EFFECT OF SUBCHRONIC FLUORIDE EXPOSURE ON IMMUNE STATUS AND HISTOPATHOLOGY IN RATS AND ITS AMELIORATION

P Krishnamoorthy,[a] NKS Gowda,[b] NC Vallesha,[b] D Rajendran,[b] G Maya,[b]
S Verma,[b] H Rahman,[c]

Bangalore, India

SUMMARY: The aim of the present investigation was to study the effects of fluoride-induced toxicity in rats on immunity and histopathology and its possible amelioration with boron, calcium chloride, and aluminium silicate. Sixty-four male Wistar albino rats, aged 8 to 12 weeks, were divided into eight groups of eight rats each. The control group, A, was given normal water while the treatment groups, B, C, D, E, F, G, and H, were given 30 ppm fluoride ion (F), 30 ppm F with 50 ppm boron, 50 ppm boron, 30 ppm F with 50 ppm calcium chloride, 50 ppm calcium chloride, 30 ppm F with 50 ppm aluminium silicate, and 50 ppm aluminium silicate, respectively. After four months, the rats were sacrificed, and blood and tissues collected for immune and histopathological studies, respectively. The effect on humoral and cell-mediated immunity was assessed by the antibody titre against sheep red blood cells and the lymphocyte proliferation assay, respectively. There was a significant decrease in cell-mediated immunity in rats given F alone and F in combination with aluminium silicate. Histopathological changes due to F toxicity were observed in the liver with vacuolar degeneration and hepatocellular necrosis, in the kidney with tubular casts, in the thyroid with loss of colloid with degeneration of epithelial cells, in the thymus with loss of lymphocytes and plasma cells, and in the spleen with loss of lymphocytes. These histopathological changes were ameliorated by supplementation with boron and calcium chloride, but not with aluminium silicate. Thus, both boron and calcium chloride, at 50 ppm, may effectively ameliorate F toxicity in the rat.

Keywords: Aluminium silicate; Boron; Calcium chloride; Fluoride toxicity; Immunity; Histopathology; Wistar albino rat.

INTRODUCTION

The fluoride ion (F), one of the main toxic minerals causing lesions in the visceral organs and affecting the immune system, is often found in ground water at levels higher than the WHO recommended maximum level guideline of 1.5 mg/L.[1] Prevalent in many parts of the world, chronic fluorosis is caused by the excessive ingestion of F over a long period and endangers the health of both human beings [2,3] and domestic animals.[4-6] Sodium fluoride is used in various pesticide formulations, including insecticides and wood preservatives.[7] It can be deposited into soil from several anthropogenic sources, both directly, through phosphate fertilizers, and indirectly, from environmental pollutants such as pesticides[7] and atmospheric pollution from industrial activities and the burning of fossil fuels,[8,9] In addition to its well known effects on the skeleton and teeth, F can also exert toxic effects on many soft tissues and organ systems, giving rise to a broad array of symptoms and pathological changes.[10,11] F toxicity in animals is

[a]National Institute of Veterinary Epidemiology and Disease Informatics, Hebbal, Bangalore-560024, India; [b]National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore-560030, India; [c]For correspondence: Dr P Krishnamoorthy, Scientist, National Institute of Veterinary Epidemiology and Disease Informatics, Hebbal, Bangalore-560024, India; E-mail: krishvet@gmail.com;

multifarious.[12,13] Even though the majority of ingested F is incorporated into calcified tissues, trace amounts of this pollutant are able to alter the activity of several enzymes and the metabolism in soft tissues including in the liver.[14,15] Few ameliorative measures have been suggested to overcome fluorosis in man[16,17] and animals.[18-20] The present study was performed to ascertain the ameliorative effect of boron, calcium chloride, and aluminium silicate on F-induced toxicity in rats.

