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Boron and calcium chloride as possible ameliorators of fluoride toxicity in Wistar rats

NKS Gowda[1]*, D Rajendran[1], P Krishnamoorthy[2], NC Vallesha[1], A Raghavendra[3], VB Awachat[1], G Maya[1] & Swati Verma[1]

[1]ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru-560 030, Karnataka, India
[2]ICAR-National Institute of Veterinary Epidemiology and Disease Informatics, Yelahanka, Bengaluru-560 064, Karnataka, India
[3]ICAR- National Bureau of Agricultural Insect Resources, Bengaluru-560 024, Karnataka, India

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Fluorosis is an endemic health problem for both humans and livestock in some parts of the world including India. This fast expanding problem needs remedial measures. Though limited reports suggest ameliorative effect of boron (B) and calcium (Ca) against fluoride (F) toxicity in animals, still there is a need for long duration study to understand the possible mechanism of action. Here, we did the same; studied the ameliorative effect of B and Ca on F toxicity in Wistar strain (*Rattus norvegicus*) rats. Forty-eight male Wistar albino rats were divided randomly into six groups. The group T1 was given normal water (control) while the groups T2-T6 were supplemented with F through water @ 30 ppm (as sodium fluoride), 30 ppm F+ 50 ppm B (as sodium borate), 50 ppm B, 30 ppm F+ 50 ppm calcium chloride and 50 ppm calcium chloride, respectively. After four months, all the rats were sacrificed and blood, teeth, femur bone and liver were collected and analyzed for mineral content. Fluoride toxicity significantly (P <0.05) reduced phosphorus content in bone, calcium content in liver, and superoxide dismutase (SOD) activity in plasma. However, F toxicity significantly (P <0.05) increased phosphorus and glucose content in plasma, and calcium and magnesium content in teeth and fluoride content in liver. Boron and calcium chloride as ameliorating agents significantly (P <0.05) reduced the fluoride content in all the internal organs and also significantly reduced the elevated level of glucose and phosphorus in plasma and improved SOD activity in plasma. It is concluded that toxic effect of F is partially ameliorated by supplementation of 50 ppm of B or $CaCl_2$.

Keywords: Fluorosis, Livestock, Mineral profile

Fluorosis is an endemic problem in some parts of India and other countries. In India, ground water in several districts of at least 19 states is reported to contain high (>1.5 ppm) fluoride (F) level[1]. Common sources of fluorosis are water having high fluoride, industrial (cement, aluminium, brick, phosphate fertilizer factories) emissions having fluoride, phosphate feed supplements and mineral mixtures, and vegetation grown on such soils having high fluoride[2]. Ground water with high F content has also been reported from parts of Africa, China, Japan and Sri Lanka[2]. Aluminium smelter factories contribute fluoride to environment as evidenced by high fluoride level in water, vegetation and prevalence of fluorosis in goats within 10 km radius[3]. In India, dental fluorosis has been recorded[5]. Gowda *et al*.[6], has indicated that about 14% of water samples in Karnataka are fluorotic (>1.5 ppm) in nature. Fluoride interferes with calcium (Ca) utilization in body and Boron (B) has a positive effect on Ca utilization. Hence, use of B and Ca is likely to have an ameliorative effect against fluorosis. The organ and biochemical changes due to fluoride exposure depends on factors, such as duration of exposure, age and nutritional status of the animal. Limited reports suggest that boron (B) has been reported as an antidote to fluoride intoxication[7,8]. Aluminium sulfate also alleviate F toxicity but the safety against free aluminium ions in water is still a matter of concern. Supplementation of B (140 ppm) to F (60 ppm) containing diet of buffaloes for 90 days showed partial amelioration against adverse effects of F[9], however the mechanism of action is largely not known.

In view of the multiple effects of fluoride on the pathophysiology in animals, here, we studied the ameliorating effect of both B and Ca for possible practical dietary strategies to prevent fluorosis in animals and humans.

