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Research Article

Fluoride-Induced Hypercholesterolemia Could Be Protective In Fluorosis: High Cholesterol Attenuates Fluoride Toxicity In *In Vitro* and *In Vivo* Assays

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ABSTRACT

Hypercholesterolemia is a consistent finding in fluorosis. The implications of fluoride-induced hypercholesterolemia are not known. In this study, we investigated the effect of high cholesterol on fluoride toxicity in in vitro and in vivo models. In vitro MTT cytotoxicity was carried out on mouse spleenocytes exposed to fluoride (3.75, 7.5, 15 and 30 µM) either in the presence (250 or 500 µM) or absence of cholesterol. Acute oral toxicity was carried out in normal and triton wr-1339 (200mg/Kg *i.p*) induced hypercholesterolemic rats using up and down procedure (OECD 425 guidelines) assisted by AOT 425 software. Cholesterol effectively countered the cytotoxicity of fluoride. The half maximal inhibitory concentration (IC_{50}) of fluoride in 250 µM cholesterol ($190.23x10^3$ ppm) and 500 µM cholesterol ($459.95x10^3$ ppm) was significantly ($P<0.05$) elevated compared to control (0.052 ppm). Triton WR-1339 induced significant ($P<0.05$) hypercholesterolemia (222.44±13.55 mg/dL) compared to control (51.92 ± 8.68 mg/dL). In acute oral toxicity test, the LD_{50} in hypercholesterolemic group was (170.20 mg/kg; 95% CL: 164.00-290.00) significantly ($P<0.05$) higher than control group (92.00 mg/kg; 95% CL: 54.07-480.00). It is concluded that fluoride induced hypercholesterolemia is a protective response against fluoride toxicity.

Key Words: Fluoride, Median lethal dose, Cholesterol, Triton WR 1339, Inhibitory concentration

INTRODUCTION

Fluorosis is a major worldwide problem affecting both human and animal life. In India, fluorosis is endemic in 17 states with an estimated 66 million people at risk and 6 million people seriously affected[1]. In some countries like UK, fluoridation of water and many products to improve oral hygiene has resulted in increased exposure to fluoride[2]. Fluoride enters the body mainly through water and excessive fluoride results in fluorosis characterized mottling of teeth, osteoporosis, osteosclerosis and physical deformities[3].

High amounts of fluoride also causes metabolic disorders such as hyperglycemia[4, 5], oxidative stress[6-8] and hypercholesterolemia[9, 10]. Hypercholesterolemia is a consistent finding in fluorosis in both humans and animals[8, 11-14]. Such an increase in cholesterol (elevated HDL cholesterol) was also observed in cadmium toxicity in chicken[15]. In case of fluoride, there is an increase in the influx of cholesterol into macrophages via scavenger CD36 receptor[16]. Further, cholesterol also accumulates in significant quantities, in erythrocyte membrane[17, 18]. The physiological implications of hypercholesterolemia in fluorosis are hitherto not investigated. Hence, in this study, we investigated the toxicity of fluoride in the presence of high cholesterol in both in vitro and in vivo assays.

MATERIAL AND METHODS

Chemicals

Water soluble cholesterol and Triton WR 1339 (Sigma, St. Louis, USA); Sodium fluoride (Merck, Mumbai, India); RPMI-1640 medium (with HEPES and 2mM glutamine), Foetal calf serum, (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide (MTT) (Hi-Media, Mumbai, India). All other chemicals used in this study were of analytical grade.

In vitro cytotoxicity assay

MTT assay for fluoride was carried out in mouse spleenocyte model[19]. Briefly, 100µL of mouse spleenocytes (cell density $1x10^7$ and cell viability more than 90 %) were incubated with serial dilutions of sodium fluoride (30µM, 15µM, 7.5µM and 3.75µM fluoride) with and without added cholesterol (250 or 500µM) in a micro titer plate. All the concentrations were maintained in triplicates with appropriate cell controls. The plate was incubated at 37°C in 5 % CO_2 incubator for 24h. 10 µL of MTT was added to all the wells four hours prior to completion of incubation. The plate was centrifuged at 1200 g for 10 min and the supernatant was discarded.

