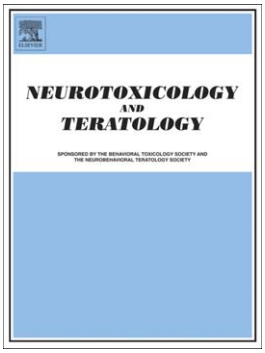

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"Locomotor activity and sensory-motor developmental alterations in rat offspring exposed to Arsenic prenatally and via lactation"

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Abstract

Arsenic (As) is one of the most toxic naturally occurring contaminants in the environment. The major source of human exposure to inorganic As (iAs) is through contaminated drinking water. Although both genotoxicity and carcinogenicity derived from this metalloid have been thoroughly studied, the effects of iAs on the development and function of the central nervous system (CNS) have received less attention and only a few studies have focused on neurobehavioral effects. Thus, in order to characterize developmental and behavioral alterations induced by iAs exposure, pregnant Wistar rats were exposed to 0.05 and 0.10 mg/L iAs through drinking water during gestation and lactation. Sensory-motor reflexes in each pup were analyzed and the postnatal day when righting reflex, cliff aversion and negative geotaxis were observed was recorded. Functional Observational Battery (FOB) and locomotor activity in an open field were assessed in 90-day-old offspring. Results show that rats exposed to low iAs concentrations through drinking water during early development evidence a delay in the development of sensory-motor reflexes. Both FOB procedure and open-field tests showed a decrease in locomotor activity in adult rats. This study reveals that exposure to the above-mentioned iAs concentrations produces dysfunction in the CNS mechanisms whose role is to regulate motor and sensory development and locomotor activity.

Key Words: Arsenic - Development exposure - Sensory-motor reflexes - Functional Observational Battery - Locomotor activity - Rat

1. Introduction

Arsenic (As) is one of the most toxic naturally occurring contaminants in the environment. The major source of human exposure to inorganic As (iAs) is through contaminated drinking water. Based on human health data, the permissible level of As, recommended by the World Health Organization (WHO) (1999) for drinking water, is 0.01 mg/L. Therefore, for humans weighing 70 kg and consuming 2 liters of water per day on average, the standard consumption of As is equivalent to ~3 x 10^{-4} mg/kg/day. Nonetheless, concentrations of iAs in drinking water exceeding the standard consumption levels recommended by WHO guidelines have been recorded in different parts of the world, e.g. concentrations of 0.20 mg/L iAs have been reported in Argentina (Concha et al., 1998), 0.40 mg/L iAs in Mexico (García-Vargas et al., 1994), 2.00 mg/L iAs in Taiwan (Yen et al., 2007) and 0.80 mg/L iAs in Indo-Bangladesh regions (Flora, 2011). Chronic exposure to iAs through contaminated drinking water can damage tissue throughout the body and it is therefore associated to a wide range of human diseases, such as hyperpigmentation and keratosis, different cancer types (bladder, lung, kidney, liver, skin), vascular pathological conditions, such as Blackfoot disease, atherosclerosis, hypertension, and diabetes (Brown and Zeise, 2004; Kapaj et al., 2006). Compared to research on genotoxicity and carcinogenicity as a result of exposure to this metalloid, the studies on the effects of iAs on the development and function of the central nervous system (CNS), in particular, have received less attention. Growing interest in the analysis of the toxic effects of iAs exposure on the CNS is evidenced by the increasing number of studies which have reported an IQ decrease as well as sensory and motor alterations in populations chronically

exposed to iAs (Calderón et al., 2001; Wasserman et al., 2004; von Ehrenstein et al., 2007; Rodriguez-Barranco et al., 2013).

Subacute exposure to iAs in adults has been found to manifest in the form of neuropsychological deficits, namely verbal memory impairment and performance deficits (Bolla-Wilson and Bleecker, 1987). In addition, significant hearing loss (Bencko and Symon, 1977), verbal skill deficits and long term memory impairments have been reported in children exposed to iAs (Calderon et al., 2001).

Acute or chronic experimental exposure to iAs during development (Hood and Bishop, 1972; Piamphongsant, 1999) or adulthood (Rodriguez et al., 2001; Shila et al., 2005) has been reported to affect the CNS. Interestingly, compared to a non-exposed control group, adult rats exposed to ~77 mg/L iAs for 60 days were found to accumulate iAs in a brain region-specific manner following the descendent order cortex-striatum-hippocampus-hypothalamus-cerebellum (Shila et al., 2005). Andean populations from northwestern Argentina who consume water with 0.20 mg/L iAs levels were observed to have iAs concentrations ranging from 9 to 11 mg/L in cord and maternal blood respectively, thus indicating the free passage of iAs to the fetus (Concha et al., 1998). Furthermore, clinical research on women chronically exposed to concentrations >0.10 mg/L iAs through drinking water during reproductive age has reported an increased risk of spontaneous abortions, stillbirth, premature births, and neonatal death (Ahmad et al., 2001; Milton et al., 2005; von Ehrenstein et al., 2006). In parallel, women exposed to iAs concentrations >0.04 mg/L during pregnancy were reported to have infants with lower birth-

weight compared to normal weight infants (Hopenhayn et al., 2003; Yang et al., 2003).

