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Genotoxic effect and rat hepatocyte death occurred after oxidative stress induction and antioxidant gene downregulation caused by long term fluoride exposure

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ABSTRACT

Studies focusing on possible genotoxic effects of excess fluoride are contradictory and inconclusive. Currently, studies have reported a probable link to oxidative stress, DNA damage and apoptosis induced by fluoride in rat hepatocytes. We developed an *in vivo* study administering three doses of fluoride by gavage given to rats for 60 day. Micronucleus test was applied to investigate genotoxic potential of fluoride. The TUNEL method determined DNA fragmentation and apoptosis. Biochemical parameters to investigate mitochondrial swelling and oxidative stress. Semi-quantitative RT-PCR and immunostaining to determine mRNA and protein expression of antioxidant enzymes. Analyses of the hepatic function and morphology were performed. Our results revealed the genotoxic potential of fluoride but did not confirm mitochondrial swelling nor an increase of positive TUNEL labelling induced by fluoride, indicating absence of apoptosis. Oxidative stress induction was confirmed and is probably associated to DNA damage. Cell death events such as empty nuclear spaces, cytoplasm degeneration, nuclear pyknosis, karyorrhexis and karyorrhexis followed by karyolysis were observed. Hepatic function did not appear to be significantly modified makes no evidence of necrosis and suggesting other cell death pathway, the autophagic. In conclusion, prolonged fluoride intake at chosen concentrations caused imbalance of the cellular oxidative state, affected DNA and disrupted cellular homeostasis. It is recommended that fluoride supplementation requires a fresh consideration in light of the current study.

1. Introduction

Many countries practice community water fluoridation (CWF) which has been used to reduce caries. Cost-benefit analysis used to support CWF in the U.S. assumes negligible adverse effects from CWF and omits the costs of treating dental fluorosis, of accidents and overfeeds, of occupational exposures to fluoride, of promoting CWF, and of avoiding fluoridated water [1].

However, metabolic, functional and structural damage caused by chronic fluorosis have been reported in many tissues [2]. It is known that the toxicity of fluoride is associated with ROS induction and the reduction of cellular antioxidant defenses against oxidative

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Abbreviations

CWF	community water fluoridation
DNA	desoxyribonucleic acid
PI3K	phosphatidylinositol-3 kinase
Akt1	serine/threonine protein kinase 1
MDA	malondialdehyde
IL-1β	interleukin 1β
IL-6	interleukin 6
TNF-α	tumor necrosis factor α
Co	control group
T1	group exposed to 1 mg of fluoride/mg/day
T10	group exposed to 10 mg of fluoride/mg/day
T15	group exposed to 15 mg of fluoride/mg/day
MMS	methyl methanesulfonate
ALT	alanine aminotransferase
CAT	catalase
-SH	reduced sulfhydryl groups
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EGTA	ethylene glycol tetraacetic acid
HE	hematoxilin/eosin
RT-PCR	Reverse Transcriptation Polymerase Chain Reaction
Mn-SOD	Mn superoxide dismutase
GSTM1	gluatione-S-transferase M1
cDNA	complementary DNA
dNTP	Deoxyribonucleotide triphosphate
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
DAB	Diaminobenzidine
MN	micronucleus
FBS	fetal bovine serum
PCEs	polychromatic erythrocytes
MNPCEs	micronucleated polychromatic erythrocytes
SEM	standard error of mean

damage. Fluoride is thought to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. Moreover, fluoride can alter glutathione levels, often resulting in the excessive production of ROS at the mitochondrial level, leading to the damage of cellular components and cell death. However, information about the mechanism of fluoride-induced mitochondrial damage is scarce [2].

Another interesting speculation about fluoride toxicity is it could cause an impact on genome integrity inducing chromosomal aberrations, sister chromatid exchanges and DNA damage in different tissues [3]. Some authors stated that fluoride does not induce DNA damage [4]. According first supposition, DNA damage could induce cell death and, when a large number of cells undergo apoptosis, it could result in organ lesion [5,6] such as hepatic and renal histological and physiological changes [2,7].

In this context, several researches have been developed. Rats were exposed to varying concentrations of fluoride (0, 50, 100, 200 mg/L) for 120 days showed fluoride-induced hepatic morphological changes and significantly increased apoptosis, DNA damage demonstrated by comet assay and relative expression of caspase-3 and caspase-9 [8]. Sprague-Dawley rats which were fed solid feed containing a fluorine content of 1.5 mg/kg and 17 mg/kg presented the expression of phosphatidylinositol-3 kinase (PI3K) and serine/threonine protein kinase 1 (Akt1) mRNA. They had significantly increased proteins in hepatocytes, as well as apoptosis and increased intracellular calcium concentration [9]. Significant increase of oxidative damage to hepatocytes, as indicated by increased MDA levels with decrease of tissue ascorbate and free radical scavenging enzymes, including catalase, superoxide dismutase and glutathione peroxidase was observed in Wistar rats with a fluoride (1 ppm) intake during 28 days [10]. Neuron apoptosis and expressions of inflammatory factors such as IL-1β, IL-6 and TNF-α were significantly increased in Wistar rat brain exposed to 60 and 120 ppm fluoride in drinking water for 10 weeks [11].

Moreover, data shown in the literature are frequently conflicting and differences in the results are possibly due to many factors, such as differences in animal species, dose, mode and time of exposure, kind of tissues examined, as well as methods used for biochemical assay.

Taking into account that genotoxicity assays are of special concern since genotoxicity has gained widespread acceptance as an important and useful indicator of carcinogenicity and that micronuclei may result from oxidative stress [12], the present study intended to look into the fluoride genotoxic potential. MN test is the most preferred *in vivo* analysis because this assay presents both wide mutagenicity range assessment (clastogenicity and aneugenicity) and high specificity in concordance with the genotoxic carcinogenicity model [13].

According a review [2], many works have concluded that fluoride induces apoptosis by elevating oxidative-stress induce lipid peroxidation, causing mitochondrial dysfunction and the activation of downstream pathways. Therefore, the aim of this study was to evaluate five major events: (1) if fluoride presents genotoxic activity, (2) if hepatic cell death process (apoptosis) occurs like a consequence of this damage and (3) if mitochondrial swelling is the possible mechanism by which fluoride could modulate liver cell death, and (4) if DNA damage has some relationship with oxidative stress and (5) tissue damage.

For this purpose, we administered sublethal concentrations of fluoride to rats by oral gavage during prolonged period and we applied the micronuclei test in bone marrow smears and the TUNEL method in the liver. Furthermore, we evaluated biomarkers of hepatic mitochondrial damage and oxidative stress induction, hepatic function and histomorphological changes.

2. Material and methods

2.1. Experimental delineation

The study was approved by the Ethics Committee of the Hermínio Ometto University Center, UNIARARAS (protocol 741/2008), and was conducted in accordance with the ethical guidelines of the Brazilian Committee of Animal Experimentation (COBEA). The animals were maintained throughout the experimental period at the Center of Animal Experimentation, Hermínio Ometto University Center, (UNIARARAS), on a 12-h light/dark cycle at a temperature of 25 °C and air humidity of 60%. Fourty-five adult male Wistar albino rats (*Rattus novergicus*) weighing 180–200 g received Purina feed and tap water *ad libitum,* as is delivered by the Municipal Water Company (SAEMA – Serviço de Água e Esgoto e Meio Ambiente do Município de Araras) to all residences and buildings in Araras, SP, Brazil, containing 0.65 ppm of fluoride.

The animals were divided into four groups with 10 animals in