Chemico-Biological Interactions 264 (2017) 25–33

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

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

F.D. Campos-Pereira [a], L. Lopes-Aguiar [b], F.L. Renosto [b], G.A.S. Nogueira [b], E.F.D. Costa [b], R. Barbieri Pulz [b], E.C.M. Silva-Zacarin [c], C.A. Oliveira [d], A.A. Pigoso [b], G.D.C. Severi-Aguiar [e, *]

[a] São Paulo State University (UNESP), Department of Biology, Institute of Biosciences, Avenida 24-A, nº 1515, Bela Vista, CEP 13506-900, Rio Claro, São Paulo, Brazil
[b] Heath Sciences Nucleus, Hermínio Ometto University Center, UNIARARAS, Avenida Dr. Maximiliano Barutto, nº 500, Jd. Universitário, Araras, São Paulo, CEP 13607-339, Brazil
[c] Structural and Functional Biology Laboratory (LABEF), Federal University of São Carlos – UFSCAR, Rodovia João Leme dos Santos, Km 110 - SP-264, CEP 18052-780, Sorocaba, São Paulo, Brazil
[d] Graduate Program in Biomedical Sciences, Hermínio Ometto University Center, UNIARARAS, Avenida Dr. Maximiliano Barutto, nº 500, Jd. Universitário, CEP 13607-339, Araras, São Paulo, Brazil
[e] Reproductive Biology Laboratory, Structural and Functional Biology Department, State University of Campinas – UNICAMP, CP. 6109, Campinas, São Paulo, CEP 13083-863, Brazil

ARTICLE INFO

Article history:
Received 6 September 2016
Received in revised form
22 December 2016
Accepted 11 January 2017
Available online 12 January 2017

Keywords:
Micronuclei
Cell death
Mitochondrial swelling
Oxidative stress
Hepatic injury

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

* Corresponding author. Laboratório de Biologia da Reprodução, Departamento de Biologia Estrutural e Funcional, Universidade Estadual de Campinas – UNICAMP, CP. 6109, Campinas, São Paulo, CEP 13083-863, Brazil.
E-mail addresses: grazielaguiar@terra.com.br, grasielaguiar@hotmail.com (G.D.C. Severi-Aguiar).

http://dx.doi.org/10.1016/j.cbi.2017.01.005
0009-2797/

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

each group, distributed in two different cages (A and B) with 5 rats in each. The animals from cage A were used for blood collection and tissue analyses (TUNEL, immunohistochemical and morphological alterations). The animals from cage B were used for bone marrow aspiration, hepatic function and oxidative damage assays, mitochondrial swelling and hepatic total RNA isolation. Five additional rats were maintained to positive control of MN assay.

2.2. Fluoride administration

The dosage of fluoride and administration route was determined according previous studies [14,15]. The experimental groups (10 rats for group) received daily by gavage 1 mg (low-dose group, T1), 10 mg (mid-dose group, T10), and 15 mg fluoride per k rat body weight (high-dose group, T15) corresponding to 2.3, 23, and 35% of LD50 values for NaF. The control group (Co n = 10) received by gavage only filtered water.

Acccording Maistro [16] for the dose selection, the Lethal Dose 50% (LD50) can be utilized. In this case, the highest dose must be 80% of the LD50 and the small ones, 50 and 25% of the LD50. For studies of a longer duration, the limit dose by day, for treatment up to 14 days is 2000 mg/kg, and 1000 mg/kg/body weight/day if treatment longer than 14 days could be performed.

In this study, the major objective was to evaluate the genotoxic potential of fluoride what justify to use of the chosen doses for a long period, although they are lower than that suggest by the cited author.

Sodium fluoride (NaF) (Ecibra, Santo Amaro, São Paulo, Brazil, Lot no. 18259) was used to prepare fluoride solutions and administered for 60 days for each T group cited above.

For the micronuclei test a positive control group (Pc $n = 5$) received water and one dose of methyl methanesulfonate (MMS - Sigma, St. Louis, MA, USA) at a concentration of 20 mg/kg 24 h before euthanasia [17].

2.3. Euthanasia and collection of material for analysis

The animals were anesthetized by intraperitoneal administration of xylazine (20 mg/ml; Rompun®, Bayer S.A., São Paulo, SP, Brazil) and ketamine (50 mg/ml; Ketalar®, Parke-Davis & Co., Wellington, New Zealand). Next, a median laparotomy was performed and the chest cavity was opened to expose the heart.