In addition, studies on animals concluded that pregnant rats maintained on drinking water containing iAs concentrations varying from 0.05 to 0.30 mg/L were observed to evidence changes in fetal brain (Nag Chaudhuri et al., 1999). Inorganic As was also reported to cross the placental barrier, thus affecting offspring during critical periods of brain development (Jin et al., 2006). One of the most common fetal malformations in iAs-exposed mice is exencephaly (Hood, 1972; Baxley et al., 1981; Nemec et al., 1998; Hill et al., 2008). Early in gestation, iAs was observed to selectively accumulate in the neuroepithelium (Lindgren et al., 1984) and As^{3+} was found to be retained in brain tissue for longer periods of time compared to other valence forms (Vahter and Norin, 1980).

Arsenic is considered to be a general toxicant of the choroid plexus, where it accumulates and causes substantial damage (Zheng, 2001). Several studies on iAs-induced embryotoxicity have demonstrated a delayed neural development in various animal species (Chaineau et al, 1990; Shalat et al., 1996). In this respect, it has been hypothesized that exposure to iAs during gestation could cause structural alterations in the neural circuit whose late effects could be manifested in the form of functional deficits.

Although developmental exposure to iAs can produce cognitive deficits in humans, the majority of the neurobehavioral studies conducted to date in animal models have focused on locomotor activity (Pryor et al., 1983; Itoh et al., 1990; Rodriguez et al., 2001; Rodriguez et al., 2002; Schultz et al., 2002; Bardullas et al., 2009). In a novel object exploration task, anxiety indices were

observed to increase in mouse offspring exposed to low iAs levels through contaminated maternal drinking water during gestation and lactation (Martinez-Finley et al., 2009). In offspring under the same exposure paradigm, the following were observed: i) a higher number of mistakes with respect to those of controls on a radial arm maze task, ii) escape latency increase during an active avoidance task, and iii) increased periods of immobility during a forced-swim task (Martinez et al., 2008).

Taking into account all the above, the purpose of the present work was to study the effects of the exposure to low levels of iAs during pregnancy and lactation on CNS functionality. Wistar rats were therefore exposed to 0.05 and 0.10 mg/L of iAs concentrations (~6.5 x 10^{-3} mg/kg/day and 1.3 x 10^{-2} mg/kg/day in rats, respectively, based on our own measurements) during pregnancy and lactation and nervous system functionality was analyzed. To this end, sensory-motor reflexes were analyzed in offspring exposed to the above-mentioned iAs concentrations during gestation and lactation, a Functional Observational Battery (FOB) was performed and locomotor activity in an open field was recorded. The postnatal day on which righting reflex, cliff aversion and negative geotaxis were reached was registered in each pup for further sensory-motor development analysis. FOB and locomotor activity assessments were performed in 90-day-old offspring.

2. Materials and Methods

2.1 Materials

Sodium arsenite ($AsNaO_2$) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and protected from sunlight.

2.2 Animals and experimental design

Parent animals were male and nulliparous female Wistar rats (90-120 days old) obtained from the animal colony at the *Bioterio* of the *Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del* Sur, Bahía Blanca, Argentina. They were maintained under constant conditions of temperature (22º ± 1º C) and humidity (50% - 60%) in a 12L:12D cycle (lights on at 7:00 h) and with food and water *ad libitum*. The evening of the females' proestrus day, male and female rats were housed overnight in couples. The presence of spermatozoa in the vaginal smears was registered as an index of pregnancy and was referred to as gestational day (GD) 0. Pregnant females were weighed and housed individually in polycarbonate cages (~860 cm^2 and 18 cm height) and were randomly assigned to one of the three following groups: a) control group, b) group treated with 0.05 mg/L of iAs in drinking water, and c) group treated with 0.10 mg/L of iAs in drinking water. There was neither bedding material nor environmental enrichment in the cages. Sodium arsenite was dissolved in deionized water and was administered to pregnant rats at concentrations levels of 0.05 and 0.10 mg/L iAs from GD 0 to postgestational day (PGD) 21. Drinking water was freshly prepared and changed daily. The control group received deionized water. Both maternal weight gain and food

intake were recorded at different GDs (GD 0, 3, 6, 9, 12, 15, 18 and 20). Drink consumption was recorded daily.

The number of pregnant dams that delivered live litters was n = 8 in the control group and n = 10 and n = 9 in the groups exposed to 0.05 and 0.10 mg/L iAs, respectively. Within 24 h after birth, all pups were weighed and litter size was determined. On postnatal day (PND) 3, litter size was randomly maintained at five males and five females whenever possible and the following data were analyzed: gestation length, litter size and body weight of pups on PND 1, 4, 7, 10, 13, 16, 19, 21 and 90. On PND 21, pups were weaned and housed in groups of six rats according to sex and treatment until PND 90. One male and female from each litter were used for the same behavioral measure. Exceptionally, it was necessary to use two males and females of the same litter. In all experiments, the observer was blind to the treatment conditions.

2.3 Sensory-motor Development

Starting on PND 3, each pup was subjected to a battery of developmental tests. One trial test per day was given to the pups on each test: righting reflex, cliff aversion, negative geotaxis and eye and ear opening. The dependent variable analyzed for each test consisted of the number of PND until each pup reached maturity of the reflex or condition according to the criteria listed below (Molina et al., 1987).

2.3.1 Righting Reflex

Each pup was placed on its back on a cloth-covered supporting surface and allowed to right itself. This reflex was registered as mature if the pup performed this response within 5 s on 2 consecutive days.

2.3.2 Cliff Aversion

Each pup was placed with their forepaws on the edge of a wooden platform and the snout protruded beyond the edge of the same platform. Latency to retract their body 1.5 cm from the edge was registered. Cliff aversion criterion was registered as mature when the pup performed this response in less than 5 s on 2 consecutive days.

