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Effect of subchronic exposure to fluoride and role of *Moringa stenopetala* on hematologic parameters in mice

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Abstract
Any toxicant gaining acess to the body and is not eliminated by liver and kidney, where these organs themselves affected by fluoride accumulation, tend to accumulate and affect the circulatory system. Fluoride accumulated in the blood circulatory system could induce abnormalities generally affecting hematologic parameters. Studies implicated that among the mechanisms by which fluorosis cause damage to tissues is through oxidative stress. Moringa (*Moringa stenopetala*, Bac.) an endogenous tree to Ethiopia is rich in antioxidants, and has potent antiodxidative effect. to investigate effects of fluorosis on hematologic parameteres, and the role of *Moringa stenopetala* leaf extract, randomized control study was conducted using 2 months old 24 (12 male and 12 female) Swiss Albino Mice, average weight 33.8±5.5g, randomly allocated in to 5 experimental groups and one control group with free access of pelleted food. The mice took different doses of fluoride, and both fluoride and *Moringa stenopetala* together for 90 days. On day 91, blood samples were collected by cardiac puncture and complete blood count was measured. There was no significant difference in hematological indices between control mice and those mice treated with fluoride and, fluoride and crude extract of *Moringa stenopetala* crude extract together (p>0.05). Exposure of this concentration for years or higher concentration for months may increase the probability of fluoride toxicity on hematological parameters.

Keywords: Fluorosis, hematologic parameters, sodium fluoride, *moringa stenopetala*

1. Introduction
Fluoride at low concentration has protective effect on dental caries. Because of its beneficial effect, many countries practice fluoridation of drinking water, where naturally occurring levels of F^- are below optimal levels [1]. According to World Health Organization (WHO), the fluoride concentration in drinking water should be 0.5 mg/l in the area with warm climate, whereas, in cooler climate it can increase up to 1.5 mg/l [2].

Despite caries prevention importance of fluoride many literatures [3, 4] described this ion as a double-edged sword, below or above the recommended values have consequences. Prolonged ingestion of fluoride in excess of the daily requirement is associated with fluoride accumulation in our body. Similarly, inadequate intake of fluoride in drinking water is known to cause dental caries [5]. Excessive fluoride intake over a long period of time results in a serious public health problem called fluorosis, which is characterized by dental, skeletal manifestations, and soft tissue damage [6]. Skeletal and dental toxicity are most common because of long term fluoride exposure, however soft tissues are also afflicted with damage by fluoride [7].

Any toxicant gaining acess to the body and is not eliminated by liver and kidney, where these organs themselves affected by fluoride accumulation [8], tend to accumulate and affect the circulatory system. The cellular constituents of this system, plasma protein, and chemical composition of blood will have qualitative and quantitative abnormalities in the exposed animals. Fluoride accumulated in the blood circulatory system could induce abnormalities in lipid peroxidation and disturb the anti-oxidative system generally affecting hematologic parameters [9].

Among the possible mechanisms of fluoride toxicity in animals, oxidative stress and lipid peroxidation that leads to initiation of apoptotic pathways was the major one. But multiple mechanisms are implicated including disruption of gene expression, damage to mitochondria, enhancing accumulation of other metallic ions like lead and alimmunium, interfering neurotransmitter activity in brain [10].

Moringa *(Moringa stenopetala*, Bac.) a tree that belongs to the family *Moringaceae* is cultivated in Ethiopia [11], *Moringa stenopetala* contains essential amino acids, carbohydrates,

fats, vitamins (E, A & C), minerals and have antimicrobial, antioxidant, antihypertensive and antidiabetics effects [12]. Among the constituents vitamins C and E, carotenoids, and antioxidant enzymes containing Mn, Fe or Zn are involved in antioxidant activity [13]. *Moringa stenopetala* which have tremendous amount of antioxidant components may have effect on fluoride induced damage [14, 15], the protective effect is yet to be investigated. The main aim of the present study is to assess effect of subchronic exposure to fluoride on hematologic parametres and to study the protective effect of *Moringa stenopetala* in mice. Because of their phylogenetic relatedness and physiological similarity to humans, the ease of maintaining and breeding them in the laboratory, as well as the availability, easyness to house them mice were used for this study.

