

Fluoride does not induce DNA breakage in Chinese hamster ovary cells *in vitro*

Flúor não induz danos ao DNA em células de ovário de hamster chinês *in vitro*

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ABSTRACT: Fluoride has been widely 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 to genetic material. Genotoxicity tests represent an important part of cancer research to assess the risk of potential carcinogens. In the current study, the potential DNA damage associated with exposure to fluoride was assessed by the single cell gel (comet) assay *in vitro*. Chinese hamster ovary 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 concentrations tested did not contribute to DNA damage as depicted by the mean tail moment and tail intensity. These findings are clinically important since they represent an important contribution to a correct evaluation of the potential health risk associated with the exposure to dental agents.

DESCRIPTORS: Sodium fluoride; Comet assay; Mutagenicity tests.

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 representam uma importante parte da pesquisa do câncer para a avaliação de risco de possíveis carcinógenos. Neste presente 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 ovário de hamster chinês foram expostas ao fluoreto de sódio (NaF) nas concentrações finais de 7 a 100 µg/ml, durante 3 h, a 37°C. Os resultados mostraram que o NaF não contribuiu para os danos no DNA em todas as concentrações testadas, conforme demonstrado pelas médias do momento da cauda e da intensidade da cauda dos cometas. Esses achados são clinicamente importantes, uma vez que representam uma importante contribuição para a correta avaliação do potencial risco à saúde associada à exposição aos agentes odontológicos.

DESCRIPTORIOS: Fluoreto de sódio; Ensaio em cometa; Testes de mutagenicidade.

INTRODUCTION

Fluoride intake in low concentrations during tooth development results in formation of more resistant to caries dental enamel⁴. It was 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 clinical appearance of adverse effects²⁶. However, some human populations are exposed to high doses of fluoride, mainly in developing countries. Studies focusing on possible genotoxic effect of excess fluoride are contradictory and inconclusive¹⁶. According to some authors, fluoride does not induce DNA dam-

age^{10,13,18,22}. However, some authors have observed the genotoxic potential of fluoride in *Drosophila melanogaster*⁷ as well as synergistic and antagonist effect with known genotoxins²⁰.

Over the past decade, the single cell gel (comet) assay in alkaline version has been a rapid, simple and reliable biochemical technique for evaluating DNA damage in mammalian cells²³. 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,

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with head (the nuclear region) and tail containing DNA fragments or strands migrating in the direction of the anode. In particular, this assay seems important for the investigation of suspected genotoxins *in vitro*²¹ and *in vivo*⁵.

As a result and because of inappropriate evidence, the purpose of this study was to investigate, by the single cell gel (comet) assay *in vitro*, whether sodium fluoride (NaF) induces DNA breakage in Chinese hamster ovary (CHO) cells or not. We chose to study the genotoxicity of CHO cells because the mechanism of induced DNA damage in these cells is well documented. The results will contribute to a better understanding of the influence of NaF on genotoxicity to cellular system.

MATERIALS AND METHODS

Treatment of cells

CHO cells were cultivated in suspension in F-10 medium (Life Sciences, NY, USA) supplemented with 10% fetal calf serum (Life Technologies, NY, USA), 100 U/ml penicillin (Life Technologies, NY, USA) and 100 U/ml streptomycin (Life Technologies, NY, USA) at 37°C with 5% CO₂. Cells were seeded into each dish of multiwells (Corning, NY, USA, 10 cm in diameter) and cultured for 3 days prior to treatment with the test substance. At the beginning of the treatment, cells were trypsinized with 2 ml (0.15%) of trypsin solution (Life Technologies, NY, USA). After 3 minutes, 2 ml of the complete medium were added and the cells were centrifuged at 1,000 rpm during 5 minutes and washed twice with fresh medium and resuspended with fresh medium. 1×10^4 cells ($\cong 10 \mu\text{l}$) were used in each treatment. Ten microliters of NaF (Sigma, St. Louis, USA) were added to the suspension of CHO cells to achieve final concentrations ranging from 7-100 $\mu\text{g}/\text{ml}$. These concentrations were defined in accordance with referenced publications^{1,24}. The same volume was added to control cultures of either negative control (distilled water) or a reference alkylating agent MMS (methylmethasulfonate, Sigma Aldrich, St. Louis, USA) at a concentration of 1 $\mu\text{g}/\text{ml}$ (positive control). Each individual substance was tested in at least three separate experiments for each individual treatment. After incubating the test tubes for 3 h at 37°C, the cells were centrifuged at 1,000 rpm (179.2 x g) during 5 minutes and washed twice with fresh medium and resuspended with fresh medium.