2.3.3 Negative Geotaxis

Each pup was placed on an inclined wire mesh ramp (angle of inclination from the base: 30º) with the head facing down. This reflex was registered as mature when the pup reached a 180º rotation of the body and climbing upwards was done within 10 s on 2 consecutive days.

2.3.4 Eye and Ear Opening

The PND on which both eyes were opened and both auditory canals were fully opened was registered.

2.4 Functional observational battery

The 90-day-old offspring were used for this test. FOB included a thorough description of the animals' appearance, behavior and functional integrity (US EPA, 1998). This was assessed through observations in the home cage while

animals moved freely in an open field and through manipulative tests. Procedural details and scoring criteria for FOB protocol have been published (McDaniel and Moser, 1993). Briefly, measurements were first carried out in the home cage. The observer recorded each animal's posture, activity and palpebral closure. The presence or absence of tremors and convulsions was noted and, if present, described. The presence or absence of spontaneous vocalizations and biting was also noted. The observer then removed the animal, rating the ease of removal and handling. The presence or absence of hindlimb flexor resistance and pressure grade was also recorded. Palpebral closure and any sign of lacrimation or salivation were rated. Other abnormal clinical signs were also recorded. The animal was next placed in FOB experimental arena having a piece of clean absorbent paper on the surface and allowed to freely explore for 3 min. During that time, the observer ranked the rat's arousal, gait score, activity level and rears as well as any abnormal postures, unusual movements and stereotypies. After a 3 min exploration, the number of fecal boluses and urine pools and the presence or absence of diarrhea on the absorbent paper were recorded. Sensorial responses were subsequently ranked according to a variety of stimuli (click stimulus using a metal clicker, approach and touch rump with a blunt object, pinch of the tail using forceps, constriction of the pupil to a penlight stimulus and touch of the corner of the eye and the inside of the ear with a fine object). Also, several motor reflexes were analyzed (flexor and extensor thrust reflexes, forelimb hopping, propioceptive positioning). Forelimb and hindlimb extensions were registered in the gait analysis. Degree of surface and aerial righting was subsequently rated. In landing foot splay, the tarsal joint pad of each hindfoot was marked with ink and

the animal was subsequently dropped from a height of 30 cm onto a recording sheet. This procedure was repeated twice. The distance from center-to-center of the ink marks was measured (cm) and the average of the two splay values was used for statistical analysis.

2.5 Open field observations

Motor activity is considered to be a test of nervous system function, and it reflects the integrated output of the sensory, motor and associative processes of the nervous system in case of the absence of systemic toxicity (Hübler et al., 2005). Locomotor activity was analyzed in an open field. Each 90-day-old offspring, different from those used in FOB, was placed in an open field of 50 cm × 50 cm × 60 cm whose floor was divided into 12 cm × 12 cm squares by black lines. The number of squares entered by each rat with all four paws, rearings (occasions on which the animals stood on their hind paws), groomings (face washing, forepaw licking and head stroking) and fecal boluses were scored each 5 min for 15 min. The number of squares crossed and the rearings performed were recorded as parameters of locomotor activity, whereas the number of groomings and the number of fecal boluses deposited were considered as parameters of emotionality (Maimanee et al., 2003). After each animal was removed, the open field was carefully cleaned with a damp cloth.

2.6 Statistics

The data about dams and their litters were analyzed by one-way ANOVA. The date of water consumption and food intake of dams as well as the weight of dams and pups were analyzed by repeated-measure ANOVA.

We used the litter as the statistical unit and we nested sex within litter for the analyses of variance for each assessment in which both sexes were tested. Each sensory-motor development test was analyzed by a two-way ANOVA (group x sex) while FOB results were analyzed according to the type of date. Continuous data (providing interval data) were tested using a two-way ANOVA followed by *post hoc* comparisons (Fisher´s Least Significant Difference test: LSD test) when differences between groups were detected. The ranked data (ranks based on a defined scale) were analyzed using Kruskal–Wallis nonparametric test followed by Mann–Whitney test. For descriptive and binary data (the presence or absence of a sign), each experimental group was compared to the control group using a chi-square test.

The data obtained in 15 total minutes of observation in the Open Field were analyzed using a two-way ANOVA followed by *post hoc* comparisons using LSD test. For the comparative analysis of the parameters analyzed every 5 min, a repeated-measure ANOVA test was used. In order to analyze the differences in each 5-min period within each group, a *t*-test for paired samples was subsequently carried out and in order to analyze the differences in each 5 min-period between groups, a *t*-test for independent samples was carried out.

Probability values lower than 0.05 were considered to be significant. All statistical analyses were carried out using an IBM SPSS 21.0 (Chicago, IL) software for Windows.

2.7 Ethics

Animal care and handling were in accordance with the internationally accepted standard Guide for the Care and Use of Laboratory Animals, Eighth Edition

(2011) as adopted and promulgated by the National Institute of Health. Experimental designs were also approved by the local standard for protecting animal's welfare, Institutional Committee for the Care and Use of Experimental Animals.

3. Results

3.1 Data about dams and their litters

There were no statistical differences in the body weight between the groups of dams on GD 0, in weight gain during all the different periods registered, and in gestational length and litter size (Table 1). Also, the body weight of pups on PND 1, 4, 7, 10, 13, 16, 19 and 21 was not affected by iAs treatment during pregnancy and lactation (Fig. 1A). No visible teratogenic malformation was observed in any of the groups analyzed.

