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Absence of DNA Damage in Multiple Organs after Oral Exposure to Fluoride in Wistar Rats

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Fluoride intake in low concentrations during tooth development results in formation of dental enamel, which is more resistant to caries (Denbesten and Thariani 1992). It was established that a concentration ranging from 0.5 to 1.5 ppm fluoride in drinking water reduces caries by 40-49% in primary teeth and 50-59% in permanent teeth, with no clinical appearance of adverse effects (WHO 1994). Nevertheless, some human populations are exposed to high doses of fluoride, mainly in developing countries. In this context, studies focusing on possible genotoxicity of excess fluoride are contradictory and inconclusive (Li et al. 1988). According to some authors, fluoride does not induce DNA damage in eukaryotic cells either in vitro or in vivo test system (Li et al. 1989; Ribeiro et al. 2004; Slamenova et al. 1992). However, some authors have described the mutagenic potential of fluoride in *Drosophila melanogaster* as well as synergistic and antagonist effect with known genotoxins (Gerdes 1971; Monsour et al. 1985). As the incidence of head and neck cancer has increased in recent years, particularly in developing countries such as India, Vietnam and Brazil, where it constitutes up to 25% of all types of cancer (Magrath and Litvak 1993), further risk factors other than tobacco smoke and the abuse of alcohol are of special concern.

According to the Center for Diseases Control, fluoridation of community drinking water for the prevention of dental caries was considered to be one of the ten most important public health achievements of the 20th century (Everett et al. 2002). After ingesting, fluoride is rapidly and extensively absorbed from the gastrointestinal tract (Whitford 1994). Owing to the action of clearance mechanisms, fluoride may be transported to the liver, kidney and urinary bladder as far as is ultimately excreted (Whitford 1999). Therefore, the oral exposure to sodium fluoride (NaF), which is the first and still-recommended fluoride compound used for fluoridation in drinking water, is important to evaluate the complete risk of fluoride. Thus, the purpose of this study was to investigate whether liver, kidney and urinary bladder are particularly sensitive organs for DNA damaging following gastrointestinal exposure to NaF by the single cell gel (comet) assay.

MATERIALS AND METHODS

Twenty female Wistar rats weighting approximately 150 g, were obtained from

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Bauru School of Dentistry, University of São Paulo, Brazil, and maintained under controlled conditions of temperature (24 ± 2°C), light-dark periods of 12 h, with free access to rat show diet (Purina, SP, Brazil) and water. The animals were randomly distributed into four groups (five animals per group): Group 1 (control) received tap water and basal diet *ad libitum*; Groups 2, 3, and 4 received 5, 15, and 100 ppm sodium fluoride (NaF), respectively, by drinking water and basal diet *ad libitum*. The lower doses were chosen based on fluoride levels currently used in city water supplies (0.5-1.5 ppm) (WHO 1994), taking into consideration rat metabolism, which is ten times faster than human metabolism in respect to fluoride (Denbesten and Crenshaw 1984). The higher dose used in this study (100 ppm of NaF) was defined as the dose producing signs of toxicity in the form of clinically detectable dental fluorosis, as previously described by other authors using this experimental design for studying dental fluorosis in rats (Denbesten and Crenshaw 1984; Denbesten et al. 2002; Smith et al. 1993). The body weight was recorded weekly.

After 75 days treatment, the rats were killed by i.p. injection of 0.5 mL/kg body weight of Anasedan (Agribrands, USA) + 1.5mL/kg body weight of ketamine hydrochloride – Vetaset (Fort Dodge, Iowa, USA). Peripheral blood (10 μL) was collected from the tail vein using a fine needle. Central fragments from the liver and kidney were collected and minced in 0.9% NaCl. The supernatant was removed and the cellular suspensions (~10 μL) were used for single cell gel (comet) assay. Urinary bladder cells were collected using disposable knife and transferred to plastic tubes containing 100 μL of cold phosphate buffer solution (PBS, Ca, Mg free). The cellular suspension (~10 μL) was used for single cell gel (comet) assay. Independent positive controls using cells from peripheral blood, kidney, liver and urinary bladder were *in vitro* treated with MMS (methylmethasulfonate) at 10μg/mL concentration for 10 min at 37 °C, in order to ensure reproducibility and sensitivity of assay. The Animal Committee of the Bauru School of Dentistry, University of Sao Paulo (USP), approved all the experimental protocols.

