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The C57BL/6J mice offspring originated from a parental generation exposed to tannery effluents shows object recognition deficits

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HIGHLIGHTS

- Tannery effluent caused memory deficit in offspring of C57Bl/6J mice.
- Tannery effluent affects the central nervous system of offspring of C57Bl/6J mice.
- The tannery effluent ingestion by the mother is a threat to the offspring.

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ABSTRACT

The main aim of the present paper is to assess whether the parental generation exposure to such discharges could cause object recognition deficits in their offspring. Male and female C57Bl/6J mice were put to mate after they were exposed to 7.5% and 15% tannery effluents or water (control group), for 60 days. The male mice were withdrawn from the boxes after 15 days and the female mice remained exposed to the treatment during the gestation and lactation periods. The offspring were subjected to the object recognition test after weaning in order to assess possible cognition losses. The results of the analysis of the novel object recognition index found in the testing session (performed 1 h after the training session) applied to offspring from different experimental groups appeared to be statistically different. The novel object recognition index of the offspring from female mice exposed to tannery effluents (7.5% and 15% groups) was lower than that of the control group, and it demonstrated object recognition deficit in the studied offspring. The present study is the first to report evidences that parental exposure to effluent of tannery (father and mother) can cause object recognition deficit in the offspring, which is related to problems in the central nervous system.

1. Introduction

Industrial processes and human activities often generate specific residues that may contain different substances and harm the environment, as well as the human health (Claxton et al., 1998; Segura et al., 2009). The problems caused by these residues constitute great threat to the quality of life nowadays, and it is in contrast to modern society commodities (Silva et al., 2012). Among

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all the generated residues, those produced by lucrative industrial activities, such as bovine skin processing, stand out, since they are some of the most pollutant anthropogenic activities in place. The inadequate tannery effluent discharge is seen as a public health issue in many countries, mainly in developing countries such as Brazil, China, Pakistan and India (Tare et al., 2003; Prabakaran et al., 2007).

The problems caused by these residues are worsened when they are directly discharged in watercourses without being subjected to any previous treatment, fact that leads to high environmental contamination risks (Gödecke et al., 2012; White, 2012; Mohan and Routray, 2015). According to Bhat (2011) and Isaac (2013), the tannery effluents discharged by leather processing industries contain high levels of organic and inorganic agents such as phenols, acids, sulphates, sulfide, as well as of toxic elements used in the tanning stages, even after they were treated.

Few studies regarding the effects of such exposure to tannery effluents on mammal experimental models have been performed (Kumar et al., 2008; Siqueira et al., 2011; Moysés et al., 2014; Silva et al., 2015; Lemos et al., 2015; Ferreira et al., 2015; Rabelo et al., 2016; Souza et al., 2016). Among the aforementioned studies, only those conducted by Siqueira et al. (2011), Moysés et al. (2014), Rabelo et al. (2016), Souza et al. (2016) and Guimarães et al. (2016) assessed the neurobehavioral effects of the exposure to tannery effluents. Moysés et al. (2014) studied neurotoxicity and hepatotoxicity induced by the chronical exposure of male Wistar rats to tannery effluents; however, they did not observe any change in the assessed variables. Accordingly, Siqueira et al. (2011), and more recently, Rabelo et al. (2016) and Souza et al. (2016) pointed out that the exposure of mice to tannery effluents may cause damages to the animals' central nervous system. Siqueira et al. (2011) reported that male Swiss mice (adults) exposed to tannery effluents (1%, diluted in water) showed anxiogenic behavior. Rabelo et al. (2016) found that male and female adult Swiss mice presented memory deficit when they were exposed to 1% of these residues diluted in water; and Souza et al. (2016) concluded that male C57BL/6J mice exposed to tannery effluents showed predictive neurobehavioral changes such as anxiety and depression. Thus, the possible neurobehavioral damages linked to memory deficits in the offspring, which are caused by parental generation exposure to tannery effluents, constitute a research field that has never been assessed before.

Experimental evidences show that the exposure to chemical substances improperly discharged in the environment may affect the epigenetic pattern and, consequently, the body (Bolton et al., 2014; Ghaderi et al., 2015; Lee et al., 2015; Kim et al., 2015; Schmitt et al., 2016). There is no information about the neurobehavioral effects of the parental generation exposure to tannery effluents on the offspring right after weaning.

Besides the findings reported in adult models, the present study assessed possible changes in the cognitive functions of C57Bl/6J mice offspring originated from a parental generation exposed to different tannery effluent concentrations in order to broaden the knowledge about the effects of the exposure to tannery effluents. By taking into consideration that the early exposure to substances may change the mechanisms that affect the development of fetuses or newborns, it is possible concluding that tannery effluents may cause object recognition deficit in the offspring of a parental generation exposed to xenobiotics.

2. Materials and methods

2.1. Animals and experimental design

Thirty-six (36) adult C57Bl/6J (3 months old), nulliparous, (18 males and 18 females) mice were stored in the bioterium of the Biological Research Laboratory at *Instituto Federal Goiano – Urutaí Campus* (Urutaí County, Goiás State, Brazil). First, the mice were randomly selected, according to body mass, and stored in polypropylene boxes (30.3 × 19.3 × 12.6 cm, at most three animals per box). All the animals were kept under 12/12 h light/dark cycle, on a ventilated shelf, and under controlled temperature and humidity conditions (22-25 °C and 55–60% humidity). All the procedures were approved by The Ethics Committee on Animal Use of Goiano Federal Institute (*Comissão de Ética no Uso de Animais do Instituto Federal Goiano*), GO, Brazil (protocol n. 17/2014). Meticulous efforts were made to assure that the animals suffered the least possible and to reduce external sources of stress, pain and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data.

The animals were initially divided in three different groups (n = 6, per group), namely: the control group, in which the animals only received potable water; and two treatment groups, in which the animals were exposed to 7.5% and 15% tannery effluent concentrations diluted in water. These animals were exposed to the treatment for 60 days, before they were put to mate. After the exposure period, the female mice were transferred to the same boxes where the males were in, thus forming six couples in each experimental group. These animals remained exposed to the treatment, in separate boxes, and they constituted the parental generation exposed to xenobiotics. Males and females were kept together for 15 consecutive days; next, the males were withdrawn from the experimental design.