Table 1. Data of dams and their litters

	Control group n = 8	**0.05 mg/L** n = 10	**0.10 mg/L** n = 9
Body weight of dams (g)			
GD 0	283.2 ± 18.6	282.8 ± 7.9	292.8 ± 8.1
Weight gain (g)			
GD 0-3	12.3 ± 1.6	12.2 ± 2.0	10.8 ± 1.5
GD 3-6	9.2 ± 2.1	6.7 ± 2.0	6.1 ± 1.4
GD 6-9	11.8 ± 2.0	8.6 ±1.6	9.9 ± 0.9
GD 9-12	18.3 ± 2.3	16.4 ± 1.9	14.2 ± 1.7
GD 12-15	14.2 ± 2.5	11.6 ± 1.5	15.0 ± 1.2
GD 15-18	31.8 ± 4.4	28.0 ± 3.5	36.4 ± 3.0
GD 18-20	24.0 ± 2.8	17.2 ±3.2	21.1 ± 3.7
GD 0-20	121.7 ± 6.0	97.7 ± 9.0	112.4 ± 8.7
Length of gestation (days)	22.0 ± 0	22.3 ± 0.2	22.0 ± 0
Litter size			
Female	4.7 ± 1.4	3.5 ± 1.2	5.8 ± 1.0
Male	6.2 ± 1.3	4.6 ± 1.0	5.1 ± 0.6
Total	10.8 ± 1.4	8.5 ± 1.7	10.9 ± 1.5
Eye Opening (days)			
Female	13.4 ± 0.5	13.7 ± 0.7	13.8 ± 0.4
Male	13.6 ± 0.5	13.7 ± 0.4	13.9 ± 0.6
Ear Opening (days)			
Female	12.0 ± 0	12.5 ± 0.6	12.2 ± 0.6
Male	12.2 ± 0.6	12.4 ± 0.5	12.1 ± 0.7

Weight PND 90 (g)			
Female	192 ± 10.4	187 ± 11.8	201 ± 9.8
Male	330 ± 15.7	341 ± 15.8	328 ± 11.8

Values are mean ± SEM. Gestational weights represent only the pregnant females that produced the live litters assessed in this study.

No statistically significant differences were observed in water and food intake during pregnancy and lactation in the dams exposed to both iAs concentrations, compared to the control group (Fig. 1B and C). As shown in Fig. 1C, the increase in drink consumption during lactation was due to the increased fluid requirements firstly of the lactating dams and subsequently of both dams and pups, particularly during the last stage of the pre-weaning period. The same was observed with food intake (Fig. 1B).

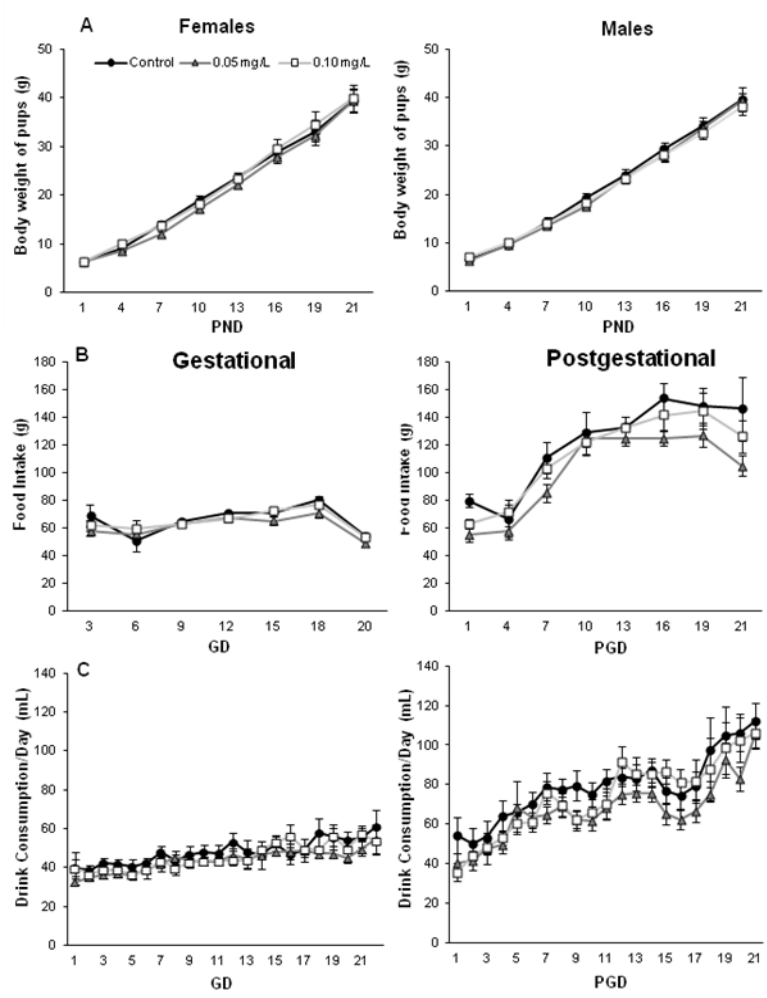

Fig. 1. A) Average of body weight on PND 1, 4, 7, 10, 13, 16, 19 and 21 of female and male offspring of each dam. B) Food intake of dams during pregnancy and lactation. Record of body weight and food intake was performed every 3 days coinciding with the record body weight of dams during pregnancy and offspring during lactation. C) Drink consumption of dams during pregnancy and lactation was recorded daily. All results are expressed as Mean ± SEM of 8-10 animals per group. PND: Postnatal Day. GD: Gestational Day, PGD: Postgestational Day.