The protocol used for peripheral blood, liver, kidney and urinary bladder cells followed the guidelines purposed by Sasaki et al. (2002) with some modifications. Namely, a volume of 5μL of peripheral blood was added to 120 μL 0.5% low-melting-point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. The supernatant (cellular suspension) (10 μl) of the liver, kidney and urinary bladder were added to 120 μL 0.5% low-melting-point agarose at 37 C, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO), for about 1 h. After lysis, slides containing urinary bladder cells were treated with proteinase K (PK) (1 mg/ml in PBS, pH 7.4), for 1h, at 37°C. The PK solution (100 μl) was layered onto the

slide and a coverslip was added to achieve equal distribution of solution. Prior to electrophoresis, the slides were left in alkaline buffer (pH>13) for 20 min and electrophoresed for another 20 min, at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized onto 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol and stored until analysis. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

A total of 50 randomly captured comets per animal (25 cells from each slide) (Hartmann et al. 2003) were examined blindly by one expertise observer at 400X magnification using a fluorescence microscope (Olympus) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Sufolk, Haverhill, UK) calibrated previously with according to manufacturer's instructions. The computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Statistical analysis for body weight data was assessed by one-way ANOVA. The results obtained in the single cell gel (comet) assay were statistically analyzed as recommended by Wiklund and Agurell (2003). For this, the tail moment data were transformed into square root and the groups exposed to NaF were compared with the controls by one-way ANOVA. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The main object of the present study was to investigate if oral exposure to NaF results in a systemic genotoxic effect with liver, kidney and urinary bladder as target organs. To our knowledge, this is the first study in which the genotoxic effects of fluoride in multiple organs have been documented in vivo. Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds which induce genetic damage such as DNA strand breaks, gene mutation, chromosomal breakage or altered DNA repair capacity. To date, a variety of assays can assess genotoxicity, including those that assess metaphase chromosomal aberrations, micronuclei, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained technicians to accurately read and interpret slides. The single cell gel (comet) assay in alkaline version was developed as a rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells (Tice et al. 2000). The basic principle of the single cell gel (comet) assay is

the migration of DNA in an agarose matrix under electrophoretic conditions. When viewed under a microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode. It is important to notice that the alkaline version of the single cell gel (comet) assay used here is sensitive for a wide variety of DNA lesions. Among them are DNA strand breaks; alkali-labile sites lesions including abasic sites and incomplete repair sites (Tice et al. 2000).

Our findings displayed that the control group and those treated with 5 and 15 ppm NaF showed no changes in external surface of fully formed rat incisor enamel. On the other hand, the group chronically exposed to the higher level of NaF (100 ppm) pointed out a disturbance of the pigmented external surface of the fully formed incisors, characterizing dental fluorosis. We have also noted no statistically significant difference in body weight among groups (data not shown), suggesting that NaF did not interfere in the host basal metabolism.

Table 1. DNA damage expressed by tail moment (mean ± standard deviation) in blood, liver, kidney and urinary bladder cells of rats exposed to fluoride.

	DNA damage (Tail moment)			
	Blood	Liver	Kidney	Urinary bladder
Negative Control	0.49± 0.18	1.50± 0.22	0.55± 0.20	0.89± 0.39
NaF 5 ppm	0.50± 0.15	1.48± 0.66	0.90± 0.43	0.79± 0.47
NaF 15 ppm	0.70± 0.32	1.23± 0.48	0.95± 0.34	0.81± 0.37
NaF 100 ppm	0.48± 0.20	1.12± 0.65	0.79± 0.12	0.42± 0.16
Positive Control[1]	3.27± 0.56*	4.87± 1.24*	3.26± 0.77*	6.77± 1.86*

[1]MMS at 10μg/mL

*P<0.05 when compared to negative control.