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Later, 100 µL of DMSO was added to dissolve the formazan formed. The absorbance was read at 530 nm after 10 min with an ELISA reader.

Cell viability (%) = Absorbance of test well x 100/ Absorbance of cell control well

Determination of half maximal inhibitory concentration (IC_{50})

Probit analysis was used for the determination of IC_{50}. The percent survivability in cytotoxicity assay was converted to probits and plotted against corresponding $\log_{10}$ concentration of fluoride. Regression analysis was used to find the best-fit equation. IC_{50} was determined form the regression equation as follows

Half maximal inhibitory concentration (IC_{50}) = (5-Y-intercept)/ (Coefficient)

Animals

A total of 15 female Wistar albino rats of 150-170 g were housed at 24 ± 1º C with 12 h light and dark cycle. The rats were maintained on standard pellet diet supplied by Hindustan Lever Ltd, India and *ad libitum* water. Permission was obtained from institutional animal ethics committee prior to the experiment.

Induction of hypercholesterolemia

Hypercholesterolemia was induced by injecting Triton WR 1339 @ 200 mg/Kg *i.p* after 18 h of fasting[18]. The control group animals were similarly fasted and were administered normal saline. After 12 h of injection, blood was collected from both the animals from tail vein and serum was separated. Serum cholesterol was determined using standard kits supplied by Span Diagnostics Pvt Ltd, Gujarat.

Acute oral toxicity testing

The oral LD_{50} of fluoride was determined using Up and Down procedure (UDP) (OECD guideline 425)[19, 20] in both control and hypercholesterolemic animals. Each animal was administered a dose of sodium fluoride, dissolved in distilled water, as suggested by Acute Oral Toxicity (AOT) 425 software[21]. After each dosing, the animals were observed for a period of 48 h. If the animal died during the observation period, subsequent animal is dosed next progressively lower dose and if the animal survived, the next animal was dosed next progressively higher dose. The experiment is continued until the stopping criteria are met as determined by the software.

Statistical Analysis

The data of serum cholesterol was presented as mean ± standard error. Median lethal dose and lethal concentrations were presented as values with 95% confidence intervals (CI). The equality of means of serum cholesterol was tested by student's t-test. Half maximal inhibitory concentration (IC_{50}) and median lethal dose (LD_{50}) were determined by probit analysis. The statistical difference between the IC_{50} and LD_{50} values was compared by ratio method as described earlier[22]. Lethal dose or inhibitory concentration ratio was considered significant based on failure of the 95% CI of the ratio to bracket the value 1. Statistical package for social sciences (SPSS 19.0 V IBM, Illinois, Chicago) was used for the analysis. The level of significance was set at $p<0.05$.

RESULTS

In vitro cytotoxicity assay

The survivability of spleenocytes decreased with progressive increase in fluoride concentration (IC_{50} = 0.052ppm). Addition of cholesterol significantly ($P<0.05$) increased the survivability of the spleenocytes in the presence of fluoride. In the presence of cholesterol, at 250 µM concentration (IC_{50} = 190.23 x 10^3 ppm) and at 500µM concentration (IC_{50} = 459.95 x 10^3 ppm) the IC_{50} was significantly elevated compared to control (Fig 1).

Acute oral toxicity

Administration of triton WR 1339 significantly ($p<0.05$) increased serum cholesterol concentration (222.44 ± 13.55 mg/dL) compared to control rats (51.92 ± 8.68 mg/dL) animals. The Up and Down procedure assisted by AOT software determined the oral LD_{50} of sodium fluoride in normal rats as 92.00 mg/kg (54.07 – 480.00). In hypercholesterolemic rats, there was significant ($P<0.05$) elevation of oral LD_{50} (170.20 mg/kg; CI: 164.00 – 290.00) compared to control group (Table 1).