MATERIALS AND METHODS

Grouping of animals and treatment: Sixty-four male Wistar albino rats, aged 8 to 12 weeks, were divided into eight groups of eight rats each. Group A was given normal water (control) and the other groups received treatment: Group B: 30 ppm F, Group C: 30 ppm F + 50 ppm boron, Group D: 50 ppm boron, Group E: 30 ppm F + 50 ppm calcium chloride, Group F: 50 ppm calcium chloride, Group G: 30 ppm F + 50 ppm aluminium silicate, and Group H: 50 ppm aluminium silicate. The fluoride (NaF), boron (BNa_3O_3), calcium ($CaCl_2$), and aluminium (Al_2SiO_3) were given to the rats through their drinking water. The dose of F (30 ppm) was chosen on the basis of our previous study in rats with different dose levels of F, viz., 10, 30, and 60 ppm.[21] The rats were housed in polypropylene cages and fed with pellet feed and purified water *ad libitum*. The animal house temperature and humidity were maintained at 23±2ºC and 50 to 70%, respectively. The present study was approved by the Institutional Animal Ethics Committee (No. IRC4.4/ IAEC/2012) and conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India.

Immunity: After a four month period of feeding the ameliorating agents and the fluoridated drinking water, the rats were assessed for their immunity. The rats were injected intraperitoneally with 200 µL of sheep red blood cells (RBC) diluted in Alsevar's solution. Fourteen days after the injection, all the rats were anesthetized and 1 to 2 mL of blood were collected by the retro-orbital plexus route using a capillary tube. The blood serum was separated by centrifuging at 2,000 rpm for 15 min and was then used for the determination of the haemagglutination antibody titre against sheep RBC to assess the humoral immunity.[22] Blood samples were collected in heparin tubes on the 120th day from four rats in each group to assess the cell-mediated immunity by the lymphocyte proliferation assay. The 3(4,5-dimethyl thiazol-2-yl) 2,5 diphenyl-tetrazolium bromide (MTT) colorimetric assay was carried out[23] with some minor modifications [24] for the proliferation of lymphocytes against concanavalin A (Sigma, USA).

Histopathology: At the end of study period, the rats were sacrificed by using an overdose of anaesthesia and the gross examination of the visceral organs was done. Tissues of liver, kidney, spleen, heart, lung, thyroid, and thymus were also collected in 10% buffered formalin (fixative) for histological study. These tissues were then processed and embedded in paraffin. Five µm tissue sections were prepared using a rotatory microtome and stained with haematoxylin and eosin stains.[25] After staining, these sections were dehydrated in an ascending series of ethanol, cleared in xylene, and mounted in the synthetic resin DPX (a mixture of

the polystyrene distyrene, the plasticizer tricresyl phosphate, and xylene). The sections were then examined under a light microscope for any histological changes.

RESULTS

The haemagglutination antibody titres against sheep RBCs in the different groups of rats are presented in Figure 1.

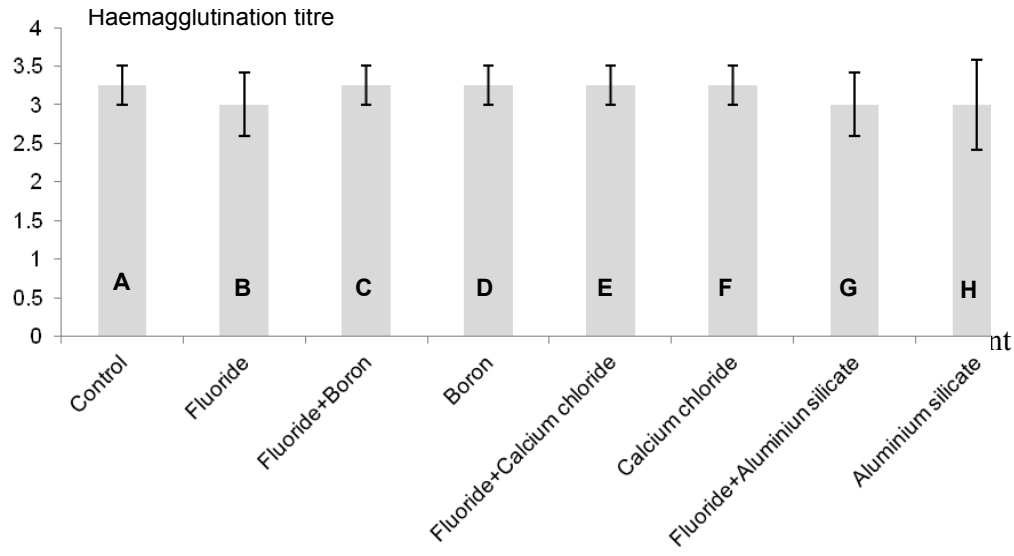


Figure 1. Mean±SE haemagglutination antibody titre against sheep RBCs in the different groups, A-H, of rats.