Blood was collected with cardiac puncture using heparin as an anticoagulant. Serum samples were separated using vacuette tubes and aliquots were kept at −80 °C before biochemical analysis. Liver slices were kept at −80 °C before oxidative damage assays.

For histopathological examination, the livers were dissected and fixed in formalin.

2.4. Bone marrow aspiration and micronucleus test (MN)

MN test is probably the most widely used test to detect both clastogenicity and aneugenicity [16]. The frequency of micronucleated polychromatic erythrocytes (MPEs) is traditionally determined from bone marrow samples [13]. For this purpose, the femur was removed from the animals, cutting the epiphysis and medullary canal, washed with fetal bovine serum (FBS) (Cultilab, Campinas, Brazil). The bone marrow sample was centrifuged, the supernatant discarded and the pellet homogenized. A smear was prepared placing a drop of the cell suspension on a slide. The slides were stained with Wright and Giemsa's solution. A total of 2000 polychromatic erythrocytes were analyzed per animal for determination of the micronuclei number. The micronuclei were identified as dark blue staining bodies in the cytoplasm of PEs. The slides were coded and analyzed blindly using a microscope equipped with an immersion objective. The results are reported as the frequency of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 polychromatic erythrocytes per animal [18,19]. Definition of a substance as mutagenic is according to the production of a significant increase ($P < 0.05$) in frequency of MNPCEs in relation to the negative control [16].

2.5. Evaluation of cell death by the TUNEL method

The TUNEL (DNA fragmentation by Terminal deoxynucleontidyl Transferase Biotin-dUTP Nick End Labeling) assay kit was employed for detection of apoptosis. Paraffin-embedded liver sections were submitted to immunodetection of DNA fragmentation indicative of apoptosis using the ApopTag® Plus kit (Chemicon Int., Temecula, CA, USA) and *In Situ* Cell Death Detection kit (ISCDDK, Roche, São Paulo, Brazil) according to the manufacturer's instructions. Both kits use anti-fluorescein peroxidase-conjugated antibody and DAB to develop the reaction. As a positive control, DNAse I was applied before the TUNEL reaction. The negative control (reaction control) consisted of omission of the TUNEL reaction enzyme. TUNEL positive nuclei were identified by brown nuclear staining and counted. In each tissue specimen, five high-power fields at × 400 magnification were randomly selected and the apoptotic index (AI) was calculated as the percentage of positive cells, using the equation: AI = (number of positive cells/total number of cells) × 100 [20,21].

2.6. Evaluation of damage to liver mitochondria

Freshly collected liver slices from all groups immediately washed in medium containing 250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2–7.3, were then homogenized in a Potter-Elvehjem tissue grinder. The supernatant was removed after centrifuging the homogenate, then centrifuged again. The medium containing 250 mM sucrose, 10 mM HEPES and 0.3 mM EGTA, pH 7.2–7.3 was used to resuspend the centrifuged precipitate. The medium containing 125 mM sucrose and 10 mM HEPES-KOH, pH 7.2–7.3 was used to resuspend the mitochondrial fraction. All procedures were performed at 4° C [20]. Thereafter, 3 mL biuret reagent received 50 μL of the sample. After 10 min, the samples were read at 540 nm, and the mean was used to calculate protein concentration in the sample with the following equation: absorbance − 0.0026/0.0073[17]. The pellet containing the mitochondrial fraction (0.5 mg protein), added to 3 mL medium containing 125 sucrose and 1 mM HEPES, pH 7.2–7.3, furnished absorbance kinetically monitored at 540 nm over a period of 15 min. After one minute, these samples were added to 40 μL $CaCl_2$ and 40 μL KH_2PO_4 and absorbance was read at 540 nm, where mitochondrial swelling is proportional to the decrease in absorbance [22].

2.7. Evaluation of hepatic function and oxidative damage

Protein concentration was measured according to the biuret method [23].

Serum activity of alanine aminotransferase (ALT) was measured employing the Synermed ALT enzymatic kit (Synermed International, Inc., Westfield, IN, USA), according to the manufacturer's instructions and absorbance was read at 532 nm.

Assay of Catalase (CAT) activity followed the method of Aebi [24], expressed as absorbance units/mg protein.

Total reduced sulfhydryls (SH) levels of liver were measured by Ellman method using liver homogenate [25]. The absorbance was measured immediately and after 15 min at 412 nm. The concentration of reduced sulfhydryl groups (-SH) was calculated using the equation ($Abs_{final} - Abs_{initial}$) x 1.57, and is reported as mM [25].

