[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

NUTRITIONAL, BIOCHEMICAL, AND HISTOPATHOLOGICAL STUDY ON DOSE AND TIME DEPENDENT EFFECTS IN FLUORIDE EXPOSED RATS

Vakdevi Validandi,[a,*] Arjun L Khandare,[a] Srinivas Dheeravath,[a] Sureka Mullapudi Venkata,[b] Srinivasu Kurella,[a] Sukesh Narayan Sinha[a]

Hyderabad, India

ABSTRACT: Aim of this study was to observe the effect of fluoride (F) in Wistar rats in a dose and time-dependent manner. Male rats (n=36) were taken and were randomly divided into six groups (group I: control, group II: 5 mg/L F, group III: 10 mg/L F, group IV: 15 mg/L F, group V: 50 mg/L F, and group VI: 100 mg/L F). Animals were examined at 3-time points of 30, 60, and 90 days for F toxicity changes. The results indicated that the F induced dose and time-dependent diet intake, body weight gain, food efficiency ratio, dental fluorosis, F level in urinary, serum, bone and teeth, and histopathological changes in the tissues of liver and kidney. The significant increase in exposure of F through water in groups V and VI as compared to other groups has shown alterations in their food efficiency ratio, body weight gain, and the significant increase in the urinary, serum, teeth, bone, liver and kidney F levels. Histopathology of the kidney revealed few dilated tubules in the kidney of animals of group VI as compared to animals of all other groups. Whereas histopathology of the liver showed microvacuolation in groups V and VI compared to groups I, II, and IV. However, there were no significant differences between the organ weight ratios among all the groups. In conclusion, the dose-dependent effect of F starts at 100, 50, and 15mg/L from first, second, and third months, respectively.However, there were no significant differences in 5 and 10 mg/L F groups after 3 months.

Keywords: Biochemical; Dose and time dependent effects; Fluoride; Fluorosis; Focal fibrosis; Histopathological; Microvacuolation; Nutritional.

INTRODUCTION

It is well established that an over exposure of fluoride (F) through fluoridated drinking water, industrial F pollution and any other F containing sources causes serious fluorosis disease not only in humans[1-3]but also in the domesticated animals.[4-6] Fluorosis is worldwide health problem and endemic in more than twenty five countries. Excess accumulation of F in teeth, bones and soft organs causes mild to severe pathological changes in them which are generally referred as dental, skeletal and non-skeletal fluorosis, respectively. The risk of developing fluorosis in an individual is minimal if exposed to 0.05-0.07 mg F/kg bodyweight by daily oral ingestion.[7, 8]The F doses of 16-64 mg/kg was found to be fatal in adults whereas, 3-16 mg/kg was harmful in neonates.[9]

The earlier studies revealed that the excess ingestion of F impairs nutritional status[10-12] like stunting.[10, 13]The effect of F on body weight gain (BWG) is still a controversial. In F-fed animals, reduced food intake and BWG have been observed in rats, rabbits, hens, and calves.[14-17] Other investigations, however, found no difference in BWG in F-fed mice.[18,19] This inconsistency could be attributable to the different species employed, the dose of F, and the length of the study. Fluoride exposure causes oxidative stress, which can lead to a reduction in growth and food utilization efficiency.[20] There are few investigations on the food efficiency ratio

[a]Food Safety and [b]Pathology Division, ICMR-National Institute of Nutrition, Hyderabad 500 007, India; *Corresponding author: Dr Vakdevi Validandi, Scientist- D, Food Safety Division, ICMR-National Institute of Nutrition, Hyderabad500 007, India; Telephone No. 040 27197391; Fax No: 040-27019074; E mail ID: vaagdevi@gmail.com

(FER), which is an important indicator of the food converted into BWG.[21, 22]Exposure to F resulted in the increased concentration of F in the blood serum and urine, [23-25]accumulation of F in the tissues and organs[23]and histopathological changes in the kidney and liver.[26]

Although there are studies in the literature on the nutritional status, F in urine and serum, and histopathological changes in the diverse organs in an F dose dependent manner, but a single comprehensive study on effect of different doses of F and different exposure periods on various nutritional, biochemical and histopathological parameters has not been studied so far.Therefore, the present study was undertaken to observe the nutritional, biochemical, and histopathological effects or changes in relation to dose and time dependent in F exposed rats.

MATERIALS AND METHODS

*Experimental animals***:** One month old thirty six male Wistar rats weighing 104.95±7.901 g (mean±SD) were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India, and were randomly distributed into 6 groups of 6 animals each and their weight was not significantly different among the groups. The feeding schedule is given in Table 1.

Table 1. Distribution of animals and feeding schedule of different groups

S.No	Group	N	Treatment
1	I	6	Normal diet and water ad libitum
2	II	6	Fluoride 5 mg/L in drinking water, normal diet ad libitum
3	III	6	Fluoride 10 mg/L in drinking water, normal diet ad libitum
4	IV	6	Fluoride 15 mg/L in drinking water, normal diet ad libitum
5	V	6	Fluoride 50 mg/L in drinking water, normal diet ad libitum
6	VI	6	Fluoride 100 mg/L in drinking water, normal diet ad libitum

All the animals were individually kept in stainless steel cages in a temperature and humidity-controlled environment with a 12-hour light/dark cycle. The Institutional Animal Ethics Committee (P9F/I-IAEC/NIN/2016/1/VAK/WNIN-38M) approved the animal care and experimental protocols, which were followed.

Food and water intake, bodyweight gain (BWG), and food efficiency ratio (FER): Food and water intake, bodyweight gain, and FER was assessed according to our earlier study.[21]

F content in urine, serum, bone, and teeth: An ion-selective electrode (Model EA 940 Orion) was used to assess urinary and serum F levels.[27] Teeth and bone F were estimated as previously mentioned.[28]

F in tissue: The tissue F levels in liver and kidney in group V and VI were determined as reported in earlier study.[23]

Dental fluorosis: Rats were assessed for dental fluorosis at 3rd month. The determination of dental fluorosis was made clinically over the entire lower incisor tooth surface according to modified Thylstrup-Fejerskov TF index.[29, 30]

Organ weight ratio: The brain, kidney, liver, testis, and heart were all collected and placed individually on pre-marked filter paper after the animals were euthanized by CO_2 inhalation. The wet organ weights were immediately weighed using Sartorius balance (BL 600, Germany, sensitivity of the balance was 0.001 g). The individual organ weight was divided by total body weight in grams and multiplied by 100 [(Organ weight/Body weight)×100=%ratio].[31]

Histopathology: Immediately after removal of the brain, kidney, and liver tissues, they were placed in 10%formalin solution and processed as per standard method[32]for histological examination.

RESULTS

Food and water intake, BWG, and FER: There was a significant decrease in food intake in animals of the group VI compared to all other groups at 3 month (Figure 1).

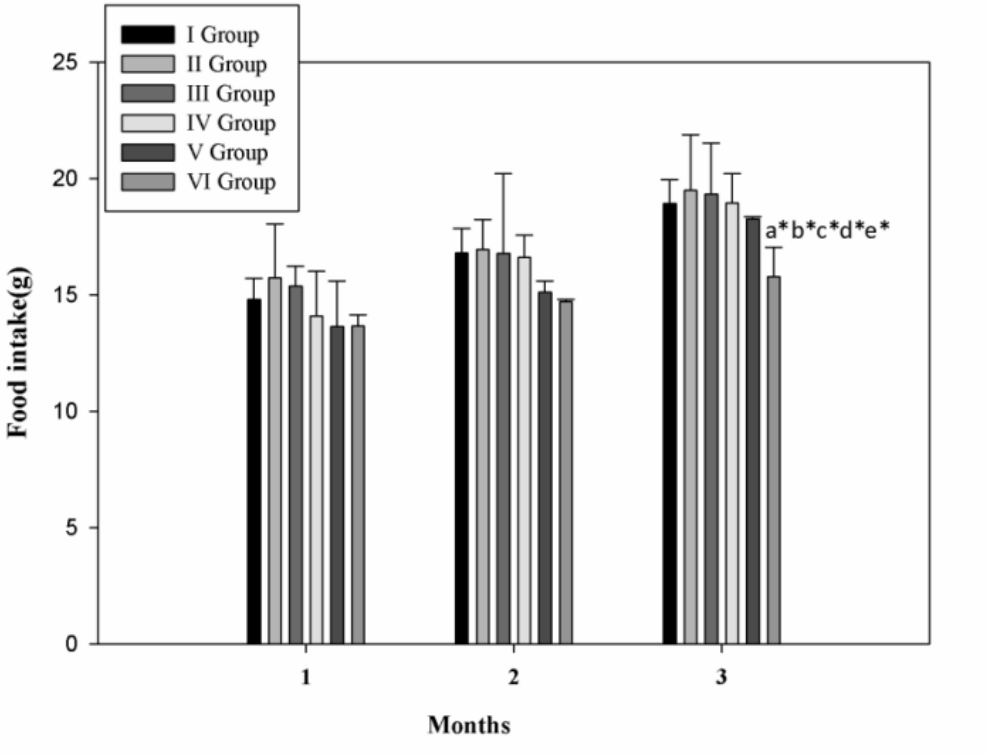


Figure 1. Food intake by rats of different groups at 1, 2, and 3 months. *Statistically significant ($p<0.05$). a=compared to group I, b=compared to group II, c=compared to group III, d=compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/group.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

The water intake in different groups is given in Figure. 2. A significant decrease in water intake was noticed in group III compared to group I at 3 month. A significant decrease in water intake was also noticed in animals of the group V and VI compared to group I, II, III, and IV at 2 and 3 months.