The pregnant females, which were kept in separate boxes, were subjected to the treatment (7.5% water and 15% tannery effluent) until offspring weaning, which occurred 28 days after birth (feed and water or tannery effluent consumption were measured daily). The daily consumption was calculated by subtracting the leftovers from the total amount of food and water/tannery effluent offered per day. Thus, the tannery effluent exposure lasted for the pregestational, gestational and lactation periods. The offspring in each experimental group (n = 36 in the control group; n = 24 in the 7.5% group; and n = 30 in the 15% group) were subjected to the object recognition test, after weaning. The object recognition is one of the most used tests to predict memory deficit in mice (Bevins and Besheer, 2006). Fig. 1 helps understanding the experimental design used in the present study.

2.2. Object recognition test

The object recognition test - modified according to Bevins and Besheer (2006) - was performed in a (30 × 20 × 13 cm^3) box, in the 29th day after the offspring's birth. The test was divided in two sessions: the training session and the test session (1 h after training). The animals were exposed to two identical objects (in size, form, and color) for 5 min, during the training session; such objects were defined as familiar objects F1 and F2 (square-shaped Lego toys). One of the familiar objects was replaced by a new one (N) during the test session (1 h after training; we used a triangular Lego toy), so that the animals could explore the familiar object and the new one for 3 min. The animals were placed in front of the objects facing the wall, at the beginning of each trial, according to Akkerman et al. (2012). The time they used to explore each object was recorded. A crossed-over design was used in all test sessions, so that the new and the familiar objects were alternately placed in order to exclude the potential preference for a certain spatial location of the objects in the box. The term exploration is herein understood as smelling and touching the objects with the nose or with the forepaws, and as having the animal standing 2 cm, or less, away from the objects (Ennaceur and Delacour, 1988; Rajagopal et al., 2014; Rabelo et al., 2016). The recognition index of each

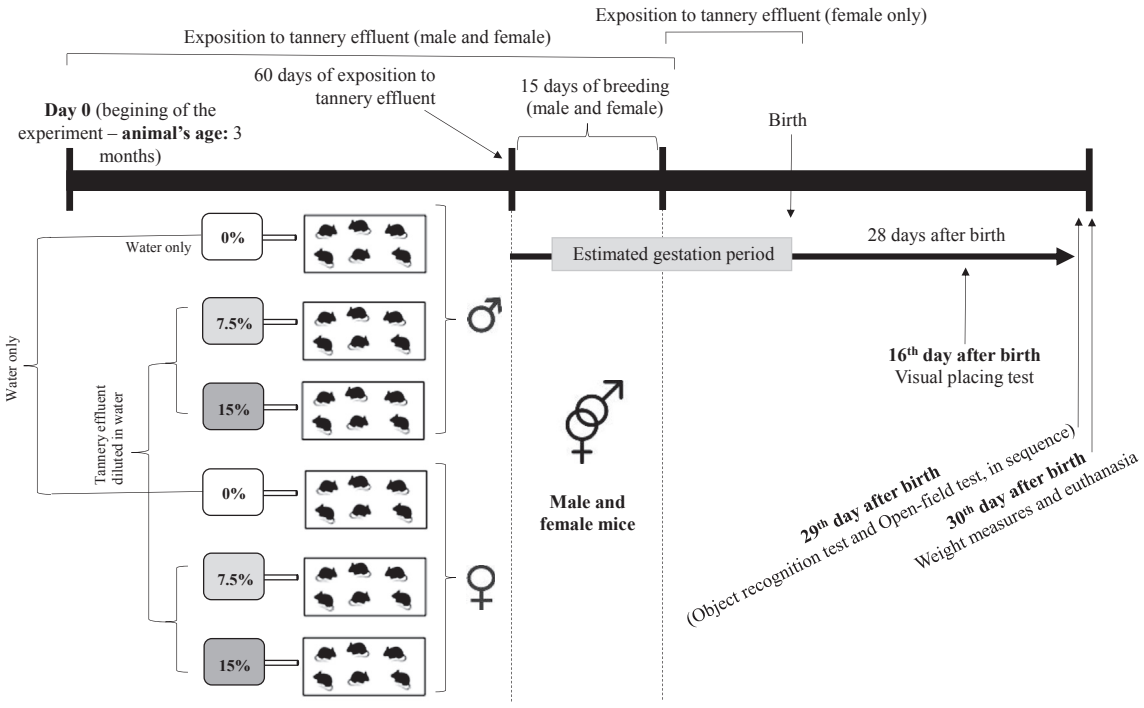

Fig. 1. Temporal distribution of the experiment and analysis.

object was calculated for each animal, as it was described by Pietá-Dias et al. (2007). The index was expressed through the ratio: TOX/(TF + TN) [TOX = time used to explore the familiar (F) or new (N) object; TF = time used to explore the familiar object; TN = time used to explore the new object]. The boxes used in the tests were cleaned with 70% alcohol between sessions.

The offspring remained with their mothers during the neurobehavioral testing in order to avoid maternal separation stress. Such test has been used for more than two decades to assess the results of early stress in mice models, including changes in the hypothalamo-hypophyseal-adrenal axis (Nishi et al., 2014; Gapp et al., 2014).

The offspring's physical development and reflex were not assessed in order to avoid offspring manipulation interference in the neurobehavioral test performed in the present study. However, two additional tests were performed to confirm whether the visual ability (Brown and Wong, 2007) and motor function of the offspring were within the normal level for recognizing, discriminating and exploring the object: visual placing and open-field tests.

2.2.1. Visual placing test

Visual placing test was performed according to Xi et al. (2009). The mice was suspended by catching the tail and slowly shifted near to an object, such as wooden stick, iron wire, etc., and the mice's actions to raise its head and caught the object with its forelimbs were recorded as a positive reaction [on postnatal day (PND) 16]. Visual placing test was measured for optesthesia.

2.2.2. Open-field test

Open-field test, a common measure of exploratory behavior and general activity in rodents (Gould et al., 2009), was performed as previously described by Souza et al. (2016). The apparatus of the open-field test consisted of a circular arena, diameter 28 cm, surrounded by a circular wall, high 45 cm, divided into 12 quadrants. The animals were placed individually in the center of the arena and videotaped for 5 min (with a camera above the apparatus). The following behavior was recorded: i) total locomotion in the quadrants, ii) rearing on the hind legs either against a vertical surface or unsupported and iii) grooming. This test was performed immediately after the object recognition test.

