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Evaluation of multi-endpoint assay to detect genotoxicity and oxidative stress in mice exposed to sodium fluoride

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ABSTRACT

Fluoride compounds are naturally present in soil, water and food. The objective of this study was to investigate the genotoxic and oxidative damage induced by chronic fluoride exposure on mammalian cells *in vivo*. For this purpose, the genotoxic potential was investigated in bone marrow cells by the micronucleus test, chromosome aberration assay and comet assay (DNA strand breaks). In addition, DNA damage was evaluated in soft tissues and organs like spleen, liver and kidney cells. The oxidative damage was assessed by selective biochemical parameters by the measurement of lipid peroxidation, reduced glutathione (GSH), glutathione S-transferase (GST) and catalase (CAT) activity in liver. Adult Swiss albino male mice were exposed to sodium fluoride in drinking water at the concentrations of 4, 12 and 20 mg/L for 30 consecutive days. Control groups (vehicle and positive) were also included. Animals were sacrificed; bone marrow and soft tissue samples were collected and subjected to series of assays respectively. We observed that NaF exposure, at the various concentrations tested caused a significant increase in the frequency of micronucleus (MN) in polychromatic erythrocytes (PCEs), structural chromosome aberrations in bone marrow cells. With the exception of the spleen cells, DNA damage was observed in bone marrow cells as well as in kidney and liver cells. We found an increase in lipid peroxidation, and catalase activity as well as decrease in glutathione activity (GSH and GST) in liver of mice respectively which were exposed to sodium fluoride. In conclusion, the data obtained clearly documents that NaF exhibits genotoxic activity and enhanced oxidative damage in mouse model.

1. Introduction

Sodium fluoride (NaF) has been used ubiquitously for decades, due to its specific and effective caries prophylactic property; as well as used for water fluoridation. Excess fluoride ingestion is the cause of fluorosis in human being. The incidence of fluorosis affecting young and old, men and women is not only confined to India, but occurs in 23 other nations around the globe [1]. In addition to well-known effects on the skeleton and teeth, fluorosis can adversely affect many tissues and organs as exhibited by a broad array of symptoms and pathological changes [2–5].

Based on the epidemiological, *in vitro* and *in vivo* studies in human, human cell lines and rodents respectively, the National Research Council-US report [6] on fluoride in drinking water noted that the genotoxic effects of fluoride at environmental concentrations are contradictory. A number of the genotoxicity studies done *in vitro* [7–11] using cell lines or *in vivo* [12,13] are contradictory to the results that showed lack of genotoxic potential [14–23]. There are reports of increased chromosome aberrations in mice/rat bone marrow and testes *in vivo*, but other studies, using similar protocols and dose ranges, have reported no induced chromosome damage [24]. Zeiger et al. [24] in their review considered chromosome damage induced by fluoride *in vivo* as an unresolved issue.

In humans [1,25,26] and in animal models [13], a close association between chronic fluoride toxicity and increased oxidative stress has been reported. Numerous studies revealed that fluoride caused extensive oxidative stress in liver, kidney, brain and heart by increasing lipid peroxidation and reduced antioxidant enzyme activities like catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) [27–31]. Other investigators

Abbreviations: CAT, Catalase; SOD, superoxide dismutase; GST, glutathione S-transferase; ROS, reactive oxygen species; MMC, Mitomycin C; OSI, organo-somatic index; MN, Micronucleus test; PCE, polychromatic erythrocyte; NCEs, normochromatic erythrocytes; MN-PCE, micronucleated-polychromatic erythrocytes; CA, Chromosome Aberration; TBARS, Thiobarbituric acid reactive species; MDA, malonadialdehyde; CDNB, Chloro-dinitrobenzene; PCE/NCE, polychromatic erythrocyte/normochromatic erythrocyte.

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[22,27,32–34] however, have reported that fluoride does not impair antioxidant systems.

Research history of fluoride so far demands for further evidences to conclude on the role of fluoride as genotoxic through ROS (reactive oxygen species) production [35]. Presented here are the results of an evaluation of multiple genotoxic endpoints (chromosome aberrations, micronucleus formations and DNA strand breaks), combined with biochemical assays in liver (measuring lipid peroxidation, reduced glutathione level-GSH, glutathione S-transferase-GST activity and catalase activity- CAT) performed *in vivo* in mice using the same dosing regimen.