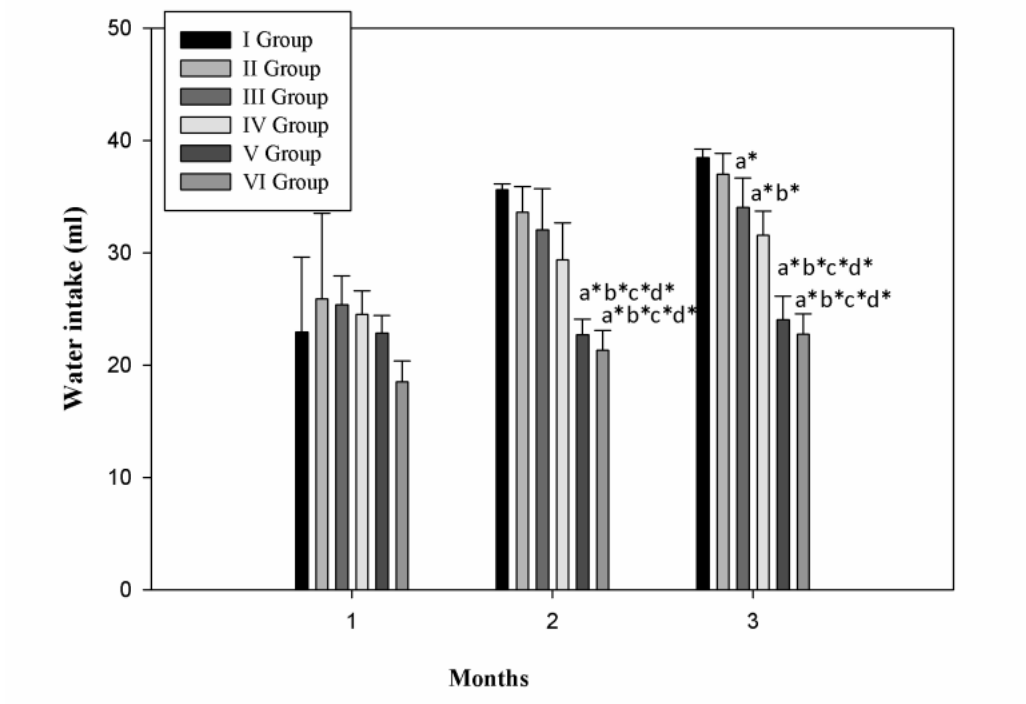


Figure 2. Water intake by rats of different groups at 1, 2, and 3 months.*Statistically significant ($p<0.05$). a=compared to group I, b=compared to group II, c=compared to group III, d=compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/group.

The BWG and FER in different groups is given in Tables 2 and 3, respectively.

Table 2. Body weight gain of rats at 1, 2, and 3 months by different groups

Group	Initial weight (g)	Body weight gain 1st month (g)	Body weight gain 2nd month (g)	Body weight gain 3rd month (g)
I	101.27 ± 9.34	115.07 ± 21.40	166.53 ± 29.58	202.40 ± 34.36
II	103.27 ± 6.98	115.73 ± 27.78	172.68 ± 37.42	202.57 ± 37.78
III	103.98 ± 6.91	112.18 ± 12.51	157.80 ± 15.52	184.35 ± 23.15
IV	104.78 ± 6.52	106.22 ± 32.88	149.52 ± 20.38	154.62 ±18.00^{a*b*}
V	105.38 ±6.85	87.78 ± 21.76	122.15 ± 25.07^{a*b*c*}	132.12 ±19.81^{a*b*c*}
VI	106.42 ± 8.65	70.75 ± 26.91^{a*b*c*d*}	85.92 ± 27.37 ^{a*b*c*d*e*}	111.25 ±13.16^{a*b*c*d*}

*Statistically significant. a=compared to group I, b=compared to group II, c=compared to group III, d=compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/group.

There was a significant decrease in BWG in the group V and VI compared to groups I, II, III and IV at 1, 2 and 3 months. A significant decrease in FER was also

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

observed in group V and VI compared to group I at 1month. A significant decrease in FER was observed in group IV and V compared to group I at 3 months.

Table 3. Food efficiency ratio (FER) of rats at 1, 2 and 3 month in different groups

Group	1 month	2 month	3 month
I	0.26 ± 0.05	0.35 ± 0.03	0.07 ± 0.03
II	0.22 ±0.06	0.32 ±0.03	0.05 ± 0.02
III	0.23 ±0.03	0.29 ±0.04	0.04 ± 0.02
IV	0.23 ±0.04	0.32 ±0.08	0.01 ± 0.02 ^{a*b*}
V	0.20 ±0.04 ^{a*}	0.29 ±0.05	0.02 ±0.03 ^{a*}
VI	0.16 ±0.03 ^{a*b*c*d*}	0.28 ±0.08 ^{a*}	0.06 ±0.05 ^{d*e*}

*Statistically significant. a=compared to group I, b=compared to group II, c=compared to group III, d= compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/group.

Urinary F: The urinary F levels in different groups are given in Figure 3. A significant increase in urinary F levels was observed as increasing of F dose and duration dependent manner.

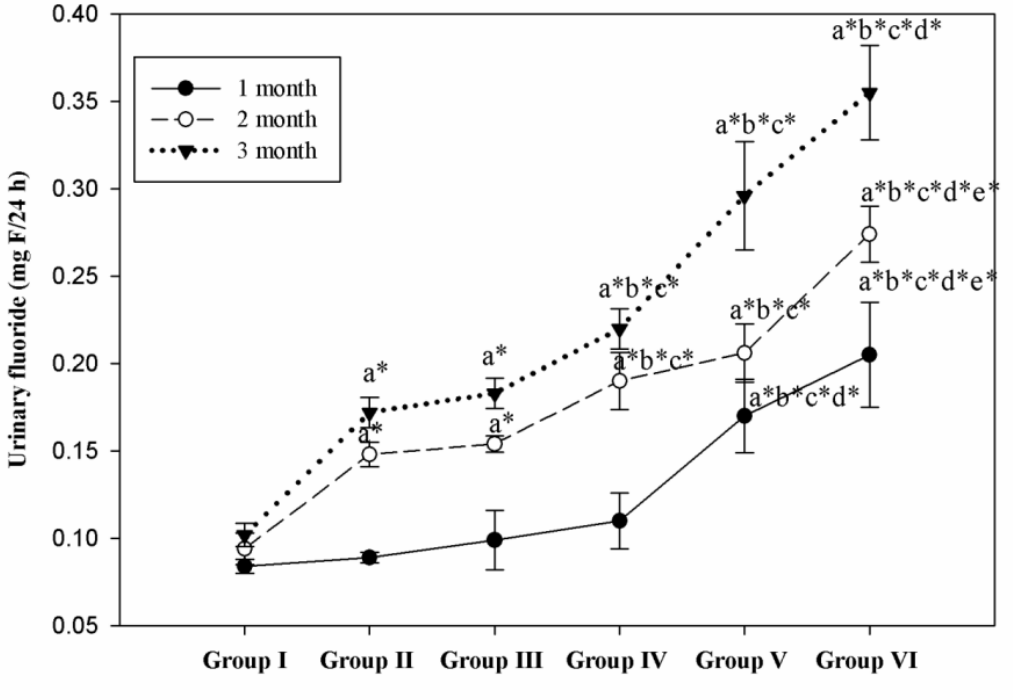


Figure 3. Urinary fluoride (mg F/24hr) levels in different groups at 1, 2, and 3 months.*Statistically significant ($p<0.05$). a=compared to group I, b=compared to group II, c=compared to group III, d=compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/ group.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

Serum F: There was a significant increase in serum F levels in group II, III, IV, V, and VI compared to group I at 1, 2 and 3 months (Figure 4). There was a significant increase in serum F levels in the group V compared to group I, II, and III at 1, 2, and 3 month. There was a significant increase in serum F levels in the group VI compared to group I, II, III, and IV at 1, 2 and 3 months (Figure 4).

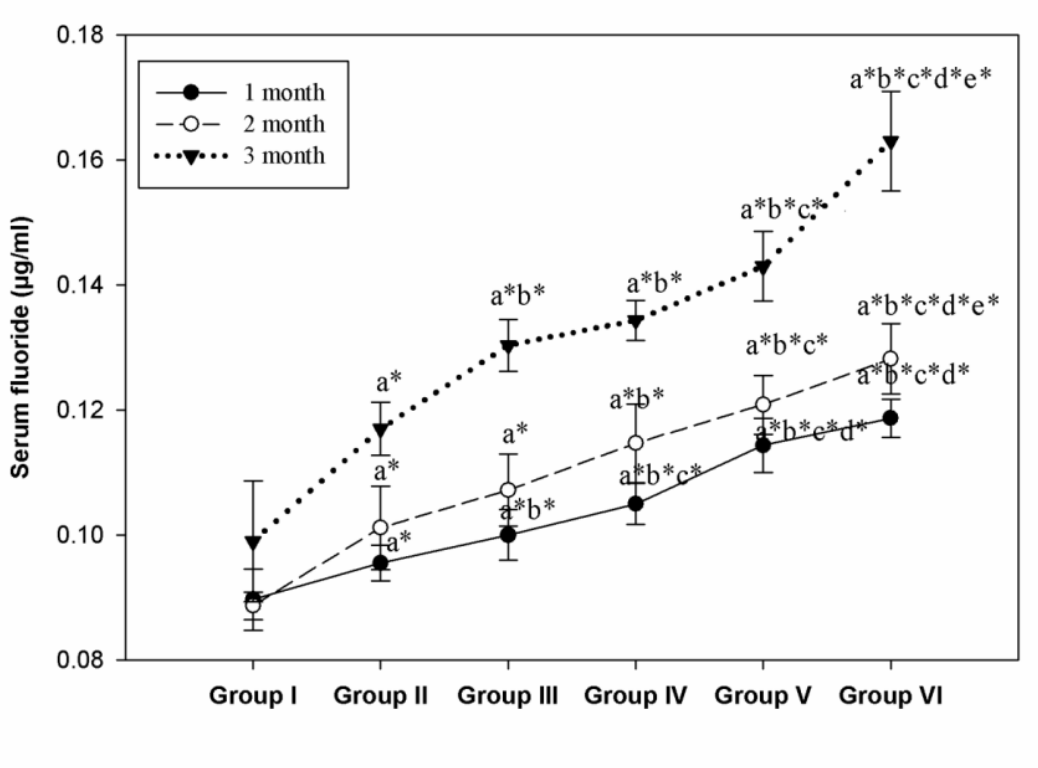


Figure 4. Serum fluoride (µg/ml) levels in different groups at 1, 2 and 3 months.*Statistically significant ($p<0.05$). a=compared to group I, b=compared to group II, c=compared to group III, d=compared to group IV, e=compared to group V. Results are expressed as mean±SD; n= 6 rats/group.