The lipid peroxidation assay used followed the description of

Buege and Aust [26].

2.8. Isolation of total RNA and semi quantitative RT-PCR to amplify hepatic antioxidants enzymes mRNA

Total RNA was isolated from approximately 100 mg of rat liver with the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), including the digestion of contaminating DNA with DNAse I, amplification grade (Invitrogen), following the manufacturer's instructions. RNA purity and concentration were determined spectrophotometrically. cDNA was synthesized from 2 μg RNA in the presence of dithiothreitol, dNTP, random primers, RNAseOUT, and SuperScript™ II Reverse Transcriptase (Invitrogen) in a final volume of 20 μl. Semi quantitative RT-PCR was used to amplify *GSTM1* and *Mn-SOD* mRNA, and to compare their expression between exposed and non-exposed rat livers.

PCR conditions were performed as reported previously by Campos-Pereira et al. [27]. Separation of the amplified products were carried out on 1.5% agarose gel, stained with ethidium bromide. The gel was photographed using the LPix-Touch® (Loccus Biotecnologia). The signal intensities of the bands were densitometrically measured using the Scion Image analysis software (Scion Corp., Frederick, MD, USA). Each value was determined as the mean of three densitometric readings. The results were expressed as average ratios of the relative expression of transcripts normalized with β-actin as the control housekeeping gene.

2.9. Histological preparation

Liver fragments were dehydrated in an increasing ethanol series and cleared for embedding in liquid paraffin. Histological liver sections at 5–7 μm thickness were obtained and stained with either hematoxylin-eosin (HE). After drying, Permount resin was used to mount the sections on slides.

Histological sections were cut from the same block with a microtome and processed for indirect immunofluorescence. Liver sections, in which the primary antibody was omitted, were used as negative control; and sections to which human liver membrane lysate (normal fetal tissue ab29880) was added served as positive control. The sections were mounted on silanated slides and antigen retrieval was performed by boiling the slides in citrate buffer at 95 °C for 1 h. The sections were incubated with the primary antibody (anti-SOD-1 or anti-GST-1 monoclonal rabbit antibody, EP1727y or EP1938y, respectively; Abcam, Cambridge, MA, USA) (1:250) overnight at 4 °C. After washing in PBS, the sections were incubated with the secondary antibody (FITC-conjugated goat anti-rabbit IgG polyclonal antibody; Abcam 6717) (1:250) for 2 h at room temperature in a dark chamber [27]. After successive washes in PBS, the slides were mounted in Permount and fluorescence was detected using an I3 filter at an excitation wavelength of 450–490 nm (Leica DM2000). The fluorometric quantification was performed using "Imaje J" software, according Jensen (2013) [28].

2.10. Statistical analysis

The results of enzyme quantification, micronucleus test and TUNEL method were analyzed by ANOVA using the Prism 3.0 program. The Tukey test was employed to compare the control and exposed groups. The results of mRNA expression are reported as the mean ± SEM and were compared by the Student *t*-test. In all cases, a P value < 0.05 was statistically significant. The results of SOD and GST immunolocalization were expressed as means ± standard deviation (SD), statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. MN frequency

The criteria to determine a result as positive for genotoxicity should include a dose-related increase in the frequency of MNPCE, or a clear increase in the frequency of MNPCEs at the highest dose, as a result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species [16].

Evaluation of mutagenicity by the micronucleus test in our study showed a high frequency of MNPCEs in animals exposed to fluoride when compared with the negative control (Cn 15 ± 3; T1 25 ± 6; T10 24 ± 4; T15 26 ± 6 - mean ± SD) (Fig. 1A–B), indicating genotoxic activity to fluoride.

3.2. Cell death by TUNEL method

The TUNEL assay was performed to examine the levels of apoptosis in each group. According Wallig et al. [29] the proportion of apoptotic cells present in a particular tissue section is usually quite low, oftentimes only 1–2% of the total cell population even in a tissue in which widespread and massive apoptotic necrosis is known to be occurring. In liver, the "background" of apoptotic cells is as low as 0.1%.

Exposed groups showed a low frequency of nuclei positive to the TUNEL method (<1% => Co = 0.82% ± 0.19; T1 = 0.74% ± 0.19; T10 = 0.36% ± 0.36; T15 = 0.44% ± 0.31) (Fig. 2 A, B). No statistically significant difference between control and exposed groups was observed demonstrating no induction of apoptosis of hepatocytes after fluoride exposition.