2. Materials and methods

2.1. Chemicals

NaF was used to subchronically expose the mice to fluoride. NaF with 98.5% purity were purchased from LOBA Chemie laboratory reagents and fine chemicals Mombi, India. A stock solution of 2000ppm fluoride prepared by dissolving 4.42g of NaF in 1L of distilled water. Water solution with 0.07ppm, 60ppm and 100ppm fluoride levels obtained by diluting the stock solution with distilled water. To prepare solution with 0.07ppm fluoride, 0.035ml of stock solution were diluted by 1L of distilled water, 60ppm fluoride: 30ml of stock solution were diluted to 1L distilled water; and 100ppm fluoride was obtained by diluting 50 ml of stock solution by 1L distilled water. The stock solution stored at 4-8 °C for 1 week. The solutions prepared each week [16].

2.2. Animals

The study was conducted using laboratory bred Swiss Albino Mice. Twenty-four (12 male and 12 female) healthy 2 months old mice weighed averagely 33.8±5.5g were collected from Tropical and Infectious Disease Research Center, Jimma University. The mice were randomly allocated in to experimental groups and control group with 4 mice in each group. The mice were kept one week in the cage for acclimatization in a group of 4 per cage and housed in polypropylene cages with sawdust to soak the excretory fluids; cages provide ample spaces for movement and kept in ambient temperature. The study animals in all groups were allowed access to pelleted food *ad libitum*.

2.3 Design

Experimental Randomized Controlled study was used to address study objective. Resource equation method was used for determining sample size, by taking existence possible attrition into consideration (10%), total sample size was 24. According to resource equation method, E- (degree of freedom) is measured as: E=Total number of animals – Total number of groups. *E = 24 – 6; E= 18* Where E: is the degree of freedom of analysis of variance (ANOVA). E between 10 and 20 is considered as adequate [17]. First, 24 Swiss Albino Mice were selected from adult healthy mice population randomly, and then these mice were randomly assigned into five experimental and one control group. Control and experimental groups were taking pelleted food ad libutum. Grouping and dosage of fluoride and *Moringa stenopetala* of each group is summarised in table 1[18].

Table 1: Grouping and dosage of fluoride and *Moringa stenopetala*

Groups (N=4)	1	2	3	4	5	6
Fluoride Dose (ppm)	0.07	60	100	60	100	0.07
Moringa Treated (mg/Kg)	-	-	-	100	100	100

2.4 Preparation of *Moringa stenopetala* aqueous extract

The fresh leaves of *Moringa stenopetala* were collected from Jimma Agricultural research center and confirmed by expert. The leaves were dried in shadow room, then powdered. 1045 gram of the dried and powdered plant material soaked in 10.45 Liter of distilled water (1:10 ratio) in conical flask and stirred intermittently every 6 hours for 72 hours at room temperature. The marc (the damp solid material) then filtered using sterile Whatman No.1, 15 cm filter paper into a clean conical flask. The filtrates were freeze-dried in a lyophilizer to yield a crude extract. The crude extract was kept in desiccators until used. 100mg/kg dose was given every morning, and dose calculation updated every two weeks according to weight. The dose was given through oral gavage with 24-26 guage gavage needles.

Twelve hours before the Euthanasia animals deprived of food and drink, anasthesia was given then transferred to dissection board. Five ml syringe was inserted anteriorly below xyphoid process directly to the heart to collect blood.

2.5 Measurement of Hematological parameters

After ansthesia blood sample collected by cardiac puncture method. Complete blood count was measured by Hematology Analyzer (HumaCount 30^{TS} Germany) after veternary modifications [19].

2.6 Statistical analysis

After data collection and cleaning data entered in to SPSS software Version 22 for statistical analysis, results expressed using descriptive statistics as tables, means and standard deviation groupwise. Statistical data analysis performed using independent t-test and one-way ANOVA with *post hoc* Benfferini test [20], after checking the assumptions, $p \leq 0.05$ considered statistically significant.