3.2 Sensory-motor reflexes

When sensory-motor development tests were analyzed (Fig. 2), two-way ANOVA detected significant differences in the interaction between factors (groups x sex) in the righting reflex ($F_{(2,27)} = 5.08$, $p<0.01$), thus indicating that iAs exposure effects were different between males and females. *Post hoc* comparisons showed that gestational and lactation exposure to 0.05 and 0.10 mg/L iAs produced a significant delay in the development of the righting reflex in female rats ($p<0.001$, in both concentrations) while no significant differences were observed in the male rats (Fig. 2A).

When cliff aversion was analyzed, two-way ANOVA also detected significant differences in the interaction between factors (groups x sex) ($F_{(2,27)} = 4.14$, $p<0.01$). *Post hoc* comparisons showed that both iAs concentrations delayed the development of this reflex in female rats ($p<0.01$ for 0.05 and 0.10 mg/L iAs). On the other hand, the delay in the acquisition of cliff aversion was only observed in males exposed to the highest concentration ($p<0.001$) (Fig. 2B).

In the analysis of negative geotaxis data, interaction between factors (group x sex) was detected by two-way ANOVA ($F_{(2,27)}$ = 4.14, p<0.01). *Post hoc* comparisons showed that only males exposed to the highest iAs concentration were observed to have delayed maturation of the reflex (p<0.001) (Fig. 2C). No statistically significant differences were observed in the development of negative geotaxis in female rats.

As to the days of eye- and ear-opening of offspring, no statistically significant differences were observed among the different groups analyzed (Table 1).

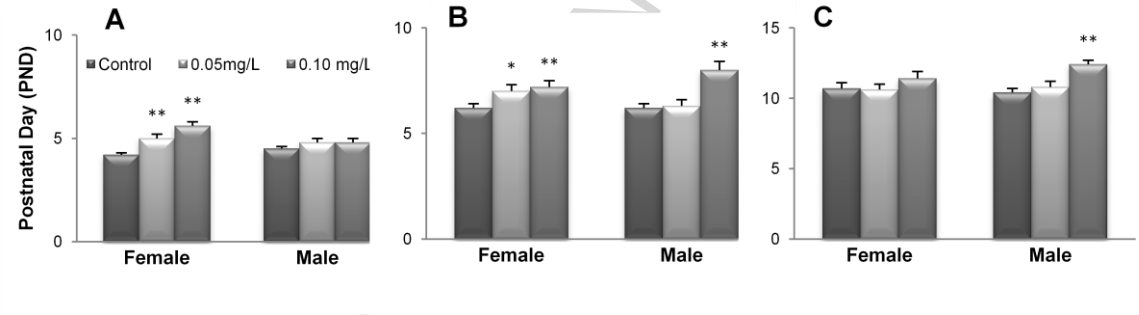

Fig. 2. Postnatal day on which control offspring, 0.05 mg/L and 0.10 mg/L As groups displayed the criterion level of A) Righting Reflex, B) Cliff Aversion and C) Negative Geotaxis. Data represent the Mean ± SEM of PND of 10 animals per group. *p<0.01 and **p<0.001 compared with the respective control group.

3.3 Functional Observational Battery

Data obtained from the Functional Observational Battery (FOB) test are shown in Table 2. We observed that adult rat offspring exposed to 0.05 and 0.10 mg/L iAs evidenced alterations in some parameters analyzed in the FOB experimental arena. In female offspring a significant decrease in the activity ($\chi^2_{(2)}$ = 11.270, p<0.01) and rearing ($\chi^2_{(2)}$ = 11.024, p<0.01) was observed. This

effect was observed in a concentration dependent manner, compared to the control group ($p<0.05$ and $p<0.01$ for 0.05 and 0.10 mg/L iAs, respectively). In male offspring, a significant decrease in the activity ($\chi^2_{(2)} = 5.916$, $p<0.05$) and rearing ($\chi^2_{(2)} = 6.071$, $p<0.05$) was observed. This effect was observed only with the highest iAs concentration compared to the control group ($p<0.05$).

According to a variety of stimuli, sensorial responses were ranked. In tail pinch response, significant differences between groups were observed in female ($\chi^2_{(2)}=5.927$, $p<0.05$) and male ($\chi^2_{(2)}=10.681$, $p<0.01$) rat offspring. In addition, the group treated with 0.10 mg/L iAs was the only one that was observed to evidence a lower response than that of the respective control group ($p<0.01$). The other parameters analyzed in the FOB experimental arena were not significantly altered in any of the groups analyzed.

Table 2. Parameters analyzed in the Functional Observational Battery test

Endpoints	Control	0.05 mg/L	0.10 mg/L	Control	0.05 mg/L	0.10 mg/L
		♀			♂	
Home cage observations:						
Normal body posture (D)	100	100	100	100	100	100
Activity (R)	1.00	1.00	1.00	1.00	1.00	1.00
Palpebral closure (R)	1.00	1.00	1.00	1.00	1.00	1.00
Clonic movements (D)	0	0	0	0	0	0
Tonic movements (D)	0	0	0	0	0	0
Biting (D)	0	0	0	0	0	0
Vocalizations (B)	0	0	0	0	0	0
Hand-held observations:						
Ease of removal from cage (R)	1.00	1.00	1.00	1.00	1.00	1.00
Ease of handling (R)	1.00	1.00	1.00	1.00	1.00	1.00
Salivation (R)	1.00	1.00	1.00	1.00	1.00	1.00
Lacrimation (R)	1.00	1.00	1.00	1.00	1.00	1.00
Piloerection (B)	0	0	0	0	0	0
Normal fur appearance (D)	100	100	100	100	100	100
Normal respiration (D)	100	100	100	100	100	100
Normal cardiovascular signs (D)	100	100	100	100	100	100
Normal limb tone (D)	100	100	100	100	100	100
Normal abdominal tone (D)	100	100	100	100	100	100
Limb grasping (B)	100	100	100	100	100	100
Experimental arena observations:						

