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Neurofunctional effects of developmental sodium fluoride exposure in rats

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Abstract. – Contrasting studies on the toxic effects of sodium fluoride (NaF) during developmental stages of Wistar rats, lead us to investigate the neurofunctional effects caused by its perinatal exposure, devoid of any overt sign of toxicity and/or gross malformation. NaF solution was administered to pregnant rats by intragastric gavage at a daily dose of 2.5 and 5.0 mg/kg from gestational day 0 to day 9 after parturition. Developmental NaF exposure caused sex and dose specific behavioural deficits which affected males more than females in the majority of the evaluated end-points. In particular, the perinatal exposure to NaF 5.0 mg/kg, significantly affected learning, memory, motor coordination and blood pressure only in male rats. Conversely, a lack of habituation upon the second presentation of the objects and failure in the ability to discriminate between the novel and the familiar object were observed only in NaF 5.0 mg/kg female rats. Finally, a significant impairment of sexual behaviour was observed in male rats at both NaF dose levels. The present data indicate that perinatal rat exposure to NaF results in long lasting functional sex-specific alterations which occur at fluoride levels approaching those experienced by offspring of mothers.

Key Words:

Sodium fluoride, Perinatal treatment, Behavioural end-points, Sexual activity, Systolic blood pressure.

Abbreviations

PND = postnatal day;
T1 = first trial;
T2 = second trial;
CS = conditioned stimulus;
US = unconditioned stimulus;
M/IL = mount intromission latency;
M/IF = mount intromission frequency;
SBP = systolic blood pressure;
r.p.m. = rotations per minute.

Introduction

Fluoride is widely used in dentistry because effective as prophylactic agent in caries. Several studies demonstrated that the oral health program significantly affected the prevalence of caries and various risk factors for caries development[1]. Since 1960, the recommended dose in drinkable water necessary to prevent caries was stated between 0.7 and 1.2 mg/litre[2]. Further fluoride sources, other than drinkable water, are drinks, tooth paste (1000-1500 ppm), mouth rinse (230-900 ppm), dietary supplements and foods in general. Alarm was given by the Sub-Committee on *"Health Effects of Ingested Fluoride"*[3] about an increased risk of toxic consequences by several sources of fluoride. Therefore, the impetus to limit total fluoride exposure is based on general desire not to expose the public to any more fluoride than the necessary amount to prevent dental caries. A proper example is present in the U.S. where alarms on fluoride prophylaxis became evident when the handling of the daily fluoride intake resulted difficult, due to several sources of fluoride, other than fluoride added to tap water. In fact, only about 5% of the world population is present in

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fluoridated areas and more than 50% of these people live in North America. Despite dental pressure, 99% of western continental Europe has rejected, banned or stopped fluoridation due to environmental, health, legal, or ethical concerns[4]. However, although the water fluoridation was discontinued, it was stated that the improved caries status among preschool children observed in some italian place like Varese, were mainly attributable to an increased ingestion of bottled mineral water with an high fluoride level (> 2 mg/L)[5] while S.I.O.I guidelines[6] stated the italian characteristics of drinkable waters related with fluoride prophylaxis for human use. Therefore, it was recommended that a community should use no more than one kind of systemic fluoride (i.e., water or salt or milk fluoridation) combined with the use of fluoride toothpastes and that the prevalence of dental fluorosis should be monitored in order to detect increased levels higher than acceptable amounts. It has been shown, in fact, that prolonged ingestion of drinking water containing high levels of fluoride (> 4 ppm) produced deleterious effects in skeletal[7-9], dental[10,11], soft tissues[12] and locomotor activity[13]. Up to date, fluoride effects on reproductive function are not yet well established. Some literature data report a compromised fertility with an impairment of spermatogenesis and steroidogenesis[14-18], a decreased natality rate[19], an increased number of fetuses with 3 or more skeletal variations[20] and a modified citoarchitectural organization of sexual accessory glands[21,22]. Other scientific reports, instead, did not show any significant impairment[23,24]. Up to now, the role of fluoride in the pathogenesis of some neoplasies[25] and neurodegenerative disorders[26] is not yet well defined. Furthermore, also, the effects of fluoride on the central nervous system (CNS) are not yet fully investigated. In fact, some scientific reports described that high levels of fluoride in drinking water affect the nervous system without causing physical malformations[27]. Conversely, in vitro studies on hippocampal neurons have shown that intracellular fluoride is able to modify the kinetic properties of calcium channels[28]. To our knowledges, only one study investigated the neurotoxic potential of fluoride[29]. On the basis of these considerations, the aim of the present project was to analyze, in rats, the neurofunctional effects produced by fluoride exposure in early developmental stages, at doses not causing neither overt structural anomalies nor evident neurotoxic symptoms. The tests were chosen with the aim to assess various components of the behavioural repertoire with different developmental patterns, all important to study CNS and its cognitive processes. Motor abilities (as evaluated with the rotarod test) were also explored in 40 day old offspring. Since the literature data on reproductive function and cardiovascular system are contradictory or absent, we also investigated the effect of perinatal NaF exposure on male sexual behaviour and systolic blood pressure of the progeny.

Materials and Methods

Animals and Exposure Conditions

Animal experimentation was performed in accordance with the EU directive 86/609 EEC, with the guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B), with the UK Animals Act 1986 and associated guidelines, and with the "Guide for the Care and Use of Laboratory Animals" as adopted and promulgated by the National Institutes of Health. According to the above guidelines, all efforts were made to minimise the number of animals and their suffering.