The mean±SE haemagglutination antibody titres in the groups were: Group A: 3.25±0.25, B: 3.0±0.41, C: 3.25±0.25, D: 3.25±0.25, E: 3.25±0.25, F: 3.25±0.25, G: 3.0±0.41, and H: 3.0±0.58. There was no significant difference in serum haemagglutination antibody titre against sheep RBCs in the various treatment groups when compared to control rats. However, there was a non-significant decrease in humoral immunity in the F, the F + aluminium silicate, and the aluminium silicate groups.

The stimulation indexes observed were: Group A: 1.05±0.07, B: 0.87±0.04, C: 0.91±0.04, D: 0.97±0.08, E: 1.05±0.01. F: 1.07±0.27, G: 0.79±0.11, and H: 1.13±0.01 (Figure 2). The lymphocyte proliferation assay revealed a significant ($p<0.05$) decrease in the stimulation index in the fluoride treatment group, Group B, and the F + aluminium silicate supplemented group, Group G, as compared to their counter parts. There was no significant difference in the stimulation index in the other treatment groups when compared to the control group. The rats in Groups C and E revealed no significant difference in the stimulation index when compared to the control rats, indicating the ameliorative effect of boron and calcium chloride on F toxicity. On gross examination, no morphological changes were observed in the visceral organs. However, diverse histopathological changes

were observed in the various visceral organs of the rats belonging to the different groups (Figure 3).

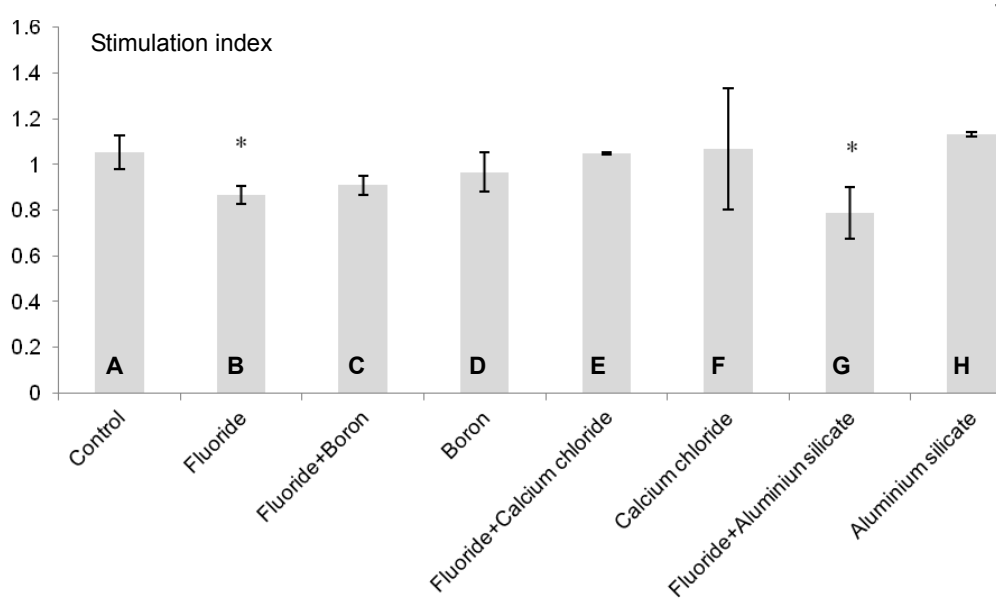
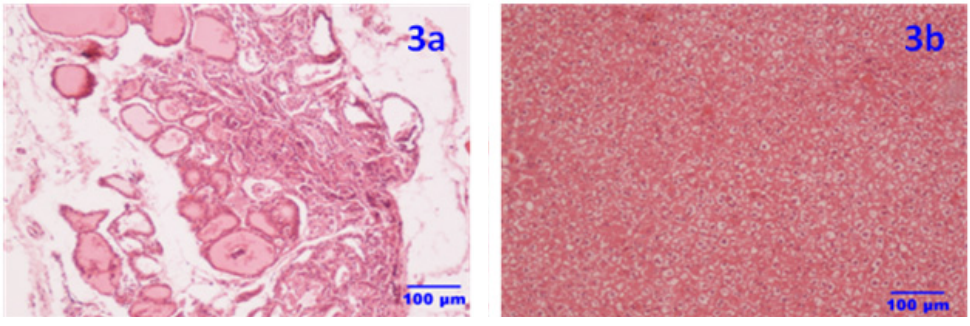