Activity level (R)	3.20	**2.88***	**2.54****	2.83	2.71	**2.50***
Rearing (R)	3.00	**2.83***	**2.54****	2.92	2.79	**2.58***
Arousal (R)	4.0	3.92	3.96	4.00	3.96	3.75
Normal gait (D)	100	100	100	100	100	100
Unusual movements (D)	1.00	1.00	1.00	1.00	1.00	1.00
Stereotyped behaviors (D)	1.00	1.00	1.00	1.00	1.00	1.00
Fecal boluses (C) (N°)	0	0	0	0	0	0
Urine pools (C) (N°)	0.40	0.42	0.20	2.52	3.50	3.25
Diarrhea (B)	0	0	0	0	0	0
Sensory reflexes:						
Approach response (R)	2.00	2.00	2.00	2.00	2.00	2.00
Touch response (R)	2.00	2.00	2.00	2.00	2.00	1.92
Click response (R)	2.00	2.13	2.00	2.00	2.00	2.00
Tail pinch response (R)	1.90	1.92	**1.50***	1.92	1.92	**1.33****
Palpebral reflex (B)	100	100	100	100	100	100
Pinna reflex (B)	100	100	100	100	100	100
Eyeblink response (B)	100	100	100	100	100	100
Motor reflexes:						
Flexor reflex (B)	100	100	100	100	100	100
Extensor reflex (B)	100	100	100	100	100	100
Forelimb hopping (B)	100	100	100	100	100	100
Propioceptive positioning (B)	100	100	100	100	100	100
Postural reactions:						
Forelimb extension (B)	100	100	100	100	100	100
Hindlimb extension (B)	100	100	100	100	100	100
Surface righting reflex (R)	1.00	1.00	1.00	1.00	1.00	1.00
Aerial righting reflex (R)	1.00	1.00	1.00	1.00	1.00	1.00
Landing foot splay (C) (cm)	5.50	5.31	5.66	6.18	6.60	5.30

Descriptive (D) and binary (B) data expressed as percentage of incidence (Chi-square test);
Ranked (R) data expressed as the mean score of the scale used (Kruskal-Wallis test);
Continuous (C) data expressed as mean value (Two-way ANOVA test)
♀: female rat; ♂: male rat. n = 10
*p<0.05 and ** p<0.01 compared to control group.

3.4 Open field

In open field observations (Fig. 3), the two-way ANOVA showed significant differences between group ($F_{(2,27)}$ = 18.88, p<0.001) and sex ($F_{(1,27)}$ = 24.03, p<0.001) in the number of squares crossed by rats during 15 min. *Post hoc* comparisons showed that female rats exposed to both iAs concentrations exhibited a significant decrease in squares crossed in a concentration dependent manner, compared to the control group (p<0.01 and p<0.001 for

0.05 and 0.10 mg/L iAs, respectively). Male rats showed a significant decrease in this parameter only with the highest concentration evaluated ($p<0.01$) (Fig. 3A).

When the number of squares crossed in each 5 min period was compared, ANOVA for repeated measures showed significant differences between group ($F_{(2,27)} = 19.98$, $p<0.001$) and between sex ($F_{(1,27)} = 24.86$, $p<0.001$). The number of squares crossed in each period of 5 min was used to evaluate the habituation of rats to the open field. All groups showed greater locomotor activity during the first 5 min period and declined in the second and in the third period ($p<0.01$ for all comparisons). This gradual and significant decrease in their locomotion activity throughout the test session indicated that all animals have habituated to the open field.

As to the other parameter of locomotor activity analyzed in the open field, two-way ANOVA showed no significant differences in the number of rearings performed by exposed rats compared to the control group although the same trend observed with the number of squares was found. However, all groups showed greater rearing activity during the first 5 min period and declined in the second and in the third period in the same way as observed with the number of squares ($p<0.01$ for all comparisons) (Fig. 3B).

The analysis of the number of groomings and fecal boluses, both considered as emotionality parameters, revealed no statistically significant differences between the control and experimental groups (data not shown).