2.3. Weight measures and euthanasia

Thirty days after birth, the body weight of the animals was measured, and then the mice were subsequently euthanized by cervical dislocation. Afterwards, we determined brain-to-body mass ratio, also known as brain-to-body weight ratio - the ratio between brain mass and body mass. In addition, we measured brain length and width of the different experimental groups (using digital caliper).

2.4. Tannery effluents and the determination of the concentrations

The tannery effluent used in the present study derived from the bovine skin tanning stage, which is classified as wet-blue, and it was provided by a tanning industry located in Goiás State, Brazil. The effluent generation data of the providing company, which were based on the company's operation and on the regulated amount of daily-treated leather, were taken into consideration at the time to set the tannery effluent concentrations of the present study. The data of two available watercourses (illegally) used as effluent receptors were also taken into account in order to calculate the tannery effluent concentrations. The 7.5% tannery effluent concentration was set to the larger watercourse contaminated with these residues, and the 15% concentration was set to the smaller watercourses. The name of the company that provided the effluents, as well as that of the receptor watercourses, was omitted for ethical reasons.

2.4.1. Physico-chemical and chemical analyses

The physico-chemical and chemical analyses of the raw tannery effluent, potable water and different concentrations of tannery

effluent on water (Table 1) were carried as presented by the American Public Health Association (APHA, 1997).

2.4.2. Analysis of organic compounds of the tannery effluent

2.4.2.1. Sample preparation. Tannery effluents samples were filtered and subjected to organic phase extraction by adding dichloromethane (DCM) (Tedia High Purity solvents - Fairfield, OH, USA) according to the method described by Soto et al. (2004) with slight variations as per our laboratory conditions. DCM was added to each aliquot at the ratio of 60 mL/L of crude sample and mixed thoroughly for 2 min, and left for 10 min for settling down the organic phase and finally the aqueous phase was separated by a separating funnel. The procedure was repeated thrice to extract organic phase and all aqueous phase was poured off. The organic phases were mixed up and concentrated under reduced pressure on a Bucchi rotatory evaporator of 2 mL/L of crude sample and the concentrated organic phase was solvent exchanged with 10% ethanol.

2.4.2.2. Electrospray ionization (−) and (+) orbitrap mass spectrometry analysis. Afterwards, the effluent sample was diluted in DCM 1:1 (v/v) in a conical bottom tube and subjected to centrifugation for 1 min. Approximately 0.5 mL of the supernatant was collected and transferred to another conical bottom tube, adding methanol (MeOH) (J.T.Baker chemicals - Swedesboro, NJ, USA) to complete a volume of 1 mL DCM/MeOH 50:50 (v/v). All solvents used had HPLC purity> 99.8%.

For the analysis on positive mode, it was added 0.1% (v/v) formic acid (HCOOH) (Sigma-Aldrich - St. Louis, MO, USA) in the solution. For the analysis on negative mode, it was added ammonium hydroxide (NH_4OH) (0.1% v/v) (Sigma-Aldrich - St. Louis, MO, USA). The resulting solutions were directly injected into the Electrospray Ionization Source (ESI) at a flow rate of 3 μL/min. Analyses were performed using a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Orbitrap MS) (Bremen, Germany) (scan range: 200 to 1,000 *m/z*; resolution: 140,000; Microscan: 3; AFG target: 2e5; maximum inject time: 50; spray voltage: 3.5; capillary temperature: 275 °C; S-lens RF Level: 50%).

The molecular formulas of the organic compounds were generated using the Xcalibur Analysis Software Package (version 2.0, Service Release 2, Thermo Electron Corporation, Bremen, Germany). The molecular formulas were accepted only when the mean differences between the theoretical and experimental masses were less than 2.0 ppm. To do so, we used the ChemSpider database. The molecular formulas were screened using the following isotopologues ions: ^{12}C, ^{1}H, ^{16}O, ^{14}N, ^{23}Na, ^{32}S e ^{39}K.

Different compounds were identified analyzing organic compounds of the tannery effluents, as shown in Fig. 2. All the contaminants detected were right in the detection limit. The identification of all contaminants in leather industry effluent extracts, as analyzed using the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer, are presented in Table 2.

2.5. Statistical analysis

The residual normality was initially checked through the Shapiro-Wilk test. The Bartlett test was used to check the residual homoscedasticity. Data regarding the body mass, brain mass and brain-to-body mass ratio, as well as the parameters relating to the visual placing and open-field tests were subjected to analysis of variance (ANOVA). The Fisher LSD test was applied at 5% probability in case of significant ($p < 0.05$) *F* value.

Regarding the object recognition test, we analyzed the data recorded during the training session of the control group (male and female mice), as well as those of the recognition index of each object (N and F) in the control groups (male and female mice) in order to validate the object recognition test. Thus, we applied the Student's t-test with Bonferroni adjustment for multiple pairwise comparisons. The resulting data about the new object recognition indices were subjected to variance analysis (ANOVA). The Fisher LSD test was applied at 5% probability in case of significant ($p < 0.05$) *F* value. The R software, version 3.0.3, was used to perform the statistical analyses.

3. Results and discussion

Our data demonstrate that exposure to xenobiotic increased tannery effluent diluted in water consumption in 7.5% (mean: 8.1 mL/day/each mice) and 15% groups (mean: 8.6 mL/day/each mice), compared to the control group (mean: 7.2 mL/day/each mice) ($F_{(2.87)} = 6.681$, $p = 0.032$), without difference in feed consumption ($F_{(2.87)} = 6.681$, 0.784 $p = 0.541$). The requirement for water is influenced by numerous factors such as the animal's activity, air temperature, humidity, respiratory rate, water intake, feed consumption, and several physiological factors such as age, reproductive status (e.g. dry, pregnant, lactating), milk production and many other factors. The key property that must be taken into consideration while assessing water quality include chemical composition, e.g.; o excess minerals or compounds such sodium, increased element in the effluent used (Table 1).

