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The impact of chronic fluoride exposure on dental integrity and growth in male *Wistar* rats at different developmental stages

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Fernanda Marlen ENRÍQUEZ-SÁNCHEZ[1], Liliana VALDEZ-JIMÉNEZ[2], Miguel Ángel LÓPEZ-VÁZQUEZ[3], María Esther OLVERA-CORTÉS[3], Paola Trinidad VILLALOBOS-GUTIÉRREZ[1], María Isabel PÉREZ-VEGA[1,2*]

[1]Laboratorio de Ciencias Biomédicas, Departamento de Ciencias de la Tierra y de la Vida, Centro Universitario de los Lagos (CULagos), Universidad de Guadalajara, Lagos de Moreno, Jalisco, México.
[2]Laboratorio de Neuropsicología, Departmento de Humanidades, Artes y Culturas Extranjeras, CULagos, Universidad de Guadalajara, Lagos de Moreno, Jalisco, México.
[3]Centro de Investigación Biomédica de Michoacán, Instituto Mexicano del Seguro Social, Morelia,Michoacán, México

***Corresponding author:**
María Isabel Pérez-Vega; Departamento de Ciencias de la Tierra y de la Vida, Centro Universitario de los Lagos (CULagos), Universidad de Guadalajara, Lagos de Moreno, Jalisco, México.

Phone: +52 474 747 8619
Email: mabelpv@hotmail.com; misabel.perez@academicos.udg.mx

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ABSTRACT

Purpose: Fluoride is a naturally occurring water contaminant. Prolonged exposure to this element has been shown to have adverse effects on different tissues, as demonstrated by numerous experimental and epidemiological studies. Additionally, fluoride can cross the placental barrier and is present in breast milk, leading to prenatal exposure in areas with endemic fluorosis. The aim of this study is to examine the impact of chronic fluoride exposure during various developmental stages on dental health and growth in male *Wistar* rats.

Methods: Adult Wistar rats were randomly assigned to fluoride exposure groups (10, 100, and 150 ppm F-) and a control group. The animals began treatment 14 days prior to mating. The litters were weaned at 21 days of age, and the males continued the same exposure concentration until they were 90 days old. Likewise, the same concentrations were used in male Wistar rats that were 21 days old until they reached 90 days of age. Food and water consumption, as well as body weight records, were kept weekly throughout the experiment. At the end of the treatment, dental evaluations were performed, and blood samples were taken to determine serum fluoride concentrations.

Results: The litters of the fluoride-treated groups showed a decrease in the number of live births and a lower weight at weaning. The offspring of the highest fluoride group exposed (150 ppm F-) had a significant reduction in both water and food consumption, while body weight gain was lower in all treated groups compared to the control group. All groups exposed to fluoride exhibited an increase in serum fluoride levels, as well as moderate to severe lesions in the incisors in a concentration-dependent manner. However, the group that was exposed to 100 ppm F- after weaning showed a lower total weight gain, a higher score on the dental evaluation scale, and a higher serum concentration of F- compared to the animals that were exposed to the same concentration since gestation.

Conclusions: This study presents evidence of the negative impact of high concentrations of F- on adult individuals, including reproductive and developmental effects in those exposed during gestation and continuing chronic consumption into adulthood.

Furthermore, the study highlights that the effects of chronic F- exposure vary depending on the developmental stage of the individual at the time of exposure.

Key-words: *Fluoride toxicity, dental fluorosis, chronic poisoning*

INTRODUCTION

Fluoride is a naturally occurring element found in soil and water as fluoride ions (F-). However, it can also be present in the air due to human activities and volcanic emissions[1].

F- has been described as a microelement necessary for normal human development due to its anti-cavity effect and ability to strengthen calcified tissues[2]. However, beneficial effects are observed at F- concentrations of 0.7-1.2 parts per million (ppm). Exceeding this concentration can cause various adverse effects on the health of the population[1,3].

People are primarily exposed to fluoride through food, air, dental products, and water[4]. The concentration of fluoride in water varies depending on the environmental characteristics of the geographical region[5]. The World Health Organization (WHO) recommends a maximum fluoride level of 1.5 ppm in drinking water for human consumption[6]. However, in some regions of the world, concentrations as high as 17.7 ppm have been recorded[7,8].