Figure 2. Mean ± SE stimulation index by lymphocyte proliferation assay in the different groups, A-H, of rats. Compared to control, *p<0.05.

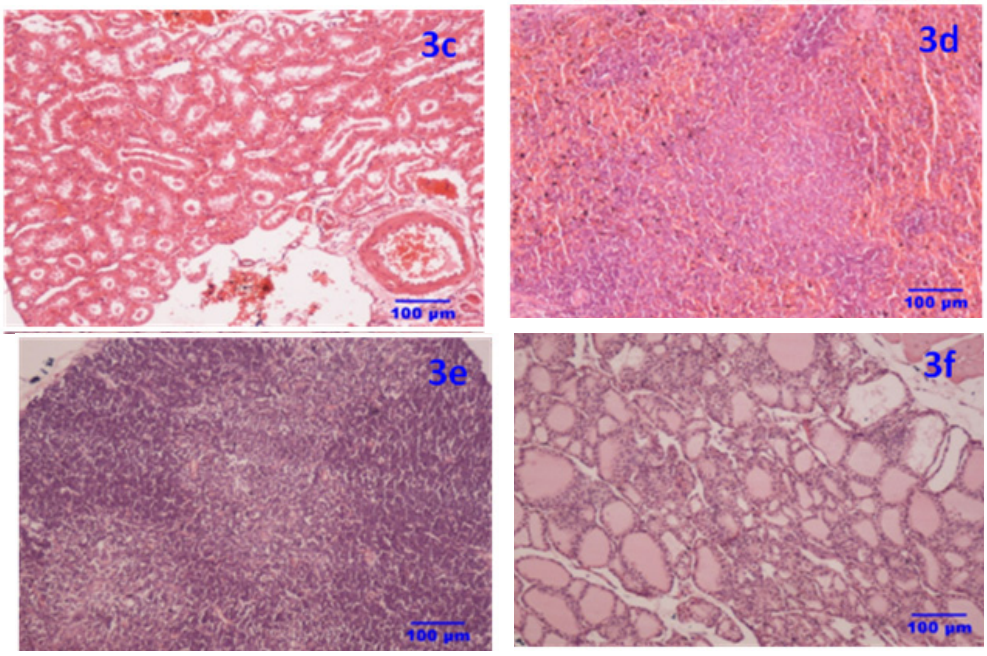
The thyroid endocrine gland in the rats of Group B revealed a loss of colloid in the follicles and degeneration of the secretory epithelial cells (Figure 3a) when compared to the control rats, which showed uniform colloid in the follicles with simple cuboidal epithelial cells. The liver of the rats belonging to Group B showed vacuolar degeneration of the hepatocytes with condensation of the nucleus, change in the hepatocyte architecture, and a focal infiltration of mononuclear cells (Figure 3b).



Figures 3a and 3b. Histopathological changes in the different treatment groups (haematoxylin and eosin, scale bar = 100 µm). 3a: Group B thyroid showing loss of colloid and degeneration of epithelial cells; 3b: Group B liver showing vacuolar degeneration, condensation of nucleus and hepatocellular necrosis.

