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*Full Length Research Paper*

# Genotoxic damage in oral epithelial cells induced by fluoride in drinking-water on students of Tula de Allende, Hidalgo, Mexico

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**Fluoride ($F^-$) compounds are present on the earth's surface, water, volcanoes and are also a product of petrochemical and cement industries. Little amounts of $F^-$ are required for the formation of bones and enamel, however, according to World Health Organization (WHO), ingestion of over 1.5 mg/L of $F^-$ may be a health hazard due to the toxic effects on the kidney, liver and it may also cause dental or skeletal fluorosis. The aim of this study is to compare the genotoxic damage in cell of oral epithelium detected by (the) comet assay, through a population of scholars from two communities located in the city of Tula; one consuming high concentration of $F^-$ from drinking-water San Miguel Vindhó (SMV) and the other with levels under the limit La Malinche (LaM). 113 (students) teenagers between the ages of 12 to 15 were selected to obtain epithelial cells by internal cheek brushing. 30 of them were selected (from) by the dental clinic service of the Health Science Institute (UAEH, Mexico) as negative and positive control groups before and after professional appliance of sodium fluoride (NaF) (2%). 31 from LaM and 52 from SMV. 200 cells per person were analyzed to measure tail moment, tail length. Visual scoring was used to classify results according to degrees of damage. These results showed a significant difference between the populations, indicating very low basic damage in both the negative control group and LaM and severe damage in both the exposed population and the positive control group. These results indicate that high levels of $F^-$ may be the cause of genotoxicity in oral epithelial cells.**

**Key words:** Epithelial oral cells, DNA damage, fluoride, comet assay, Tula.

## INTRODUCTION

Fluoride ($F^-$) is a trace essential element for bones and teeth development in human beings and animals (Ling and Jian, 2006; Griffin et al., 2007). It is also potentially toxic with a low margin of safety (ASTDR, 2003). The human's consumption from drinking water to prevent dental decay is advised to be between 0.7 to 1.2 mg/L (Kaseva, 2006). Chronic exposure to $F^-$ over the limit established by the World Health Organization (WHO) (1.5 mg/L) is the cause of dental and skeletal fluorosis. It also increases the probability of renal diseases (WHO, 2004), liver and parotid glands damage (Shanthakumari et al., 2004), as well as brain damage, and is also linked to decreasing IQ in school children (Wang et al., 2007). Toxic effect over cells of soft tissues such as endothelium, gonads and neurological system has also been reported, including modifications in cell proliferation, membrane permeability and induction of apoptosis (National Research Council, 2006; Yan et al., 2007).

Studies in China and India reported an increased prevalence of skeletal fluorosis linked to the absorption of

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drinking water containing $F^-$ above the level of 1.4 mg/L (Jolly et al., 1968; Choubisa et al., 1997; Xu et al., 1997). However, such studies suffer from limitations: the diagnostic criteria are not always specified or consist of self-reported symptoms and only drinking water is considered as a source of exposure. If the intake of $F^-$ totals is between 6 to 14 mg/day, there is a clear risk of skeletal adverse effects (Li et al., 2001).

$F^-$ can be found naturally in mineral deposits of non-metallic compounds, in sedimentary, hydrothermal, metamorific and also volcanic soils, which determine the nature of the water source (Hernández, 1997). In Mexico, high levels of $F^-$ in water is chronic and endemic in the zone called "fluorite belt" that includes the states of Coahuila, Durango, Zacatecas, San Luis Potosí, Guanajuato and Queretaro (Ortega, 2009). The State of Hidalgo is not in this area; nevertheless, the geological properties of Tula de Allende which are characterized by natural deposits of limestone, kaolin, gypsum, dolomite, quartz, clay and silica are associated with very high levels of $F^-$ in underground water (Geological Monograph, 1992).

Tula de Allende is located over the geological volcanic fault of El Doctor (Segerstrom, 1961), constituted principally by limestone deposits. Those deposits are used in the production of cement, which is one of the main economic activities of this municipality, as well as, thermo electrical, the petroleum industry and agricultural production. Tula is located north 20°10´, south 19° 57´ latitude, longitude east 99° 15´, and west 99° 30´ and at an altitude of 2477 m above sea level. In a previous report, Vazquez-Alvarado et al. (2010) showed high levels of $F^-$ found in Ex-Hacienda well which provides drinking water to the community of San Miguel Vindhó (SMV), with an average yearly concentration of 1.41 mg/L (April, 2008 to April, 2009), and a maximum of 1.99 mg/L observed in April, 2009. The control well Manzanitas-I, had an average of 0.62 mg/L with a maximum level of 0.78 mg/L on April of the same year, this well supplies tab water to the community of La Malinche (LaM). A correlation between the high concentration of $F^-$ found in the water of SMV and the presence of dental fluorosis with 85% of prevalence in its community was established. Meanwhile, a prevalence of only 4% was observed in the community of LaM.

