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Lack of DNA Damage Induced by Fluoride on Mouse Lymphoma and Human Fibroblast Cells by Single Cell Gel (Comet) Assay

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Fluoride has widely been used in Dentistry because it is a specific and effective caries prophylactic agent. However, excess fluoride may represent a hazard to human health, especially by causing injury on genetic apparatus. Genotoxicity tests constitute an important part of cancer research for risk assessment of potential carcinogens. In this study, the potential DNA damage associated with exposure to fluoride was assessed by the single cell gel (comet) assay *in vitro*. Mouse lymphoma and human fibroblast cells were exposed to sodium fluoride (NaF) at final concentration ranging from 7 to 100 μg/mL for 3 h at 37°C. The results pointed out that NaF in all tested concentrations did not contribute to DNA damage as depicted by the mean tail moment and tail intensity for both cellular types assessed. These findings are clinically important because they represent a valuable contribution for evaluation of the potential health risk associated with exposure to agents usually used in dental practice.

Key Words: comet assay, sodium fluoride, mouse lymphoma cells, human fibroblasts.

INTRODUCTION

Fluoride intake in low concentrations during tooth development results in the formation of a more caries-resistant enamel structure (1). It has been established that a concentration of 0.7 ppm fluoride reduces caries by 40-49% in primary teeth and 50-59% in permanent teeth, with no clinically detectable adverse effects (2). However, some human populations are exposed to high doses of fluoride, mainly in developing countries. In this context, studies focusing on possible genotoxic effect of excess fluoride are contradictory and inconclusive. According to some authors, fluoride does not induce DNA damage (3-6). However, some authors have observed the mutagenic potential of fluoride in Drosophila melanogaster (7) as well as synergistic and antagonist effect with known genotoxins (8).

Over the past decade, the single cell gel (comet) assay in alkaline version has been used as a rapid, simple and reliable biochemical technique for evaluating DNA damage in mammalian cells (9). The basic principle of the single cell gel (comet) assay is the migration of DNA in an agarose matrix under electrophoretic conditions. 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. This assay is particularly important for investigation of suspected genotoxins in vitro (10,11).

The purpose of this study was to investigate whether sodium fluoride (NaF) can induce DNA breakage in mouse lymphoma and human fibroblast cells by the single cell gel (comet) assay *in vitro*. The results would contribute to a better understanding of the mechanism of NaF concerning genotoxicity upon the cell system.

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MATERIAL AND METHODS

All procedures in this study were approved by and performed in compliance with the Ethics Committee of the Faculty of Medicine of Botucatu, UNESP, Brazil.

Cell Culture. L5178Y mouse lymphoma cells were cultivated in suspension in RMPI 1640 glutamax medium (Amersham Life Sciences Inc., Arlington Heights, IL, USA) supplemented with 10% heat-inactivated horse serum and penicillin/streptomycin (Life Technologies, Grand Island, NY, USA) at 100 µg/mL at 37° C with 5% CO_2 according to Rothfuss et al. (12). Human fibroblasts were cultivated as described by Stanley et al. (13). Namely, a fragment biopsy was extracted from skin and fibroblasts were seeded into each dish of multiwells (Corning, 10 cm in diameter) at 37°C in a 95% air/5% CO_2 humidified incubator in Dulbecco's Modified Essential Medium (DMEM; Life Technologies) supplemented with 20% fetal calf serum and 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). Cells were cultured for 3 days prior to treatment with the test substance.

NaF Treatment. 1 X 10^5 cells (~10 µL) were used in each treatment. Ten microliter of NaF (Sigma-Aldrich Corp., St. Louis, MO, USA) were added to the suspension of cells to give a final concentration that ranged from 7-100 µg/mL. These concentrations were defined as described in previous studies (14,15). The same volume was added to control cultures of either negative control (distilled water) or a reference alkylating agent methylmethane sulfonate (MMS) (Sigma-Aldrich Corp.) at 10 µg/mL concentration (positive control). Each substance was tested in at least 3 separate experiments for each individual treatment. After incubation for 3 h at 37°C, the cells were centrifuged at 1000 rpm during 5 min and washed twice with fresh medium and ressuspended with fresh medium.