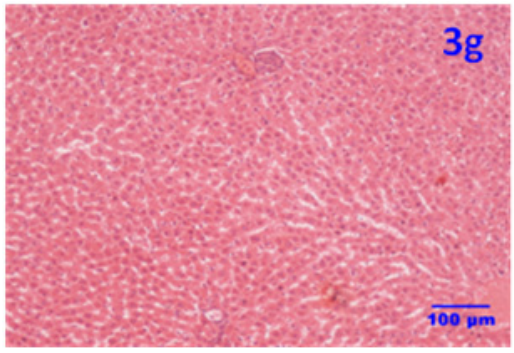
Kidney revealed infiltration of mononuclear cells with loss of tubular epithelial cells with degeneration and casts in the tubular lumen (Figure 3c). Spleen

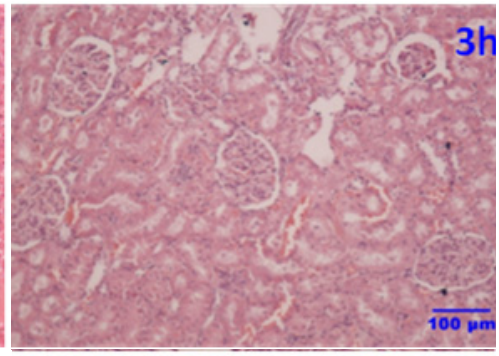
revealed loss of lymphocytes from white pulp and mild reticulum cell hyperplasia (Figure 3d) whereas thymus showed loss of lymphocytes and plasma cells from the germinal centres (Figure 3e) in rats exposed to fluoride ions. In the Group C rats, the thyroid showed normal secretory epithelial cells with colloid in the follicles (Figure 3f).

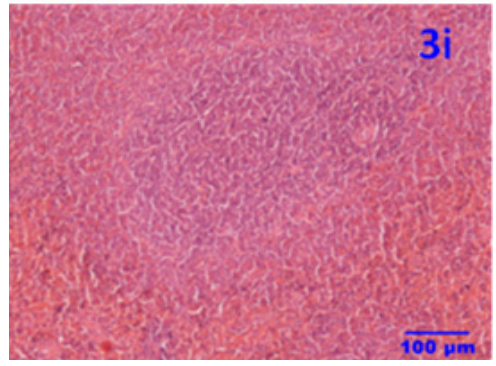


Figures 3c-3f. Histopathological changes in the different treatment groups (haematoxylin and eosin, scale bar = 100 µm). 3c: Group B kidney showing formation of casts stained pink colour in the lumen of tubules; 3d: Group B spleen showing mild loss of lymphocytes with reticulum cell hyperplasia; 3e: Group B thymus showing loss of lymphocytes and plasma cells from the germinal centres; 3f: Group C thyroid showing appearance of colloid with normal secretory epithelial cells.

The liver of the Group E rats showed hepatocytes with a vesicular nucleus and pink stained cytoplasm (Figure 3g) which was similar to that of the control rats. The kidney of the Group C rats revealed normal glomerular architecture without any casts in the tubular lumen (Figure 3h) as seen in the rats of Group B. The spleen in the Group E rats revealed germinal centres with lymphocytes and normal architecture (Figure 3i) as seen in the control rats. The rats in Groups D, F, and H who were given boron, calcium chloride, and aluminium silicate alone, respectively, showed no histopathological changes in these organs as compared to the rats of the control group. The rats in Groups C and E revealed amelioration from the toxic effects of F in terms of the histological changes in the liver, kidney, spleen, thyroid, and thymus. However, the rats in Group G given F + aluminium silicate revealed similar changes in these visceral organs to those of the rats in Group B, indicating no ameliorative effect of aluminium silicate on F toxicity. The heart and lung tissues did not show any histopathological changes in any of the rats in the treatment groups as compared to the control rats.







Figures 3g-3i. Histopathological changes in the different treatment groups (haematoxylin and eosin, scale bar = 100 µm). 3g: Group E liver showing normal appearance of hepatocytes with vesicular nucleus and pink stained cytoplasm; 3h: Group C kidney showing disappearance of casts in the tubules and normal glomerular architecture; 3i: Group E spleen showing normal architecture with germinal centre containing lymphocytes.

DISCUSSION

The present findings indicated that while humoral immunity was affected only slightly, to a statistically nonsignificant extent, the cell-mediated immunity was greatly affected by F ingestion at 30 ppm through the drinking water. In physiological amounts, boron has been shown to increase serum antibodies in broilers and rats.[26] The thymus gland cultivates mainly T lymphocytes and mediates the cellular immunity. The cellular immunity was affected by F toxicity and concurred with the pathological changes in the thymus. The bone marrow mainly cultivates B lymphocytes and mediates humoral immunity. In addition to storing red blood cells, the spleen is a peripheral immune organ and generates an immune response. The visceral organs like liver, kidney, spleen, thymus, and thyroid showed toxic effects on F exposure.