3.3. Hepatic function, oxidative damage and mitochondrial swelling

Data are presented on Fig. 3. -SH levels were significantly increased in the T15 group after 60 days of treatment (Fig. 3D). These results show that fluoride affects thiol homeostasis [2]. According Barbier et al., interference with disulfide-bond formation can result in the accumulation of misfolded proteins in the endoplasmic reticulum (ER) causing ER stress and ROS production [2].

ALT (Fig. 3A), MDA (Fig. 3B), CAT levels (Fig. 3C) and mitochondrial swelling (Fig. 3E) did not show any significant difference for the exposed groups compared to the control group.

3.4. Mn-SOD and GSTM1 RNA transcripts quantification

The RT-PCR analysis revealed decreased expression levels of *Mn-SOD* and *GST-M1* transcripts in the T15 group. The expression of the genes studied remained unchanged in the other groups (Fig. 4).

3.5. Hepatic immunolocalization of SOD and GST

Immunohistochemical analysis using antibodies against the antioxidant enzymes SOD and GST respectively (Fig. 5A and B) showed a significantly decreased expression for both proteins in the exposed groups.

3.6. Liver histopathology

Analysis of histological liver sections obtained from control animals revealed a normal cytoarchitecture of the organ (Fig. 6A). In contrast, were observed progressive changes accompanying the increasing doses of sodium fluoride in exposed groups. Nuclear pyknosis and karyorrhexis were observed in T1 (Fig. 6B). Empty nuclear spaces, cytoplasm

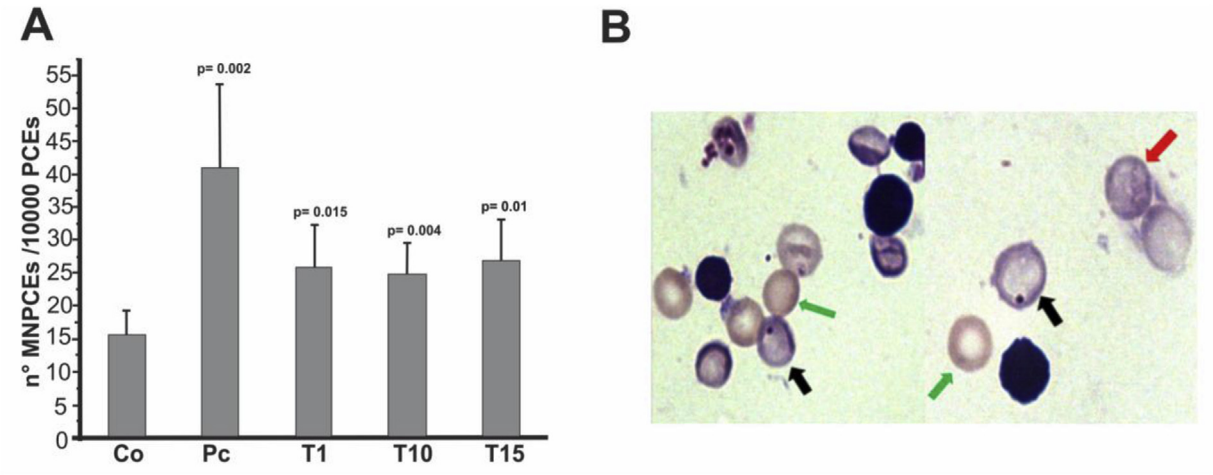

Fig. 1. A - Comparison of the number of micronucleated polychromatic erythrocytes (MNPEs) among 2000 polychromatic erythrocytes per animal. Co = control; Pc = positive control; T1 = 1 mg/kg/day; T10 = 10 mg/kg/day; T15 = 15 mg/kg/day; n = 5. B - Bone marrow smears stained with Wright and Giemsa's solution and analyzed with immersion objective; green arrow - normochromatic erythrocyte; red arrow - polychromatic erythrocyte; black arrow - micronucleated polychromatic erythrocyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degeneration and karyorrhexis followed by karyolysis were stated in T10 and T15 (Fig. 6C). Microinfiltrations were present in T15 (Fig. 6D).

4. Discussion

MN test revealed that fluoride induced DNA damage in rat erythrocytes in all groups which demonstrates its genotoxic potential and the risk of exposition. This is the first description of fluoride positive response to MN test. Significant damaging effects of fluoride on cellular DNA have already been observed by comet assay [8,30] and SMART test (Somatic Mutation and Recombination Test) [31]. Besides, fluoride has been reported to cause a depression in DNA and RNA synthesis in cultured cells [3].