F in bone and teeth: A dose dependent increase in the bone and teeth F levels were observed in the study. There was a significant increase in the bone and teeth F levels in the group IV compared to group I. There was a significant increase in bone and teeth F levels in the group V and VI compared to group I, II, and III (Figure 5).

F in liver and kidney: There was a significant increase in F accumulation in the liver in the group VI compared to group I and V. An F dose dependent increase in the accumulation of F in the kidney was observed. However, there is an increase in the F accumulation in the kidney in the group V and VI compared to group I but not significant (Table 4). Since there was no significant difference in the tissue F levels in the liver and kidney in group V compared to group I, hence, the tissue F levels in the group II, III and IV were not determined.

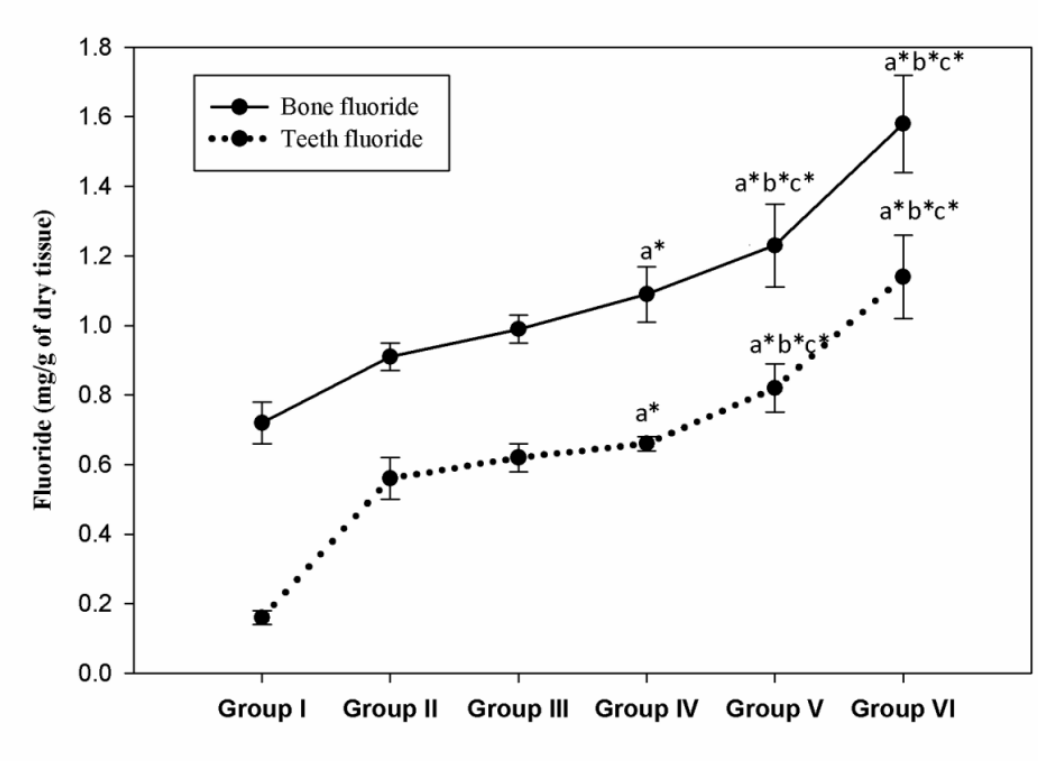


Figure 5. Bone and teeth fluoride levels in the different groups at 3 months. *Statistically significant ($p<0.05$). a=compared to group I, b=compared to group II, c=compared to group III. Results are expressed as mean±SD; n= 6 rats/group.

Table 4. Accumulation of fluoride (F) in the liver and kidney tissue

Group	Liver F (mg F/g tissue)	Kidney F (mg F/g tissue)
I	0.3167±0.04320	2.9960±0.88401
IV	ND	2.5800±0.78823
V	0.3100±.03082	3.3400±0.24980
VI	0.7925±0.19805^{a*b*}	3.7300±0.38184

ND – Not determined.
*Statistically significant. a=compared to group I, b=compared to group II. Results are expressed as mean±SD; n= 6 rats/group

Dental fluorosis: An F dose dependent increase in the severity of dental fluorosis was observed in the study. The mild dental fluorosis was observed in group V and VI at 3 month which is shown in Figure 6. Dental fluorosis in rats was characterized with modified Thylsrup-Fejerskov TF index.[29, 30]

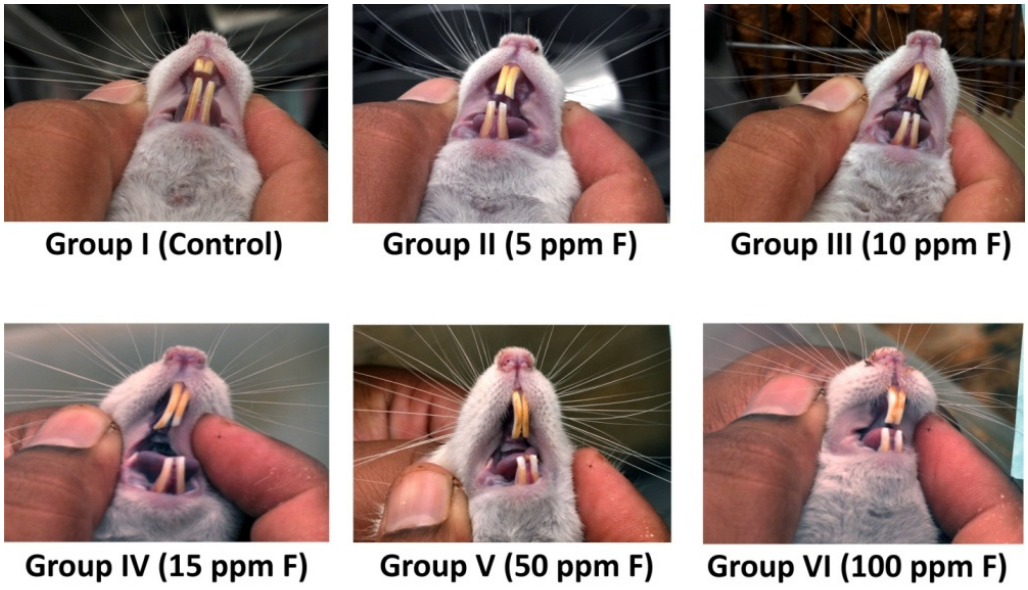


Figure 6. Dental fluorosis in rats at 3 months in different groups characterized with modified Thylsrup-Fejerskov TF index.[29, 30]

Organ weights: There were no significant difference was found among all the groups in organ weight ratio for brain, kidney, heart, testes, and liver (Figure 7).

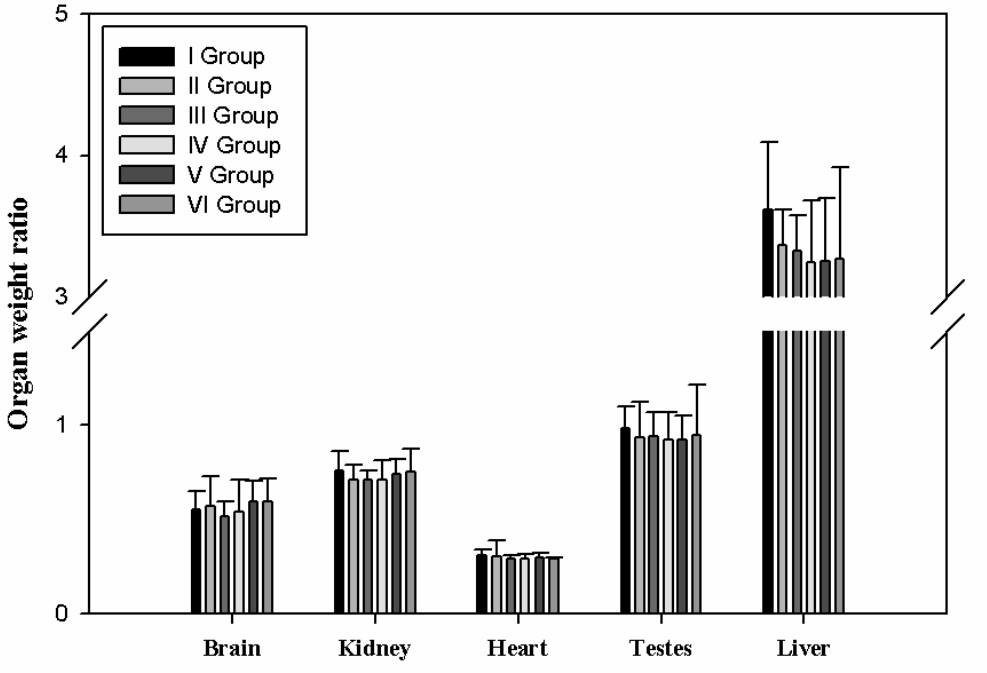
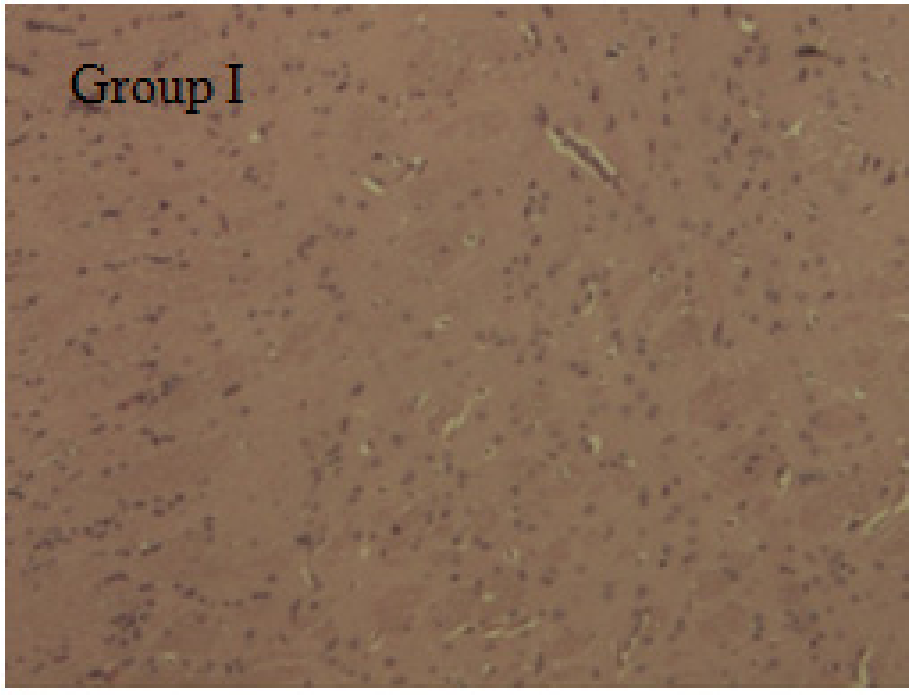