2.7 Ethical considerations

In the administration of substances, an appropriate technique in a professional manner, enabling the achievement of the anticipated results whilst causing minimum distress to the animals were applied. The physico-chemical properties of administered substances and their vehicle were in accordance with the biocompatibility criteria for the route of administration.

To minimize the study animals suffering, humane procedures followed in handling the animals and during sample collection. Enriching of the cages in which the animals were kept, using anesthesia during sample collection and scarification of the study animals were among the steps to decrease the distress and suffering. Disposal of contaminated laboratory utensils and waste chemicals handled according to laboratory safety guidelines. Ethical clearance was obtained from Jimma University Institutional Review Board.

3. Result and Discussion

Following maceration, sedimentation, filtration and freeze drying processes of 1045g powder of *Moringa stenopetala*

leaves using distled water as solvent, 105g of crude extract was obtained and the percentage yield of the extract was about 10.05%. Total powder, yield and percentage of yield obtained from aqueous extraction of leaves of *Moringa stenopetala* is given in table 2.

Table 2: Total powder, yield and percentage of yield obtained from aqueous extraction of leaves of *Moringa stenopetala*

Part of plant	Weight of powder	Weight of crude extract	Percentage
Leaves	1045g	105g	10.5%

General clinical observations were made once daily, taking into consideration the appearance of different signs. No mice showed changes in the following parameters at all doses of fluoride and *Moringa stenopetala*. The parameters used to observe were pilo-erection, salivation, locomotion, food & water intake, lacrimation, diarrhea, urination, depression, breathing and excitement. No effects were observed in the given parameters in all groups as compared to control groups within 90 days of treatment.

Initially 24 mice 12/12 male/female with initial average weight of 33.8±5.5g, with no significance difference between groups, table 3.

Table 3: Body weights of mice initially showing there was no significant difference between groups (p>0.05).

Group (N=4)	1	2	3	4	5	6
Initial	29.75±7.22	37.75±4.7	33.25±5.5	31±5.2	35.25±4.2	36.25±4.1

The mean and standard deviation of some hematologic indices is indicated in table 4A: White blood cell count (WBC) of experimental group was increased, however platelet count of experimental groups were decreased as compared to the control group. Generally, there was increasing trend of WBC as the dose of fluoride increased in groups taking fluoride only, whereas groups taking both fluoride and moringa stenoptella, the WBC were not increasing. Administration of moringa stenoptela caused a decrease in platelet count, while these changes are not statistically significant.

The lymphocyte number (LY Mnumber) of experimental groups showed no change compaired to the controls, while the percent of lyphocytes (LYMpercent) showed a slight decreament in fluoride only treated groups. The percentage decreament was probably caused by the inceased number of granulocytes (GRAnumber) as well as percent of granulocytes (GRApercent) in this group. Again these parametres were statistically insignificant (p> 0.05), table 4A.

Table 4A: Hematologic indices of mice, mean and standard deviation for different groups, shows no significant difference (p> 0.05)

Group	1	2	3	4	5	6
WBC (10^3/µl)	3.18±0.854	4.51±2.57	7.18±6.57	3.51±3.06	1.96±1.33	3.43±1.28
LYMnumber	2.17±1.03	2.1±0.57	2.61±1.27	2.65±2.87	1.75±0.25	1.93±0.87
LYMpercent	66.55±14.4	55.28±23.7	48.27±22.7	66.42±17.86	59.75±22.27	55.47±11.86
MIDnumber	0.46±0.17	0.79±0.48	0.90±0.73	0.44±0.10	0.36±0.05	0.66±0.36
MIDpercent	15.07±6.30	17.35±2.50	12.72±4.28	17.9±9.69	12.25±4.73	19±6.19
GRAnumber	0.54±0.44	1.62±2.28	3.66±5.38	0.43±0.19	0.95±1.02	0.82±0.29
GRApercent	18.37±15.84	26.82±23.22	39±25.57	15.92±9.2	28±27.01	25.52±9.94
PLT(10^3/µl)	439±331	386±349	394±352	114±52.62	265.7±340	209±291

Red blood cell count (RBC) and the RBC indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) have showed no change between control group and different experimental groups. The hemoglobin concentration of experimental groups was decreased, sa as to the red blood cell number, MPV, HCT, but MCH, and MCHC were increasing. All these parametrs are not significantly associated with the control and experimental groups, as well as, by the concentration fluoride or *Moringa stenopetala* they took, (p> 0.05, table 4B.