F- has a cumulative effect that leads to chronic intoxication known as fluorosis. The most obvious manifestation of fluorosis is observed in the subject's teeth. However, epidemiological and experimental studies have shown that this condition also affects other organs and tissues, such as the nervous system, gastrointestinal tract, kidneys, liver, and even the reproductive system[9–11]. Additionally, due to its ability to cross the placental barrier and be excreted in breast milk[12,13], individuals residing in areas with endemic fluorosis are exposed to F- during the prenatal stage. However, there is limited literature on the effects of chronic consumption of F- in adults who were exposed to it during the embryonic period and it is unclear whether there are differences for those who begin exposure at other stages of development.

The aim of this study is to examine the impact of chronic fluoride exposure during various developmental stages on dental health and growth in male *Wistar* rats.

MATERIAL AND METHODS

Sixteen *Wistar* strain rats with an average weight of 239 ± 2.5 g (females) and 334 ± 24.9 g (males) from the Centro de Investigación Biomédica de Michoacán del Instituto Mexicano del Seguro Social (IMSS) were randomly assigned to the different exposure groups: 10, 100, and 150 ppm F- (F10, F100, and F150, respectively) and a control group (C, 1.5 ppm F-). After 14 days of treatment, females were exposed in contact with the male (female/male ratio 3:1) for two weeks for mating. The pregnant females were separated and continued the same treatment during pregnancy and lactation. Litters were adjusted to 8 animals per cage when possible.

The offspring were weaned at 21 days of age and only males (n=12 per group) were selected randomly from the litters and continued exposure to fluoridated water until 90 days of age. Offspring not selected for this work were either sacrificed or assigned to other experiments.

Additionally, this study examined the impact of beginning fluoride exposure during gestation and lactation versus starting fluoride consumption only until weaning. For this purpose, the data used were obtained from an earlier study conducted by our team[14] in which male *Wistar* rats were exposed to the same doses of F- (10, 100, and 150 ppm) from 21 days postnatal to 90 days of age. The parameters measured in both studies were the same, making the data viable for statistical analysis.

During the study, rats were kept in standard biotherium environmental conditions (temperature 22-26°C, relative humidity 40-70%, 12-hour light/dark cycles). Commercial balanced feed for laboratory rodents was provided to them at free access (Nutricubos, Agribrands Purina México S.A. de C.V.; protein 23% - fat 3% - fiber 6% - ash 7% - calcium 1% - phosphorus 0.6% - nitrogen free extract 55%). Fluoridated water prepared with deionized water and sodium fluoride (Meyer® Chemistry) was administered as drinking water.

All experimental procedures were carried out by the Guide for the Care and Use of Laboratory Animals[15].

General measurements

Food and water consumption was measured every third day per group, to do this, the amount of water and food administered to each cage was weighed and the amount remaining 24 hours later was subtracted from the initial amount. The daily average individual consumption was calculated by dividing the 24-hour consumption by the number of animals per cage. Statistical analysis was performed using data obtained in the same week. Body weight was recorded individually each week.

Dental evaluation

At 90 days of age, dental evaluation of the incisors was performed on each rat using the method described by Ekambaram and Paul (2001)[16] (Table 1).

Table 1. Dental evaluation scale used.

Score	Characteristics of the incisors
0	Normal shape, bright orange color, smooth glaze
1	Slight enamel whitening
2	Faint horizontal band of enamel, calcareous spots, slight erosion
3	Chalky enamel, moderate tip erosion, staining
4	Torn and chipped edges, loss of enamel color
5	Extended and eroded tips, abnormal curvature

Determination of F- concentration in blood serum

The ion-selective electrode method was used to determine the concentration of F- in serum. Serum was obtained by centrifuging blood (2500 rpm for 15 minutes) collected via intracardiac puncture from anesthetized animals (n=6) using sodium pentobarbital (50 mg/kg IP). A solution was prepared by mixing equal parts of serum and TISAB II. The F- concentration was measured using a Thermo Scientific™ model 9609BNWP ion-selective electrode and an Orión Star™ model A324 multiparameter pH and ISE meter. The concentration was expressed in parts per million (ppm). Each sample was measured in triplicate at a constant temperature of 23°C ± 3°C with constant agitation.