Primiparous Wistar female rats (Harlan SRC, Milan, Italy), weighing 200-260 g were used. Animals were allowed free access to food and water, housed at constant room temperature (20-22°C) and exposed to a light cycle of 12 h day (08:00-20:00 h) for 2 weeks before the experiment. Pairs of females were placed with a single male rat in the late afternoon. Each female rat was inspected for vaginal smears on the following morning at 09:00 h. The day on which sperm were present was designated as day 0 of gestation. The route chosen in this study for exposure was via drinking water to mimic human exposure. Starting from day 1 of gestation and daily through postnatal day 9, experimental female rats were administered, by intragastric gavage, a solution of sodium fluoride (NaF, Novartis S.p.a., Origgio, VA, Italy) dissolved in deionized water. Drinking water was deionized water throughout the study. Preliminary experiments were performed with the following experimental groups: 1) undisturbed animals (no gavage at all); 2) 1 ml/kg of deionized water by gavage; 3) solution containing NaF 2.5 mg/kg/ml dissolved in deionized water by gavage; 4) solution containing NaF

5.0 mg/kg/ml dissolved in deionized water by gavage. Once comparisons between first and second group gave no statistical differences in all tests performed during the preliminary part of this research, which considered doses up to 20 mg/kg/ml, the treatment schedule was set up without the control group which was scheduled without any manipulation (gavage). Doses over 10 mg/kg were not considered due to the detection of some structural anomaly found in the offspring. Consequently, the doses of NaF employed in the present study were 2.5 and 5.0 mg/kg which respectively correspond to the upper level and twofold greater than the concentration level of the fluoride recommended dose (1,2 mg/ litre in drinking water). On the day following parturition (PND 1), all litters were reduced to a standard size of four male and four female pups per litter, when possible. Pups were then examined, weighed, sexed and marked with black ink on their backs for individual identification. They were weaned on PND 21 and the four males and four females of each litter were housed separately in cages identical to the home cage. One pup per litter from different litters per treatment group was then used in all experiments. Each pup was used for a single test and tested only once.

Reproduction Data

Body weight of each dam (10 females/group) was taken on days 0 and 20 of gestation. The number of dams giving birth and the length of pregnancy were determined. Litter size at birth and postnatal mortality (number of male and female pups died before weaning) were evaluated. The body weights of male and female rats (one pup per litter from 10 different litters per treatment group) were taken on postnatal day one (PND 1) and afterwards on PND 10 up to PND 120.

Locomotor Activity Test

According to a previously described method[30], motor activity was measured using an "*Opto-Varimex*" apparatus (Columbus Instruments, Columbus, OH, USA) linked to an IBM PC. The apparatus consisted of a square shaped apparatus provided with 15 × 15 infrared emitters located on the *x* and *y* axes and an equivalent amount of receivers located on the opposite walls, 1-2 cm above the floor, where was located the transparent plastic cage (42 × 42 × 30 cm; height 20 cm) with a cover provided of several little holes for passive ventilation. Vertical activity was measured by two further lines of 15 infrared emitters/receivers, positioned at different levels in order to monitor rearings (lower position) or jumps (higher position). At different ages, all levels of the infrared light beams were modified, according to the size of the animals. Each interruption of a beam generated an electric impulse which was send to PC software and simultaneously visualized on a digital counter. Motor activity was then analyzed by PC using the *"Auto-Track"* software (Columbus Instruments, Columbus, OH, USA) and defined as a trespass of three consecutive photo-beams, while other movements (e.g. repeated interruption of the same photo-beams) were considered as stereotypy-like movements. Resting time was calculated as the amount of time during which there was neither ambulatory nor stereotypic movement. Vertical activity was recorded as the number of beams interruptions belonging to two upper beam lines (rearings and/or jumps of the animals). The following parameters were measured: *ambulatory time* (AT, seconds); *stereotypic time* (ST, seconds); *resting time* (RT, seconds); *distance travelled* (DT, centimeters); *total ambulatory counts* (TAC, number of interrupted beams); *rearings* (number of 2nd line interrupted beams); *jumps* (number of 3rd line interrupted beams). All tests were carried out in a sound attenuating cabin (size: 1 × 1 × 2 mt) illuminated by a fluorescent 20-W light suspended 2 m above the apparatus. Background noise of 42 decibels was produced by a fan. Motor activity was measured on males and females on PNDs 21, 40 and 60 (n = 10 for each experimental group). At the beginning of the test sessions, each rat was placed in the middle part of the central square and its activity was recorded during a 30-min period.

Novel Object Exploration Test

The novel exploration object test used in the present study was a modified version[31] of that previously described by Ennaceur and Delacour[32]. Briefly, 40-day-old male and female rats (males: n = 12, females: n = 6 for each experimental group) were submitted to two habituation sessions (intersession interval: 24 h) where they were allowed 5 min to explore the apparatus (circular arena, 75-cm diameter, height = 40 cm). Twenty-four hours after the last habituation session, each rat was placed in the arena and given two 3-min trials with a 1-min intertrial interval.

In the first trial (T1) rats were exposed to two identical objects (black and white striped plastic bottles), which constituted samples A1 and A2. During the second trial (T2) rats were exposed again to two objects, one of which was familiar but the other was a new object, B. Object exploration was quantified as: (1) exploratory activity: total time spent exploring both objects during each trial (T1 and T2); (2) index of global habituation: it is measured by comparing the total time spent exploring the two objects in T1 to that spent in T2; (3) discrimination between the new and the familiar objects: it is measured in T2 by comparing the time spent exploring the familiar object with the time spent exploring the new object.

Rotarod Test

Motor coordination and balance were assessed by using a computerized electronically controlled system (TSE system, Bad Homburg, Germany), consisting of a four-lane rotating drums (6.5 cm in diameter) suspended 30 cm above the stainless steel floor grid, whose surface was manufactured to provide grip for the animals. Animal falls were detected by light-beam sensors mounted into each compartment and with the unit equipped with an escape preventing transparent cover. The apparatus was set up in an environment with minimal disturbances. Rats were acclimated to the moving rod for 3 min on the day before the test. Two experimental paradigms were performed:

A. A single session at constant rotation speed mode (15 r.p.m.)
B. A single session at accelerating rotation speed mode (5-40 r.p.m.)

In the first procedure (constant speed session), rats were placed on the rotating rod at 15 rotations per minute (r.p.m.) and four trials were conducted monitoring the latency to fall up to 300 sec. Inter trial interval for each animal was 20 min.

In the second procedure (accelerating rotation speed mode) rats, previously trained during the constant speed session, were tested as follows: each rat was placed on the stationary rod for approximately 30 sec.; then rod rotation accelerated from 5 to 40 r.p.m. up to 300 sec. Time from the beginning of the acceleration to the animal fall was automatically recorded. The test was performed in 40 day old male offspring. Each experimental group consisted of 10 animals for males and of 6 animals for females (one pup per litter from different litters per treatment group). All rotarod experiments were conducted between 10.00 and 14.00 h.