The ameliorative effect of boron and calcium chloride at 50 ppm level on F toxicity was observed with the haemagglutination antibody titre, the lymphocyte proliferation assay, and the histopathological examination. Extensive ballooning degeneration, hepatocellular necrosis, and infiltration of mononuclear cells have also been observed in the livers of mice fed with 500 ppm of NaF in drinking water.[27] The lesion observed in the present study concurred with the previous report [27] but was less severe and this might have been due to the lower dose of F used in the present study. High doses of boron (>200 mg/L) in drinking water can significantly inhibit the growth of immune organs and lead to the exhibition of toxic effects in broilers.[28] Another study indicated that birds fed with 100 mg/L of boron had a rapid development of the thymus at 4 weeks of age, with an increased

number of lymphocytes within the thymic parenchyma, and gradual enlargement of the lobules which were well developed by 6 weeks of age.[28] Boron plays a vital role in regulating enzymatic activity in pathways involved in energy substrate metabolism, insulin release, and the immune system. It modifies insulin release by altering the metabolism of nicotinamide adenine dinucleotide phosphate (NADP) and may affect glucose, lipid, and protein metabolism.[26] However, toxic effects of boron have also been reported when fed at high levels (>400 mg/L) in broilers.[28] Thus, low doses of boron may be used in animals to ameliorate the toxic effects of F as observed in this study.

The aluminium silicate did not show any amelioration on F toxicity. A toxic effect of aluminium chloride along with F in mice testis has been reported previously.[9,29] This may be the reason for aluminium silicate not having an ameliorative effect on F toxicity and also having an additional toxic effect as observed in the cell-mediated immunity and the histopathological changes in the various organs in the Group G rats. However, aluminium silicate is being widely studied for its effect on the amelioration of various mycotoxins in poultry.

We found calcium chloride overcame the toxic effects of F on immunity and the various organ pathologies and this concurred with a previous report which indicated that calcium neutralizes the F bioavailability in a lethal model of F poisoning in mice.[30] The spleen and thymus showed histopathological changes in Groups B and H which corresponds to the decreased stimulation index in the lymphocyte proliferation assay. The histopathological changes observed in the rats of the different treatment groups corresponded to the immunity results.

Thus, boron and calcium chloride may be conveniently used for ameliorating F toxicity. However, more experimental studies are highly recommended in order to understand the changes occurring at the molecular and subcellular levels in F toxicity and their amelioration by various agents.

ACKNOWLEDGEMENTS

The authors thank the Director, National Institute of Animal Nutrition and Physiology, Bangalore, India, for providing the necessary facilities for carrying out this research work as an Institute research project.

REFERENCES

1 Swarup D, Dwivedi SK. Research effects of pollution on livestock. Indian J Anim Sci 1998;68:814-24.
2 Choubisa SL, Choubisa DK, Joshi SC, Choubisa L. Fluorosis in some tribal villages of Dungarpur district of Rajasthan, India. Fluoride 1997;30(4):223-8.
3 Choubisa SL. Endemic fluorosis in southern Rajasthan (India). Fluoride 2001;34(1):61-70.
4 Choubisa SL. Some observations on endemic fluorosis in domestic animals of southern Rajasthan (India). Vet Res Commun 1999;23(7):457-65.
5 Choubisa SL. Fluoridated ground water and its toxic effects on domesticated animals residing in rural tribal areas of Rajasthan (India). Int J Environ Stud 2007;64(2):151-9.
6 Choubisa SL. Fluoride toxicosis in immature herbivorous domestic animals living in low fluoride water endemic areas of Rajasthan, India: an observational survey. Fluoride 2013;46(1):19-24.