Table 1
Physico-chemical and chemical features of the water, 100% tannery effluents, and 7.5% and 15% tannery effluents used in the present study.

Parameters	Tannery effluent (100%)	Potable water	Tannery effluent (7.5%)	Tannery effluent (15%)	WHO guidelines for potable water quality[a]	
					Normally found in fresh water/surface water/ ground water	Health-based guideline by the WHO
pH at 25 °C (UpH)	4.05	7.19	5.17	4.87	No guideline	6.5–8.5
Total dissolved solids ($mg.L^{-1}$)	37,380.00	80.00	2877.5	5675.0	No guideline	No guideline
Zn ($mg.L^{-1}$)	0.30	0.03	0.05	0.07	No guideline	3.00 $mg.L^{-1}$
Na ($mg.L^{-1}$)	9690.00	5.01	731.4	1457.76	<20 $mg.L^{-1}$	200 $mg.L^{-1}$
Ca ($mg.L^{-1}$)	601.20	4.00	48.8	93.58	No guideline	No guideline
Mg ($mg.L^{-1}$)	364.80	2.43	29.6	56.79	No guideline	No guideline
Pb ($mg.L^{-1}$)	0.32	<0.01	<0.01	0.05	No guideline	0.01 $mg.L^{-1}$
As ($mg.L^{-1}$)	<0.01	<0.01	<0.01	<0.01	No guideline	0.01 $mg.L^{-1}$
Cr ($mg.L^{-1}$)	859.00	<0.05	64.4	128.85	<0.002 $mg.L^{-1}$	0.05 $mg.L^{-1}$
Cd ($mg.L^{-1}$)	0.95	<0.001	0.1	0.14	<0.002 $mg.L^{-1}$	0.003 $mg.L^{-1}$
Ni ($mg.L^{-1}$)	5.50	<0.01	0.4	0.83	<0.02 $mg.L^{-1}$	0.02 $mg.L^{-1}$

[a] The WHO's guidelines for potable-water quality, set up in Geneva, 1993, are the international reference point for standard setting and for potable-water safety (http://www.lenntech.nl/toepassingen/drinkwater/normen/who-s-drinking-water-standards.htm).

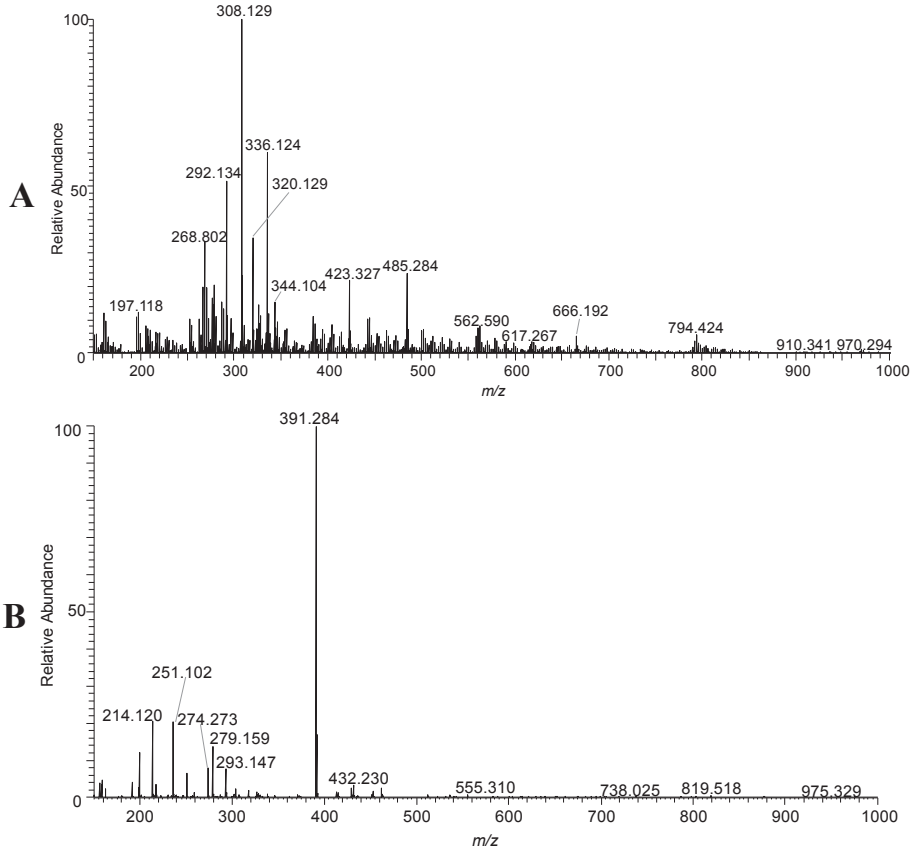

Fig. 2. (A) Mass spectrum of raw tannery effluent by Electrospray Ionization (−) Orbitrap MS and (B) Electrospray Ionization (+) Orbitrap MS.

Table 2
General information from the analysis of organic compounds of the tannery effluents.