DISCUSSION

Fluoride is highly reactive and easily crosses cell membrane in the form of hydrofluoric acid by passive nonionic diffusion[23]. After entering the cell, fluoride affects cellular metabolism and physiology depending on the cell type, concentration, and time of exposure[7]. Fluoride can induce inflammation, cell contraction, inhibit protein synthesis and cell cycle progression, oxidative stress, and DNA damage[7]. Fluoride is toxic to several in vitro and in vivo systems. Sermatozoa incubated with 250µM of fluoride showed a significant decline in motility, glutathione level and altered lysosomal activity[24]. Sodium fluoride caused cell arrest from S to G2/M stage and inhibited the activities of 5'-nucleotidase, succinate dehydrogenase and acyl-carrier-protein phosphodiesterase in rat cerebral cortex astrocytes [25]. Fluoride is also cytotoxic to cultured human pulp cells and inhibits cell growth, proliferation, mitochondrial activity and protein synthesis[26]. Fluoride induced apoptotic was reported in several organs like lungs, kidneys, liver, brain, bone marrow, erythrocytes[7].

MTT assay measures living cells, which convert colorless MTT into a colored formazan through mitochondrial reduction[27]. This assay is successfully employed to assess the cytotoxicity of variety of compounds[28-30]. Fluoride in a dose dependent fashion decreased the survivability of spleenocytes in MTT assay. The addition of cholesterol at 250 and 500µM significantly attenuated the cytotoxicity of fluoride. In earlier works, enrichment of media with cholesterol increased the resistance of PC12 cells to the cytotoxicity of amylin peptides (AβP: 1-40; 1-42)[31]. Similarly, high cholesterol in acute myeloid leukemia cells prevented the cytotoxicity of chemotherapy[32]. In the above studies, the protective effect diminished upon depleting cholesterol from plasma membrane.

In this study, UDP was used to determine the median lethal dose of sodium fluoride in normal and hypercholesterolemic rats. UDP is a stepwise procedure,

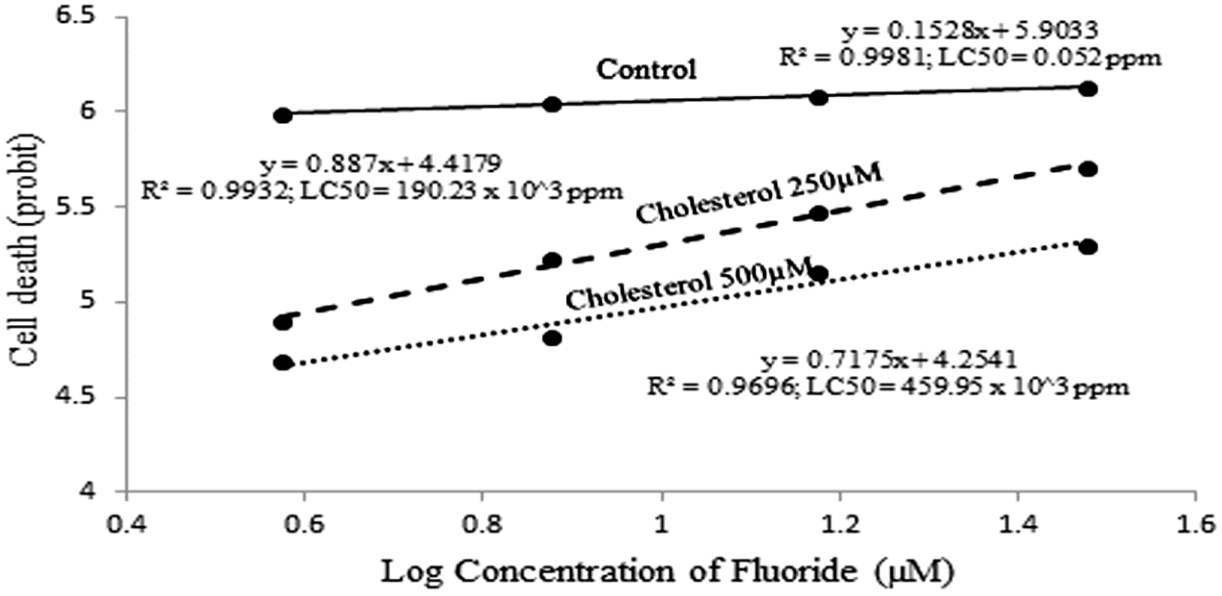

Figure 1: Half maximal inhibitory concentration (IC_{50}) of fluoride in the presence (250 and 500µM) and absence of cholesterol