The *in vivo* rodent micronucleus (MN) assay is the primary test in a battery of genotoxicity tests and is recommended by the regulatory agencies around the globe to be conducted as part of product safety assessment [16]. It is especially relevant to assessing genotoxicity hazard because it allows the consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair process although these may vary among species, among tissues, and among genetic endpoints [16], reinforcing our results about fluoride genotoxicity.

Additionally, our data showed no significant difference in the frequency of TUNEL positive nuclei between control and exposed groups. Therefore, in conditions of our study no apoptosis induction was characterized. In contrast with our data, Jothiramajayam et al. [6] demonstrated that NaF at lower concentrations (<1 μg/mL) induced oxidative stress leading to apoptotic cell death.

In this way, take into account the data of our research we can hypothesize that DNA damage did not induce apoptosis although inverse situation can be observed for some toxicants such as cisplatin.

da Silva Faria et al. [32] employed MN assay and detected the genotoxic activitty of cisplatin, a chemotherapeutic agent. As result, occurs intrastrand and interstrand cross-links with purine bases of DNA, with consequent formation of DNA-cisplatin adducts and distortion of the DNA double helix. This distortion interferes with the normal mechanisms of DNA transcription and replication, therefore inhibiting cell proliferation and causing cell death (apoptosis), which is an important mechanism of cell death associated with the renal damage induced by cisplatin. They also characterized apoptosis by TUNEL assay.

It is know that changes in mitochondrial membrane permeability can cause the mitochondria mediated apoptosis signal pathway start [33,34]. However, our results showed that fluoride did not induce mitochondrial swelling of hepatocytes.

At this point the following question arises: what would be the

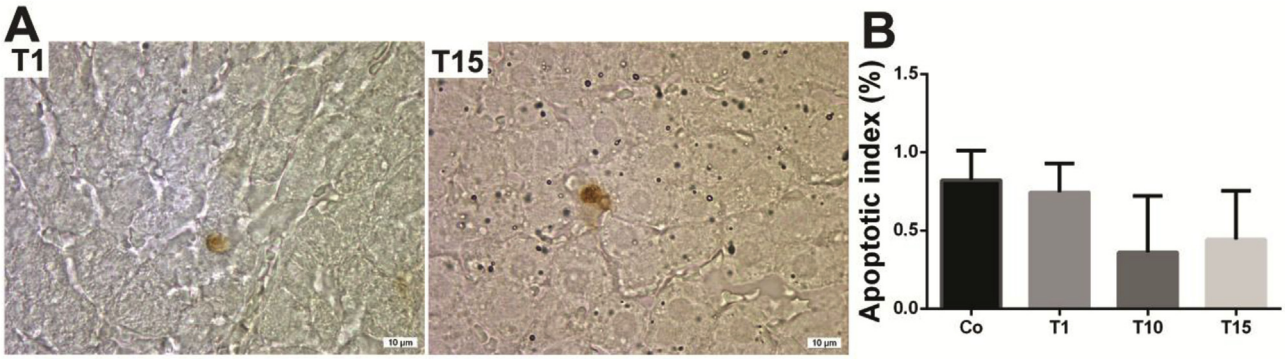

Fig. 2. A - Liver sections submitted to immunodetection of DNA fragmentation indicative of apoptosis by TUNEL method. TUNEL positive nuclei were identified by brown nuclear staining. B - Apoptotic index (AI) in percentage obtained for all groups (Co = control; T1 = 1 mg/kg/day; T10 = 10 mg/kg/day; T15 = 15 mg/kg/day). Values are expressed as the mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