ID	*m/z*	Error (ppm)	RDB[a]	Molecular formula	Name
By ESI(−)-Orbitrap MS					
1	197.11803	−1.461	3.5	$C_{11}H_{17}O_3$	5-Cyclohexyl-5-oxopentanoic acid
2	292.13458	0.950	11.5	$C_{19}H_{18}NO_2$	1-benzoyl-2,2,4-trimethyl-1,2-dihydro-6-quinolinol
3	308.12958	1.178	11.5	$C_{19}H_{18}NO_3$	(S)-3-Hydroxy-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester
4	320.12949	0.853	12.5	$C_{20}H_{18}NO_3$	2-(4-Butoxy-phenyl)-quinoline-4-carboxylic acid
5	336.12433	0.590	12.5	$C_{20}H_{18}NO_4$	(2S)-1-(9H-fluoren-9-ylmethoxycarbonyl)pyrrolidine-2-carboxylic acid
6	344.10432	0.741	15.5	$C_{20}H_{14}N_3O_3$	(5,6-Diphenyl-furo[2,3-*d*]pyrimidin-4-ylamino)-acetic acid
7	423.32705	0.463	8.5	$C_{29}H_{43}O_2$	4,4′-Methylenebis[2,6-bis(1,1-dimethylethyl)phenol]
8	485.28424	−0.200	9.5	$C_{28}H_{41}N_2O_3S$	1,2-Naphthalenediol, 5,6,7,8-tetrahydro-6-[[6-[[2-[(2-methoxyphenyl)thio]ethyl]amino]hexyl]propylamino]
9	617.26770	0.226	8.5	$C_{26}H_{41}N_4O_{13}$	3-Amino-3-désoxy-β-D-glucopyranoside de (1S,2R,3S,4S,6R)-4,6-diamino-3-[(6-{[(benzyloxy)carbonyl]amino}-6-désoxy-β-D-glucopyranosyl)oxy]-2-hydroxycyclohexyle
10	666.19287	0.989	31.5	$C_{44}H_{28}NO_6$	N-[3′-Hydroxy-6'-(1-naphthylmethoxy)-3-oxo-3H-spiro[2-benzofuran-1,9'-xanthen]-5-yl]-4-biphenylcarboxamid
11	794.42456	1.545	15.5	$C_{43}H_{60}N_3O_{11}$	2-propenoic acid, 3-(4-hydroxyphenyl)-, (3aR,4R,6S,7aR)-6-[[[4-[[(1,1-dimethylethoxy)carbonyl]amino]-1-[[[4-(octyloxy) phenyl]amino]carbonyl]butyl]amino]carbonyl]hexahydro-6-hydroxy-2,2-dimethyl-1,3-benzodioxol-4-yl ester, (2E)-
By ESI(+)-Orbitrap MS					
12	214.12059	1.657	4.5	$C_{12}H_{17}NONa$	Diethyl toluamide
13	251.10241	−0.890	6.5	$C_{12}H_{15}N_2O_4$	Ethyl 6-(2-furyl)-2-hydroxy-4-methyl-1,6-dihydropyrimidine-5-carboxylate
14	274.27359	−1.698	−0.5	$C_{16}H_{36}NO_2$	N-Lauryldiethanolamine
15	279.15901	−0.271	5.5	$C_{16}H_{23}O_4$	Dibutyl phthalate (plasticizer contaminant)
16	293.14761	1.472	3.5	$C_{13}H_{22}N_2O_4Na$	Ethyl 1-(4-morpholinylcarbonyl)-4-piperidinecarboxylate
17	391.28418	−0.271	5.5	$C_{24}H_{39}O_4$	Dioctyl phthalate (plasticizer contaminant)
18	432.23013	0.796	14.5	$C_{29}H_{31}NONa$	5-(4-*tert*-butylphenyl)-2,2-dimethyl-1,3,5,6-tetrahydrobenzo[*a*]phenanthridin-4-one

[a] RDB − ring/double bond equivalent. The ions were detected as deprotonated [M-H]- protonated [H+H]+ and aduccts [M+Na]+ and [M-S]-.

Regarding the object recognition test, in general, it consists of two phases. In the first phase, the animal is presented with the to-be-familiarized object (commonly referred to as the “sample object”) in a distinct environment that typically differs from the housing cage. Following sample-object exposure, the animal is returned to the home cage/colony for a retention period. In the second phase, the animal is returned to the environment and presented with two objects: the previously experienced sample object and a novel object. In this novel-object test, object recognition is distinguished by the animal spending more time exploring

the novel object (Antunes and Biala, 2012). This object-recognition procedure takes advantage of the tendency of the animal to approach and explore novelty. The test does not require extensive preliminary training, can be conducted in one session (e.g., high-throughput potential), does not require exposure to aversive stimuli, does not require food or water restriction, and has been replicated in many laboratories using a variety of apparatus designs and objects with mice and rats (Bevins and Besheer, 2006).

According to Bevins and Besheer (2006), the ability of discriminating a familiar object from a novel one indicates "recognition" of the previously experienced object, as well as detection of differences between the objects. This recognition, by definition, requires intact memory of the previously experienced object. As such, that task is a useful tool for assessing the behavioral and neural processes mediating storage and/or subsequent recall of the features that comprise the familiar object.

The object recognition test showed that the recognition level of familiar objects (F1 and F2) in the training session was different from zero and did not present difference, according to the Student's t-test with Bonferroni adjustment ($p > 0.05$) (Fig. 3A). Similar studies also considered these results as the test validation index, because they demonstrated that the exploration of random objects in the trials resulted in equal exploration of both objects, besides excluding potential influences due to the preference for certain spatial location where the objects were placed in the testing box (Rabelo et al., 2016). Also, the training session showed that the novel object recognition index (N) of the control group animals was greater than the familiar object recognition index (F), and this result constituted the index of other validation tests herein performed (Fig. 3A).

On the other hand, the analysis of the novel object recognition indices from the test session (performed 1 h after the training session) applied to offspring from different experimental groups showed statistical variations. The novel object recognition index of the offspring from female mice exposed to tannery effluents (7.5% and 15% groups) was lower than that of the control group ($F_{(2.87)} = 3.890$, $p = 0.047$) (Fig. 3B), and it demonstrated object recognition deficit in the studied offspring.

Considering that investigators have suggested that performance of mice in behavioral tasks may depend on visual ability (Robinson et al., 2001; Voikar et al., 2001; Carman and Mactutus, 2002; Brooks et al., 2005; Clapcote et al., 2005), we realized visual placing test on postnatal day (PND) 16. Our data do not show statistical differences among offspring ($F_{(2.87)} = 0.266$, $p = 0.612$). Furthermore, to confirm whether the motor or exploratory function of the offspring were within the normal level for recognizing and discriminating and exploring the object, open-field test was performed. Our data also showed no statistical differences for behavioral parameters directly related to motor or exploratory function of the animal (Fig. 4) [total locomotion in the quadrants ($F_{(2.87)} = 0.019$, $p = 0.890$); rearing on the hind legs either against a vertical surface or unsupported [time - ($F_{(2.87)} = 1.050$, $p = 0.320$) and frequency - ($F_{(2.87)} = 0.112$, $p = 0.741$)]; grooming [time - ($F_{(2.87)} = 1.728$, $p = 0.206$) and frequency - ($F_{(2.87)} = 1.992$, $p = 0.177$)].