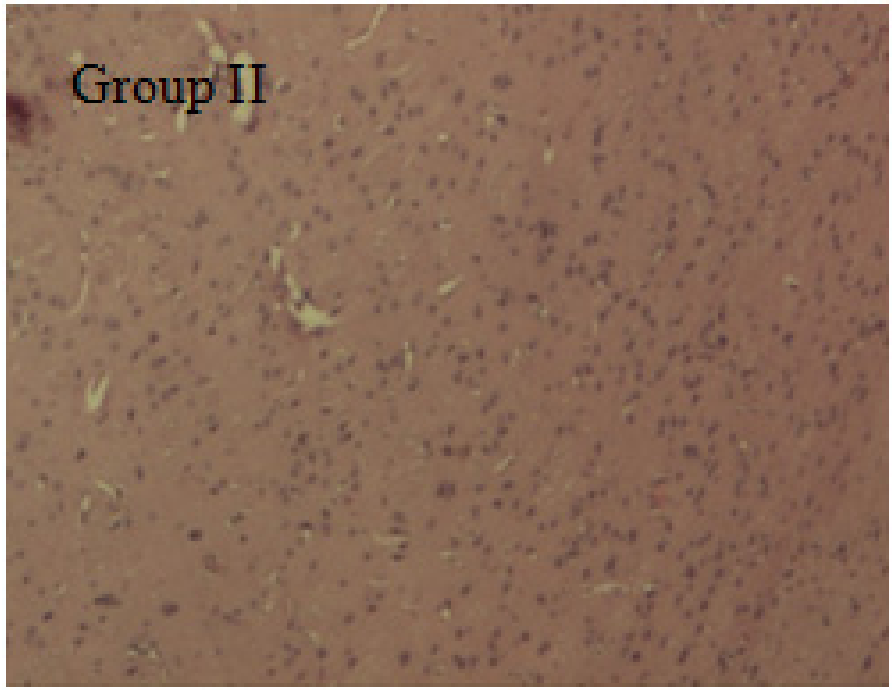
Figure 7. The organ weight ratio of brain, kidney, heart, testes, and liver among different groups at 3 months.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

Histopathology: The dose dependent changes due to F were studied in the histopathology of brain, kidney, and liver. We did not observe any histopathological changes in the brain among all the groups (Figures 8A–8F).



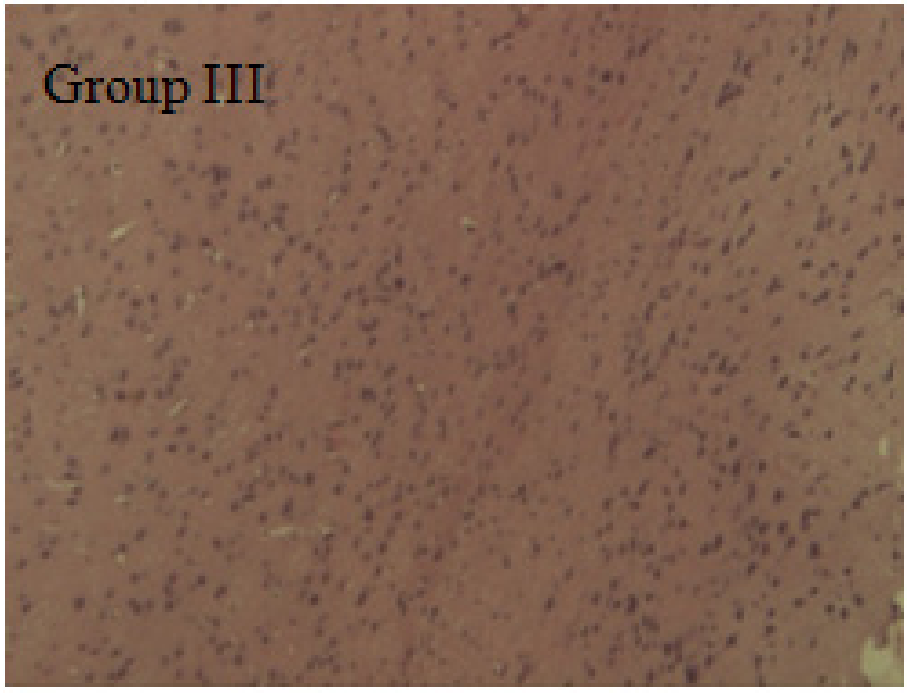
8A



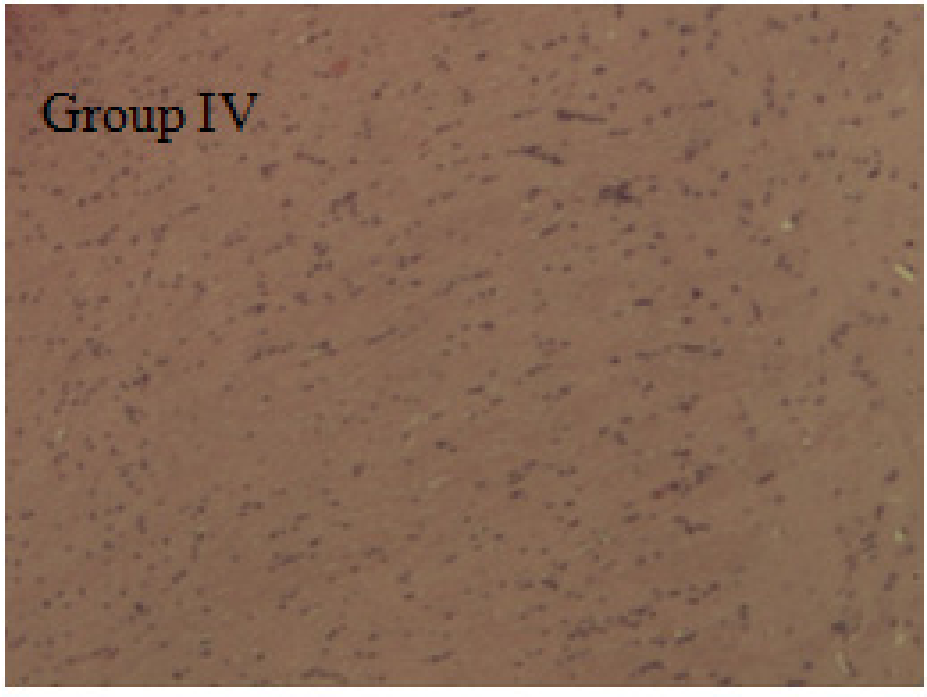
8B

Figures 8A and 8B. Histopathology of brain (H&EX 10) from rat exposed to different F doses for 3 months. 8A: Group I; 8B: Group II.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at
www.fluorideresearch.online/epub/files/189.pdf]



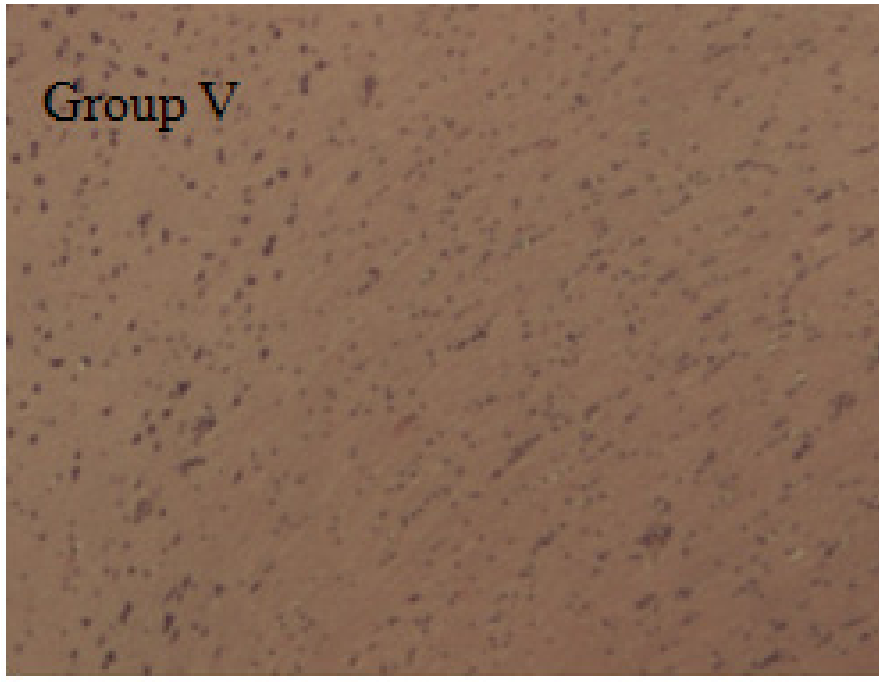
8C



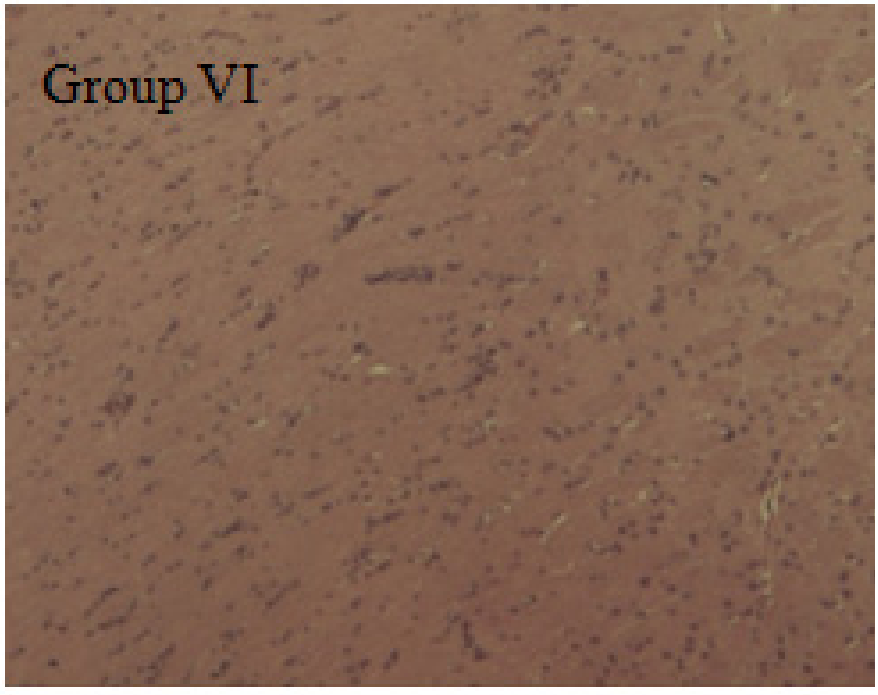
8D

Figures 8C and 8D. Histopathology of brain (H&EX 10) from rat exposed to different F doses for 3 months. 8C: Group III; 8D: Group IV.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]