Passive Avoidance Test

A "step down" apparatus was used according to the previously described method[33]. It consisted of a compartment (25 × 24 × 24 cm) constructed of black Plexiglas and equipped with a grid floor to which an elevated compartment (13 × 24 × 16 cm) with a solid Plexiglas floor was attached. A guillotine door (9 × 10 cm) separated the opening between the elevated compartment and the large compartment. A 25 Watt lamp illuminated the elevated compartment while the large compartment remained dark. Scrambled foot shocks were delivered from a Letica shock generator (LI 2750 Unit, Barcelona, Spain). The experiments were performed in a sound-attenuating cabin (Amplifon G-type cabin) which was into complete darkness except for illumination of the elevated compartment of the step-down apparatus.

Each animal was removed from its home cage and placed in a holding cage near the apparatus. Two minutes later, the rat was placed into the lighted compartment. After a 10 sec. delay, the guillotine door was raised and the time up to the complete entrance (all feet) of the rat into the dark compartment was calculated (*approach latency*), taking its measure as an index of emotional, non-associative behaviour. A single 2 sec. inescapable scrambled foot shock (0.5 mA) was delivered immediately after the rat entered the dark compartment (acquisition trial). Twenty four hours later, each animal was tested for memory retention. The animal was placed in the elevated compartment and the latency to re-enter the dark compartment was recorded and assumed as a measure of memory retention (*avoidance latency*). Both acquisition and retention trials lasted for a maximum observation time of 180 sec. Experiments were conducted in 60-day old male and female offspring (n = 8 for each experimental group).

Active Avoidance Conditioning Test

The apparatus consisted of a *"two-way avoidance"* boxes housed inside a sound attenuating chamber (*Amplifon G-type cabin (Size: 1 × 1 × height 2 mt*). Each avoidance box (*size: 50 × 20 × height 20 cm*) was divided into two compartments connected by an opening of 9 × 12 cm and

operated by an electromechanical programming equipment. A white 3-W light lamp (conditioned stimulus, CS) was alternatively switched on in each compartment. An unconditioned stimulus (US, 0.5 mA scrambled foot shock) was delivered 12 sec after the onset of the CS, which remained switched on until a maximum of 18 sec during the presentation of the US. Afterwards, both CS and US were automatically turned off. The "*intertrial interval (ITI)*" period was 60 sec. A conditioned avoidance response (CAR) was recorded when an animal avoided the US by crossing over to the opposite compartment during the first 12 sec when only the CS was switched on. An *"escape response"* consisted of the animal moving into the opposite compartment following the onset of the US. This response terminated both the CS and the US. Sixty-day old animals were subjected to a 100 trial session (four 25-trial blocks) with a 60-s intertrial interval. Each experimental group consisted of six animals (one male and one female rat/litter from different litters/treatment group).

Blood Pressure Measurement

In all groups of animals, non invasive systolic blood pressure (SBP) was measured using a tail-cuff (Letica 5100, PanLab, Barcelona, Spain) according to the standard procedures previously described[34,35]. Before BP recording, all animals were trained to reduce the stress associated with the BP measurements. Training consisted of three sessions over 3 days during which the tail cuff was inflated three times in quick succession. For better detection of tail pulse, the animals were preheated to 37° C for 20 min. BP readings were considered successful when the animal did not move and a clear initial pulse was observed. SBP values reported are the average of three sequential blood pressure measurements that were within 10 mmHg of each other.

Sexual Behaviour and Related Ultrasonic Vocalization

Naive male rats were tested for sexual behaviour at 60 days of age (10-min session); thereafter, the animals were subjected to a further five 10 min sessions (intertrial interval = 15 days). Rats achieving ejaculation in one of the six 10 min sessions were not retested in the remaining 10 min sessions. Finally, one more test was performed at 150 days of age (30-min session). As stimulus females were used bilaterally ovariectomized rats in which oestrus had been induced by subcutaneous injection of estradiol benzoate (8 µg/rat) and progesterone (200 µg/rat) dissolved in 0.2 ml of sesame oil, 52 and 4 h before the test session, respectively. Sexual behaviour was recorded by a Canon MV500i digital video camera connected to a Sony Trinitron Show View apparatus (TV and tape-recorder: all in one). Ultrasonic calls, detected by a QMC ultrasonic microphone connected to a receiver (QMC Bat Detector S200) which transformed, in real time, the ultrasonic calls into audible sounds, were sent to the videotape-recorder through the microphone connection socket of the video camera. The experiment was carried out in a sound-attenuating chamber (amplifon G-type cabin) and the arena was a plastic cylinder (diameter = 50 cm; height = 40 cm) placed on a square transparent glass floor at a height of 130 cm from the floor of the cabin. A 50 cm square mirror was placed at 45° below the floor of the cylinder in order to have the inferior sight of the arena and to record the sexual behaviour with a video-camera placed orizontally outside the cabin, looking the mirror through a sound attenuated glass-camera window. The experiment started leaving each male rat alone for 5 min; an oestrous female was then introduced into the centre of the arena and the behaviour of the couple was recorded together with their ultrasonic vocalizations. Video tape-recordings were later replayed and analyzed (in slow motion when necessary) and the following sexual parameters were measured:

- (M/IL) *mount-intromission latency* (time between the introduction of the female into the mating cage and the first mount or intromission in each ejaculatory series);
- (M/IF) *mount-intromission frequency* (number of mounts or intromissions in each ejaculatory series);
- (EjL) *ejaculation latency* (time between the first intromission and ejaculation in each ejaculatory series);
- (EjF) *ejaculation frequency* (number of ejaculations in each 30 min test session);
- (PEjL) *post-ejaculatory interval* (interval between each ejaculation and the next intromission in each ejaculatory series);
- (TM/IF) *total mount/intromission frequency* (total number of mounts or intromissions during the test session);
- *(ICI) inter-copulatory interval (ejaculation latency/intromission frequency in each ejaculatory series).*

- *(L50) Latency to the first 50 kHz call (time between the introduction of the stimulus female into the mating cage and the appearance of the first 50 kHz call);*
- *(PEjVL) postejaculatory vocalization latency (time from ejaculation to the beginning of the 22 kHz postejaculatory vocalization);*
- *(PEjVD) postejaculatory vocalization duration (time from the beginning until the end of the 22 kHz postejaculatory vocalization).*

A total number of 30 rats were used. Each experimental group consisted of 10 animals (one male/litter from 10 litters/treatment group).