Currently, lymphocytes of peripheral blood are the most used biomarkers in human; nevertheless, it is also important to use other tissues such as oral epithelium which is located in one of the most common site of malignant transformation (Szeto et al., 2005). Comet assay is useful to evaluate genotoxcity. It is a fast and simple technique, which analyses and quantifies with accuracy DNA damage in nucleated cells (Singh et al., 1988; Horváthová et al., 1998). Cell damage can be measured individually, and DNA breaks can be determined on cells obtained from diverse tissues of a single organism (Obwald et al., 2003; Tovalin et al., 2006). Comet assay has been used in humans (Lee et al., 2004) to establish the genotoxic effect in individuals exposed to different damaging contributors including radiation, chemical and oxidative stress (Tice et al., 2000; Dusinka and Collins, 2008). The first place in contact with xenobiotics, including the food, are the epithelial cells of the mouth (Winning and Townsed, 2000), making these cells an attractive target with the potential to determine *in vivo* DNA damage (Glei et al., 2005; Landi et al., 2003; Kleinsasser et al., 2006). The aim of this study is to determine if the $F^-$'s concentration in drinking water is a significant factor in the induction of genotoxicity on the exposed community of SMV, by the comet assay on oral epithelium cells.

## MATERIALS AND METHODS

### Chemicals

Low melting point agarose (LMPA), dimethyl sulfoxide (DMSO), ethylene diamine tetraacetic acid (EDTA) disodium salt, Triton 100X, Trizma, ethydium bromide and blue trypan solution 0.4%, were obtained from Sigma-Aldrich (St. Louis MO., USA.). Agarose, trypsine 1:250 of porcine pancreas from Gibco In vitrogen were obtained from Carisbad Ca. USA. Sodium chloride, potassium phosphate, sodium phosphate, sodium hydroxide and methanol, were obtained from JT Baker Mexico city; deionized water was purchased from Hycel (Mexico city). Saline isotonic solution and distillated water were purchased from PiSA Laboratory (Mexico City). Interdental brushes with no additives were obtained from Oral B (Braun, Mexico city). Commercial sodium fluoride gel (2%) for professional application was supplied by Medicom (Montreal, Quebec, Canada).

### Geographical characteristics

SMV is an urban location in the south of the municipality of Tula de Allende lying at an altitude of 2,100 m. It has 10,488 inhabitants. LaM is in the north of the city, 2030 m above sea level, it has 2,000 inhabitants. Both communities studied are located in the same municipality and have very similar geographic and socio-economic conditions (Delimitation of metropolitan areas in Mexico, 2005).

### Water sample

The wells of Ex- Hacienda and Manzanitas-I, were sampled for this study on March, 2009 according to Mexican Official Norm (NMX by its abbreviations in Spanish) (NMX-AA-051-SCFI-2001) by personal of Development and Research Laboratory of Studies of Quality of the Water (IDECA, S.A. de C.V., México, city). One litre (1 L) of water was taken in polyethylene bottles previously washed with HCl 10% and rinsed with distilled water, the recipients were labeled with identification codes including: date, time, location, air temperature, water temperature, name and signature of the person that performed the sample.

Temperature was measured with a check temperature pocket thermometer (HANNA instruments) *in situ*, calibration number H198501. pH was taken with a digital meter (OAKTON 1234251). Samples were transported in an icebox to maintain the temperature at 4°C (39.2 °F). Once in the laboratory, water samples were distillated according to Standard Methods (APHA-WPCF-AWWA, 1998). This took place before the start of the procedure to

determine procedure to determine $F^-$'s concentration in order to eliminate any interference due to color, turbidity and presence of other substances. Measurements were performed with a spectrophotometer by SPANDS method in acid media, based on Mexican regulations (NMX-AA-077-SCFI-2001), and concentrations of $F^-$ were determined by comparing with a standard calibration curve. In addition, quantification of carbonates (NMX-AA-036-SCFI-2001) and aluminum (NMX-AA-051-SCFI-2001) were realized to avoid possible interferences in $F^-$ determination.