7 Budavari S. The Merck Index: an encyclopedia of chemicals, drugs, and biologicals. 11th ed. New Jersey: Merck, Rahway; 1989.
8 Arnesen AKM. Effects of fluoride pollution on pH and solubility of Al, Ca, Mg, K and organic matter in soil from Ardal (Western Norway). Water Air Soil Pollut 1998;103:375-88.
9 Stevens DP, McLaughlin MJ, Alston AM. Phytotoxicity of aluminium-fluoride complexes culture by *Avena sativa* and *Lycopersicon esclentum*. Plant Soil 1977;192:81-93.
10 Waldbott GL. Nonskeletal fluorosis [editorial review]. Fluoride 1978;11:111-4.
11 Choubisa SL. Fluoride in drinking water and its toxcosis in tribals, Rajasthan, India. Proc Natl Acad Sci India Sect B Biol Sci 2012;82(2):325-30.
12 Choubisa SL. Status of fluorosis in animals. Proc Natl Acad Sci India Sect B Biol Sci 2012;82(3):331-9.
13 Choubisa SL. Fluorotoxicosis in diverse species of domestic animals inhabiting areas with high fluoride in drinking waters of Rajasthan, India. Proc Natl Acad Sci India Sect B Biol Sci 2013;83(3):317-21.
14 Holland I. Fluoride inhibition of protein synthesis. Cell Biol Int Rep 1979;3:701-4.
15 Wang YN, Xiao KQ, Liu JL, Dallner G, Guan ZZ. Effect of long term F exposure on lipid composition in rat liver. Toxicology 2000;146:161-9.
16 Choubisa SL, Choubisa L, Choubisa D. Reversibility of natural dental fluorosis. Int J Pharmacol Biol Sci 2011;5(20):89-93.
17 World Health Organization. Fluoride and human health, Monograph series No. 59. Geneva: World Health Organization; 1970.
18 Ekambaram P, Paul V. Modulation of fluoride toxicity in rats by calcium carbonate and by withdrawal of F exposure. Pharmacol Toxicol 2002;90:53-8.
19 Choubisa SL. Natural amelioration of fluoride toxicity (Fluorosis) in goats and sheep. Curr Sci 2010;99(10):1331-2.
20 Choubisa SL, Mishra GV, Sheikh Z, Bhardwaj B, Mali P, Jaroli VJ. Food, fluoride, and fluorosis in domestic ruminants in the Dungarpur district of Rajasthan, India. Fluoride 2011;44(2):70-6.
21 Krishnamoorthy P, Gowda NKS, Vallesha NC, Rajendran D, Maya G, Verma S et al. Effect of long term fluoride toxicity on immunity and pathology in Wistar albino rat. Indian J Vet Pathol 2014;38(3):164-8.
22 Kamran AS, Rahimi SH, Karimi Torshizi MA. Effect of dietary oil seed on n-3 fatty acid enrichment, performance parameters and humoral immune response of broiler chickens. Iranian J Vet Res 2009;10:158-65.
23 Bounous DI, Campagnolid RP, Bram J. Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. Avian Dis 1992;36:1022-7.
24 Krishnamoorthy P, Rao SBN, Dineshkumar D, Rahman H. Effect of feeding detoxified karanj seed cake on immunity and pathology in growing lambs. Iranian J Vet Res 2014;15:69-71.
25 Bancroft JD, Stevens A. Theory and practical of histological staining techniques. 6th ed. London: Churchill Livingstone; 1996.
26 Hunt CD. Dietary boron: an overview of the evidence for its role in immune function. J Trace Elem Exp Med 2003;16:291-306.
27 Bouaziz H, Ketata S, Jammoussi K, Boudawara T, Ayedi F, Ellouze F, Zeghal N. Effects of sodium fluoride on hepatic toxicity in adult mice and their suckling pups. Pestic Biochem Phys 2006;86:124-30.
28 Jin E, Gu Y, Wang J, Jin G, Li S. Effect of supplementation of drinking water with boron on performance and immune organ parameters of broilers. Italian J Anim Sci 2014;13:205-14.
29 Chinoy NJ, Sorathia HP, Jhala DD. Fluoride + aluminium induced toxicity in mice testis with giant cells and its reversal by vitamin C. Fluoride 2005;38(2):109-11.
30 Heard K, Hill RE, Cairns CB, Dart RC. Calcium neutralizes fluoride bioavailability in a lethal model of fluoride poisoning. J Toxicol Clin Toxicol 2001;39(4):349-53.