Table 4B: Hematologic indices of mice, mean and standard deviation for different groups, shows no significant difference (p> 0.05)

Group	1	2	3	4	5	6
HGB (g/dl)	14.95±1.43	11.225±4.5	12.52±2.91	13.02±0.55	11.05±5.34	10.1±4.19
MCH (pg)	16.90±0.63	17.375±2.50	15.3±1.29	16.17±0.92	14.77±0.83	15.5±0.70
MCHC (g/dl)	35.6±1.25	36.35±1.27	36.22±1.45	37.07±1.92	35.17±1.4	36.62±1.47
MCV (fl)	47.6±1.82	48.02±8.60	42.37±4.83	43.60±0.28	42.05±0.75	42.5±1.71
PCT (%)	0.27±0.18	0.23±0.21	0.22±0.20	0.06±0.03	0.15±0.23	0.11±0.16
MPV (fl)	6.57±0.56	6.02±0.38	5.52±0.43	5.8±0.16	5.82±0.09	5.72±0.62
HCT (%)	42.17±5.03	30.765±11.87	34.81±9.20	35.18±2.03	31.02±14.4	27.74±12.04
RBC (10^3/µl)	8.86±0.973	6.58±2.98	8.12±1.35	8.07±0.48	7.34±3.36	6.52±2.75

Platelet Distribution Width coefficient of variation (PDWcV) and Platelet Distribution Width standard deviation (PDWsD) have slightly decreased in fluoride only treated groups (group 2 and group 3). Red cell distribution width - coefficient of variation (RDWcV), red cell distribution width - standard deviation (RDWsD) platelets larger than 12 femtoliter and smaller than 30 femtoliter (PLCC) and Platelet larger cell ratio (PLCR) given in table 4C shows no significant difference (p> 0.05).

Table 4C: Hematologic indices of mice, mean and standard deviation for different groups, shows no significant difference ($p > 0.05$)

Group	1	2	3	4	5	6
PDWsD (fl)	9.37±1.71	8.47±1.66	6.5±1.04	7.87±0.25	7.92±0.56	7.85±1.93
PDWcV (%)	38.05±2.39	36.67±2.78	32.7±2.6	36.05±0.5	36.22±1.15	34.97±3.71
RDWs (fl)	18.9±1.62	19.7±12.49	12.5±7.17	15.15±1.17	16.02±2.82	15.15±4.90
RDWc (%)	17.67±0.52	17.4±1.88	19.72±4.55	17.47±0.63	19±1.65	18.05±2.14
PLCC (10^3/µl)	79.25±43.34	64.25±63.66	48.75±52.32	13.5±8.34	30.25±43.96	26±34.06
PLCR (%)	21.98±7.2	11.61±7.13	8.38±6.59	11.03±3.04	9.42±2.51	12.77±9.39

Sub chronic effects of fluoride on hematological parameters of mice showed that, there were no significant difference in hematological indices between control mice and those mice treated with fluoride and, fluoride and crude extract of *Moringa stenopetala* crude extract together ($P>0.05$).

Metabolic, functional and structural damage caused by subchronic fluorosis have been reported in many tissues. WBC count of experimental group was increased, however hemoglobin of experimental groups were decreased as compared to the control group. Sub chronic effects of fluoride on hematological parameters of mice showed that, there were no significant difference in hematological indices between control mice and those mice treated with fluoride and, fluoride and crude extract of *Moringa stenopetala* crude extract together ($P>0.05$).

The circulatory system plays an important role in maintaining homeostasis in animals. The cellular constituents, plasma protein, and chemical composition of blood have vital roles in the different metabolic activities. Any toxicant gaining access into the body that is not eliminated by the liver is distributed throughout the body by the circulatory system. When the concentration of a toxicant increases sufficiently, it may cause qualitative and quantitative abnormalities in the exposed animals.