2. Material and methods

2.1. Chemicals

Sodium fluoride (NaF, CAS No. 7681-49-4), Bovine serum albumin (BSA), normal melting point agarose (NMA), low melting point agarose (LMPA), thiobarbituric acid (TBA, CAS no. 504-17-6), ethidium bromide (EtBr, CAS no. 1239-45-8), triton X-100 were purchased from Sigma–Aldrich Co. (USA). Roswell Park Memorial Institute medium (RPMI 1640), Fetal bovine serum (FBS), Phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free), glutathione reduced (CAS No. 70-18-8), 1-chloro-2, 4-dinitro benzene (CDNB, CAS No. 97-00-7), Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB, CAS No. 69-78-3], 2-nitrobenzioc acid (CAS No. 69-78-3), dithiothreitol (CAS No. 3483-12-3), di-sodium salt of ethylenediaminetetra acetic acid (EDTA), were purchased from Hi Media, Mumbai, India. Giemsa (CAS No. 51811-82-6), May-Gruenwald (CAS No. 62851-42-7), tris buffer (CAS No. 77-86-1), dimethyl sulfoxide (DMSO), trichloroacetic acid, methanol, hydrogen peroxide, acetic acid, potassium chloride, sodium citrate monohydrate, sodium hydroxide (NaOH), sodium chloride (NaCl) were purchased from Merck, India.

2.2. Animal handling and care

Healthy male Swiss-albino mice (8-12 weeks old and weighing 25–30 g) that were randomly bred at the institutional animal house were used for the study. The animals were kept in cages with autoclaved paddy husk for bedding and maintained under standard laboratory conditions (14 h: 10 h dark/light cycle, a temperature of (22 ± 2 °C), and 50–70% humidity). The animals were fed on with standard rodent pellets (consisting of crude protein, and fiber) and drinking water (containing NaF) *ad libitum* throughout the study. The ethical clearance for the use of animals in the study was obtained from the institutional animal ethics committee. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India.

2.3. Dose selection

According to World Health Organization (WHO) guidelines for drinking water, a fluoride level of 1.5 mg/L is the desirable upper limit and for the US Environmental Protection Agency [36] it is 4.0 mg/L. India reduced the upper limit of fluoride in drinking water from 1.5 to 1.0 mg/L with a rider that less is better [1]. In India, water sample analysis reveals fluoride contaminant level range from 0.2 to 20 mg/L [37]. Based on the above information following concentrations viz. 4, 12 and 20 mg/L of NaF were selected for the current study.

2.4. Dose preparation

Daily fresh solution of sodium fluoride was prepared in double distilled water. The animals had free access to drinking water, containing different concentrations of NaF (4, 12 and 20 mg/L) prepared in distilled water. Control set of animals were provided with distilled water. To avoid additional fluoride contamination from the water used, quantification of fluoride ion was performed using fluoride ion selective electrodes (WTW, Germany). The effective concentration of fluoride ion thus obtained was <0.087, 1.6, 6.1 and 8.6 mg/L for control, 4, 12 and 20 mg/L NaF concentrations respectively. Mitomycin C (MMC), dissolved in physiological saline was used as positive control.

2.5. Treatment schedule

The animals were divided into 5 experimental groups, each of six male mice as follows:

Group 1 – negative controls received distilled water in drinking water.

Groups 2, 3, 4 – Animals were exposed to NaF in drinking water at the concentrations 4, 12 and 20 mg/L, respectively.

Group 5 – Positive controls received a single ip. injection of MMC (2 mg/kg body weight) 24 h before sacrifice.

All animals except those of Group 5 were sacrificed on the thirtieth day of the experiment. Animals were sacrificed by cervical dislocation and the femur and other organs were removed. Micronucleus test and comet assay in multiple organs were conducted in same set of animals ($n = 6$), whereas chromosome aberration test and the enzyme assays were performed in another set of animals ($n = 5$) having identical groups.