Previous study conducted by our group has observed that NaF did not induce a significant increase in the level of DNA strand breaks in peripheral blood cells after oral administration (Ribeiro et al. 2004). In the current study, no increase in the level of DNA strand breaks for the all doses of NaF tested were detected in peripheral blood cells (Table 1). These data are in accordance with those described by of Li et al. (1995) and Kahlil and Da'dara (1994) in lymphocytes and rat bone marrow cells, respectively. In contrast, other authors have observed increased DNA breakage in human lymphocytes after in vitro incubation with NaF (Kleinsasser et al. 2001). Taken together, our results support the hypothesis that NaF does not induce genotoxic effect (DNA strand breaks) in blood cells.

According to the in vivo single cell gel (comet) assay guidelines (Tice et al. 2000), it is recommended to analyze cells from liver, since it is the main organ for metabolism. In our analysis of DNA damage, we were able to exclude cells that presented "clouds" of DNA considering that these cells could represent dead cells, resulting from putative cytotoxic effects of NaF rather than primary DNA-damage induced by direct interaction between DNA and genotoxic agent. One possible explanation for detected NaF genotoxicity is based on the hypothesis that NaF and its related compounds may induce lipid peroxidation through generation of free radical species, which contributes to alterations of cellular functions, genotoxic damage and tumour initiation (Anuradha et al. 2001; Wang et al. 2000). However, it is not clear how NaF absorbed by the gastrointestional tract could be able to exert these biological actions. Our data show no noticeable DNA damage in liver cells of rats exposed to NaF (Table 1).

Kidney and urinary bladder are the main pathways for the elimination of absorbed fluoride ions and the main target organs for fluoride toxicity as well (Kono et al. 1986; 1987). In this way, some authors have addressed that high doses of fluoride may cause nephrotoxicity as well as coagulation necrosis of the renal proximal tubules (Takagi and Shiraki 1982). Furthermore, studies indicate that the ingesting of excess fluoride facilities calcium oxalate crystalluria and promote the formation of bladder stones in rats, leading to extensive inflammatory process (Anasuya 1994). While inflammation helps to eradicate these pathologic processes, it is inevitable to expose target organs to certain endogenous genotoxic agents. This may be a reason why NaF can contribute either to kidney carcinogenesis or to the increased bladder cancer risk (Grandjean et al. 1992). Nevertheless, our results pointed out that NaF does not induce DNA damage in kidney and bladder cells at all doses tested (Table 1).

It is generally accepted correlation between genotoxic and carcinogenic effects of a variety of chemicals (Auletta and Ashby 1988). Whether the DNA damage is repaired or persists is important to the fate of organs targeted by chemical carcinogens. However, the development of tumors in target organs depends not only on the initial levels of induced DNA damage and its repair, but also on other contributing factors including the production of reactive metabolites, their distribution, and their effect on cell proliferation (Sasaki et al. 2002). For this reason, genotoxicity tests do not always reflect carcinogenicity. Moreover, in vitro and in vivo genotoxicity tests detect compounds that induce genetic damage directly or indirectly by various mechanisms. Therefore, no single test is capable of detecting all genotoxic agents. For a more detailed judgment on the genotoxic potential of NaF, a battery of tests is necessary.

REFERENCES

Anasuya A (1982) Role of fluoride in formation of urinary calculi: studies in rats. J Nutr 112:1787-1795

Anuradha CD, Kanno S, Hirano S (2001) Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Rad Biol Med 31:367-373

Auletta A, Ashby J (1988) Workshop on the relationship between short-term information and carcinogenicity; Williamsburg, Virginia, January 20-23, 1987. Environ Mol Mut 11:35-45

DenBesten PK, Crenshaw MA (1984) The effects of chronic high fluoride levels on forming enamel in the rat. Arch Oral Biol 29:675-679

Denbesten PK, Thariani H (1992) Biological mechanisms of fluorosis and level and timing of systemic exposure to fluoride with respect to fluorosis. J Dent Res 71:1238-1243

Denbesten PK, Yan Y, Featherstone JD, Hilton JF, Smith CE, Li W (2002) Effects of fluoride on rat dental enamel matrix proteinases. Arch Oral Biol 47:763-770