Single Cell Gel (Comet) Assay. The protocol used for single cell gel (comet) assay for each treatment and controls followed the guidelines recommended by Tice et al (9). Briefly, 10 µL of cells (~1 X 10^5 cells) were added to 120 µL of 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 the slides were immersed in a lysing solution consisting of 2.5 M sodium chloride, 100 mM ethylenediaminetetraacetic (EDTA), 10 mM Tris-HCl buffer at pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% dymethylsulfoxide (DMSO) for about 1 h.

Prior to electrophoresis, the slides were left in alkaline buffer containing 0.3 mM NaOH and 1 mM EDTA (pH>13) for 20 min and electrophoresed for another 20 min at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored at room temperature until blind analysis in a fluorescence microscope (Olympus; Optical Co. Ltd, Tokyo, Japan) at X400 magnification. To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

DNA Damage. An automated analytical software (Comet Assay 2.2; Perceptive Instruments, Haverhill, UK) was used to determine DNA damage. Two parameters were estimated: tail moment (product of tail DNA/total DNA by the center of gravity) and tail intensity (percentage of DNA in the tail) from 50 cells *per* treatment.

Cytotoxicity Assay. Cell viability test for mouse lymphoma and human fibroblast cells was performed using Tripan blue staining before treatment (9). Namely, a freshly prepared solution of 10 µL Tripan blue (0.05%) in distilled water were mixed to 10 µL of each cellular suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Non-viable cells were blue-stained. At least 200 cells were counted.

Statistical Methods. Parameters in triplicate from the single cell gel (comet) assay for treated cells versus control cells were compared by one-way ANOVA followed by a post-hoc analysis at 5% significant level using the SPSS statistical software package, version 1.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The single cell gel (comet) assay was used to measure DNA damage in mouse lymphoma and human fibroblast cells *in vitro*. DNA strand breaks were represented by the mean tail moment and tail intensity for 50 comets *per* sample.

In all tested concentrations, NaF did not induce strand breakage in DNA of either mouse lymphoma cells (Table 1) or human fibroblast cells (Table 2). The mean cell viability was approximately 95% for both cell types assessed.

DISCUSSION

The aim of this study was to evaluate *in vitro* the NaF-induced genotoxic damage in different cell types using the single cell gel (comet) assay. Mouse lymphoma cells were chosen to investigate fluoride genotoxicity because the mechanism of DNA damage induced in these cells has been well documented. Human fibroblast cells were utilized because they represent ordinary cells. We believe that cell cultures have advantages over animal experimentation because they exclude the complex homeostatic mechanisms that occur *in vivo*.

It is noteworthy 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 including abasic sites and incomplete repair sites. According to the proposed *in vitro* single cell (gel) comet assay testing guidelines (9), cells should be exposed for 3-6 h. Herein, in this study, NaF was exposed for 3 h. Considering that alkylating agents are expected to be the most potent and abundant chemical DNA-damaging agent in our environment (16), MMS was used for alkylation damage, serving as a positive control.

Fluoride is well known as a specific and effective caries prophylactic agent and its systemic or local application has therefore been recommended widely over the past decades. However, excessive concentrations may cause extensive damage to biological systems. It has been established that therapeutic exposure to xenobiotics may result in their covalent binding to DNA, which may lead to genetic damage and could be an initial event in the process of chemical carcinogenesis (17). NaF, the first and still most recommended fluoride compound for fluoridation of drinking water, was able to induce morphological and neoplastic transformation of Syrian hamster embryo cells (18), as well as to increase chromosomal aberrations in Chinese hamster ovary cells (19). Furthermore, studies have shown that, although it is a non-oxidant ion, NaF caused oxidative stress indirectly leading to DNA breakage (19). The findings of the present study clearly demonstrated that NaF in all tested concentrations did not cause damage to DNA of either mouse lymphoma or human fibroblast cells. It is assumed that these negative results are because fluoride is not capable of forming adducts on the bases of DNA or those that intercalate into DNA secondary structure. These results are consistent with those of previous studies (3-6,15). An earlier study conducted by our research group revealed that NaF did not induce DNA damage in oral mucosa cells *in vitro* as well (11). On the other hand, it has been shown that NaF inhibited both protein and DNA synthesis in cultures of mammalian cells (20). It may be speculated that this inhibition of DNA synthesis is a secondary effect on DNA because there is no apparent mechanism by which the reported genotoxic effects can be induced by direct interaction of fluoride with DNA.