There were no differences on body mass ($F_{(2.87)} = 0.393$, $p = 0.681$), brain mass ($F_{(2.87)} = 1.790$, $p = 0.200$) and brain-to-body mass ratio ($F_{(2.87)} = 0.865$, $p = 0.358$) (Fig. 5), suggesting that the object recognition deficit was not related to any physical/anatomical effect. Thus, our data allows one to inferring that the object recognition deficit is associated with effects of organic and/or inorganic constituents of the xenobiotic about neural mechanisms related to cognition.

The present findings are the first reports on the object recognition deficit of offspring from a parental generation exposed to tannery-effluent feeding. They indicated cognitive impairment in

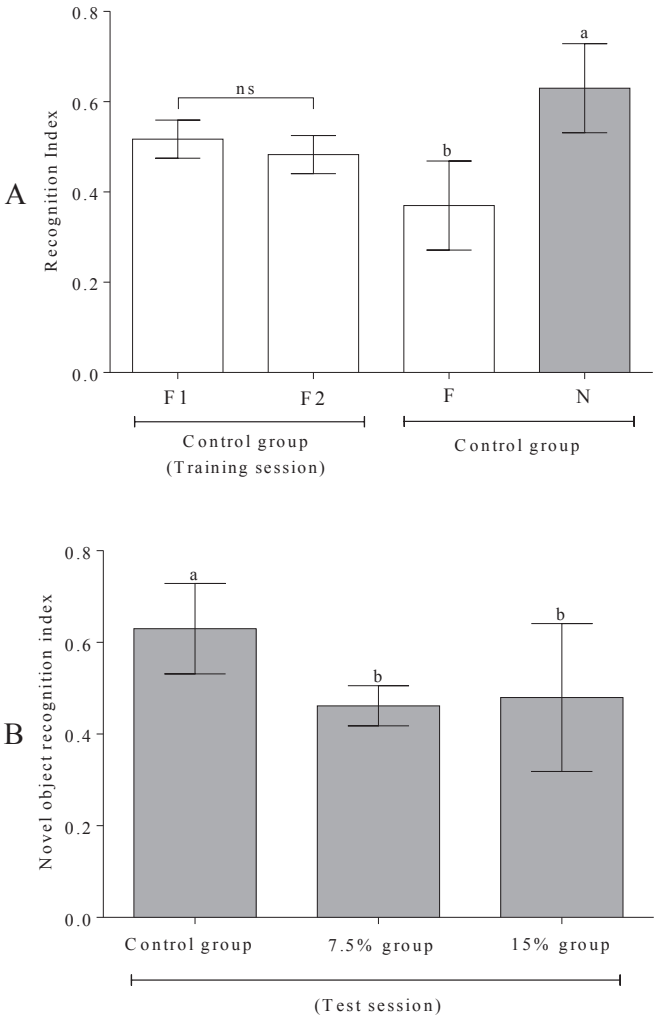

Fig. 3. (A) Recognition indices of offspring from C57Bl/6J control mice calculated 1 h after the training session and (B) new object recognition indices from C57Bl/6J mice exposed or not to tannery effluents diluted in water, at different concentrations (control, 7.5% and 15%), calculated 1 h after the training session of the object recognition test. Bars indicate mean ± standard deviation. F1: familiar object 1 of the training session; F2: familiar object 2 of the training session; F: familiar object of the test session; N: new object of the test session. In "A", different letters represent statistically significant difference between groups, according to the Student's t-test, with Bonferroni adjustment. In "B", different letters represent statistically significant differences between the new object recognition indices of the experimental groups, according to the variance analysis (ANOVA). The Fisher LSD test was applied at 5% probability.

the assessed newborns. Although the present study did not focus on assessing the adverse effects of the tannery effluent exposure on the reproductive skill or capacity of the assessed matrices, the data presented changes caused by xenobiotics in the cognitive capacity of the offspring and showed that these substances may affect the development of the animals after weaning.

Increasing evidences have shown that many pollutants may alter the epigenetic programming and/or raise the risk of generations F1, F2 and even F3 to present some type of disease or neurobehavioral changes due to pre- and neonatal exposure to them (Perera and Herbstman, 2011). These evidences include the effect of the exposure to heavy metals (Ahmad et al., 2013; Niu et al., 2014; Saini et al., 2014; Ghaderi et al., 2015), to tobacco (Singh et al., 2011; Orellana et al., 2014; Lee et al., 2015), to air pollutants (Weldy et al., 2013, 2014; Bolton et al., 2014), and to endocrine disrupters discharged in the environment (Kalb et al., 2016; Kim et al., 2015;