Statistical analysis

Data are presented as the mean ± standard error of the mean (M ± SEM). The data obtained in this study were analyzed using one-way analysis of variance (ANOVA) and the Tukey multiple comparison test was used as a *post hoc* test. The data obtained from the dental evaluation were analyzed using the Kruskall-Wallis test and the Dunn's test for comparisons between groups. Finally, correlation tests were performed between the data obtained.

To compare the groups exposed after weaning, the analysis was carried out between the groups exposed to the same concentration of fluoride. A Mann-Whitney U test was used for the dental evaluation data and a Student's t-test for the other parameters.

Statistical tests were conducted using R version 4.3.1 and GraphPad Prism version 8.3.0.

RESULTS

In both male and female parents, food consumption was lower in the three groups exposed to fluoride before and during mating compared to the control group (C). Additionally, the groups exposed to 100 and 150 ppm F- showed lower water consumption in the two weeks before and during mating. However, no statistically significant differences in body weight were observed among the different study groups during treatment, and no changes in teeth were detected.

The groups exposed to F- had a lower number of pups born alive per litter, as shown in Figure 1. This difference was statistically significant in the F150 group compared to C ($p<0.05$). Additionally, there was a negative correlation between the concentration of F- in the drinking water and the number of pups born alive ($r=-0.74$, $p<0.001$).

In the F150 group, three additional females were assigned to obtain more litters and complete the 12 males per group. This was necessary because the pregnancy success and number of live births per litter were lower than in the other groups. The pregnancy success rate in this group was 66.67%, compared to 100% in the other groups of this study.

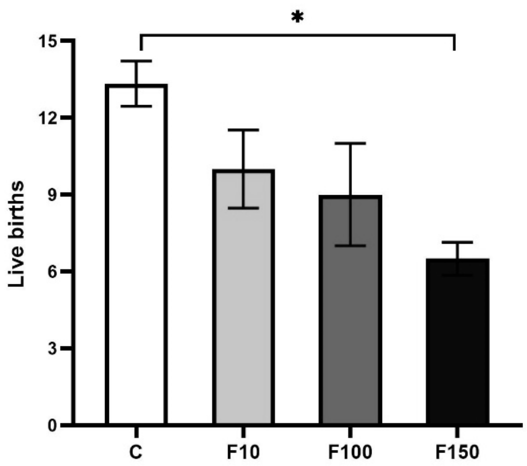

Fig. 1. Live births per litter
Each bar represents M ± SEM, *n=3* for C, F10 and F100; *n=4* for F150. * *p*<0.05 compared to C.

Figure 2 displays the weaning weights of males. Groups F10 and F100 exhibited lower weaning weights than C (*p<0.001*).

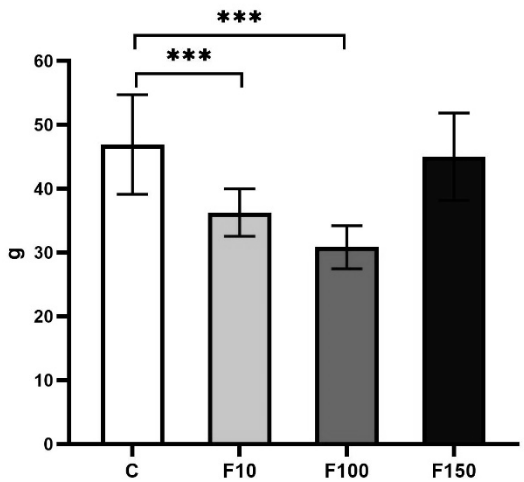

Fig. 2. Weaning weights of the offspring males
Each bar represents M ± SEM, *n=12*. *** *p*<0.001 compared to C.

At week 8 postweaning, F10 showed a significantly lower food intake than C (*p<0.05*), and F100 showed a statistically significant decrease (*p<0.05*) compared to C at weeks 2 and 6 postweaning. Conversely, F150 exhibited a significantly lower food intake than C (*p<0.05-0.001*) at weeks 6-10 postweaning (see Fig. 3A).

At week 7 PW, water consumption (Fig. 3B) was significantly lower (*p<0.05*) in F10 and F100 offspring compared to C. Additionally, F150 showed significantly lower consumption (*p<0.05-0.001*) compared to C at weeks 7-10 PW.