Statistical Analysis

General reproduction data were analysed by overall one-way analysis of variance (ANOVA). Two-way ANOVAs for repeated measures were performed on progeny body weight gain, active avoidance, blood pressure measurements and locomotor activity tests. Data relative to motor coordination (pooling data of the four trials at constant rotation speed mode), passive avoidance, novel object exploration and sexual behaviour tests were analysed by Kruskal-Wallis ANOVA. Individual comparisons were performed by Tukey's or Dunn's Multiple Comparison tests, where appropriate. Fisher's exact-test was also used when appropriate.

Results

Reproduction data

Reproduction data are reported in Table I. An overall one-way ANOVA showed that dam weight gain, pregnancy length, number of dams giving birth and litter size at birth were not significantly affected by perinatal treatment with sodium fluoride. Moreover, perinatal NaF treatment did not induce a significant decrease of male and female pup weight at birth and afterward. Fisher's exact test revealed that fluoride exposure did not induce an increase of postnatal mortality.

Novel Object Exploration Test

As far as the novel exploration object test, Kruskal-Wallis ANOVA for total exploratory activity during T1 showed the following results: males: $H = 0.46$; $df = 2$; n.s.; females: $H = 2.10$; $df = 2$; n.s. As regards global habituation, within group comparisons showed that 40-day old control and both doses of NAF treated-rats in males and 2.5 mg/kg NaF treated-rats in females exhibited lower levels of exploratory activity during T2 compared to those found in T1, whereas no significant difference in the exploration time between T2 and T1 was found in 40-day old female rats treated with 5.0 mg/kg of NaF (Figure 1).

Kruskal–Wallis ANOVA for discrimination showed the following difference:

males: $H = 0.66$; $df = 2$; n.s.; females: $H = 0.45$; $df = 2$; n.s. Within-group comparisons showed that time spent by control and both doses of NAF-treated rats in males and by control and 2.5 mg/Kg NaF-treated rats in females in exploring the familiar object during T2 was significantly lower than the time spent exploring the novel object.

Conversely, 5.0 mg/kg NaF-treated female rats did not show any significant difference in the exploration time of the novel with respect to the familiar object (Figure 2).

Rotarod Test

Kruskal-Wallis ANOVA for the latency to fall showed the following results: (*constant speed mode*) males: H = 13.69; df = 2; $p < 0.005$; females: H = 1.99; df = 2; n.s. (*accelerating speed mode*) males: H = 13.08; df = 2; $p< 0.005$; females: H = 0.56; df = 2; n.s. Between groups comparisons (Dunn's test) showed that falling latencies of the NaF treated male rats, at the highest dose level (5.0 mg/kg), was significantly ($p < 0.001$) shorter than those of control animals in both experimental paradigms, while no difference was observed in female treated groups (Figure 3).

Locomotor Activity Test

Two-way repeated measures ANOVAs for locomotor activity showed that perinatal treatment with NaF did not significantly affect this parameter either at 2.5 mg/kg or at 5.0 mg/kg dose levels in both males and females offspring at all ages tested (data not shown).

Passive Avoidance Test

During the first (acquisition) trial, statistical analysis (Kruskal-Wallis ANOVA) for the approach latencies did not reveal any significant difference between groups (male: H = 1.09; df = 2; n.s; female: H = 0.59; df = 2; n.s). However, when the trial was repeated 24 h later (retention trial), Kruskal-Wallis ANOVA for the avoidance

Table I. Reproduction parameters.

Groups	Dam weight gain_(%)	Pregnancy length (days)	Litter size at birth (n)	Pup weight gain	PND 1	PND 10	PND 15	PND 20
Vehicle	54.40 ± 1.00	21.00 ± 0.12	11.25 ± 0.55	Males	6.48 ± 0.15	23.32 ± 0.4	38.00 ± 0.7	51.18 ± 0.99
				Females	6.60 ± 0.13	23.28 ± 0.35	34.38 ± 0.62	47.60 ± 1.00
NaF 2.5 mg/kg	51.37 ± 1.25	21.00 ± 0.15	10.40 ± 0.60	Males	6.19 ± 0.14	24.46 ± 0.53	36.92 ± 1.20	51.50 ± 1.58
				Females	6.87 ± 0.07	23.12 ± 0.89	35.30 ± 1.01	48.73 ± 1.68
NaF 5.0 mg/kg	50.70 ± 2.50	20.80 ± 0.13	12.30 ± 0.60	Males	6.34 ± 0.16	25.56 ± 1.02	37.74 ± 1.37	53.20 ± 2.18
				Females	6.28 ± 0.29	23.28 ± 0.38	35.20 ± 1.16	49.20 ± 1.76

No differences were observed in dam weight gain, pregnancy length, litter size at birth and pup weight gain between treatment groups. Data are expressed as mean values ± S.E.M. [a]% from GD 0 to delivery.

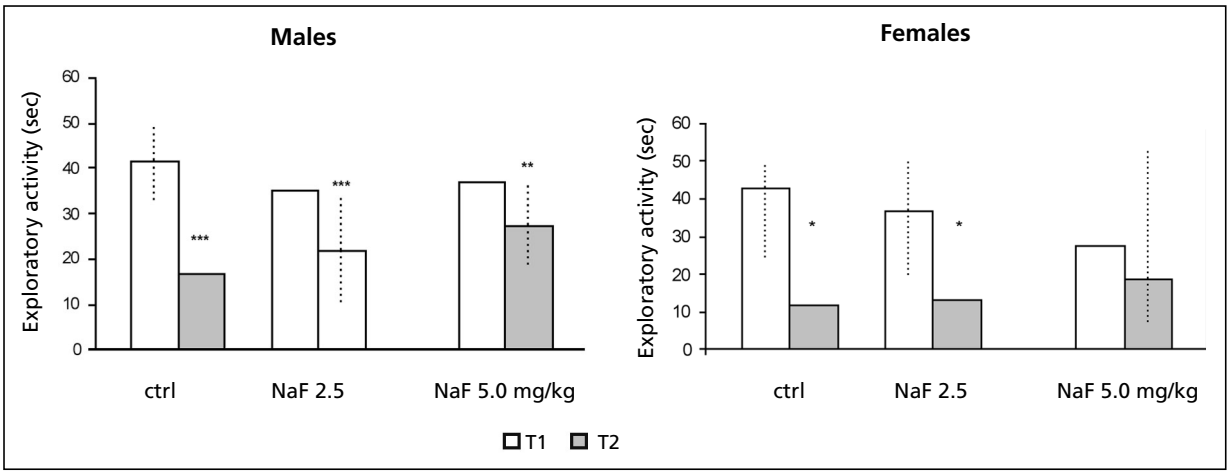

Figure 1. Novel object exploration test. No difference was observed in exploratory activity during Trial 1 (T1) in both male and female NaF-treated rats with respect to controls at both dose levels (2.5-5.0 mg/kg). Data were expressed as median values and interquartiles (dashed line) and refer to twelve (male) or six (female) rat group at PND 40. A lack of habituation (lower levels of exploratory activity during T2 compared to those found in T1) was observed in female treated rats at the highest dose level with respect to controls. Statistical analysis (Kruskal-Wallis ANOVA) showed the following results: males: H = 1.09; df = 2; n.s.; females: H = 0.59; df = 2; n.s.; *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$ vs T1 of the same group (Wilcoxon Paired Signed-Rank test).