Single cell gel (comet) assay

The protocol used for single cell gel (comet) assay followed the guidelines proposed by Tice *et al.*²³ (2000). Briefly, a volume of 10 μl of 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 (Life Technologies, Auckland, New Zealand), and covered with a coverslip. After brief solidification of agarose in refrigerator, the coverslip was removed and slides immersed in lysis solution (2.5 M sodium chloride - Merck, Darmstadt, Germany, 100 mM ethylenediaminetetracetic - EDTA - Merck, Darmstadt, Germany, 10 mM Tris-Chloridric acid - HCl buffer, pH 10 - JT Baker, Mexico City, Mexico, 1% sodium sarcosinate - Sigma, St. Louis, USA - with 1% Triton X-100 - Mallinckrodt, Kentucky, USA - and 10% dymethylsulfoxide - DMSO - Mallinckrodt, Kentucky, USA) for about 1 hour. Prior to electrophoresis, slides were left in alkaline buffer (0.3 mM NaOH - Merck, Darmstadt, Germany - and 1 mM EDTA - Merck, Darmstadt, Germany, pH > 13) for 20 minutes and electrophoresed for another 20 minutes, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (Life Technologies, Auckland, New Zealand) (pH 7.5), fixed in absolute ethanol (Merck, Darmstadt, Germany) and stored at room temperature until blind analysis in a fluorescence microscope at 400 X magnification. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

DNA damage

An automatized analysis system (Comet Assay 2.2 - Perceptive Instruments, Haverhill, UK) was used to determine DNA damage. Two parameters were estimated: tail moment (tail DNA/total DNA x center of gravity) and tail intensity (percentage of DNA in tail) from 50 cells per treatment⁸.

Cytotoxicity assay

Cytotoxicity assessment is an integral part of the single cell gel (comet) assay. Since cytotoxicity produces strand breaks that show up as increased DNA migration, it is recommended not to perform the single cell gel (comet) assay on samples with more than 30% cytotoxicity. Thus, cell viability test for CHO cells was performed using Tripan blue staining¹⁹ (Merck, Darmstadt, Germany) before

TABLE 1 - Mean \pm standard deviation of DNA damage (tail moment and tail intensity) in Chinese hamster ovary cells exposed to NaF.

NaF ($\mu\text{g/ml}$)	DNA damage ^a	
	Tail moment	Tail intensity
0 ¹	0.27 \pm 0.16	2.99 \pm 1.06
7	0.53 \pm 0.15	2.97 \pm 0.47
28	0.33 \pm 0.20	3.01 \pm 1.20
56	0.47 \pm 0.17	2.94 \pm 1.84
100	0.52 \pm 0.12	3.23 \pm 1.22
Positive control ²	3.50 \pm 1.23*	22.59 \pm 6.48*

^aData of three independent repeats; *p < 0.05; ¹distilled water; ²methylmethasulfonate (1 $\mu\text{g/ml}$).

the beginning of the treatment. Namely, a freshly prepared solution of 10 μl Tripan blue (0.05%) in distilled water was mixed to 10 μl of each cellular suspension during 20 minutes, and then 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 performed as recommended by Wiklund, Agurell²⁵ (2003). Namely, the migration data were transformed into root square and analyzed by one-way ANOVA using the software SPSS (version 1.0, SPSS Sigma Stat, Chicago, USA) for Windows. For statistic significance, p < 0.05 was considered.