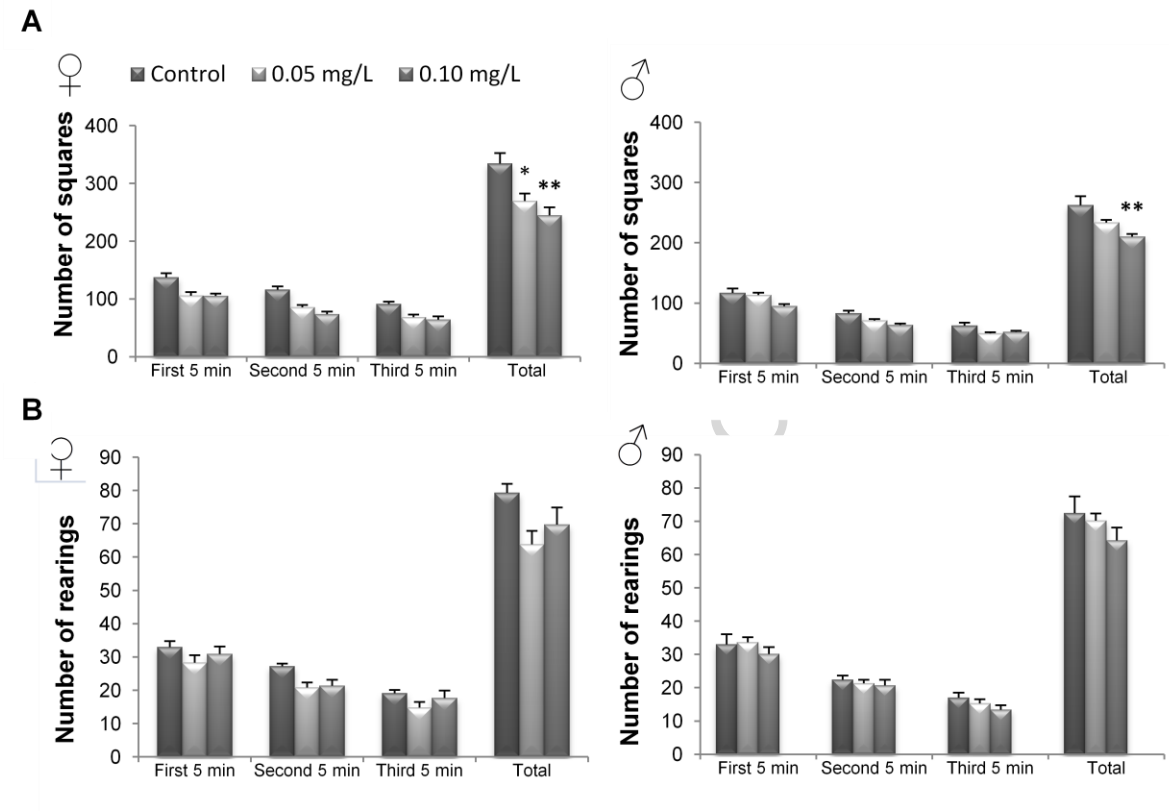

Fig. 3: Locomotor activity of female and male adult rats exposed to 0.05 mg/L and 0.10 mg/L iAs in water during gestation and lactation. A) Number of squares crossed in the open field recorded every 5 min for a total of 15 min. B) Number of rearings made in each period from 5 min to ~15 min showing how all groups in the second and third 5 min decreased locomotor activity compared to the first 5 min, thus indicating habituation to the new environment. Data represent the Mean ± SEM of 10 animals per group. #p<0.01 compared to the number of squares crossed and number of rearings made during the first 5 min. *p<0.01 and ** p<0.001 compared to the respective control group.

4. Discussion

The present study shows that exposure to low levels of iAs during prenatal period and lactation affects neither maternal weight gain during pregnancy nor gestational length, litter size and pup body weight on different PNDs. However, persistent sensory-motor changes were observed in rat offspring exposed to iAs through maternal drinking water during gestation and lactation. A delay in the development of the righting reflex and cliff aversion was observed in female offspring whose dams had been exposed to 0.05 and 0.10 mg/L iAs concentrations. In contrast, in male offspring whose dams had been exposed to 0.10 mg/L iAs concentrations, only a delay in cliff aversion was observed. It was also found that males exposed to high iAs concentrations showed a delay in the maturation of the negative geotaxis reflex. Further research revealed that exposure to higher doses of iAs (10 mg/kg/day) during GDs 15-18 caused a delay in the development of the pinal reflex and eye opening in female mice (Colomina et al., 1997). In contrast, no changes in eye opening, startle reflex and negative geotaxis were observed in rats exposed to 1.5 - 4.5 mg/kg/day iAs during GDs 8-22 (Gandhi et al., 2012). Righting reflex, cliff aversion and negative geotaxis response of rats are indicative of motor activity and sensory development, both of which are regulated by the vestibular system and the cerebellum (Altman and Sudarshan, 1975; Kreider and Blumberg, 2005) and a delay in their development may be indicative of primary damage to neuronal myelination (Wu et al., 2008). In particular, the regulation of postural adjustments include vestibular, exteroceptive (e.g., tactile) and propioceptive systems (Altman and Sudarshan, 1975). Except for the observations regarding

the negative geotaxis, sensory-motor reflexes were found to take longer to develop in females exposed to the two iAs concentrations analyzed, thus indicating higher sensitivity to both iAs concentrations with respect to males.

FOB tests showed both in female and male adult rats a low nociceptive reflex response after exposure to 0.10 mg/L iAs concentrations during gestation and lactation, thus indicating a possible analgesic effect. In addition, *in vitro* studies have suggested that iAs modulates macrophage activity producing an over-expression of cyclooxygenase-2 (COX-2) resulting in an increase in prostaglandin E2 (PGE2) concentrations in endothelial cells, and that these effects may lead to an *in vivo* enhancement of inflammatory and pain responses (Tsai et al., 2002). Evidence on *in vivo* experiments with high iAs concentrations administered acutely in mice demonstrated that iAs on its own produces neither pain nor significant inflammation (Aguirre-Bañuelos et al., 2004). On the other hand, different toxicological effects have been associated with single doses and chronic exposure to iAs. For example, in humans, while single dose exposure to iAs has been related to initial nausea, vomiting, abdominal pain and severe diarrhea (Chappell et al., 1997; Morales et al., 2000; Ratnaike, 2003), chronic exposure has been associated with multisystem diseases, such as inflammation-related atherosclerosis and other cardiovascular diseases (Tsai et al., 2002; Tchounwou et al., 2004; Navas-Acien et al., 2005). However, no studies have been carried out to date on the relationships between iAs exposure and intensity of tissue inflammation and related pain. Likewise, no findings on the effects yielded by the exposure to low iAs levels during gestation and lactation have been obtained to date.