latencies gave the following significant difference: male: H = 16.67; df = 2; $p < 0.001$; female: H = 5.20; df = 2; n.s. Between group comparisons (Dunn's test) showed that avoidance latencies of the NaF treated-male rats at the highest dose level (5.0 mg/kg) was significantly shorter ($p < 0.001$) than those of control animals, while no difference was observed in female-treated groups (Figure 4).

Active Avoidance Test

An over-all two way repeated measures ANOVA for CARs gave the following results: *male*: Ftreatment = 6.27, df = 2/15, $p < 0.02$; Fsession = 26.67, df = 3/45, $p < 0.001$; Ftreatment x session = 7.20, df = 6/45, $p < 0.001$; *female*: Ftreatment = 1.13, df = 2/15, n.s.; Fsession = 17.25, df = 3/45, $p < 0.001$; Ftreatment x session = 2.08, df = 6/45, n.s. Post hoc test (Tukey's multiple comparison

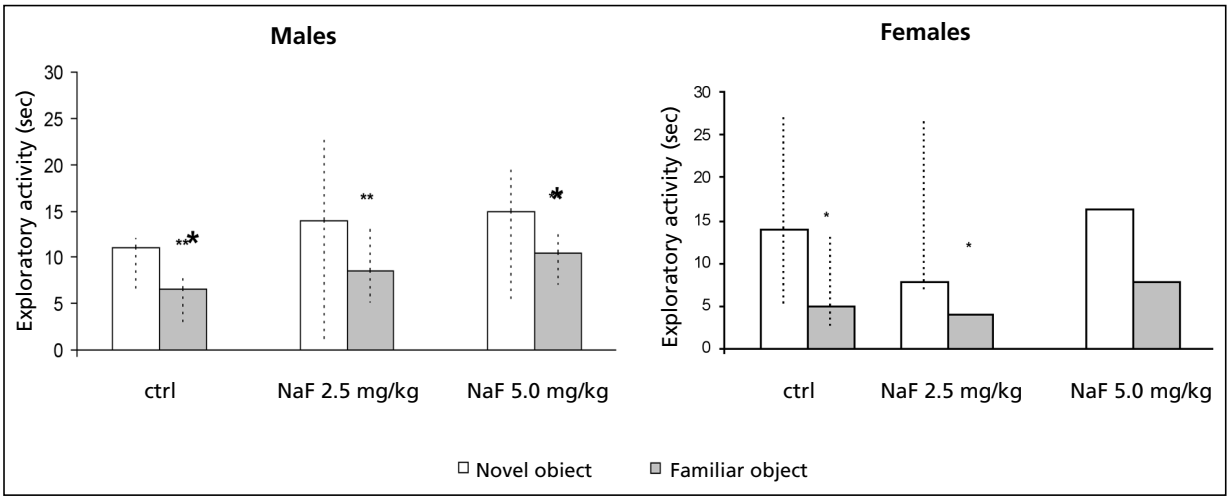

Figure 2. Novel object exploration test. Object recognition, which is assessed by the preference displayed from normal rats for investigating novel rather than familiar objects was evaluated during Trial 2 (T2) in 40 day old male (n = 12) and female (n = 6) rats. Data were expressed as median values and interquartiles (dashed line). Perinatally NaF female treated rats, at the highest dose level (5.0 mg/kg) apparently have lost the capacity to discriminate. Statistical analysis (Kruskal-Wallis ANOVA) showed the following data: males: H = 0.66; df = 2; n.s.; females: H = 0.45; df = 2; n.s.; *$p < 0.05$; **$p < 0.001$ vs. novel object (Wilcoxon Paired Signed-Rank test).