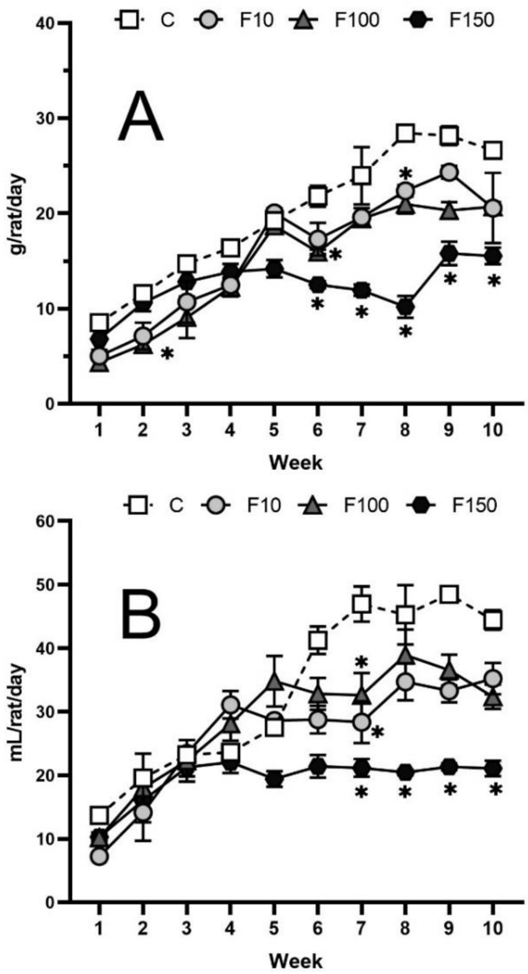

Fig. 3. Food (A) and water (B) consumed by offspring over 10 weeks PW.
Each point represents the estimated average weekly consumption per rat over 24 hours ± SEM, *n=12*. * *p*<0.05 compared to C

Feed intake was slightly lower in the groups exposed to F- from weaning throughout the 10 weeks post-weaning (PW), but this was statistically significant only in the groups exposed to 10 ppm F- at week 6 PW (*p*<0.01) and at week 8 PW in the animals exposed to 100 ppm F- (*p*<0.01). On the other hand, water consumption was lower in the groups exposed to F- from the prenatal stage, but significant differences (*p*<0.01) were observed only in the groups treated with 10 ppm F- at week 3 PW.

Throughout the treatment, the highest fluoride-treated groups had a significantly lower average body weight (*p*<0.05) compared to the C group (Fig 4). Additionally, F10, F100, and F150 had lower total weight gain (17.0%, 14.9%, and 46.5%, respectively) compared to C (*p*<0.05-0.001).

Rats that were exposed to 10 and 100 ppm F- from the prenatal stage had a significantly lower mean body weight compared to groups that were exposed to the same dose from weaning at weeks 2-5 (p<0.05-0.001) and 1-2 PW (p<0.05-0.001), respectively. Rats that were exposed to 150 ppm F- from the prenatal stage had a higher mean body weight compared to those that started treatment from weaning at weeks 1-3 PW (p<0.05-0.01).

Table 2 shows the total weight gain of the groups exposed from gestation and from weaning onwards. A significant difference was found in the group exposed to 100 ppm F- (p<0.05).

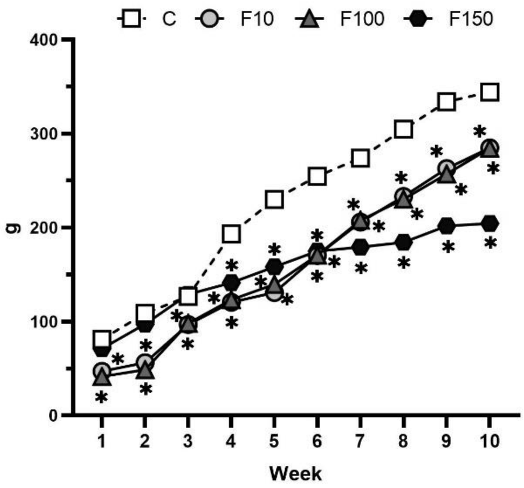

Fig. 4. Body weight of offspring from weaning to the end of treatment

Each point represents M ± SEM, *n=12*. * p<0.05 compared to C.

Table 2. Total weight gain (M ± SEM), *n=12*.