2.6. Organo-somatic index of liver

The body weight of each animal was recorded before the treatment and also on the thirtieth day of the treatment. The weight of whole liver of respective group of animals was recorded. From these values the organo-somatic index (OSI) of liver was calculated by the following formula [38]:

Organo-somatic index = (Weight of the organ/Total body weight on day 30th) × 100

2.7. Micronucleus test (MN)

The micronucleus test was carried out in mouse femoral bone marrow cells and frequencies of micronucleated-polychromatic erythrocytes (MN-PCE) were evaluated according to the method of Schmid [39], with minor modifications [40]. Animals were sacrificed by cervical dislocation on thirtieth day of the experiment. The femoral bone marrow cells were aspirated using syringe and needle (21 G) with 3.0 ml of fetal bovine serum and centrifuged at 800 × *g* for 10 min. The supernatant was discarded; pellet was mixed, smeared on clean glass slides and fixed in methanol for 5 min. The fixed smear was stained with undiluted May–Gruenwald stain for 5 min followed by diluted May–Gruenwald stain (1:1, v/v in distilled water) for 3 min. The slides were washed with distilled water, stained with Giemsa (10%, v/v in Sorenson buffer) for 10 min and observed under light microscope (Carl, Zeiss, Berlin, Germany). All slides were coded and scored blind. The incidence of micronucleated (MN) cells per 500 polychromatic erythrocyte (PCE) was determined for each animal and the percentage of PCEs with MN was calculated. Thousand erythrocytes were scored from each animal to calculate the ratio of polychromatic erythrocyte (PCEs) to normochromatic erythrocytes (NCEs) and the toxic effect of NaF to bone marrow cells was evaluated.

2.8. Single cell gel electrophoresis (Comet assay)

The alkaline comet assay was performed according to the method of Singh et al. [41] with minor modifications. Animals were sacrificed, liver, kidney, spleen and femurs were removed and then single cell suspension was prepared as previously described by Sasaki et al. [42]. Briefly, the spleen, kidney and liver tissues were minced in 0.075 M NaCl solution containing 0.024 M Na_2EDTA (pH 7.5). Followed by the centrifugation, the cells were resuspended in phosphate buffered saline. The bone marrow cells were washed in 0.075 M NaCl solution containing 0.024 M Na_2EDTA (pH 7.5) and resuspended in phosphate buffered saline. Slides were prepared by mixing the cell suspension with 1% low melting point agarose; layered on the slide base coated with 1% normal melting point agarose and placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 10% DMSO, and 1% Triton X-100, pH 10.0) at 4 °C for 1 h. Then the slides were subjected to DNA unwinding in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH >13) for 20 min and subsequently electrophoresis was performed at 0.7 V/cm and 300 mA at 4 °C for 25 min in freshly prepared electrophoresis buffer (1 mM EDTA disodium salt and 300 mM NaOH). After electrophoresis the slides were neutralized with Tris buffer (400 mM, pH 7.4). Slides were stained with 20 μg/ml ethidium bromide (EtBr) and stored at 4 °C in a humidified slide box until scoring. Slides were scored at a final magnification of 400 × using an image analysis system (Komet 5.5, Kinetic Imaging, Andor technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Germany) equipped with an attachment of a CCD camera. The comet parameters used to measure DNA damage in the cells were tail DNA (%). Images from 150 random cells (per animal) were analyzed as per the guidelines [43].

2.9. Chromosome Aberration test (CA test)

The chromosome aberration test was performed on mouse bone marrow cells with slight modifications [44]. Animals were sacrificed by cervical dislocation on the thirtieth day of the experiment. One and half hours prior to sacrifice, animals were injected intraperitoneally with colchicine (4.0 mg/kg bw). The femoral bone marrow cells were aspirated in RPMI 1640 media and centrifuged at 800 × *g* for 10 min, the pellet was incubated in 8.0 ml of KCl (0.075 M) at 37 °C for 30 min, followed by centrifugation at 800 × *g* for 5 min. The cells were fixed in Carnoy's fixatives (glacial acetic acid/methanol, 1:3, v/v), washed thrice with Carnoy's fixative at intervals of 10 min. The pellet was resuspended in fixative and dropped on chilled slides from the height of 2–2.5 ft, and air dried. The slides were stained with freshly prepared Giemsa stain (8%, v/v in Sorenson buffer) for 10 min followed by washing with distilled water. A total number of 50 metaphases per animal were evaluated for chromosomal aberrations. The types of aberrations were scored and recorded strictly in accordance with the method of Tice and Ivett [45]. The metaphase cells were scored at 1000× magnification, with selection being based on uniform staining quality, lack of overlapping chromosomes and chromosome number (40 ± 2 chromosomes). Each chromosome aberration recorded was of the following types: G′, G″, as chromatid and isochromatid gaps; B′, B″, as chromatid and chromosome breaks, RR as chromatid rearrangement. Responses were evaluated as the percentage of