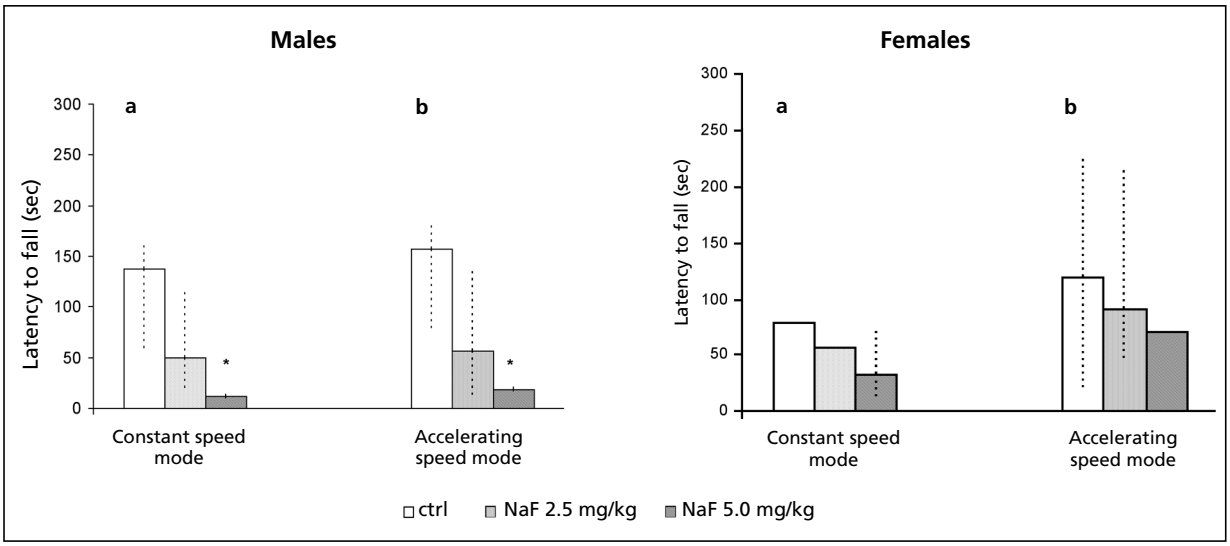

Figure 3. Rotarod test. The latency to fall from the rotating rod of male and female rats perinatally exposed to vehicle (ctrl) or NaF (2.5-5.0 mg/kg) during a single session at (*a*) constant rotation speed mode (15 r.p.m.; pooling data of four trials) and at (*b*) accelerating rotation speed mode (from 5 to 40 r.p.m.) was recorded at PND 40. Data are expressed as median values and interquartiles (dashed line) and refer to ten (male) or six (female) rat group. A significant impairment in motor coordination performance was evident at the highest dose level (5.0 mg/kg) only in perinatally NaF-exposed male rats in both experimental paradigms. In particular, statistical analysis (Kruskal-Wallis ANOVA) provided the following results (constant speed mode: males: H = 13.69; df = 2; $p < 0.005$; females: H = 1.99; df = 2; n.s.; (accelerating speed mode: males: H = 13.08; df = 2; $p < 0.005$; females: H = 0.56; df = 2; n.s. *$p < 0.001$ vs controls (Dunn's Multiple Comparison's test).

test) revealed a significant impairment in the acquisition of an active avoidance task in 60 day old male rats only at the dose of NaF 5.0 mg/kg (1st 25 trials block = n.s.; 2nd and 3rd 25 trials blocks = $p < 0.05$; 4th 25 trials block = $p < 0.001$). Female rats didn't show any significant impairment in the acquisition of conditioning responses neither at dose of 2.5 mg/kg nor at 5.0 mg/kg (Figure 5).

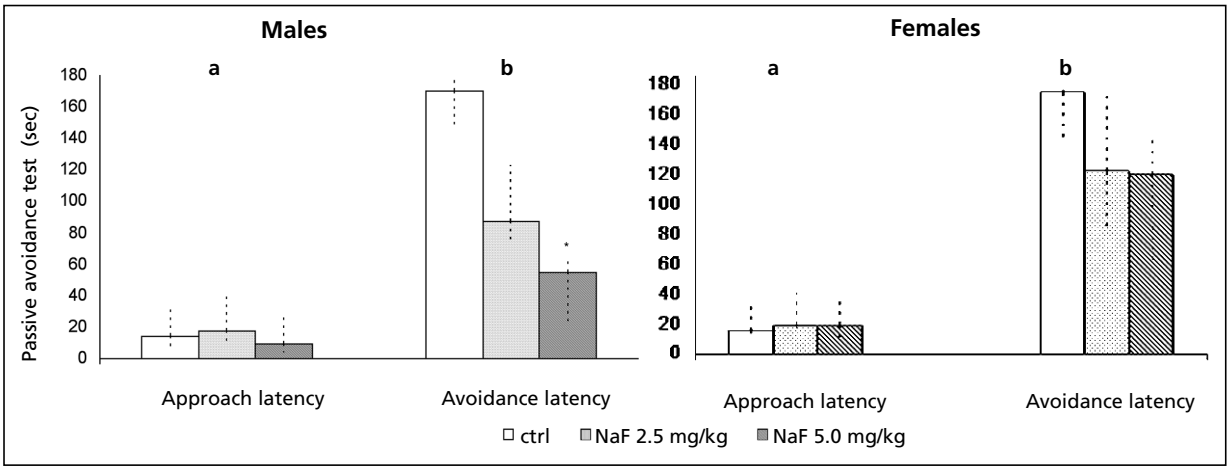

Figure 4. Passive avoidance test. (*a*) Approach latency and (*b*) avoidance latency measured 24 h later (retention) of 60-day-old male and female NaF exposed offspring. Groups of eight rats were scored. Data are expressed as median values and interquartiles (dashed line). Statistical analysis (Kruskal-Wallis ANOVA) showed no differences in approach latencies between groups in both genders (males: H = 1.09; df = 2; n.s.; females: H = 0.59; df = 2; n.s.). A significant reduction in avoidance latencies was observed in the case of males. In particular, Kruskal-Wallis ANOVA provided the following data: males: H = 16.67; df = 2; $p < 0.001$; females: H = 5.20; df = 2; n.s. *, $p < 0.001$ vs controls (Dunn's Multiple Comparison's test).