F- (ppm)	Beginning of exposure		p
	Prenatal	After weaning	
10	248.7 ± 5.2	254.8 ± 6.7	0.476
100	253.5 ± 6.8	206.2 ± 19.2	0.036*
150	159.3 ± 17.2	153.8 ± 21.1	0.842

* p<0.05 compared to the group at the same concentration after weaning.

In F100 and F150, changes in the incisors were mainly characterized by loss of enamel coloration, overgrowth, and tooth fractures.

The lesions were observed in a concentration-dependent manner, as shown in Fig 5. Group C did not exhibit any changes in their teeth (Fig 6A-B), while F10 showed mild lesions (score 0-1) (Fig 6C). On the other hand, F100 (Fig 6D) and F150 (Fig 6E-F) had significantly higher dental assessment scores (2-3 and 3-5, respectively) compared to Group C (p<0.001).

The dental evaluation results are comparable to the study where exposure began after weaning. However, the group exposed to 100 ppm F- showed a significantly higher score ($p<0.05$) than the group exposed to the same concentration of F- since the prenatal period (Fig 5).

Furthermore, a negative correlation was found between the dental score and total body weight gain in the rats with prenatal exposure (rho=-0.82; $p<0.001$) and after weaning (rho=-0.72; $p<0.001$).

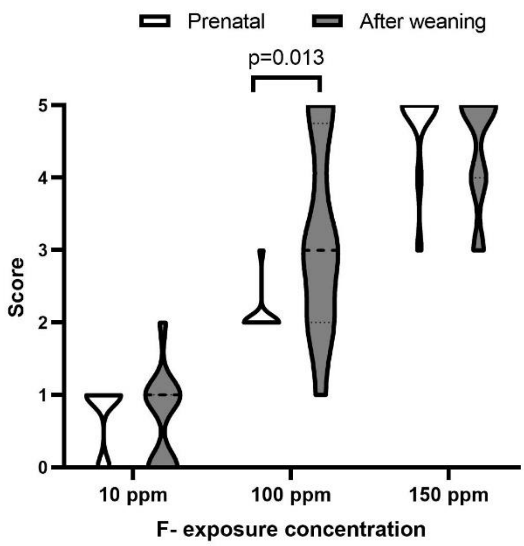

Fig. 5. Dental evaluation at 90 days of age

Comparison of groups exposed to the same F- concentration from gestation and after weaning. Median and range are shown, *n=12*.

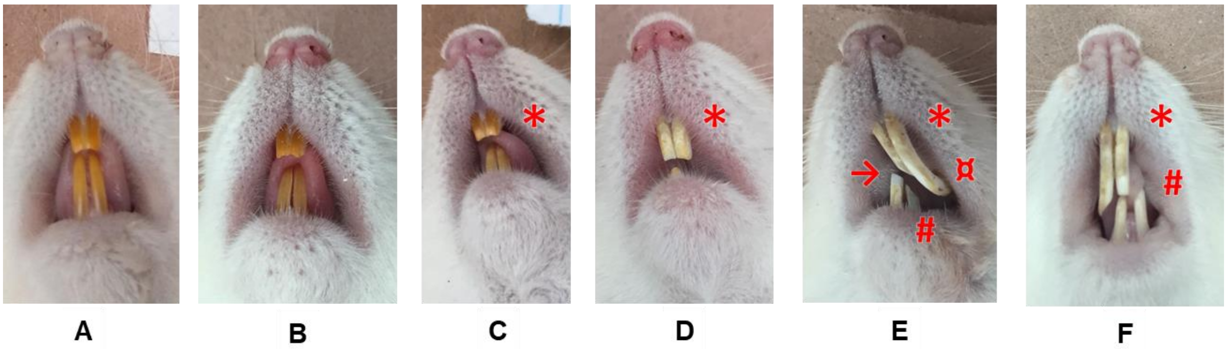

Fig. 6. Dental lesions in the offspring of the different exposure groups
A and B. Group C and with normal incisors; **C.** Predominant lesions in group F10; **D**. Predominant lesions in group F100; **E and F.** Predominant lesions in group F150. ***loss** of enamel coloration; **→**overgrowth; **#** fracture; **¤** abnormal curvature.