8E

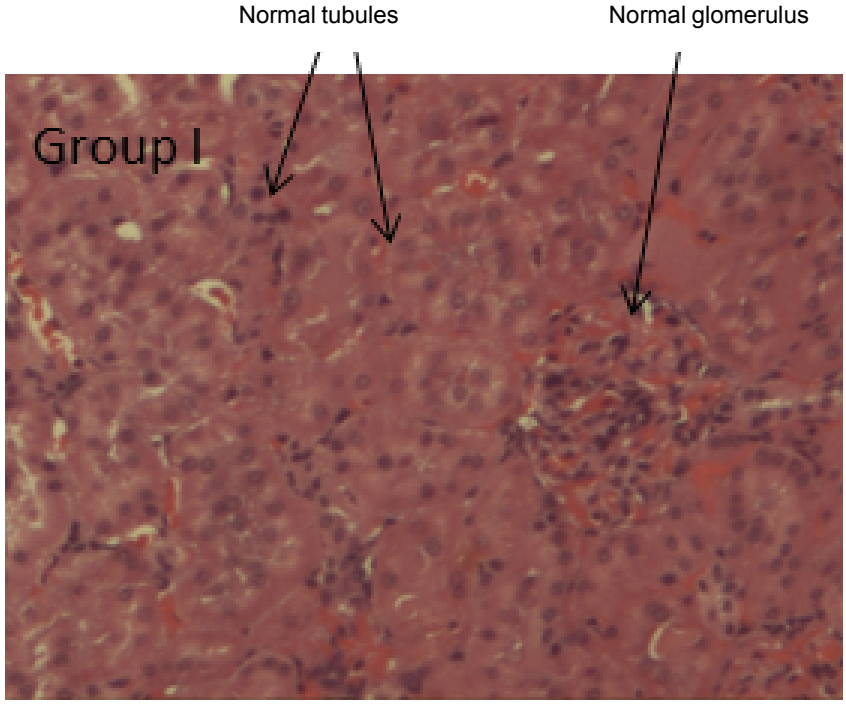


8F

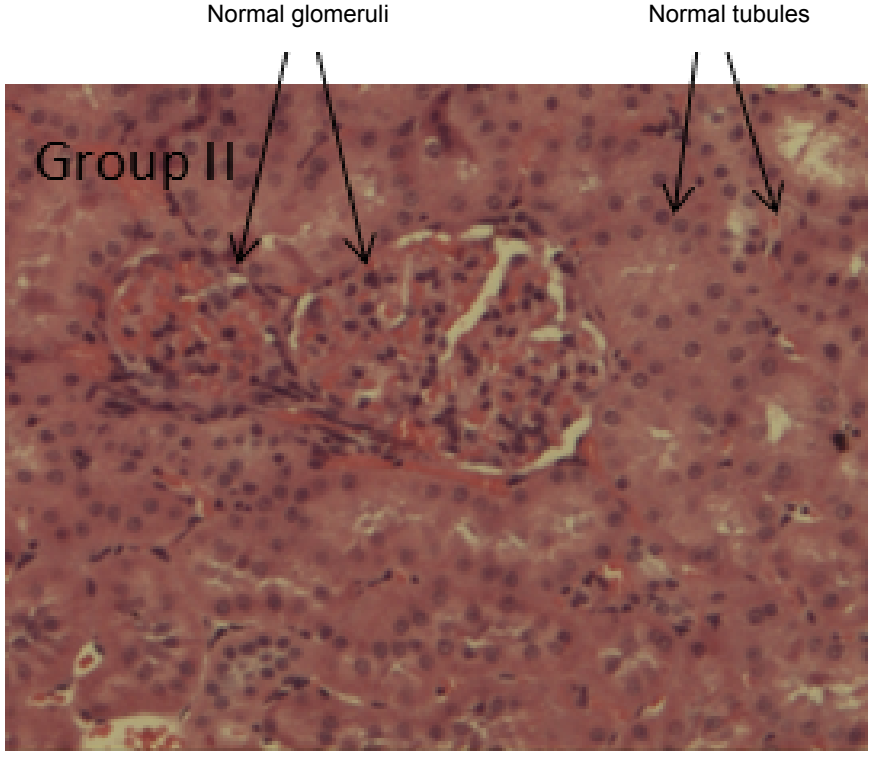
Figures 8E and 8F. Histopathology of brain (H&EX 10) from rat exposed to different F doses for 3 months. 8E: Group V; 8F: Group VI.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at
www.fluorideresearch.online/epub/files/189.pdf]

However, in kidney few focal dilated tubules were observed in group VI compared to all other groups (Figure 9A–9F).



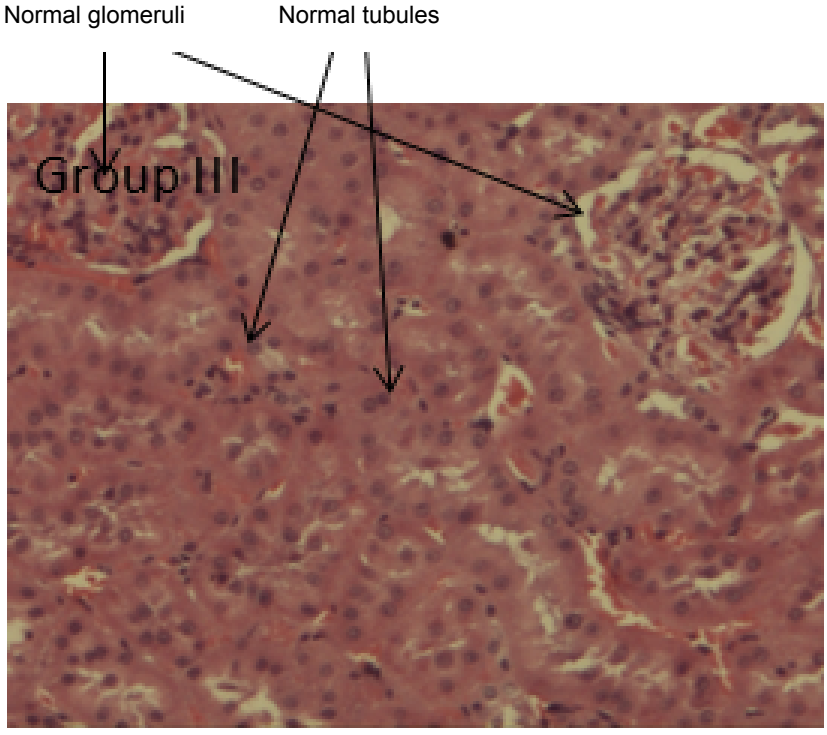
9A



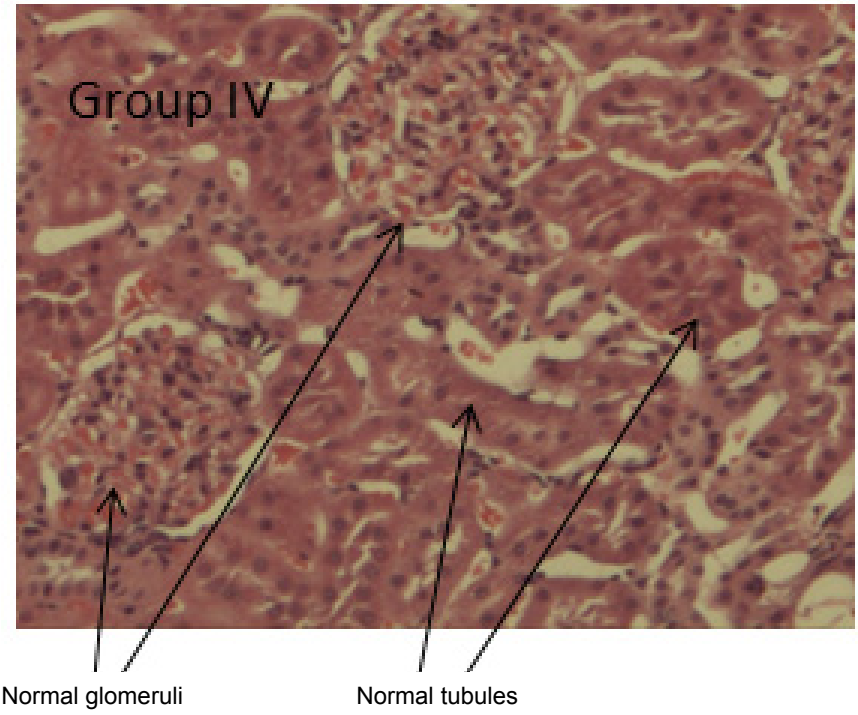
9B

Figures 9A and 9B. Histopathology of kidney (H&EX 10) from rat exposed to different F doses for 3 months. 9A: Group I; 9B; Group II.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at
www.fluorideresearch.online/epub/files/189.pdf]