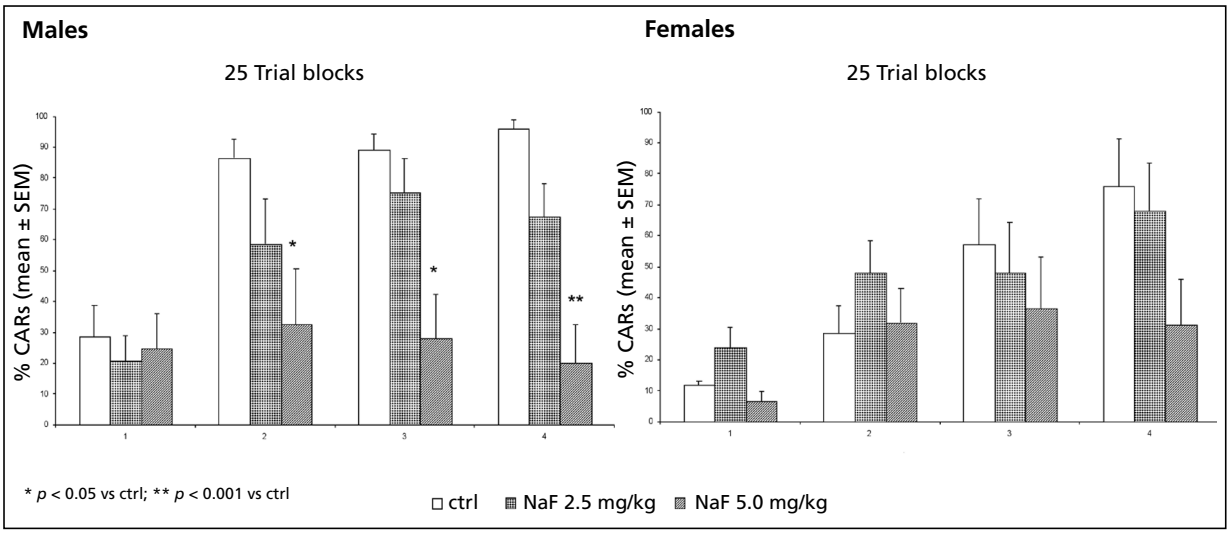

Figure 5. Active avoidance conditioning test. CAR = Conditioned Avoidance Response. Data are expressed as mean values (% of CARs) ± SEM for groups of six 60 day-old male and female rats. A significant impairment in learning performance was evident in male rats at the highest NaF dose level (5.0 mg/kg) Two-way repeated measures ANOVA for CARs gave the following results: males: F treatment = 6.27, df = 2/15, $p < 0.02$; F session = 26.67, df = 3/45, $p < 0.001$; F treatment × session = 7.20, df = 6/45, $p < 0.001$; females: Ftreatment = 1.13, df = 2/15, n.s.; F session = 17.25, df = 3/45, $p < 0.001$; F treatment × session = 2.08, df = 6/45, n.s.; *$p < 0.05$ (2nd and 3rd 25 trials blocks);** $p < 0.001$ (4th 25 trials block) with respect to control (Tukey's multiple comparison test).

Blood Pressure Measurements

To explore the possible effect of perinatal NaF exposure on cardiovascular function, systolic blood pressure (SBP) was measured in male and female 120 day old rats. When assessed with unpaired t-test analysis, SBP was not significantly different between male or female animals from both control and NaF 2.5 groups (Figure 6) ($p > 0.43$). However, when compared with respective female group, a substantial increase in SBP was observed in male Wistar rats treated with NaF 5 mg/kg ($p < 0.05$). Indeed, in male rats, two-way ANOVA analysis confirmed higher values of SBP in the group exposed to 5 mg/ml NaF concentration with respect to control and NaF 2.5 mg/ml treated animals ($p < 0.05$, $p < 0.01$, respectively). By contrast, no significant change in SBP was observed in female rats of the various groups ($p > 0.8$). Thus, perinatal exposure to NaF leads to a dose-dependent, long-lasting and gender specific functional impairment in hemodynamic control.

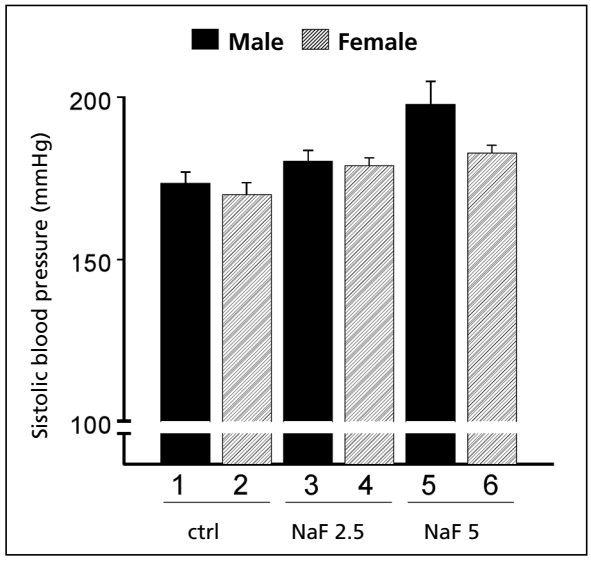

Figure 6. Systolic blood pressure. Systolic blood pressure was measured with a tail cuff in male (filled columns) and female (dashed columns) 120 day old Wistar rats perinatally exposed to either vehicle (ctrl), 2.5 mg/kg sodium fluoride (NaF 2.5), or 5.0 mg/kg sodium fluoride (NaF 5 mg/kg), as described in Methods. Systolic blood pressure was not significantly different between male or female Wistar rats from ctrl and NaF 2.5 groups. However, when compared with respective ctrl group, a significant increase in SBP was observed in male (but not in female) Wistar rats treated with NaF 5 mg/kg ($p < 0.01$). Values shown are the average ± SEM of 3 independent experiments for each group of animals.

Sexual Beviour and Related Ultrasonic Vocalization

Results indicate that perinatal exposure to NaF notably affected copulatory activity of rat offspring. As far as the latency to the first

mount/intromission, Kruskal-Wallis ANOVA gave the following significant difference: H = 11.64, df = 2; $p < 0.005$. Individual comparisons (Dunn's Multiple Comparison test) showed a significant increase in mount/intromission latency of 60 day old male rats perinatally exposed to both doses of NaF (2.5 mg/kg; $p < 0.05$; 5.0 mg/kg, $p < 0.01$) with respect to controls (Figure 7 panel A).

Kruskal-Wallis ANOVA for mount/intromission frequency gave the following significant difference: H = 8.81, df = 2; $p < 0.05$. Post hoc test (Dunn's Multiple Comparison test) showed that 60 day old fluoride-treated rats exhibited a significant ($p < 0.05$) decrease in mount/intromission frequency at both dose levels (2.5-5.0 mg/kg) as compared to control animals (Figure 7 panel B).

The percentage of rats achieving at least one ejaculation within the 6th 10 min sessions (130 days of age) was significantly lower at both dose levels (2.5 mg/kg; $p < 0.005$; 5.0 mg/kg, $p < 0.001$) in NaF treated rats than in the control group (control: 80%; 2.5 mg/kg: 10%; 5.0 mg/kg: 0%). However, when the animals were retested during a 30 min session (150 days of age) the percentage of rats achieving at least one ejaculation was significantly lower ($p < 0.005$) only at the highest NaF dose level (5.0 mg/kg) with respect to the control group (control: 80%; 2.5 mg/kg: 50%; 5.0 mg/kg: 10%). Statistical analysis for all other sexual and ultrasonic parameters did not show any significant difference.