Everett ET, McHenry MAK, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, Martinez-Mier EA, Warrick JM, Stookey GK (2002) Dental fluorosis: variability among different inbred mouse strains. J Dent Res 81:794-798

Gerdes RA (1971) The influence of atmospheric hydrogen fluoride on the frequency of sex-linked lethal and sterility in Drosophila melanogaster. Fluoride 4:25-29

Grandjean P, Olsen JH, Jensen OM, Juel K (1992) Cancer incidence and mortality in workers exposed to fluoride. J Natl Cancer Inst 84:1903-1909

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR (2003) 4th International Comet Assay Workshop. Recommendations for conducting the in vivo alkaline comet assay. 4th International Comet Assay Workshop. Mutagenesis18:45-51

Khalil AM, Da'dara AA (1994) The genotoxic and cytotoxic activities of inorganic fluoride in cultured rat bone marrow cells. Arch Environ Cont Toxicol 26:60-63

Kleinsasser NH, Weissacher H, Wallner BC, Kastenbauer ER, Harreus UA (2001) Cytotoxicity and genotoxicity of fluorides in human mucosa and lymphocytes. Laryngorhinootologie 80:187-190

Kono K, Yoshida Y, Yamagata H, Tanimura Y, Takeda Y, Harada A, Doi K (1986) Fluoride clearance in the aging kidney. Stud Environ Sci 27:407-414

Kono K, Yoshida Y, Yamagata H, Watanabe M, Shibuya Y, Doi K (1987) Urinary fluoride monitoring of industrial hydrofluoric acid exposure. Environ Res 42:415-20

Li Y, Liang CK, Katz BP, Brizendine EJ, Stookey GK (1995) Long-term exposure to fluoride in drinking water and sister chromatid exchange frequency in human blood lymphocytes. J Dent Res 74:1468-1474

Li YM, Dunipace AJ, Stookey GK (1988) Genotoxic effects of fluoride: a controversial issue. Mut Res 195:127-136

Li YM, Zhang W, Noblitt TW, Dunipace AJ, Stookey GK (1989) Genotoxic evaluation of chronic fluoride exposure: sister-chromatid exchange study. Mut Res 227:159-165

Magrath I, Litvak J (1993) Cancer in developing countries: opportunity and challenge. J Natl Cancer Inst 85:862-874

Monsour PA, Kruger BJ, Smid JR (1985) Effects of a single intravenous dose of sodium fluoride on plasma electrolytes and metabolites in rats, rabbits and cockerels. J Dent Res 64:1281-1285

Ribeiro, DA, Marques ME, Assis GF, Anzai A, Poleti ML, Salvadori DMF (2004) No relationship between subchronic fluoride intake and DNA damage in Wistar rats. Caries Res 38:576-579

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S (2002) The comet assay with 8 mouse organs: results with 39 currently used food additives. Mut Res 519:103-119

Slamenova D, Gabelova A, Ruppova K (1992) Cytotoxicity and genotoxicity testing of sodium fluoride on Chinese hamster V79 cells and human EUE cells. Mut Res 16:99-115

Smith CE, Nanci A, Denbesten PK (1993) Effects of chronic fluoride exposure on morphometric parameters defining the stages of amelogenesis and ameloblast modulation in rat incisors. Anat Rec 237:243-258

Takagi M, Shiraki S (1982) Acute sodium fluoride toxicity in the rat kidney. Bull Tokyo Med Dent Univ 29:123-130

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mut 35:206-221

Wang YN, Xiao KQ, Liu JL, Dallner G, Guan ZZ (2000) Effect of long term fluoride exposure on lipid composition in rat liver. Toxicology 146:161-169

Whitford GM (1999) Fluoride metabolism and excretion in children. J Public Health Dent 59:224-228

Whitford GM (1994) Intake and metabolism of fluoride. Adv Dental Res 8:5-14

Wiklun SJ, Agurell E (2003) Aspects of design and statistical analysis in the comet assay. Mutagenesis 18:167-175

World Health Organization (WHO). Available from URL: http://home.iae.nl/users/lightnet/health/fluoride.htm. 1994