The concentration of serum F- was significantly higher (*p*<0.01) in F100 and F150 (0.076±0.007 and 0.318±0.067, respectively) compared to C (0.031±0.002). Additionally, F150 had a higher serum F- concentration (*p*<0.001) compared to F100 and F10 (0.060±0.005). Serum F- concentration increased in a dose-dependent manner. However, in the group exposed to 100 ppm F-, the increase in serum fluoride concentration was significantly higher (*p*<0.05) in the group exposed after weaning (Fig 7).

There was a positive correlation found between serum F- concentration and the severity of dental lesions in the rats with prenatal exposure (rho=0.92, *p*<0.001) and after weaning (rho=0.88, *p*<0.001).

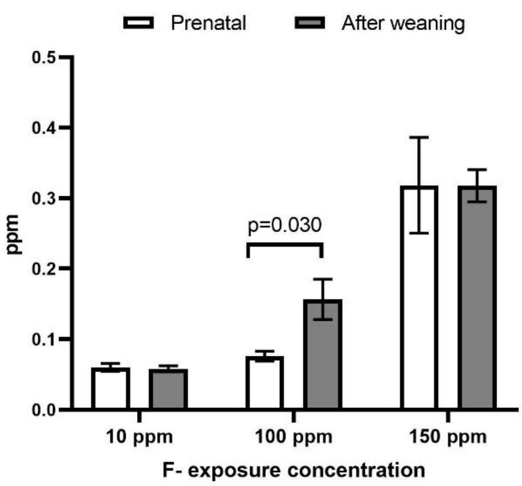

Fig. 7. Serum F-concentration at 90 days of age
Comparison of groups exposed to the same F-concentration from gestation and after weaning. Each bar represents M ± SEM, *n=6* for prenatal exposure; *n=7* for after weaning exposure.

DISCUSSION

The present study employed concentrations of 10, 100, and 150 ppm F-. The use of high concentrations of F- for studying its toxic effects in rodents is a subject of debate in the literature. It is argued that such concentrations of F- are rarely found in water for human consumption, even in areas with endemic fluorosis[2]. However, studies have shown that rats require ingesting 10 to 20 times higher concentrations of F- than humans to reach similar circulating levels of F-[17–20].

Similarly, the correlation between serum F- concentration and the severity of incisor lesions observed in this study indicates that the manifestation of dental lesions is closely related to the amount of circulating F-[21]. According to Bronckers et al. (2009), changes in teeth occur in both rats and humans at similar serum F- concentrations in both species[19]. This suggests that in rats, as in humans, the same level of F- accumulation is required in other tissues for toxic effects to occur. This explains why such high concentrations of F- are used when rodents are used as experimental subjects.

The present investigation's results indicate that parents exposed to F- experienced a decrease in water and food consumption, as well as a reduction in the number of live births and lower weaning weights of their offspring.

Previous studies have reported that F- treatment in rats leads to a decrease in pregnancy success rates, a lower number of viable fetuses, and an increase in resorptions[22,23]. The toxic effects of this element on the female reproductive system have been attributed to structural changes in the uterine tissue and ovaries, as well as a deterioration of the follicles and a decrease in sex hormone concentration. In male animals exposed to F-, alterations in sperm density and motility have been reported, resulting in poor fertilization and embryonic development[10].

In this study, the weaning weight of the group exposed to the highest concentration (150 ppm F-) was not affected compared to the other exposure groups. This may be because the litters in this group were smaller than in the others due to the decrease in live births, which favored that the offspring had less competition for maternal milk. However, the offspring of this group showed a reduction in food and water consumption compared to the C group throughout the treatment after weaning. This reduction was observed in the F10 and F100 groups only during certain weeks.

The groups exposed to the highest doses of F- showed a decrease in water and feed consumption compared to the control group, both in animals exposed since gestation and in those that began treatment at weaning. However, this effect was greater in those that did not have prenatal exposure at the 100 ppm F- dose.

The reduction in food and water consumption is in line with reports from authors who have administered F- for extended periods[16,22,24]. F- ingestion has been shown to have effects on the gastrointestinal tract, including gastritis, nausea, delayed gastric emptying, and damage to the gastrointestinal mucosa. These effects lead to a decrease in appetite and, consequently, a reduction in intake[16,25].