Discussion

The results of the present study indicate that perinatal exposure to sodium fluoride (NaF), at dose levels below those associated with gross malformations and/or overt neurotoxic effects, produces both short and long term sex and dose specific neurobehavioural alterations in rat offspring. Lack of significant changes in maternal and pup weight gain suggests that nutritional deficiency was not a factor in the fluoride offspring outcome. Spontaneous locomotor activity was not significantly affected by developmental fluoride exposure. However, the present findings show that perinatal exposure to low doses of NaF produce learning and memory impairment only in male-treated rats at the highest dose level. The learning impairment, tested in an active avoidance task, does not appear to be attributable to alterations of a non associative nature. In fact, change in emotionality seems to be absent as indicated by the evaluation of escape response latencies which were not affected by developmental sodium fluoride exposure. Moreover, spontaneous crossings during intertrial interval were not significantly influenced by perinatal NaF treatment. Furthermore, memory impairment in

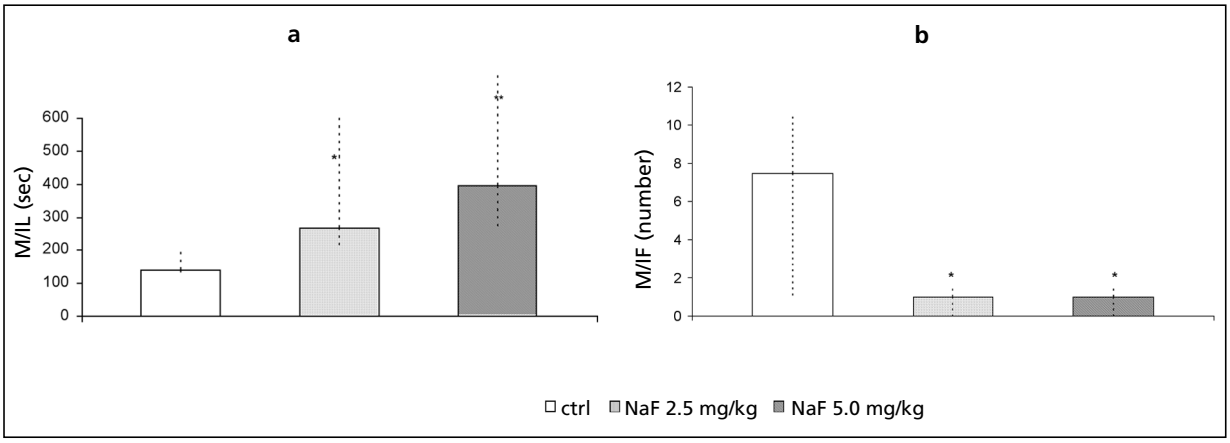

Figure 7. Sexual behaviour. (*panel a*) Mount/Intromission Latency (MI/L) and (*panel b*) Mount/Intromission Frequency (M/IF) in 60 day old male rats during a 10 min session. Each experimental group consisted of 10 animals. Data are expressed as median values and interquartiles (dashed line). NaF exposed male rats at both dose level showed a significant increase in M/IL and a significant decrease in M/IF. In particular, Kruskal-Wallis ANOVA showed the following results: M/IL: H = 11.64, df = 2; $p < 0.005$; M/IF: H = 8.81, df = 2; $p < 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$ with respect to control (Dunn's Multiple Comparison test).

male perinatal NaF-exposed rats, assessed by the disruption in the retention of a passive avoidance task, does not appear to be attributable to alterations of a non associative nature, because approach latency, measured during the acquisition trials of the learning task, remained unchanged. Our data are in agreement with recent findings showing a learning-memory behaviour impairment in mice after drinking different concentration of sodium fluoride[36]. Therefore, several surveys of persons, chronically exposed to industrial fluoride pollution, reported symptoms related to impaired central nervous system functioning with cognition and memory deficits[13]. Finally, recent findings have shown a decrease in the number of neuronal nicotinic acetylcholine receptors (nAChRs) in the brain of rats receiving either 30 or 100 ppm fluoride. Since nAChRs play major roles in cognitive processes such as learning and memory, the decrease in the number of nAChRs caused by fluoride toxicity may be an important factor in the mechanism of cognitive dysfunction[37]. Concerning the "*novel exploration object"* test, object recognition, which is assessed by the preference displayed from normal rats for investigating novel rather than familiar objects, provides a valid and relatively pure measure of non spatial working memory[31,32]. Developmental fluoride exposure did not influence exploratory activity in T1 in both male and female NaF-treated rats with respect to controls at both dose levels whereas a lack of habituation (lower levels of exploratory activity during T2 compared to those found in T1) was observed in female treated rats at the highest dose level with respect to controls. Habituation failure exhibited by female NaF-exposed animals could be the consequence of altered mechanisms involved in attention and/or short-term memory. At both dose levels, male-treated rats were able to discriminate between the novel and the familiar object. On the contrary, female treated rats, at the highest dose level, apparently have lost this capacity to discriminate.

To our knowledge, no study investigated the effects of developmental fluoride on object recognition performance; thus, our study provides the first evidence that perinatal fluoride exposure produces this sex specific neurobehavioural impairment which may be related to hormonal changes. It is known, in fact, that the brain function could be differently affected by drugs depending on type and hormones level present at the time of drug exposure[38-40].

Acute and chronic exposure to NaF may differentially affect cardiovascular function. While acute fluoride intoxication leads to the progressive fall in arterial blood pressure responsible for cardiovascular damage[41], prolonged ingestion of fluoride may directly induce histopathological and biochemical changes in myocardial tissue[42]. Our results provide the first evidence that perinatal fluoride exposure, at the highest doses used in this study, may significantly increase systemic blood pressure in male Wistar rats. Although future work needs to address the potential implications of these findings, it remains nevertheless possible that alteration in hemodynamic homeostasis may be one mechanism by which NaF may participate in cardiovascular dysfunction long after the end of fluoride exposure.

As far as motor coordination, the present findings show an impairment of motor performance only in male offspring at the highest dose level in both experimental paradigms. Previous studies, using very high dose of NaF (500 ppm in drinking water), have shown a shortening of rotarod endurance time in sodium fluoride treated rats[43]. In this study, however, was observed a suppression of spontaneous locomotor activity due to an impaired central cholinergic mechanism. In the absence of muscular deficits, evaluated by the hanging test (data not shown) the low score level exhibited on the rotarod test, either at constant or in the accelerating speed modes, specifically reflects an impairment in motor learning and spatio-temporal organisation of movement.

Furthermore, the results of the present study show that developmental NaF exposure causes a significant impairment of sexual behaviour in male offspring at both dose levels. However, sexual impairment was long lasting (up to 5 months of age) only at the highest NaF dose level (5 mg/kg) with respect to control group. These results are in agreement with a recent scientific report, which describes an impairment in spermatogenesis and steroidogenesis of NaF treated male rats[18].

In summary, the present findings show that perinatal rat exposure to NaF results in long lasting functional sex-specific alterations which occur at fluoride levels approaching those experienced by offspring of mothers. In fact, humans occasionally are exposed to high amounts of fluoride and, since fluoride readily penetrates into the brain[29], a neurotoxic risk should be taken in account.

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