RESULTS

The single cell gel (comet) assay was used to measure DNA damage in CHO cells *in vitro*. DNA strand breaks were represented by the mean tail moment and tail intensity for 50 comets/sample. As seen in Table 1, NaF did not induce strand breaks in DNA in any of the concentrations tested. The mean cell viability for CHO cells was approximately 95% (data not shown).

DISCUSSION

The aim of this study was to evaluate the NaF-induced genotoxic damage in CHO cells *in vitro*. The investigation was conducted utilizing the single cell gel (comet) assay. We have postulated that cell cultures have advantages over animal

experimentation since they afford highly defined culture conditions, thereby avoiding the complex homeostatic mechanisms that occur *in vivo*. It is important to notice that the alkaline version of the single cell gel (comet) assay used is sensitive for a wide variety of DNA lesions, such as DNA strand breaks, alkali-labile sites lesions including abasic sites and incomplete repair sites. These lesions were estimated by the software Comet Assay II through tail moment and tail intensity. Tail moment is a virtual measure calculated by the computerized image analysis system considering both the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the major indices of induced DNA damage among the various parameters calculated using this method. Tail intensity was calculated considering the percentage of DNA in the tail of comets. According to the proposed *in vitro* single cell gel (comet) assay testing guideline²³, cells should be exposed for 3-6 h. Herein, cells were exposed to NaF for 3 h. Considering that alkylating agents are expected to be the most potent and abundant DNA-damaging chemical found in our environment¹⁴, we used MMS for alkylation damage characterizing the 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 in 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 carcinogenesis⁶. NaF, the first and still-recommended fluoride compound used for fluoridation of drinking water, was able to induce morphological and neoplastic transformation of Syrian hamster embryo cells^{11,15}, as well as to increase chromosomal aberrations in CHO cells¹. Furthermore, studies have shown that although NaF is a non-oxidant ion, it caused oxidative stress indirectly leading to DNA breakage^{2,3}. Our results clearly demonstrated that NaF could not contribute to the damage of DNA. We assumed that these negative results were obtained because fluoride is not capable of forming adducts on DNA bases or those that intercalate in DNA secondary structure. Our results are consistent with those of other studies^{12,13,17,24}. An earlier study conducted by our group reported that NaF did not induce DNA damage in oral mucosa cells *in vitro* either²¹. On the other hand, studies conducted by some researchers have

reported that NaF inhibits both protein and DNA synthesis in cultures of mammalian cells⁹. Probably, the inhibition of DNA synthesis is a secondary effect on DNA and therefore there is no apparent mechanism by which the reported genotoxic effects can be induced by a direct interaction of fluoride on DNA. It is important to stress that the single cell gel (comet) assay does not necessarily provide evidence on a mutagenic potential of substances. The ability of this assay for detecting DNA damage makes possible to determine if a compound has the potential to induce DNA breakage as strand breaks; however this condition may or may not lead to true mutations.

According to the proposed *in vitro* single cell gel (comet) assay testing guideline²³, highly damaged DNA may suggest dead cells; the latter were excluded from the analysis, since they could reflect possible cytotoxicity. Thus, the single cell gel (comet) assay expert group recommends a concurrent assessment of cellular viability²³. Our results indicated that CHO cells were tested for cytotoxic-

ity by trypan blue exclusion, and constantly more than 95% of cells excluded trypan.

CONCLUSION

In summary, our results indicate that NaF did not induce DNA lesions in CHO cells *in vitro*. Since genotoxicity tests form an important part of cancer research and risk assessment of potential carcinogens, our study represents an important contribution to the correct evaluation of the potential health risk associated with agents usually used in dental practice.

ACKNOWLEDGMENTS

CNPq (National Council for Scientific and Technological Development) (grant number: 141501/2002-2) and TOXICAN (Center for Genotoxins and Carcinogens Evaluation) supported this study.

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Received for publication on Mar 01, 2004
Accepted for publication on May 20, 2004