9C

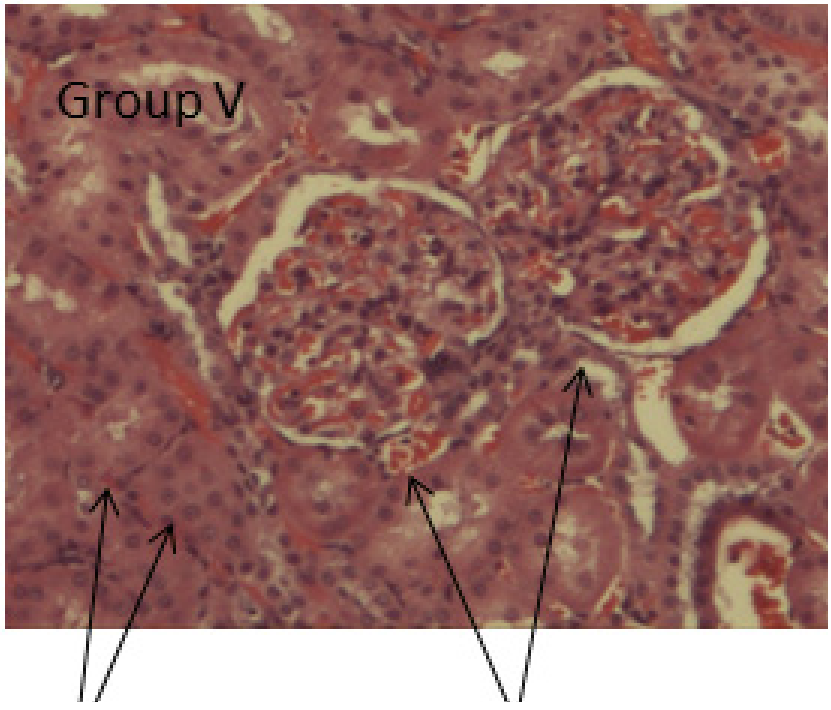


9D

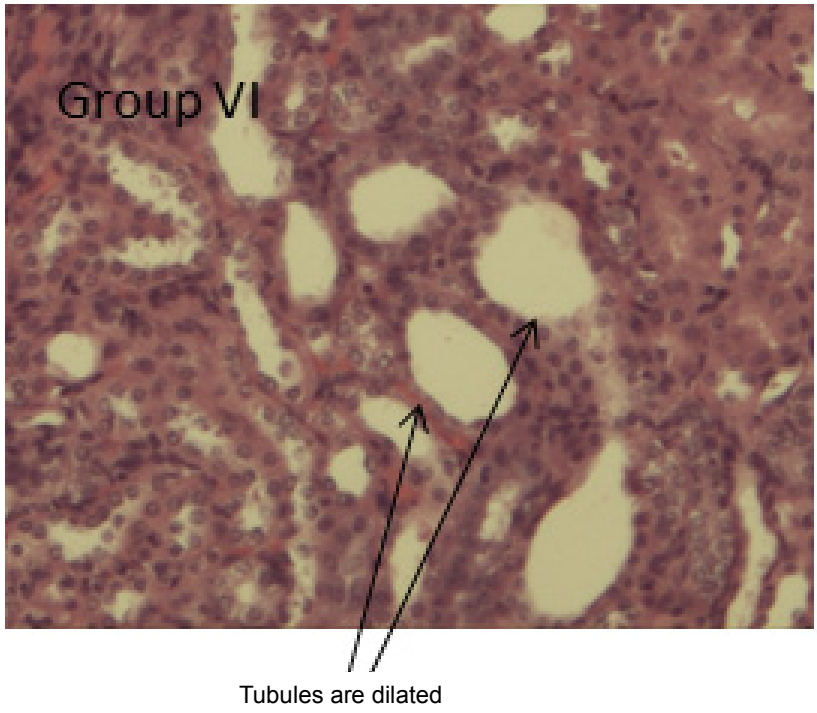
Figures 9C and 9D. Histopathology of kidney (H&EX 10) from rat exposed to different F doses for 3 months. 9C: Group III; 9D; Group IV.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

9E



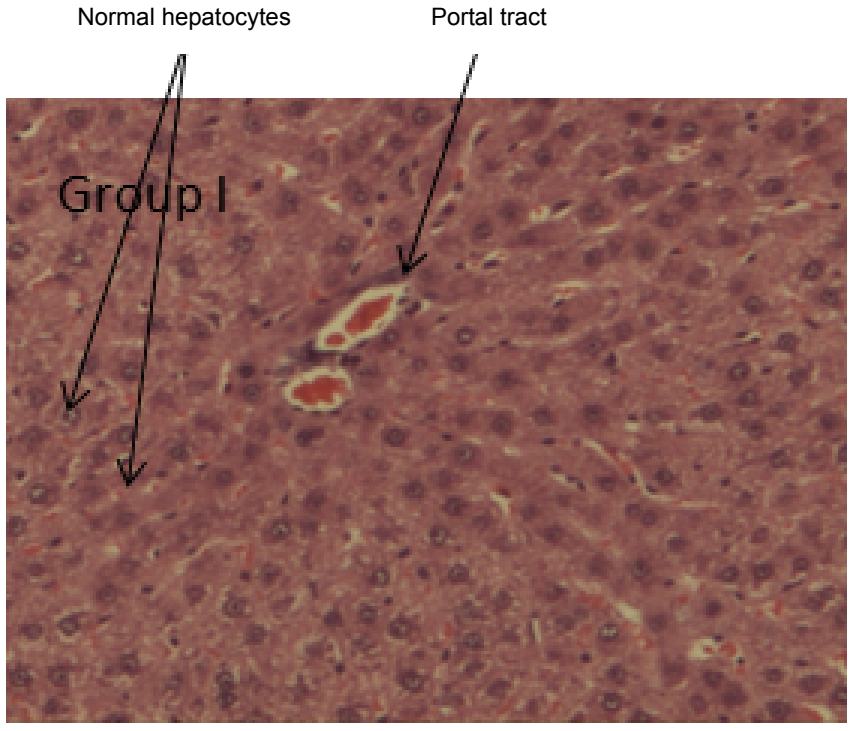
9F



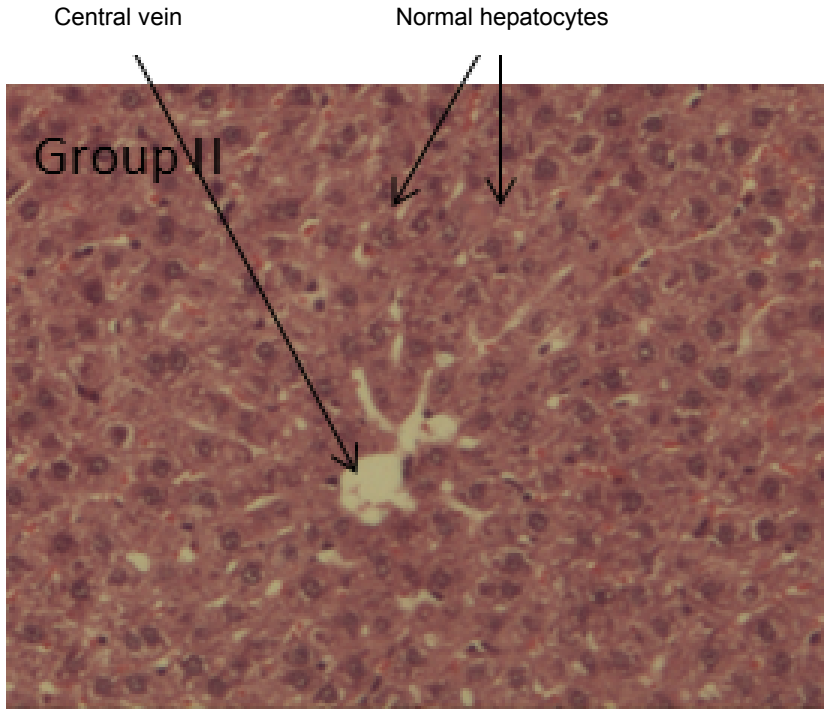
Figures 9E and 9F. Histopathology of kidney (H&EX 10) from rat exposed to different F doses for 3 months. 9E: Group V; 9F; Group VI.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at
www.fluorideresearch.online/epub/files/189.pdf]

In the liver, microvacuolation in 33-66% was observed in group V compared to group I, II, III, and IV. Microvacuolation in >90% liver was observed in group VI compared to group I, II, III, and IV (Figure 10)