*Correspondence:
Phone: +91 80 25711304; Fax: +91 80 25711420
E-mail: nksgowda@rediffmail.com

Materials and Methods

Experimental animals

Forty-eight male Wistar strain (*Rattus norvegicus*) albino rats aged 8-10 weeks were divided into six groups of eight rats each with two rats in each cage. The group T1 was given normal water (control), groups T2-T6 were supplemented with F through water @ 30 ppm (as sodium fluoride, AR grade, SD fine chemicals, Mumbai), 30 ppm F+ 50 ppm B (as sodium borate, AR grade, SD fine chemicals, Mumbai), 50 ppm B, 30 ppm F+ 50 ppm calcium chloride (A R grade, SD fine chemicals, Mumbai) and 50 ppm calcium chloride, respectively. The dose of F (30 ppm) in water was decided based on our previous study with different dose levels of F (10, 30, and 60 ppm) in rats[10]. The B and calcium chloride levels were selected based on the earlier findings[9]. All rats were housed in polypropylene cages and fed with standard rat pellet feed and offered purified water *ad libitum*. Temperature and humidity were maintained at 23±2ºC and 50-70%, respectively, in the animal house. The animal experiment protocol was approved by Institutional Animal Ethics Committee (IAEC) (No. 11,2012) and carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India, New Delhi.

Sample collection

All the rats from each group were sacrificed using overdose of chloroform anaesthesia and blood was drawn from heart and serum was separated by centrifuging at 2000 rpm for 15 min. The serum samples were preserved at −20°C till further analyses for minerals, Superoxide dismutase (SOD) and glucose. Internal organs (teeth, femur bone and liver) were collected and rinsed with normal saline and preserved in ice for laboratory analysis.

Mineral and biochemical analysis

Serum glucose was estimated as per glucose oxidase-peroxidase method[11] using Span diagnostic kit. The SOD activity was determined by a modified method of pyrogallic acid autooxidation[12]. The change in absorbance per minute in control and test were recorded to calculate the SOD activity. One unit of SOD activity was expressed as amount of enzyme required to inhibit the pyrogallol autooxidation by 50%. Samples of bone and teeth were boiled in water and soft tissue adhering were removed. Whole femur bone, teeth and liver were dry digested in a muffle furnace at 600°C for 3 h and prepared the mineral extract with 5N HCl and analyzed for minerals (Ca, Mg) in atomic absorption spectrophotometer using air-acetylene flame (Perkin Elmer AA Analyst 300, USA). For estimation of Ca and Mg acid extracts and serum were suitably diluted with 0.1% lanthanum chloride to avoid interference from phosphate and other minerals. Copper and zinc in serum samples were analyzed after dilution with deionised water. Phosphorus in bone, teeth and liver[13] and serum P was estimated by colorimetric method[14]. Certified mineral standards were run in each analysis to ensure the accuracy of estimation. The fluoride content in water and mineral extracts of bone, liver and teeth was estimated in ion analyzer (Jenway 3345, UK).

Statistical analysis

Data obtained were analyzed statistically by one way analysis of variance (ANOVA) as per Snedecor and Cochran[15] and using SAS (Statistical Analysis System) Enterprise Guide software version 4.2, SAS India Limited, India[16].

Results

The bone mineral profile in rats supplemented with different fluoride ameliorating agents varied significantly among the treatment groups (Table 1). Supplementation of B @ 50 ppm (T4) level significantly (P <0.05) enhanced the Ca deposition in bones compared to control rat (T1). Among the treatment groups the Ca content (%) varied significantly (P <0.05) in the range of 19.25 ± 0.90 in group supplemented with fluoride and $CaCl_2$ (T5) to 25.62 ± 0.99 in group supplemented with B alone (T4), whereas the phosphorus content was lower in group supplemented with fluoride and boron (T3) and higher in group with boron supplementation (T4). Bone magnesium and fluoride contents were higher in boron (T4) and fluoride (T2) supplemented groups, respectively. Fluoride supplementation alone (T2) significantly (P <0.05) reduced the bone phosphorus content, on contrary to increased F content.

Teeth mineral profile in rats supplemented with different fluoride ameliorating agents is presented in Table 1. There was a significant variation in Ca, Mg and F content in teeth among the treatment groups. The calcium content (%) ranged from 20.80 ± 0.74 to 24.82 ± 0.48. There was a non-significant variation observed in phosphorus content in teeth among the treatment groups. Contents of calcium, magnesium

and fluoride were significantly (P <0.05) higher in fluoride supplemented group (T2), whereas phosphorus was higher in the control group (T1).