The effect of fluoride on hematologic indices was quit ambiguous with different extremes of research findings. In line with the current study Kamalpreet *et al*. [21] reported there was no significance difference in PCV, MCV, MCH, MCHC and WBC between cases treated with sodium fluoride and controls, and a decrease in hemoglobin level was noted by oral exposure of calves. Erdal Mustafa *et al*. [22] after treating mice with 100 ppm fluoride, also agreed in insignificant difference in RBC, Hgb, MCV, RDW, and MCHC but a decrease in WBC count in 100 ppm (0.07ppm control) sodium fluoride treated group of mice. Uslu *et al.* studied rats and did not observe anemia after 45 days of exposure to 30 and 100 ppm fluoride in their drinking water [23]. Similarly, Khandare *et al*. reported that fluoride had no adverse effects on hematological parameters (hemoglobin, packed cell volume, leukocyte count, neutrophils (%), lymphocytes, eosinophils, monocytes) in dogs [24]. Zerwekh *et al*. examined the calcitropic hormones and biochemical markers of bone turnover, serum chemistry, and blood hematology in 75 postmenopausal women allocated to two groups: placebo plus calcium citrate or intermittent slow-release sodium fluoride plus calcium citrate. They found no alterations in peripheral blood hemoglobin, hematocrit, and WBC and RBC counts after 2 years of therapy [25].

On the other hand Eren *et al*. reported that WBC counts were significantly higher in flourized group than control group in rats [18]. They also showed eosinophilia and dysplastic changes on granulocytes in the bone marrow samples in the fluorosis group. In another study, it has been reported that cattle afflicted with fluorosis developed anemia and eosinophilia of leukocytes [26].

Similar studies showed significant decrease in RBC, WBC, and neutrophil counts, as well as the hemoglobin and hematocrit values [27]. Cetin *et al.* reported a decrease in RBC count, hemoglobin value, and packed cell volume and an increase in total leukocyte count and in the percentage of lymphocyte in rabbits with chronic fluorosis [28] Recently, Karadeniz *et al*. reported significant increase in WBC and decrease in RBC, and neutrophil counts, as well as the hemoglobin and hematocrit values in mice [29]. Kant *et al*. reported a decrease in Hb, packed cell volume, WBC count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in goats [30]. In study of sodium fluoride toxicity on hematological parameter, Kamble and Velhal indicate sodium fluoride accumulated in the blood causes reduction in the blood components and disturbed the protein level in the body. They found a decrease in Hb, RBC and WBC in the experimental animals [31].

Atmaca, *et al.* found treatment with fluoride caused a significant decrease in WBC, RBC, PLT counts and neutrophil ratio in fluoride treated group as compared to control group [19], and other study revealed that significantly lowered hematological parameters of calves (Hb content, PCV and TLC) were observed in fluorotic calves than control group [32]. Some factors have been mentioned for the toxic effect of fluoride on hematologic parameters especially anemia. It has been proposed that inhibition of globulin synthesis, depression of erythropoiesis [33], Machalinski *et al.* observed that sodium fluoride has marked negative effects on hematopoiesis [34]. Direct toxic effects of fluoride on bone marrow have also been indicated.

This study found that subchronic fluorosis has no effect on hematological parameters in agreement with some the above mentioned previous studies but not others. The concentration of fluoride in drinking water [35], exposure time [36], and the phylogenic level of animals [37] influence the toxic effect of fluoride on hematological parameters, and might account for different findings. Perhaps exposure of this concentration for years or higher concentration for months may increase the probability of fluoride toxicity on hematological parameters.

4. Conclusion

Subchronic fluoride exposure has no effect on hematological parameters, the effect of *Moringa stenopetala* on hematological parameters were inconclusive. Exposure of this concentration for years or higher concentration for months may increase the probability of fluoride toxicity on hematological parameters. Detailed chronic toxicity studies of fluoride should be carried out on hematological parameters on higher animals.

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