, despite a slight increase in their daily food intake compared to control group.

Most of the available experimental studies employ intraperitoneal injections or oral gavage of Δ^9-THC to perform exposures and access the toxic effects of *Cannabis sativa* on pregnancy and fetal outcomes. The idea of our model was to represent as near as possible "realworld" conditions of human marijuana use. This means that in this study animals were exposed not only to Δ^9-THC, but to the whole smoke produced by marijuana pyrolisis. For this, we developed an inexpensive, efficient and reproducible exposure chamber to conducted controlled exposures of marijuana smoke.

Smoke inhalation exposures using experimental animals are rarely used, due to many difficulties to control and standardize the exposure process (Moir et al., 2008; Lichtman et al., 2001; Ho et al., 1970). Our exposure was conducted based on Lichtman's et al. (2001) work. In their study mice were exposed, via inhalation, to burning of marijuana cigarette (200 mg) during 5 min in a controlled air-smoke flow. Our exposure model has already proven successful, once it was confirmed the presence of THC–COOH in murine urine. Moreover, it also proved to be safe and not stressful method, causing no deaths during the exposure process and no changes in feeding behavior.

Recent reviews highlight the importance of the endocannabinoid system for implantation and placentation, and although the mechanisms are not completely understood, data suggest that controlled concentration of endocannabinoids is essential for successful embryo development (Chan et al., 2013; Melford et al., 2014). Since the Δ^9-THC can bind to the same receptors as anandamide (AEA), which is an endogenous cannabinoid that participates in the control of many reproductive functions such as fetal implantation and development (Maccarrone, 2015), the THC could act on the system equilibrium or its control, thereby affecting the embryo development and placentation. The levels of endogenous cannabinoids decrease during pregnancy progression (Habayeb et al., 2004); low systemic levels are necessary for a healthy progression of pregnancy (Maccarrone, 2015), and any increase in the levels of anandamide impacts pregnancy, increasing the risk for abortion, pregnancy loss, and growth restriction in humans and mice (Zuckerman et al., 1989; Paria and Dey, 2000). Besides, AEA increases the activity of NOS (nitric oxide synthase), which acts as a potent local vasorelaxant that is important for the maintenance of low vascular resistance in the fetoplacental circulation (Aban et al., 2013).

Depending on the route of administration, dose, time of exposure, the animal model, and how *Cannabis* toxicity is tested (prepared extracts, specific components, or by pyrolysis), the results from the studies vary. Joneja (1976), Harbison and Mantilla-Plata (1972), and Mantilla-Plata et al. (1975) noted that depending on the gestational period of exposure to Δ^9-THC, the outcomes are different. Exposures during initial stages are associated with increases in fetal reabsorptions and pregnancy loss, depending on the dose, and exposures in mid gestation lead to fetal growth retardation. In contrast to Abel (1985) that pointed out that administration of cannabinoids reduces food and water consumption (and this might also influence maternal and fetal weights), food intake was not altered in our study. Studies reviewed by Abel (1985) used gavage to administrate the drug and this may have caused some discomfort (gastric mucosa injury) and thus could have depressed the food intake. Trezza et al. (2008) and Campolongo et al. (2011) used gavage to treat pregnant rats (2.5–5 mg/kg THC) from GD 15 to the end of pregnancy, and no difference in maternal weight gain and fetal birth weight was observed; however food intake was not monitored.

However, the effects observed in our study should be interpreted and attributed to inhalation of the smoke produced by the pyrolysis of *Cannabis sativa* and not to any specific components (e.g., THC). There are several noxious chemicals present in marijuana smoke, NO,NOx, CO, hydrogen cyanide, aromatic amines, ammonia, toluene, benzene, and polycyclic aromatic hydrocarbons (Moir et al., 2008), with potential effects on maternal and fetal health (Hudak and Ungváry, 1978; Sladek et al., 1997; Longo, 1977; Choi et al., 2008; Farhi et al., 2014).

The observation of higher rates of post implantation losses and increased number of male pups per litter in *Cannabis* group allowed us to suggest another sex-specific endpoint: early in pregnancy female embryos are more susceptible (period of implantation) to the effects of smoke. Male and female embryos present differences not only in sex chromosomes but in their metabolism due to differences in sex and autosomal-related genes expression and this may consequently determine gender susceptibility (Kobayashi et al., 2006; Gardner et al., 2010; Donjacour et al., 2014).

Regarding differences in placental weight, our data indicate that inhalation of *Cannabis* smoke during pregnancy compromised placental efficiency. Placentas from the *Cannabis* group were

heavier, although fetal weight was reduced, compared to control group. This implies that more grams of placental/gram fetal tissue were needed to support development. This effect was marked in males, but with a borderline significance in females; the mechanisms involved need to be investigated, although hypoxia and maternal metabolic changes might be involved (Wilson and Ford, 2001; Fowden et al., 2009).

Many mechanisms could underline the compromised fetal development observed in our study. Reduced weight at birth and restricted intrauterine growth are effects commonly associated with exposure to *Cannabis sativa* during pregnancy (Mantilla-Plata et al., 1975; Murthy et al., 1986; Zuckerman et al., 1989; Hayatbakhsh et al., 2012); however, no previous study has reported increased susceptibility of male fetuses to this effect. Two studies found similar results of increased number of male pups per litter of exposed dams (Hutchings et al., 1987; Fried and Charlebois, 1979). In our study, estimation of the ponderal index $[(BW/CRL)^3 \times 100)]$ for the *Cannabis* group indicated that fetal growth is restricted at the end of pregnancy.

There were some limitations in this study. Our evaluation of the presence of THC–COOH in murine urine is an indicator of exposure and not dose. The small volume of urine that we could sample after the exposures, and the low-dose exposure that we adopted in this study, imposed many difficulties to determinate its quantities in this biological matrix. In the study of Lichtman et al. (2001), mice were exposed, via inhalation, to smoke produced by burning 50, 100, and 200 mg of marijuana, containing 3.4% of Δ^9-THC, during 5 min. The estimated Δ9-THC doses were 2.0, 3.5, and 5.6 mg/kg, respectively. Our exposure was conducted in a very similar manner, we burnt 200 mg of marijuana, during 5 min, however our sample contained 10 times less Δ^9-THC (0.3%), which allows us to suggest a dose of exposure equivalent to 0.5 mg/kg.

In summary, our results indicate that smoking marijuana during pregnancy even at low doses can be embryotoxic and fetotoxic, increasing implantation failures and compromising fetal development. The intrauterine environment is a determinant for fetal development, and any perturbation that occurs during this critical period of life can predispose individuals to later life diseases (Gluckman et al., 2005). Therefore, more studies are needed to recognize and better understand the impacts of smoking marijuana during pregnancy and its impacts on future health. These aspects and the spreading legalization of recreational use of this drug deserve critical evaluation.

Conflict of interest

None.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.05.020.

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