The liver mineral profile (%) in rats supplemented with different fluoride ameliorating agents showed that levels of calcium, phosphorus and fluoride varied significantly (P <0.05) among the treatment groups, whereas magnesium (P>0.05) content showed no difference (Table 1). The calcium content(%) varied from 0.15 ± 0.01 to 0.38 ± 0.04, phosphorus(%) varied from 1.12 ± 0.06 to 1.32 ± 0.04, magnesium (%) from 0.10 ± 0.01 to 0.17 ± 0.01 and fluoride (ppm) varied from 35.87 ± 4.19 to 93.75 ± 6.53 among the different treatment groups.

The plasma mineral profile in rats supplemented with different fluoride ameliorating agents is presented in Table 2. Significant differences (P <0.05) in phosphorus (%), magnesium (%), activity of SOD (u/min/mg protein) and glucose (mg %) were recorded among the treatment groups, whereas calcium, copper and zinc varied insignificantly (P >0.05). The SOD activity was higher in control (T1) and lower in the fluoride group (T2). Phosphorus and glucose contents were significantly (P <0.05) higher in fluoride supplemented group (T2) and lower in group supplemented fluoride with $CaCl_2$ (T5). Magnesium content was lower in group supplemented fluoride alone and with fluoride and boron (T3).

Table 1 — Bone, teeth and liver mineral profile in rats supplemented with fluoride ameliorating agents (DM basis)

Groups	Calcium %	Phosphorus %	Magnesium %	Fluoride (ppm)
Bone mineral profile				
T1	21.00^{bc} ± 1.13	10.76^{a}± 0.33	1.59^{b}± 0.07	125.3^{c}± 6.33
T2	22.75^{abc} ± 0.86	8.82^{bc}± 0.29	1.55^{b}± 0.06	586.7^{a}± 25.80
T3	23.37^{ab} ± 1.12	8.80^{bc}± 0.55	1.61^{b} ± 0.40	291.8^{b}± 8.96
T4	25.62^{a}± 0.99	10.79^{a} ± 0.50	2.47^{a} ± 0.19	113.1^{c} ± 5.17
T5	19.25^{bc} ± 0.90	9.45^{ab}± 0.49	1.48^{b}± 0.15	348.7^{b}±38.79
T6	22.50^{abc} ± 0.91	8.88^{bc} ± 0.36	1.46^{b}± 0.20	106.6^{c} ± 15.21
Teeth mineral profile				
T1	21.65^{bc} ± 0.65	13.77 ± 0.73	1.14^{b}± 0.16	43.62^{d} ± 3.55
T2	24.82^{a} ± 0.48	12.62 ± 0.40	1.85^{a} ± 0.22	165.1^{a}± 12.02
T3	21.65^{bc}± 0.41	12.86 ± 0.55	1.67^{ab}± 0.11	80.62^{c} ± 6.84
T4	23.50^{ab} ± 0.54	12.61 ± 0.53	1.43^{ab}± 0.05	47.50^{d} ± 3.72
T5	20.80^{bc}± 0.74	12.53 ± 0.34	1.78^{a}± 0.12	91.12^{bc} ± 5.75
T6	23.25^{ab} ± 0.70	12.12 ± 0.31	1.70^{ab}± 0.08	32.25^{d}± 4.55
Liver mineral profile				
T1	0.25^{bc}± 0.02	1.12^{b}± 0.06	0.17 ± 0.01	43.87^{bc} ± 3.69
T2	0.15^{c}± 0.01	1.18^{ab} ± 0.04	0.12 ± 0.01	93.75^{a}± 6.53
T3	0.28^{ab}± 0.02	1.19^{ab}± 0.08	0.13 ± 0.02	65.00^{b}± 8.66
T4	0.26^{b}± 0.02	1.18^{ab}± 0.05	0.11 ± 0.01	39.37^{c}± 5.74
T5	0.33^{ab}± 0.02	1.20^{ab}± 0.02	0.10 ± 0.01	53.75^{bc} ± 3.37
T6	0.38^{a} ± 0.04	1.32^{ab}± 0.04	0.12 ± 0.01	35.87^{c} ± 4.19

$^{a, b, c}$ means with different superscript within the column differ significantly (P <0.05). [T1, control; T2, 30 ppm F; T3, 30 ppm F+50 ppm boron; T4, 50 ppm boron; T5,30 ppm F+50 ppm $CaCl_2$; and T6, 50 ppm $CaCl_2$. DM, Dry matter]