In all groups treated with F-, there was a decrease in body weight gain during treatment, with the most significant decrease observed in the F150 group. These animals had lower weaning weights due to prenatal exposure to F- and continued consumption of F- after weaning. This was caused by the effects of F- on the gastrointestinal tract and alterations in the teeth, which made feeding difficult. However, the study only found lower body weight in the first weeks of post-weaning exposure compared to animals exposed to the same doses during gestation. As the treatment progressed, the groups treated with the highest doses not exposed prenatally showed a lower body weight compared to those exposed during gestation, particularly in the groups that consumed 100 ppm F-.

Changes in enamel are the most obvious indicator of chronic F- intoxication. The presence of F- during the enamel maturation phase leads to hypomineralization of the tooth[20]. The rats in this study also showed overgrowth of their incisors, likely due to the lack of wear that should occur in rodents as a result of reduced food consumption. This overgrowth made feeding more difficult, resulting in lower weight gain. However, according to Amira et al. (2005), F- has been reported to decrease nutrient absorption in the intestine due to morphological changes in the mucosa and an increase in intestinal motility[25]. Additionally, at the cellular level, F- has adverse effects on protein synthesis, which ultimately leads to a decrease in weight gain in individuals[16].

In this investigation, animals treated with F- showed a dose-dependent increase in serum fluoride concentration. According to Buzalaf and Milton (2011), most of the absorbed F- is deposited in calcified tissues, with a smaller amount in soft tissues. The concentration of F- in the bloodstream is dependent on the time elapsed since ingestion, as well as the dynamics of F- in the deposited tissues, as it does not bind permanently and can return to circulation. Thus, it is suggested that circulating F- maintains a different ratio with the accumulated depending on the type of tissue, making it a reliable biomarker of exposure[26].

In animals exposed to F- both the degree of dental lesions and serum F- concentration were dose-dependent and the positive correlation obtained between these two parameters in both treatment initiation schedules confirms that the severity of the changes in the teeth is indicative of the degree of exposure of the individual as described by other authors[3,27]. The study found that rats exposed to 100 ppm F- from weaning had higher serum F- concentrations and dental evaluation scores compared to those exposed to the same concentration from gestation.

The observed differences between groups exposed to the same concentrations from gestation and weaning onwards are mainly found in animals exposed to 100 ppm F-. These findings suggest that subjects exposed to F- since gestation may be susceptible to adverse effects early on and may go through a stage of adaptation to the element that allows them to develop tolerance to its harmful effects. As a result, they may present a lower effect in the postnatal stage. However, animals exposed to fluoride after weaning may experience adverse effects due to the accumulation of F- in their bodies. It seems that their ability to adapt and tolerate the element may be lower than that of animals exposed to it prenatally.

Nevertheless, these differences were not observed in the groups exposed to the highest concentration of 150 ppm F-. This suggests that the adaptive capacity mentioned earlier will be reduced as the concentration of F- ingested increases. No other studies were found that evaluated the harmful effects of chronic F- consumption in adult subjects exposed before or after gestation and lactation in a comparative manner. Further studies are necessary to verify the potential adaptation to this toxicant and the underlying mechanisms.

Ni et al. (2020) and Zhang et al. (2023) suggest that fluoride-resistant cells can be obtained in vitro by exposing them to fluoride concentration gradients[28,29]. It is hypothesized that a similar mechanism may occur in vivo, where exposed mothers pass small amounts of fluoride to their offspring through the maternal-fetal circulation and subsequently in breast milk, leading to resistance or adaptation and attenuating the toxic effects.

CONCLUSIONS

Exposure to high concentrations of F- had a negative impact on the number of live births per litter and male weaning weights. Additionally, the offspring experienced concentration-dependent changes in feed intake, water consumption, body weight, incisor integrity, and serum fluoride concentration. At the 100 ppm dose of F-, differences were observed between animals exposed since gestation and those that began exposure after weaning. This suggests the initiation of possible adaptive mechanisms to F- exposure in subjects exposed from the prenatal stage, possibly tending to reduce the harmful effects of F-. However, further studies are required to verify the possible adaptation to this toxicant and to understand the cellular mechanisms involved.

This study presents evidence of the negative impact of high concentrations of F- on adult individuals, including reproductive and developmental effects in those exposed during gestation and continuing chronic consumption into adulthood.

Furthermore, the study highlights that the effects of chronic F- exposure vary depending on the developmental stage of the individual at the time of exposure.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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