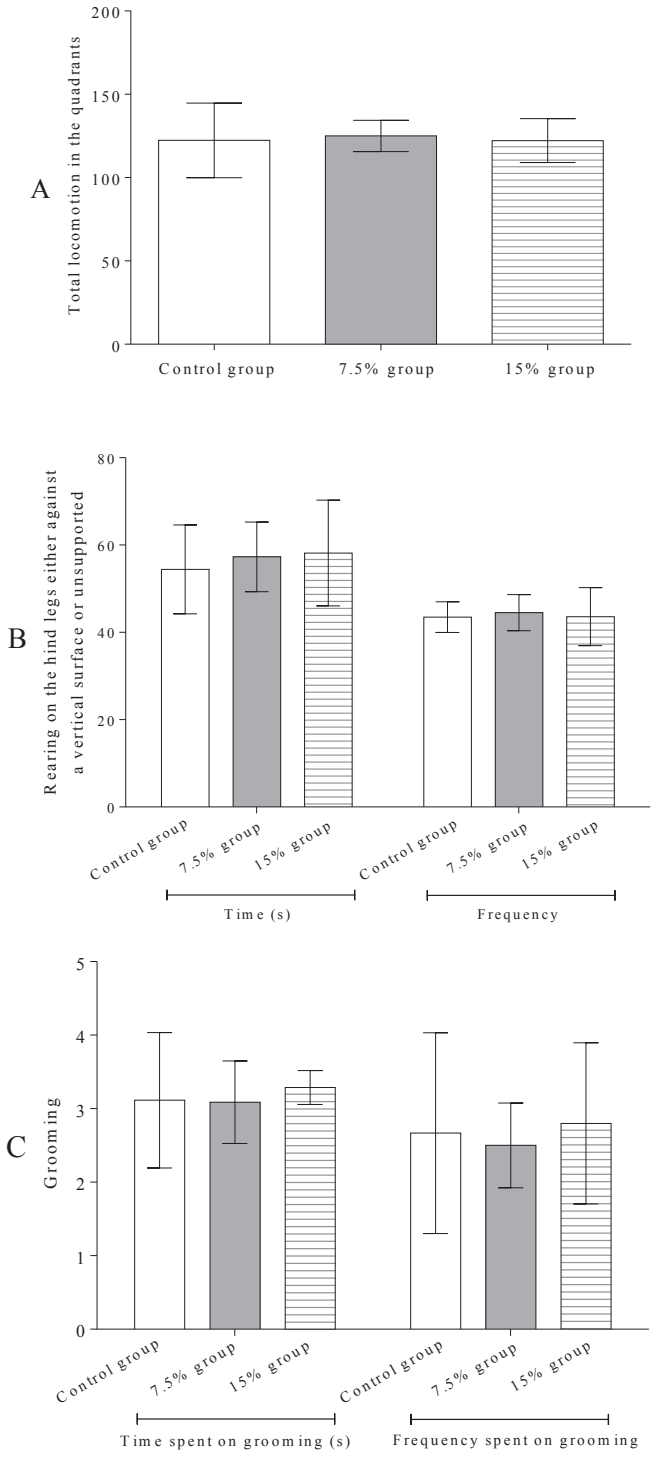

Fig. 4. (A) Total locomotion in the quadrants, (B) rearing on the hind legs either against a vertical surface or unsupported and (C) grooming from C57Bl/6J mice exposed or not to tannery effluents diluted in water, at different concentrations (control, 7.5% and 15%). Bars indicate mean ± standard deviation.

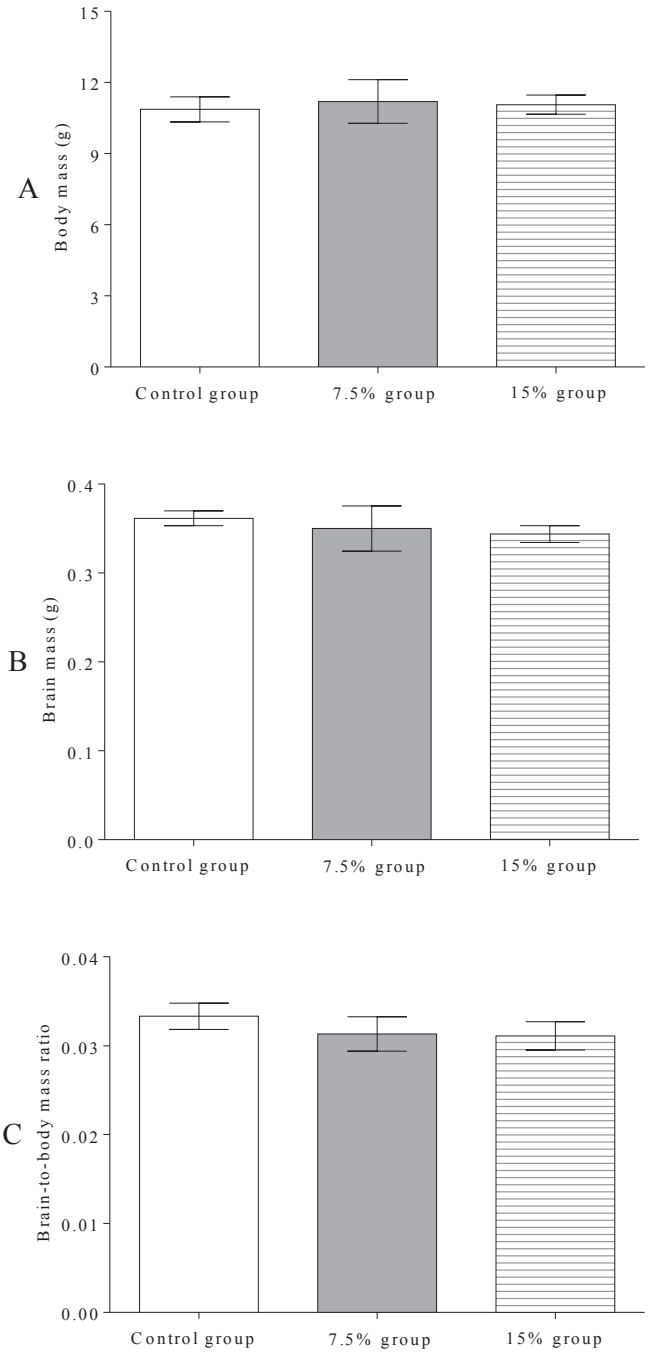

Fig. 5. (A) Body mass, (B) brain mass and (C) brain-to-body mass ratio from C57Bl/6J mice exposed or not to tannery effluents diluted in water, at different concentrations (control, 7.5% and 15%). Bars indicate mean ± standard deviation.

Schmitt et al., 2016).

Few studies about mammal exposure to tannery effluents and about its neurobehavioral effects have been published (Siqueira et al., 2011; Moysés et al., 2014; Rabelo et al., 2016; Souza et al., 2016). Furthermore, the existing studies assessed adult experimental models, which did not originate from a parental generation exposed to tannery effluents (differently from the present study), as well as other features that make the comparison between results complicated. Siqueira et al. (2011) showed that male Swiss mice (adult) exposed to 1% tannery effluent diluted in water, for 21 days, presented predictive anxiety behavior, and it suggests that this type of effluent negatively affects the central nervous system of the animals.

Moysés et al. (2014), on the other hand, investigated possible neuro hepatotoxic effects induced by the chronical exposure to tannery effluents and also investigated the predictive anxiety behavior, as well as depression and memory deficit in male-adult-Wistar rats. The authors did not observe any change in the assessed

parameters and suggested that the studied experimental model may not be the suitable species for toxicological studies about tannery effluents. Therefore, the present study reported that male and female Swiss mice exposed to 1% water-diluted and gavage-offered tannery effluents for 15 days (differently from the previously mentioned studies, in which the animals were offered water-diluted tannery effluents in dispensers) presented memory deficit (Rabelo et al., 2016).