**Populations of study**

The population of study was 113 scholar children and the criteria of inclusion for the pupils were:

(1) No gender discrimination
(2) Age group between 12 and 15 years old.
(3) Must be born and must live in the community of which water supply was being sampled.
(4) Have informed and signed parental consent.
(5) Voluntary participation in the study.
(6) Have not undergone orthodontic treatments.

Social-demographic data was obtained from a survey completed by the population of the study. Volunteers were questioned about smoking and alcohol consumption habits, viral diseases, history of exposure to X-rays and recent vaccinations. In addition, each volunteer was examined to evaluate the presence of lesions or infections in the oral cavity to rule out any possibility that could interfere with the results.

Ethical aspects were strictly followed to protect the dignity and the wellbeing of the subjects involved, ensuring respect, confidentiality and the protection of human rights. The ethical considerations were based on the General Law of Health of Mexico (2007) which deals with studies related to the inspection of human beings.

Thirty (30) volunteers were selected as the control group from the dental clinic service of the Health Science Institute (Autonomus University of Hidalgo State), for professional application of sodium fluoride (NaF) (2%) to prevent tooth decay. Samples of epithelial cells were obtained before and after application of the $F^-$ to provide negative and positive control groups, respectively. $F^-$ was applied in special trays for the duration of 4 min. Once the trays were removed, patients were asked to spit and at the same moment samples were taken with the interdental brush on the left and right side of the mucosa of the cheek, avoiding contact with teeth, tongue and gums. This process was carried out in less than 60 s. Concerning the communities, 31 students were sampled from the non-exposed community (LaM) and 52 from the exposed area (SMV).

**Comet assay**

Samples were obtained in the morning before recess at a time when students had not done any previous physical activity. Epithelial cells of buccal mucosa were collected from each student according to Besarati-Nia et al. (2000) by repeatedly washing out the mouth with tepid distillated water to remove exfoliated death cells and then gently brushing the internal part of both the right and the left cheeks with an interdental brush. Two samples per volunteers were taken. The brushes were immersed in 200 µl phosphate-buffered saline (PBS) 37°C and stirred in a vortex for 5 s to obtain the cell suspension. Viability of the cells was determined by Trypan blue dye exclusion, with 95% of cell survival. 10 µl of cell suspension were mixed with 75 µl of LMPA (0.5%). The mixture was expanded with a cover slide over a microgel of normal agarose (1%) and placed in-between microscope slides previously coded and left over ice for gelation. Five minutes (5 min) later, a second layer of LMPA was added. The micro-slides were immersed for 30 min in a solution of trypsine 0.25% at 37°C (Szeto et al., 2005) before being rinsed in saline isotonic solution and immersed in solution of lysis (2.5 M NaCl, 100 mM $Na_2$ EDTA,10 mM Tris, Triton 100X 1%, DMSO 10% pH 10) at 4°C for 4 h. Slides were then rinsed and placed immersed in buffer (NaOH 300 mM, 1 mM $Na_2$ EDTA, pH > 13) in an electrophoresis chamber for 30 min, after what electrophoresis was performed for 20 min (300 mA, 25 V). Slides were washed three times with 0.4M Tris pH = 7.5, and dehydrated with absolute methanol for 5 min for their preservation (Tice et al., 2000).

**Qualitative evaluation**

Slides were dye with 50 µl of ethidium bromide (0.02 mg/ml). 100 cells per sample were observed and classified in a visual scale according to Browne (2009). The criteria to determine the percentage of damaged nucleus and to calculate the damage index (DI) in order to obtain a numeric value for statistical analysis following this formula:

DI = % of nucleus grade 0 (0) + % of nucleus grade 1 (1) + % of nucleus grade 2 (2) + % of nucleus grade 3 (3)+ % of nucleus grade 4 (4)

(#): number of times the size of the head appeared to migrate on the tail.

**Quantitative evaluation**

From every sample, 100 cells were measured to determine tail moment (TM) and tail length (TL) using Metasystem Image Analyzer and the COMET 2.0 software, with fluorescence Microscope Carl Zeiss Axioimager using 20 X/0.065 dry, exciter filter 515 to 560 nm and barrier filter 590 nm.

**Statistical analysis**

Data were analyzed with Instat 3.0 software using non-parametric Wilcoxon test for damage index. Student T-test was used for comparing TM and TL among populations and controls, for both, were considerate $P < 0.05$, CI of 95% and standard error (SE).