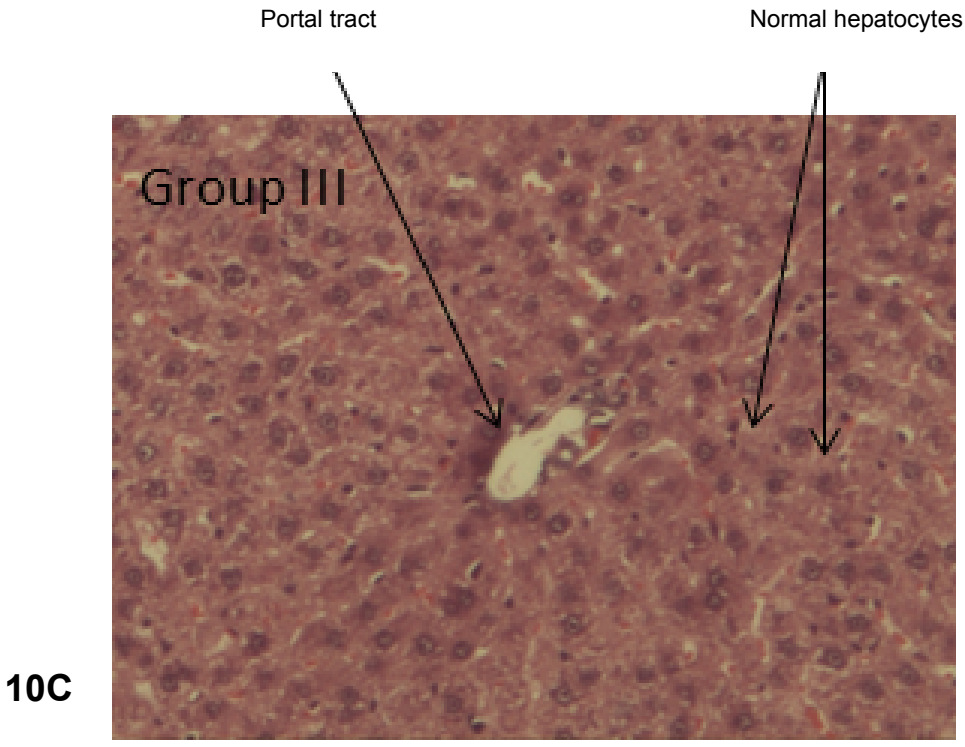


10A

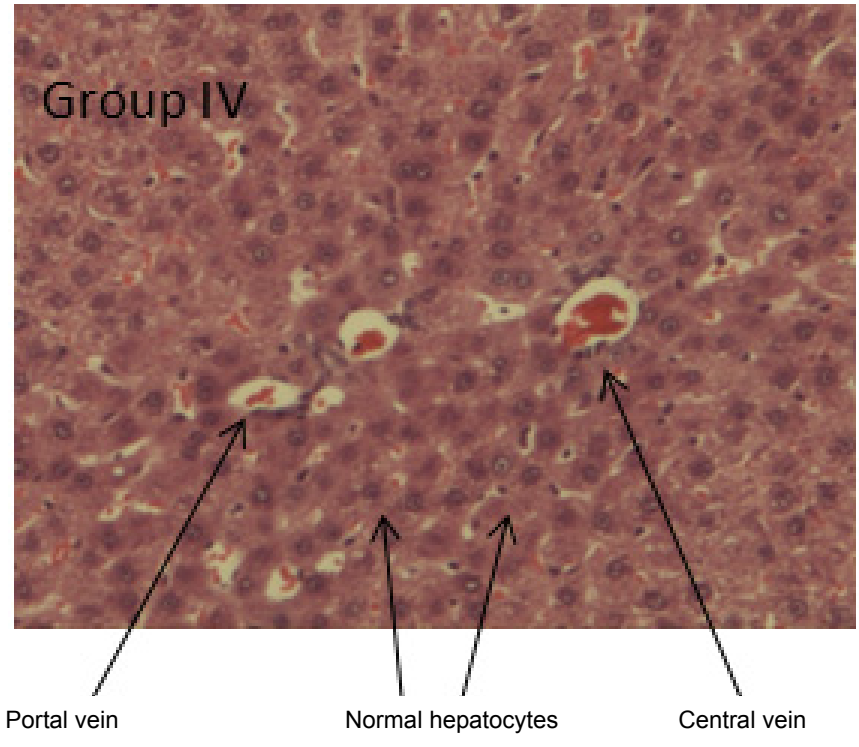


10B

Figures 10A and 10B. Histopathology of livery (H&EX 10) from rat exposed to different F doses for 3 months. 10A: Group I; 10B; Group II.



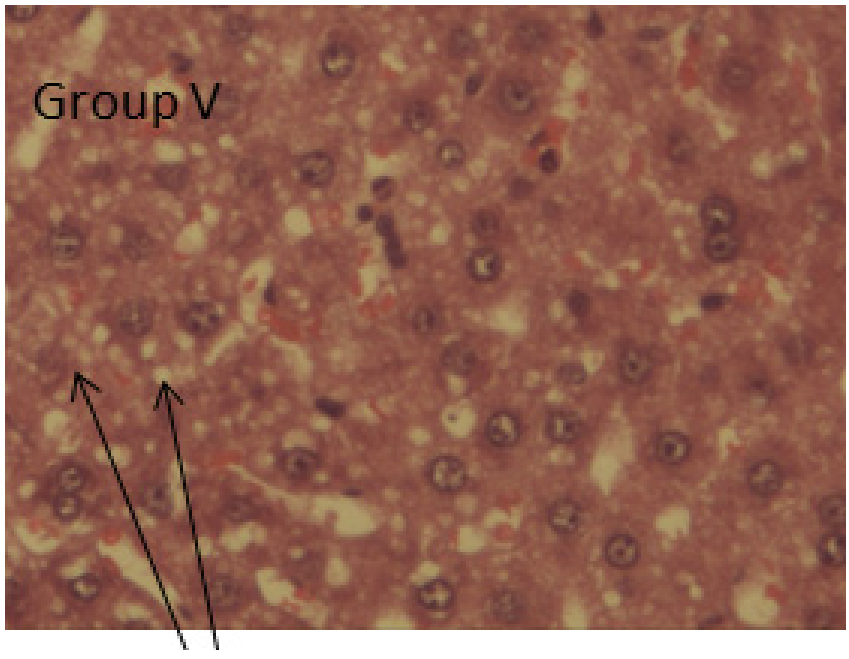
10C



10D

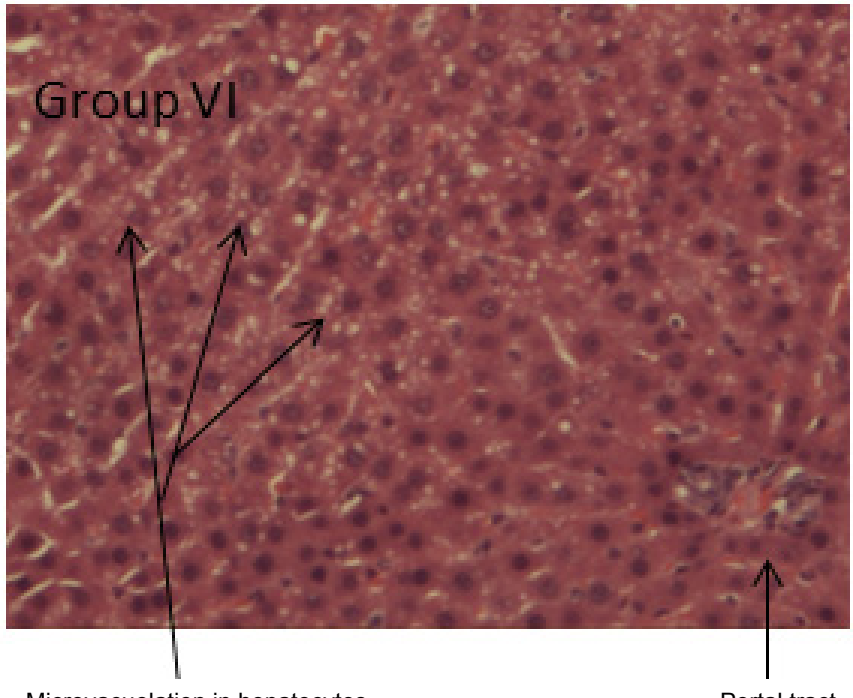
Figures 10C and 10D. Histopathology of livery (H&EX 10) from rat exposed to different F doses for 3 months. 10C: Group III; 10D; Group IV.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at
www.fluorideresearch.online/epub/files/189.pdf]



10E

Microvacuolation in hepatocytes



10F

Microvacuolation in hepatocytes

Portal tract

Figures 10E and 10F. Histopathology of livery (H&EX 10) from rat exposed to different F doses for 3 months. 10E: Group V; 10F; Group VI.

[Now published in full after the initial publication as an Epub ahead of print May 18, 2022 at www.fluorideresearch.online/epub/files/189.pdf]

DISCUSSION

The effect of F in Wistar rats was investigated in this study in a dose and time-dependent manner. The decrease in food intake in the group VI compared to all other groups at 3 month corroborates with the earlier studies.[14-17]Dental lesions may inevitably impair the ability of rats to masticate food prior to swallowing, which might contribute to a decreased food intake in F treated rats.[33] Fluoride administration reduced body weight increase in rats in a dose and time-dependent manner, according to a previous study.[34]The poor food and water consumption may be a contributing factor for reduced BWG.[35]Other investigations, however, found no difference in BWG[19] in F-fed animals. This inconsistency may be explained by the fact that different species were employed; the dose of F and also the research was conducted over different time durations. The significant decrease in FER in the group V and VI agrees with the earlier studies.[21]The reduced FER has been attributed to reduced food intake, reduced digestibility, and improper utilization of food due to altered metabolic effects.[36]The group V and VI showed an effect on FER from the first month onwards might be due to the highest concentration of F intake. The significant increase in urinary F levels in groups II, III, IV, V, and VI increased in a dose-dependent manner from 2 month onwards as reported in earlier studies.[23-25] The significant increase in serum F levels in group II, III, IV, V, and VI compared to group I at 1, 2, and 3 month is in a dose dependent manner as reported in earlier studies.[23-25]The increased serum F levels in the present study represents the true F exposure in the rats, thus leading to the toxic effects of F on different organs.In the present study, the increase in the severity of dental fluorosis in rats is F dose dependent manner. Our results agree with the earlier studies where the F dose-dependent changes in the teeth were observed.[37,38] Plasma F concentrations in humans range from 1 to 10 µmol/l after long-term intake of 1–10 ppm F in drinking water.[39] Fluorotic alterations can be seen in incisors of rodents after drinking water containing 25–100 ppm fluoride; these doses also enhance the plasma F levels to 3-10 µmol/l, which are similar to those observed to cause fluorosis in humans.[40,41] The increase in accumulation of F in bone and teeth in the present study is in a dose dependent manner and is in concurrence with the earlier reports.[42,43] In the present study, the organ weight ratios were not affected as reported in earlier studies.[44-46] The histopathological changes in the kidney and liver in the high F groups are in accordance with the similar studies conducted in rats.[26,47,48] In conclusion the dose dependent effect of F starts at 100, 50, and 15 mg/L from first, second, and third months, respectively, however there were no significant differences in 5 and 10 mg/L F groups after 3 months. In the present study, the dose of F concentrations that would cause F toxicity is 15 mg/L in rats. This toxic dose i.e., 15 mg/L in rats would cause plasma F levels equivalent to those in humans consuming fluoridated water levels of 3 mg/L. According to ICMR and WHO above the 1.0 or 1.5 mg/L level of fluoride causes toxicity in human and animals.[50] However, earlier study conducted by Turner et al. (2001)[51], suggested a NOAEL of 0.94 mg F/Kg bw/day with a LOAEL at 3.2 mg F/Kg bw/day which correspond to drinking water concentrations for humans of 3 mg F/L and 10 mg F/L respectively.[51] Hence, the present study also suggests the human consumption of 3 mg/L F in drinking water might cause the F toxicity in the population.

ACKNOWLEDGEMENTS

The authors acknowledge the encouragement and guidance of Director, ICMR-National Institute of Nutrition, Hyderabad, India.

CONFLICT OF INTEREST

The authors declare that they do not have conflict of interest.

REFERENCES

[1] Adler P, Armstrong WD, Bell ME, Bhussry BR, Büttner W, Cremer H-D, et al. Fluorides and human health. World Health Organization Monograph Series No. 59. Geneva: World Health Organization, 1970.