Table 1: Median Lethal Dose of Sodium Fluoride (LD_{50} mg/kg) in normal and hypercholesterolemic rats

Group	No. of Rats	Body weight (g)	Serum Cholesterol (mg %)	Median lethal dose (Oral LD_{50}) (mg/kg)
1.Control	N = 6	158.56 ± 21.15	51.92 ± 8.68	92.00 (54.07 – 480.00)
2. Triton	N= 9	153.33 ± 15.28	222.44 ± 13.55**	170.20(164.00 – 290.00)*

Values are Mean ± S.D ; Student's t-test assuming unequal variance using SPSS Software 19.0 (V); LD_{50} values (95% Confidence Intervals); Median lethal dose was determined using AOT software
* P<0.05; ** P<0.01

in which individual animals are progressively dosed depending on the outcome from previous animal. The doses are generally increased or decreased by a factor of 3.2. This process continues until there is a reversal of the initial outcome (i.e., the point where an increasing dose results in death rather than survival, or decreasing dose results in survival rather than death). Further, additional animals are dosed until a stopping criterion is achieved or if selected upper (2000 or 5000 mg/kg) limit dose is reached[33]. This method has the advantages of using single animals of one sex and species (usually female rodents) with an average number of animals used between 6 to 9 and expected deaths between 2 to 3. Further, this procedure provides a point-estimate of LD_{50} with confidence intervals which helps in classifying the chemical according to Globally Harmonized System (GHS). The 95% confidence interval for the LD_{50} are ±32% in UDP using 6 to 9 animals, compared to ±15% in conventional studies using 40 to 50 animals[19].

The LD50 of sodium fluoride obtained by UDP in control group was 92.00 mg/kg (54.07 – 480.00), which is agreement with the previous reported value of 52mg/kg (43.16 - 60.84) for oral route[34]. Large doses of soluble fluoride forms corrosive hydrofluoric acid, which interferes with ion gradients in excitable cells, and/or precipitates divalent cations in serum and interferes with various enzymes. The symptoms of acute fluorosis are generally manifested as gastroenteritis, cardiac arrhythmias, and collapse[35]. Further, the inhibitory effect on cholinesterase is also associated with the toxic effects of fluoride[36]. In addition, fluoride is also reported to interfere with cellular respiration, generation of reactive oxygen species, apoptosis and interference with transport proteins of calcium, phosphate, glucose, Na-K ATPase[37, 38].

Hypercholesterolemia induced by Triton WR-1339 is a result of inhibition of endothelial lipase - in the initial period of injection (up to 3 h)- and due to increased hepatic synthesis by stimulating HMG-CoA reductase activity- in the later (from 9-12 h) period[39, 40]. The blocking of uptake of lipoproteins by extrahepatic tissues also contributes to increased cholesterol in blood[39,41,42]. In the hypercholesterolemic group, there was a significant elevation of oral LD_{50} (170.20 mg/kg; CI: 164.00 – 290.00) compared to 92.00 mg/kg (CI: 54.07 – 480.00) control group. Elevation of LD_{50} of fluoride in hypercholesterolemic group indicates protective effect of cholesterol against fluoride toxicity. Cholesterol is an antioxidant[43] and cholesterol derivatives such as glucocorticoids, testosterone, progesterone, and estrogen can act as free radical scavengers[44]. Hence, it is possible for high cholesterol to prevent fluoride induced oxidative stress and therefore prevent damage. In previous reports, induced-hypercholesterolemia offered significant protection against infection[45-47], neutralized *Staphylococcus aureus* α toxin[48], inhibited endotoxin

mediated cytokine release[49,50] and improved the survivability of rats injected with endotoxin [51,52.]

In conclusion, high cholesterol attenuated fluoride toxicity in both in vitro and in vivo systems. Hence, fluoride induced hypercholesterolemia could be a protective response against fluoride toxicity. The antioxidant and free radical scavenging properties of cholesterol might be responsible for protection against fluoride-induced free radical damage. However, further studies are required to corroborate and substantiate the present findings.

Conflict of interest

The authors declare that there is no conflict of interests with respect to this research work.

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