Table 2 — Plasma mineral profile, SOD and glucose level in rats supplemented with fluoride ameliorating agents

Groups	Calcium (mg%)	Phosphorus (mg%)	Magnesium (mg%)	Copper (ppm)	Zinc (ppm)	SOD (u/min/mg protein)	Glucose (mg%)
T1	10.40±0.30	2.51^{b}±0.18	2.23^{ab}±0.14	1.17±0.06	0.94±0.08	40.85^{a}±1.51	163.4^{b}±6.94
T2	10.14±0.19	3.46^{a}±0.14	2.21^{ab}±0.08	1.37±0.13	1.03±0.09	19.76^{c}±1.05	227.4^{a}±11.22
T3	9.96±0.17	2.72^{b}±0.19	2.05^{b}±0.16	1.51±0.14	1.06±0.04	25.92^{bc}±2.21	175.5^{b}±8.55
T4	9.97±0.70	2.71^{b}±0.12	2.78^{a}±0.25	1.19±0.12	0.97±0.08	34.72^{ab}±2.26	153.1^{b}±7.50
T5	9.51±0.62	2.43^{b}±0.14	2.32^{ab}±0.37	1.18±0.16	0.89±0.07	30.79^{abc}±3.01	132.4^{b}±9.66
T6	9.53±0.52	2.47^{b}±0.11	2.46^{ab}±0.30	1.45±0.15	0.91±0.07	31.84^{ab}±4.29	138.8^{b}±13.61

$^{a, b, c}$ means with different superscript within the column differ significantly (P <0.05). [T1, control; T2, 30 ppm F; T3, 30 ppm F+50 ppm boron; T4, 50 ppm boron; T5,30 ppm F+50 ppm $CaCl_2$; and T6, 50 ppm $CaCl_2$. SOD, Superoxide dismutase]

Discussion

The bone mineral profile of experimental rats supplemented with fluorosis ameliorating agents showed that fluoride toxicity (T2) significantly (P <0.05) reduced the bone phosphorus content, on contrary to the increased F content. After absorption, F is readily attracted by the calcium rich tissues such as bone and teeth[17]. This might be the reason for higher level of F in bones of fluoride supplemented group (T2). Supplementation of B alone @ 50 ppm (T4) level significantly (P <0.05) enhanced the calcium deposition in bones compared to control group (T1). This indicates that B has a role in calcium utilisation as well as bone mineralization process[18]. This was evident from the reports of previous researchers[19-21]. Franke *et al.*[22] reported that the formation of fluoride-boron complex, which is preferably excreted through the kidney and less markedly stored in bone, theoretically might explain boron as an antidote to fluoride. Though F alone did not reduce the calcium content in the bone, supplementation of F and $CaCl_2$ also influenced the bone calcium content. Boron or $CaCl_2$ supplementation to ameliorate excess fluoride intake did not have any effect on bone phosphorus content. Similarly, Armstrong and Spears[23] also reported that apparent absorption and retention of Ca and P were not affected (P >0.05) by dietary B supplementation.

Generally magnesium content of bone is low as compared to calcium and phosphorus[24]. However, boron supplementation alone significantly (P <0.05) improved the magnesium content of bone. Nielsen[25] reviewed that combined deficiency of boron and magnesium caused detrimental changes in bones of animals.

Fluoride content of bone in rats supplemented with 30 ppm F was significantly (P <0.05) increased. Excess dietary intake of F causes deposition in bone, teeth and other internal organs, including kidney[24]. The ameliorative effect against excess F intake has been noticed with supplementation of 50 ppm boron as well as calcium chloride in terms of reduction in bone fluoride content. Similarly, Sharma *et al.*[26] reported that fluoride induced adverse effects on reproductive and other organs in female rats were ameliorated with vitamin C, vitamin D and calcium supplementation. When 2 mg sodium fluoride (NaF) per kg body weight per day was administered with 0.8 mg boron per kg body weight, fluoride level in bone and kidney was reduced which suggested that boron could antagonize the fluoride toxicity and also the extent of recovery was more pronounced in boron treatment than with strontium administration[27].