[2] Choubisa SL. A brief and critical review of endemic hydrofluorosis in Rajasthan, India. Fluoride 2018;51(1):13-33.

[3] Choubisa SL, Choubisa D, Choubisa A. Fluoride contamination of groundwater and its threat to health of villagers and their domestic animals and agriculture crops in rural Rajasthan, India, Environ Geochem Health 2022.doi.org/10.1007/s10653-022-01267-z

[4] Swarup D, Dwivedi SK. Environmental pollution and effect of lead and fluoride on animal health. Indian Council of Agricultural Research, New Delhi.2002, pp 68-106.

[5] Choubisa SL, Choubisa D. Status of industrial fluoride pollution and its diverse adverse health effects in man and domestic animals in India. Environ Sci Pollut Res 2016,23(8):7244-54.doi:10.1007/s11356-016-6319-8.

[6] Choubisa SL. A brief and critical review on hydrofluorosis in diverse species of domestic animals in India. Environ Geochem Health 2017;40(1):99-114. doi:10/1007/s 10653-017-9913-x

[7] Burt BA. The changing patterns of systemic fluoride intake. J Dent Res 1992;71:1228–37.

[8] Wei ZD. Fluoridation in China: a clouded future. Fluoride 2002;35(1):1–4.

[9] Wang LF, Huang JZ. Outline of control practice of endemic fluorosis in China. Soc Sci Med 1995;41(8):1191–5.

[10] Irigoyen-Camacho ME, García Pérez A, Mejía González A, Huizar Alvarez R. Nutritional status and dental fluorosis among school children in communities with different drinking water fluoride concentrations in a central region in Mexico. Sci Total Environ 2016;541:512–9.

[11] Del Carmen AF, Javier FH, Aline CC. Dental fluorosis, fluoride in urine, and nutritional status in adolescent students living in the rural areas of Guanajuato, Mexico. J Int Soc Prev Community Dent 2016;6(6):517–22.

[12] Rugg-Gunn AJ, al-Mohammadi SM, Butler TJ. Effects of fluoride level in drinking water, nutritional status, and socio-economic status on the prevalence of developmental defects of dental enamel in permanent teeth in Saudi 14-year-old boys. Caries Res 1997; 31(4):259–67.

[13] Correia Sampaio F, Ramm von der Fehr F, Arneberg P, Petrucci Gigante D, Hatloy A. Dental fluorosis and nutritional status of 6- to 11- year old children living in rural areas of Paraiba, Brazil. Caries Res 1999;33:66–73.

[14] Lohakare J, Pattanaik A, Khan SA. Effect of long-term fluoride exposure on growth, nutrient utilization and fluoride kinetics of calves fed graded levels of dietary protein. Biol Trace Elem Res 2010;138:148–62.

[15] Khandare AL, Kumar PU, Shankar HN, Kalyanasundaram S, Shanker Rao G. Effect of calcium deficiency induced by fluoride intoxication on lipid metabolism in rabbits. Fluoride 2007;40:184–9.

[16] Dunipace AJ, Edward JB, Wilson ME, Zhang W, Katz BP, Stookey GK. Chronic fluoride exposure does not cause detrimental, extra skeletal effects in nutritionally deficient rats. J Nutr 1998;128:1392–1400.

[17] Coetze CB, Casey NH, Meyer JA. Fluoride tolerance of laying hens. Br Poult Sci 1997;38:597–602.

[18] Balaji B, Kumar EP, Kumar A. Evaluation of standardized Bacopamonniera extract in sodium fluoride-induced behavioural, biochemical, and histopathological alterations in mice. Toxicol Ind Health 2015;31(1):18-30.

[19] Turner CH, Garetto LP, Dunipace AJ, Zhang W, Wilson ME, Grynpas MD, et al. Fluoride treatment increased serum IGF-1, bone turnover, and bone mass but not bone strength in rabbits. Calcif Tissue Int 1997;61:77–83.

[20] Chen J, Cao J, Wang J, Jia R, Xue W, Li Y, et al. Effects of fluoride on growth, body composition, and serum biochemical profile in a freshwater teleost, Cyprinuscarpio. Environ Toxicol Chem 2013;32(10):2315–21.

[21] Shankar P, Ghosh S, Bhaskarachary K, Venkaiah K, Khandare AL. Amelioration of chronic fluoride toxicity by calcium and fluoride-free water in rats. Br J Nutr 2013;110(1):95-104.

[22] Maha AH, Haneen HM. The effect of pomegranate leaves powder on biological, biochemical and histological changes of induced obese rats. J Am Sci 2017;13(1):62-70.

[23] Inkielewicz, Krechniak J.Fluoride content in soft tissues and urine of rats exposed to sodium fluoride in drinking water. Fluoride 2003;36(4):263–6.

[24] Yu-e Song, Hao Tan, Ke-jian Liu, Yu-zeng Zhang, Yun Liu, Cui-rong Lu, et al. Effect of fluoride exposure on bone metabolism indicators ALP, BALP, and BGP. Environ Health Prev Med 2011;16:158–163.

[25] Pereira HA, Leite Ade L, Charone S, Lobo JG, Cestari TM, Peres-Buzalaf C, et al. Proteomic analysis of liver in rats chronically exposed to fluoride. PLoS One 2013; 8(9):e75343.

[26] Khadar Basha S, Jayantha Rao K. Sodium fluoride induced histopathological changes in liver and kidney of albino mice. Acta Chim Pharm Indica 2014;4(1):58–62.

[27] Tusl J. Direct measurement of fluoride in human urine using fluoride electrode. Clin Chem Acta 1970;27(1):216-8.

[28] Singer L, Armstrong WD. Determination of fluoride in bone with the fluoride electrode. Anal Chem 1968;40:613–4.

[29] Thylstrup A, Fejerskov O. Clinical appearance of dental fluorosis in permanent teeth in relation to histologic changes. Community Dent Oral Epidemiol 1978;6:315-28.

[30] Fejerskov O, Larsen MJ, Richards A, Baelum V. Dental tissue effects of fluoride. Adv Dent Res 1994;8:15-31.

[31] Huang MC, Chao A, Kirwan R, Tschanz C, Peralta JM, Diersen-Schade DA, et al. Negligible changes in piglet serum clinical indicators or organ weights due to dietary single-cell long chain polyunsaturated oils. Food Chem Toxicol 2002;40:453–60.

[32] The Merck Index, 7th edition, Merck & Company Inc. Rahway, New Jersey. page 460, 1960

[33] Shupe JL, Olson AE, Peterson HB, Low JB. Fluoride toxicosis in wild ungulates. J Amer Vet Assoc 1984;185:1295–1300.

[34] Shanthakumari D, Srinivasalu S, Subramanian S. Effects of fluoride intoxication on lipid peroxidation and antioxidant status in experimental rats. Toxicology 2004;204:219–28.

[35] Ekambaram P, Vanaja P. Modulation of fluoride toxicity in rats by calcium carbonate and by withdrawal of fluoride exposure. Pharmacol Toxicol 2002;90:53–8.

[36] Gardiner EE, Winchell KS, Hironaka R. The influence of dietary sodium fluoride on the utilization and metabolizable energy value of a poultry diet. Poult Sci 1968;47(4):241-4.

[37] Zhang Y, Zhang Y, Zheng X, Xu R, He H, Duan X.Grading and quantification of dental fluorosis in zebra fish larva. Arch Oral Biol 2016;70:16–23.

[38] Hong F, Zheng C, Xu DG, Qian YL. Chronic combined effects of fluoride and arsenite on the Runx2 and downstream related factors of bone metabolism in rats.Zhonghua Yu Fang Yi Xue Za Zhi 2013;47(9):794–8.

[39] DenBesten P, Wu Li. Chronic fluoride toxicity: dental fluorosis. Monogr Oral Sci 2011;22: 81–96.

[40] Angmar-Mansson B, Whitford GM. Environmental and physiological factors affecting dental fluorosis. J Dent Res 1990:706–13.

[41] Angmar-Mansson B, Whitford GM. Enamel fluorosis related to plasma F levels in the rat. Caries Res 1984;18:25–32.

[42] Liu L, Zhang Y, Gu HF, Zhang KQ, Ma L, Cheng RB, et al. The effect of fluoride on the metabolism of teeth and bone in rats. Shanghai Kou Qiang Yi Xue 2014;23(2):129–32.

[43] Vieira AP, Mousny M, Maia R, Hancock R, Everett ET, Grynpas MD. Assessment of teeth as biomarkers for skeletal fluoride exposure. Osteoporos Int 2005;16(12):1576–82.

[44] Chattopadhyay A, Podder S, Agarwal S, Bhattacharya S. Fluoride-induced histopathology and synthesis of stress protein in liver and kidney of mice. Arch Toxicol 2011;85(4):327–35.

[45] Collins TF, Sprando RL, Black TN, Shackelford ME, Bryant MA, Olejnik N, et al. Multigenerational evaluation of sodium fluoride in rats. Food Chem Toxicol 2001;39(6):601–13.

[46] Bird DM, Carriere D, Lacombe D.The effect of dietary sodium fluoride on internal organs breast muscle and bones in captive American kestrels (Falco sparverius). Arch Environ Contam Toxicol 1992;22:242–246.

[47] Song GH, Huang FB, Gao JP, Liu ML, Pang WB, Li Wb, et al. Effects of Fluoride on DNA Damage and Caspase-Mediated Apoptosis in the Liver of Rats. Biol Trace Elem Res 2015;166(2):173–82.

[48] Song GH, Gao JP, Wang CF, Chen CY, Yan XY, Guo M, Wang Y, Huang FB. Sodium fluoride induces apoptosis in the kidney of rats through caspase-mediated pathways and DNA damage. J Physiol Biochem 2014;70(3):857–868.

[49] Dunipace AJ, Brizendine EJ, Zhang W, Wilson ME, Miller LL, Katz BP, et al. Effect of Aging on Animal Response to Chronic Fluoride Exposure. J Dent Res 1995;74(1):358–68.

[50] World Health Organization (WHO). Guidelines for drinking water quality. 3rd ed. Geneva: WHO; 2008. p. 375-377.

[51] Turner CH, Hinckley WR, Wilson ME, Zhang W, Dunipace AJ. Combined effects of diets with reduced calcium and phosphate and increased fluoride intake on vertebral bone strength and histology in rats. Calcif Tissue Int 2001;69:51–7.