According to the proposed *in vitro* single cell gel (comet) assay testing guidelines (9), highly damaged DNA may correspond to dead cells; the latter were excluded from the analysis because they could reflect

Table 1. Mean (±SD) of DNA damage (tail moment and tail intensity) in mouse lymphoma cells exposed to NaF.

NaF (μg/mL)	DNA damage[1]	
	Tail moment	Tail intensity
0	0.25 ± 0.19	2.05 ± 1.89
7	0.21 ± 0.11	1.90 ± 1.25
28	0.52 ± 0.15	2.02 ± 0.47
56	0.24 ± 0.12	2.12 ± 1.13
100	0.22 ± 0.10	1.85 ± 0.16
MMS[2]	3.87 ± 1.40*	17.00 ± 5.29*

[1]Data from three independent experiments. [2]Methylmethasulfonate at 10 μg/mL concentration. * indicates statistically significant difference at $p<0.05$.

Table 2. Mean (±SD) of DNA damage (tail moment and tail intensity) in human fibroblast cells exposed to NaF.

NaF (μg/mL)	DNA damage[1]	
	Tail moment	Tail intensity
0	0.46 ± 0.13	3.69 ± 1.66
7	0.53 ± 0.17	6.77 ± 3.27
28	0.36 ± 0.15	5.40 ± 1.22
56	0.20 ± 0.15	2.59 ± 1.39
100	0.44 ± 0.14	5.36 ± 1.90
MMS[2]	3.50 ± 1.98*	22.59 ± 6.48*

[1]Data from three independent experiments. [2]Methylmethasulfonate at 10 μg/mL concentration. * indicates statistically significant difference at $p<0.05$.

possible cytotoxicity. Therefore, it is recommends that cellular viability is assessed concurrently with the single cell gel (comet) assay (18). In the present study, mouse lymphoma and human fibroblast cells were tested for cytotoxicity by trypan blue exclusion, and it was noticed that over 95% of cells excluded trypan constantly.

In conclusion, the results of this study indicated that NaF is not a genotoxin. Since genotoxicity tests constitute an important part of cancer research for risk assessment of potential carcinogens, the findings hereby reported represent a valuable contribution for evaluation of the potential health risk associated with agents usually used in dental practice.

RESUMO

O flúor tem sido amplamente usado na Odontologia, pois é um agente profilático efetivo e específico contra a cárie dentária. Entretanto, o flúor em excesso pode representar perigos à saúde humana, especialmente por causar agressão ao material genético. Testes de genotoxicidade constituem uma parte importante da pesquisa do câncer para a avaliação de risco de possíveis carcinógenos. Neste estudo, danos ao DNA associados à exposição ao flúor foram avaliados pelo teste de células individualizadas em gel de agarose (teste do cometa) *in vitro*. Células de linfoma murino e fibroblastos humanos foram expostas ao fluoreto de sódio (NaF) nas concentrações finais de 7 a 100 µg/mL durante 3 h a 37ºC. Os resultados mostraram que o NaF não contribuiu para os danos ao DNA em ambos os tipos celulares estudados e em todas as concentrações testadas, conforme demonstrado pelas médias do momento da cauda e intensidade da cauda dos cometas. Estes achados são clinicamente importantes, uma vez que representam uma importante contribuição para a avaliação do risco potencial à saúde associada à exposição a agentes geralmente empregados na prática odontológica.

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