Teeth mineral profile showed that Ca, Mg and F contents in teeth were significantly increased due to fluoride ingestion. After absorbion into the blood, the fluoride readily retained in calcium rich tissues such as bone and teeth[17]. The elevated F level in bone and teeth was ameliorated with the supplementation of either 50 ppm of boron or $CaCl_2$. However, F content was not restored to that of control group. Further, it is also reported that fluoride-boron complex formation resulted in more excretion than deposition in bone and teeth[22].

Fluoride toxicity caused significant reduction in liver calcium content and increased fluoride content. Chiony and Memon[28] reported that not only the mineral content but also changes in liver function due to significant increase in serum transaminases, accumulation of glycogen and inhibition of phosphorylase activity after toxic ingestion of NaF @10 mg/kg body wt. for a month in rats. In contrary, there was no significant increase in liver F content when 2 mg NaF/kg body wt. per day was administered to rats for a period of 6 months[27]. The extent of fluoride toxicity depends on factors like mineral content of water, feed and vitamin content of feed besides gastric acidity and stress[22,28,29]. Boron supplementation alone without fluoride ingestion did not cause any changes in liver Ca, P, Mg and F contents as compared to control group (T1). Phosphorus and magnesium contents in the liver were not affected due to fluoride toxicity. Formation of fluoride-boron complex, which was excreted through the kidney and not stored in bone, will explain boron as an antidote to fluoride[22], and hence the reason for changes occurred in the present study.

Plasma mineral profile, SOD activity and glucose content of experimental rats supplemented with different fluoride ameliorating agents showed that Ca, Cu and Zn contents in the plasma was not influenced by the fluoride and also by ameliorating agents. However, phosphorus and glucose contents were increased significantly due to fluoride toxicity, however B and $CaCl_2$ significantly (P <0.05) ameliorated this effect. In contrary, SOD activity was reduced due to F toxicity and it was partially ameliorated by supplementation of either B or $CaCl_2$. Fluoride is reported to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase[17]. Zhan *et al*.[30]

also found reduced hepatic SOD1 mRNA in young pigs fed diets supplemented with high fluoride concentration. The reason for reduced SOD might be due to over production of free radicals and/or inhibition of SOD by fluoride[31,32].

Dose dependent effect of fluoride ingestion on immunity related mRNA expression of cytokines in mice has been observed[33]. Fluoride inhibited the catalase antioxidant enzyme activity by interacting with di or trivalent metals located in the active site of this antioxidative enzyme[34]. Significantly (P <0.01) increased bone turnover markers and oxidative stress were observed with decreased levels of antioxidant enzymes activity in postmenopausal women residing in the fluorotic villages[35]. Supplementation of jambul fruit extract could reverse the heapto-renal pathological changes in mice caused due to fluoride exposure[36]. The above reports clearly demonstrate the prooxidant nature of fluoride through which pathological and immunity related changes are produced. The antioxidant property of boron through modulation of hepatic superoxide dismutase activity under abiotic stress in rats has be recently reported[37]. This could be one of reasons of observed ameliorative effect of boron against fluoride ingestion in the present study.

Fluoride intake may result in impaired glucose tolerance or elevated level of blood glucose[38,39]. Komatsu *et al.*[40] evaluated insulin secretion in RINm5F cells exposed to fluoride and found increased insulin release up to 60 min. Adverse effect of F on serum glucose in the present study was effectively ameliorated by supplementation of either 50 ppm boron or $CaCl_2$. Fluoride intake beyond 30 ppm through drinking water in rats for more than 90 days showed reduced immunity and pathological changes in liver, kidney and thyroid[10] and were ameliorated by supplementation of either boron or $CaCl_2$ at 50 ppm level[11,41]. These reports evidently support the ameliorative changes observed in the present study due to boron (sodium borate) or Ca ($CaCl_2$) supplementation in rats induced fluoride toxicity.

Conclusion

It is evident from the findings that fluoride toxicity significantly (P <0.05) reduced the phosphorus content in bone, calcium content in liver, superoxide dismutase activity in plasma. However, F toxicity significantly increased phosphorus and glucose content in plasma, calcium and magnesium content in teeth and fluoride content in liver. Boron and calcium chloride as ameliorating agents significantly reduced the fluoride content in target organs and also significantly reduced the elevated glucose and phosphorus level in plasma and restored the SOD activity in plasma. Hence, it is concluded that the toxic effect of 30 ppm of F is partially ameliorated by supplementation of 50 ppm of either boron or $CaCl_2$.

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