The experimental design of the studies conducted by Siqueira et al. (2011), Moysés et al. (2014) and Rabelo et al. (2016) comprised different types of tannery effluents. The experimental strain model (the present study was the only one that used *inbred* mice lineage) and the xenobiotic exposure period are some of the particularities of the present study.

According to Shakir et al. (2012), the tannery effluents contain xenobiotics, and their chemical composition is complex and dramatically changes in tanning industries. Moysés (2014) identified more than 20 chemical organic compounds in the effluent in order to analyze the effect of tannery effluent exposure *in vitro* on different enzymes in mice and in *Drosophila melanogaster*. We have identified high concentrations of heavy metals in the tannery effluent (Table 1) and 18 different organic compounds (Table 2).

Cadmium, which has well known neurotoxic effects, was one of the chemical elements found at high concentrations in the tannery effluents used in the present study (Table 1). Many studies have shown that offspring exposed to cadmium during the gestational and/or lactation periods presented memory deficit (Petering et al., 1979; Baranski et al., 1983; Baranski, 1984, 1986; Lehotzky et al., 1990).

Nickel is another heavy metal found at high concentrations in the herein employed effluent (Table 1). However, there is still no study investigating the effect that nickel exposure during the gestational and/or lactation periods has on the cognitive functions of mice offspring. There is evidence that the ingestion of this element by the mothers may harm the offspring. Saini et al. (2014) demonstrated that Swiss mice exposed to Ni^{2+} and to nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) presented significant decrease in the offspring size and body mass, higher mortality rates and morphological anomalies in the eyes, limbs and tale of the offspring.

Lead, whose neurotoxic effect is well known, was another important element found in the effluent. Harmful effects on the structural plasticity of mice's neurons have already been reported; such effects lead to the weakening of the spatial memory and of the learning skills of the animals (Xiao et al., 2014). Yu et al. (2015) recently demonstrated changes in important synaptic proteins (such as PSD-95, nNOS and SYP) found in the hippocampus of offspring exposed to lead, and it suggested that such protein change may have influenced the properties of the synaptic transmission or even caused deficit in their learning capacity or memory. By considering that the hippocampus is an important brain region for memory and object recognition, Broadbent et al. (2010), Clarke et al. (2010), Yu et al. (2015), among other aforementioned authors, reinforced the hypothesis that memory impairment in offspring originated from the 7.5% and 15% tannery effluent contamination groups may be associated with the hippocampus damage caused by one or more elements found in the effluent.

Regarding the analysis of organic compounds carried in the tannery effluent, the variety of compounds was surprising. The signal intensity in the spectrum (Fig. 2) shows the relative abundance for some compounds known to have neurotoxic effects. The effects of plasticizer contaminants identified in the tannery effluent on the cognitive function of rodents and humans has been demonstrated in previous studies (Ning et al., 2010; Ma et al., 2013; Li et al., 2013; Miodovnik et al., 2014). Dibutyl phthalate (DBP), for example, is widely used as plasticizer in numerous kinds of products such as plastic packaging in food industries. There is a high risk of DBP exposure for human; it can easily migrate into the human bodies through food plastic packaging and be a potential hazard for human health.

Recently, Farzanehfar et al. (2016) investigated in mice neurobehavioral effects of oral DBP for 14 days (6.25, 12.5, 25, 50, 100 and 200 mg/kg), using different behavioral tests, including memory deficit predictive test. The results showed that DBP could reduce total distance movement, impair memory function and could induce anxiety in mice. Other studies that exposed rodents to organic compounds found in tannery effluent [e.g. diethyl toluamide (Abdel-Rahmana et al., 2001) and N-Lauryldiethanolamine (Craciunescu et al., 2006)], reinforce the hypothesis that the constituents of tannery effluents were responsible for object recognition deficit observed in offspring.

It is worth highlighting that the effluent ingestion effects on health are many and depend on the assessed species and strains, on the way and period of exposure, on the age and gender of the assessed animals, and on whether the animals result from parental generations, which were exposed (or not) to xenobiotics. The present findings indicate that the effects of tannery effluents go beyond the oral ingestion of residues by adult animals. The present study found cognitive losses in the offspring exposed to tannery effluent during the fetal development (gestational) and lactation periods.

Although we have not performed chemical evaluation (organic or inorganic) of the placenta or mother's milk (which is a limitation of the present study), our paper opens perspectives for further researches in this field. It is possible that the effluent components (inorganic and/or organic) transferred through the placenta, or through the mother's milk, may have caused disorders in the production and performance of neurotransmitters or of central nervous system organs correlated to the offspring learning process and memory.

On the other hand, the effects observed in the offspring may be related to paternal exposure (not just maternal exposure). Relatively recent findings have sparked interest in epigenetic alterations of paternal genomes and its effects on offspring. This emergent field focuses on how environmental influences can epigenetically alter gene expression and ultimately change the phenotype and behavior of progeny (Curley et al., 2011; Marczylo et al., 2012; Braun and Champagne, 2014; Day et al., 2016)

The study of parental influences, in both epidemiological and laboratory contexts, suggests that there are several ways through which parents can shape their offspring's development (Curley et al., 2011). In mammals, the intense prenatal and postnatal investment of mothers in the care of their offspring and the rarity of bi-parental care have directed much of the research on parental effects to the role of mother–infant interactions in promoting survival and adaptive development. However, even among species whose paternal investment in offspring care is limited, there is evidence for paternal effects. In the literature, “paternal effects” on offspring development can have a variety of meanings (Curley et al., 2011; Bohacek et al., 2013). Thus, the study of environmentally induced parental germline epigenetic effects is currently expanding and may provide an explanation for the transgenerational influence of parents's experiences on offspring development.

4. Conclusion

The C57Bl/6J mice offspring originated from a parental generation exposed to tannery effluents (7.5% and 15% concentrations) presented object recognition deficit, right after weaning. The present study is the first to demonstrate that the xenobiotic toxicity

may be transferred to the next generation, from paternal and maternal exposure to tannery effluent. It allows inferring that the tannery effluent ingestion by the parents (father and mother) is a threat to the offspring. Further studies in this field may help elucidating the mechanisms that may lead to object recognition deficit in offspring originated from a parental generation exposed to tannery effluents.

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