## RESULTS

### Water quality

Concentration of $F^-$ was equal to 1.67 mg/L in the Ex-Hacienda well which is the source of drinking water to the SMV community. This can be considered over the maximum advisable limit of Mexican regulation (NMX-AA-077-SCFI-2001) for allowed level of $F^-$ (1.5 mg/L). Water temperature was 20°C, air temperature 29.2°C, pH *in situ* 7.69, aluminum < 0.2 mg/L, alkalinity 287 mg/L. For the Manzanitas-I well which provides drinking water to LaM community, the level of $F^-$ was equal to 0.69 mg/L, water temperature 24.1°C, air temperature 29.2°C, pH 7.4, aluminum < 0.2 mg/L and alkalinity 217 mg/L.

**Table 1.** Comet categories for visual scoring grade 0 to 4 in scholar aged 12 to 15.

| School population | Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 | Average mean and SE | Confidence intervals (CI) |
|---|---|---|---|---|---|---|---|
| Negative control | 95.2 | 0.3 | 1.0 | 0.8 | 2.7 | 15.36 ± 2.52 | (10.20, 20.53) |
| Positive control | 41.7 | 3.3 | 3.0 | 7.0 | 45.0 | 210.97 ± 11.09[a] | (188.27, 233.66) |
| LaM (control population) | 97.0 | 0 | 0 | 0.1 | 2.7 | 12.83 ± 1.76 | (9.22, 16.43) |
| SMV (exposed population) | 40.0 | 7.0 | 2.0 | 5.0 | 46.0 | 190.50 ± 9.71[ab] | (170.97, 210.03) |

The damage index was determined using non-parametric Wilcoxon test. [a]Statistical difference when compared to negative control versus all groups, P < 0.0001; [b]Statistical difference when La Malinche is compared with San Miguel Vindhó, P < 0.0001.

**Table 2.** Measurement of TM and TL in controls and both communities of Tula.

| School population | Average mean and SE | Confidence Intervals 95% | P value |
|---|---|---|---|
| **Negative control** | | | |
| Tail moment (TM) | 6.54 ± 1.40 | (3.66, 9.42) | |
| Tail length (TL) | 10.29 ± 2.25 | (5.69, 14.89) | |
| **Positive control** | | | |
| Tail moment | 118.37 ± 6.58[a] | (104.91, 131.83) | |
| Tail length | 128.47 ± 7.25 | (113.64, 143.30) | |
| | | | <0.0001* |
| **LaM (control population)** | | | |
| Tail moment | 4.81 ± 0.64 | (3.53, 6.10) | |
| Tail length | 3.89 ± 0.79 | (2.29, 5.48) | |
| **SMV (exposed population)** | | | |
| Tail moment | 104.01 ± 4.90[ab] | (94.27, 113.75) | |
| Tail length | 111.45 ± 5.14 | (101.23, 121.67) | |

The measurement of the Tail Moment and Tail Length were considered with the test of Student-Newman-Keuls. [a], Presents statistical difference when compared to negative control versus all groups, P < 0.0001; [b], presents statistical difference when compared La Malinche with SMV, P < 0.0001.

## Comet assay

Table 1 shows the results of the percentage of damaged nucleus observed for the negative control, positive control, LaM and SMV, graded according to Browne´s scale. Grade zero represents intact nucleus; Grade one minimum damage, Grade two moderate damage, Grade three severe damage and Grade four highly damaged. From those results, we can observe that in the negative control and non-exposed population most of the nucleuses are intact with 95.2 and 97%, respectively graded zero. On the other hand, the positive control 41.7% and SMV 40% could be considered intact and 45 and 46% highly damage with a classification on grade four, respectively. Statistical analysis of DI showed significant differences between positive control and SMV when they were compared to the negative control, indicating an increase in DNA damage. For LaM, there are not significant differences in this parameter.

Results in Table 2 show average values and standard error of TM and TL for controls and communities. TM 118.37 and TL 128.47 nm of the positive control had a significant increase when compared with the negative control TM 6.54 and TL 10.29 nm. For LaM, the basal genotoxic damage with TM average equal to 4.81 and TL 3.89 nm, was similar to the one observed in the negative control, nevertheless, for the exposed community, values of TM reached 104.01 and TL 111.45 nm, similar to those observed in the positive control group; this data indicates that the induction of genotoxic damage in the epithelial cells may be directly related to the high concentration of fluoride in water from the Ex-Hacienda well consumed by SMV.

## DISCUSSION

In all the statistical tests that were conducted in this study, the confidence intervals (CI) denoted a strong statistical power and all P values (<0.0001) were statistically

significant. Dusinska and Collins (2008) mentioned that when an individual measurement of comets is used for a population, SE value must be low. The number of cells evaluated per person (200 cells) gave a total of 6,000 for control groups and 6.200 for non-exposed community (LaM); 10,400 for exposed population (SMV), giving enough statistical creditability to establish a possibility of genotoxic effect of $F^-$ under the experimental conditions.