FOB tests also showed that exposure to low iAs concentrations through contaminated drinking water during gestation and lactation produced in adult rats a decrease in locomotor activity in the experimental arena. In female rats, this effect was observed with the two iAs concentrations tested. In contrast, in male rats, hypoactivity was only observed in the animals exposed to the highest concentrations, thus indicating a higher sensitivity to iAs in females. When locomotor activity was analyzed in the open field, the same effect was observed, i.e. hypoactivity in females exposed to the two concentrations tested and in males exposed only to the highest concentration. No consensus has been reached to date on what the effects of iAs exposure are on locomotor activity. In rodents, Bardullas et al. (2009) observed a no-linear dose/behavioral response relationship to the effects of iAs exposure. They, in fact, found that C57BL6/J adult female mice exposed to 0.05, 0.50 and 5.00 mg/L iAs evidenced hyperactivity while female mice exposed to 50 mg/L iAs showed no differences with respect to the control group. In contrast, C57BL6/J male mice exposed to 50 mg/L iAs were observed to evidence hypoactivity. In adult albino rats, chronic exposure to 0.5 or 50 mg/L resulted in locomotor hypoactivity (Bardullas et al., 2013). In addition, open field locomotion was observed to increase in rats of 3, 4, 10, 13 and 17 weeks of age, exposed to 36.7 mg/L iAs through contaminated maternal drinking water from GD 15 to adulthood (Rodriguez et al., 2002).

Arsenic exposure causes alterations in several neurotransmitter systems, such as monoaminergic (Nagaraja and Desiraju, 1993; Tripathi et al., 1997; Mejia et al., 1999; Rodriguez et al., 2001; Jana et al., 2006; Lin et al., 2007), cholinergic (Valkonen et al., 1983; Kobayashi et al., 1987), GABAergic and glutamatergic

(Nagaraja and Desiraju, 1993). Previous research reported dysfunctions in cerebral nitric oxide (NO) production (Zarazua et al., 2006). Arsenic exposure also affects brain development, particularly cerebellar Purkinje cells (Dhar et al., 2007), adversely affecting cognitive development (Rodriguez et al. 2001, 2002). In addition, observations of increased levels of dopamine (DA) in the striatum, though not in the nucleus accumbens or prefrontal cortex (Rodriguez et al. 2010), agree with findings of alterations in striatal and mesencephalic monoaminergic markers after iAs exposure. The latter has been also found to increase mesencephalic DA (Rodriguez et al., 2001) and striatal 3,4-Dihydroxyphenylacetic acid (DOPAC) (Mejia et al., 1997) and to reduce 3H-spiperone binding to striatal membranes (Yadav et al., 2009). Although iAs doses, exposure time, and species used in these studies are not the same as those analyzed in the present work, Yadav et al. (2009) suggest that nigrostriatal dopaminergic system becomes target for iAs exposure. On the other hand, an *in vitro* study has shown that As^{+3} and DA act synergistically to enhance toxicity in human dopaminergic neuroblastoma SH-SY5Y cells (Shavali et al., 2008). Therefore, elevated striatal DA contents associated to high iAs exposure have the potential to damage this brain region in particular. Rodriguez et al. (2010) observed a significant decrease in all behavioral parameters only in the group of rats exposed to 50 mg/L iAs during a 1-year-long exposure. This may indicate that the hypoactivity observed is thus a consequence of a dysfunction of the dopaminergic neurotransmitter system. On account of the fact that in the present study rats were exposed to low iAs concentrations through contaminated drinking water during gestation and lactation, both critical developmental and highly sensitive periods, hypoactivity

in adulthood could be interpreted as derived from alterations produced in the nigostriatal dopaminergic neurotransmitter system. Furthermore, in the open field tests, our control group as well as that of rats exposed to iAs were found to habituate to new environments, thus confirming that the concentrations used did not affect habituation. Habituation to a novel habitat, which is in general studied in an open field or a similar environment, is believed to be one of the most elementary forms of learning that involves the hippocampus where decreasing exploration as a function of repeated exposure to the same environment is taken as an index of memory (Thiel et al., 1998; Thiel et al., 1999).

Further findings from the present study show that the two iAs concentrations tested delayed the maturation of some reflexes and produced hypoactivity in female rats. In contrast, in male rats, delayed maturation of reflexes and hypoactivity were observed only in the animals exposed to the highest iAs concentrations, thus indicating that sensitivity to this metalloid is higher in females than in males. Likewise, Bardullas et al. (2009) also reported different effects between sexes in C57BL6/J adult mice exposed to similar and higher iAs concentrations (0.05, 0.5, 5.0 or 50 mg/L iAs of drinking water) during 4 months. They observed that, compared to males, the females exposed to the lowest iAs concentrations tested evidenced changes in locomotor activity.

Taken together, findings from the present study reveal that although early exposure to low iAs concentrations through contaminated maternal drinking water produces no alterations to progeny development, it does cause dysfunction in the CNS mechanisms and, as a consequence, alter locomotor activity and motor and sensory development. Further research on neurotransmission systems and oxidative stress in different brain areas will

pave the way to elucidate the mechanisms involved in hypoactivity and delays in sensory-motor development.

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Highlights

- **Arsenic exposure at an early period causes neurobehavioral alterations in offspring.**
- **Arsenic causes delayed development of sensorimotor reflexes.**
- **Arsenic produces less response in nociceptive reflex.**
- **Arsenic results in decreased locomotor activity.**
- **Female rats are more sensitive to arsenic exposure.**