Basal damage of epithelial cells in normal conditions is very low as observed in the negative control group where most of the nucleus were classified with no damage, and had low values on TM and TL. This indicates that the manipulation of the cells as biomarkers during the sample and experimental procedure was correct, and avoided false positive results. For human studies, cells such as peripherial lymphocytes (Yañez et al., 2003; Jasso et al., 2007) or spermatozoids (Codrington et al., 2004) are used as biomarkers of genotoxicity caused by environmental xenobiotics, nevertheless, the use of other type target cells is needed. Other studies using oral epithelial cells have indicated that they can be used as a good biomarker since they are the first in contact with ingested xenobiotics, the sample is easy to obtain, and the sensitivity to detect genotoxicity is high (Szeto et al., 2005; Ribeiro et al., 2004; Winning and Townsend, 2000).

The increase on DNA damage of epithelial cells when they were in contact with NaF made evident the high genotoxic potential of $F^-$, since most of the cells of this group were classified with the maximum degree of damage (45%). Considering that the samples were taken from the same students used for negative control, we can infer that the topical appliance of fluoride gel on teeth is able to induce a very significant DNA damage on the oral epithelial cells in an acute exposure.

For LaM community, genotoxic damage is as low as the one observed in the negative control group, and even though the city of Tula has an important pollution problem due to its industrial activities (ATSDR, 2003; Shashia, 2003; Wania, 2004). The life style and socio-demographic conditions of the students did not influenced the basal level of DNA damage of oral epithelium cells, validating this community as a good population of reference.

A marked genotoxic effect was found in the exposed community with similar distribution observed in the positive control group, for grade of damage, damage index, TM and TL. This indicates that it is very possible that the induction of DNA damage observed in the SMV is directly related with the presence of $F^-$ in the water consumed in the area; since social-demographic conditions were very similar to those observed at LaM. On March 2009, levels of $F^-$ at the Ex-Hacienda well (1.67 mg/L) were above the level permitted by the Mexican regulation. This was enough to have a very similar response to the DNA damage found in the positive control where the concentration of $F^-$ was more higher, indicating the low security margin of $F^-$. Genotoxicity of $F^-$ evaluated with comet assay using hippocampus neuronal cells *in vitro* was reported by Zhang et al. (2008), there was an increase in TM for exposition to 2.1 and 4.2 $F^-$ mM for 24 h. Also in fetal teeth exposed to $F^-$ (10 and 20 mM) for 48 h there was a decrease in cellular proliferation and an increase on apoptosis (Yan et al., 2007).

Exposure to $F^-$ affects cells of hard tissues such as teeth and bones, as well as in soft tissues such as gonads, kidney, endothelium (National Research Council, 2006). Exposure to $F^-$ increases the production of superoxide as a consequence of hydrogen peroxide metabolism, inducing peroxinitrate, hydroxyl radicals increasing oxidative stress (Barbier et al., 2010; Garcia-Montalvo et al., 2009; Chinoy, 2003), and inducing lypoperoxidation of membranes, apoptosis and DNA damage (Wang et al., 2007). The clinical application of NaF induced genotoxicity on the oral epithelium in acute exposition according to Zeiger et al. (1993). Tiwari and Rao (2010) considered $F^-$ as a mutagenic agent that even in low concentrations can increase the induction of chromosomal aberrations. In a previous study, the exposed community was observed to have dental fluorosis and a correlation with the high concentration of $F^-$ in the well water (Vazquez-Alvarado et al., 2010). However, the high concentration of $F^-$ also affected DNA of epithelial cells, making them susceptible to mutations, transformation and neoplasia development (Guachalla and Ascarrunz, 2003), if the DNA damage is unable to be repaired. Evidence that cytogenetic biomarkers are correlated with cancer risk has been strongly validated in recent results from both cohort and nested case-control studies, showing that Comet assay is a marker of cancer risk. Reflecting both, the genotoxic effects of carcinogens and individual cancer susceptibility (Bonassi et al., 2007; Smerhovsky et al., 2001; Rekhadevi et al., 2007)

In conclusion, $F^-$ in water for human consumption over the limit of 1.5 mg/L is able to induce a strong genotoxic effect; therefore, other epidemiological studies must be done to establish the toxic impact over the health of an open population chronically exposed to this agent.

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