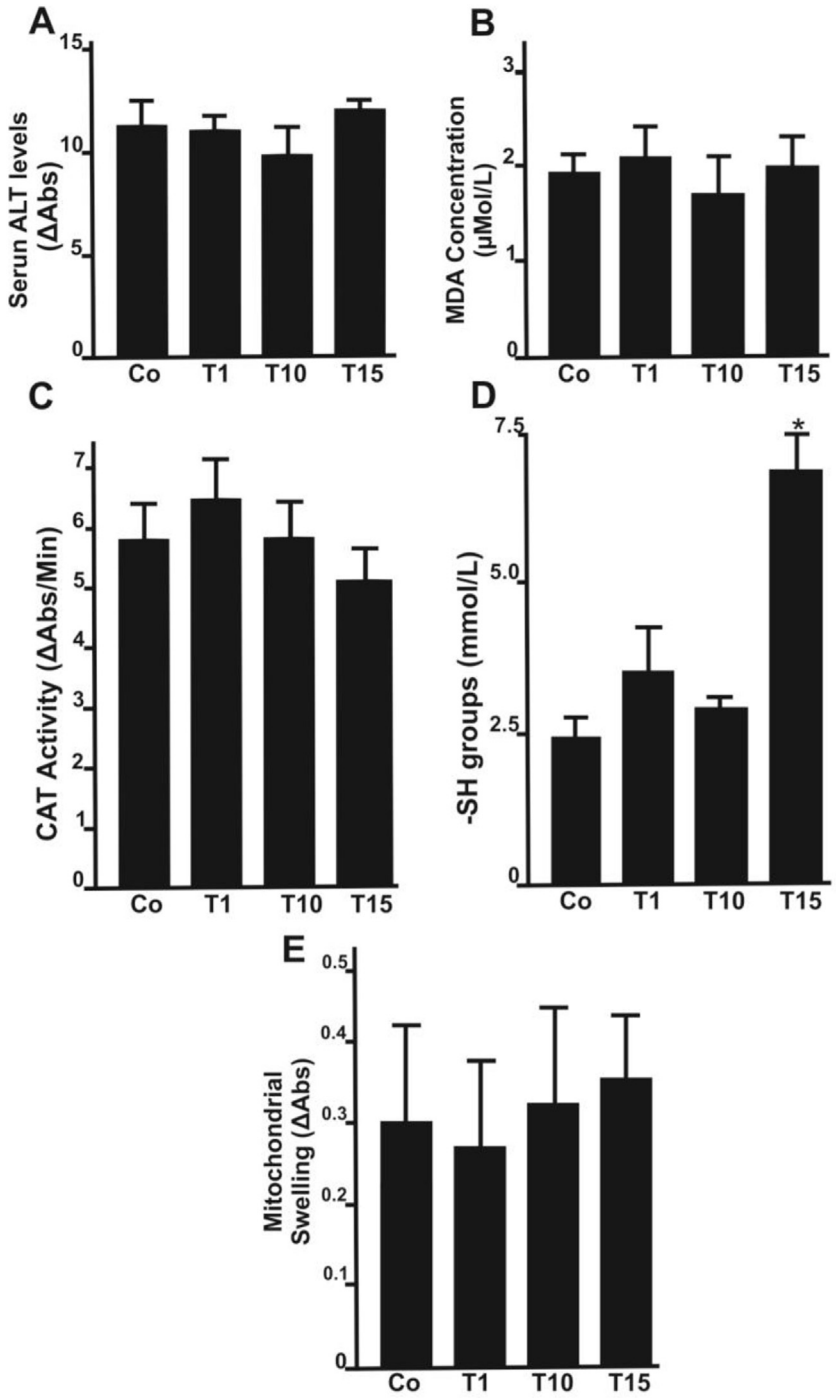

Fig. 3. Effect of *in vivo* administration of fluoride on the serum levels of ALT (A), and MDA concentration (B), catalase activity (C), −SH groups levels (D) and mitochondrial swelling (E) in rat livers. Values are expressed as the mean ± SD (n = 6). (*) significant difference from the control group.

cause of the damage detected in the DNA?

As a major source for DNA damage is the oxygen-derived free radicals which can cause strand breaks and base alteration in DNA [3]. Our results indicate that fluoride affected thiol homeostasis and oxidative process. Protein- SH group levels were assayed as indicators of the redox status of thiols similarly a study developed by Kowalczyk-Pachel et al. [35]. We observed an increase on reduced sulphydryl (-SH) levels after fluoride exposition which correlated well with the reduced expression and activity of MnSOD and GSTM1. Similarly, Milnerowicz et al. [36] in a review about effects induced by metals present in tobacco smoke, determined that these contaminants can cause oxidative stress by weakening the oxidative mechanisms due to binding to thiol groups in enzymes or metabolically-important protein molecules.

SOD and GST are major antioxidant players that can be modulated by different xenobiotics and their gene expression analysis could demonstrate any up or downregulation of the antioxidant system [37]. Besides, GST is the enzyme responsible for the conjugation of GSH with certain endogenous metabolites or xenobiotics [35]. Fluoride is thought to inhibit the activity of

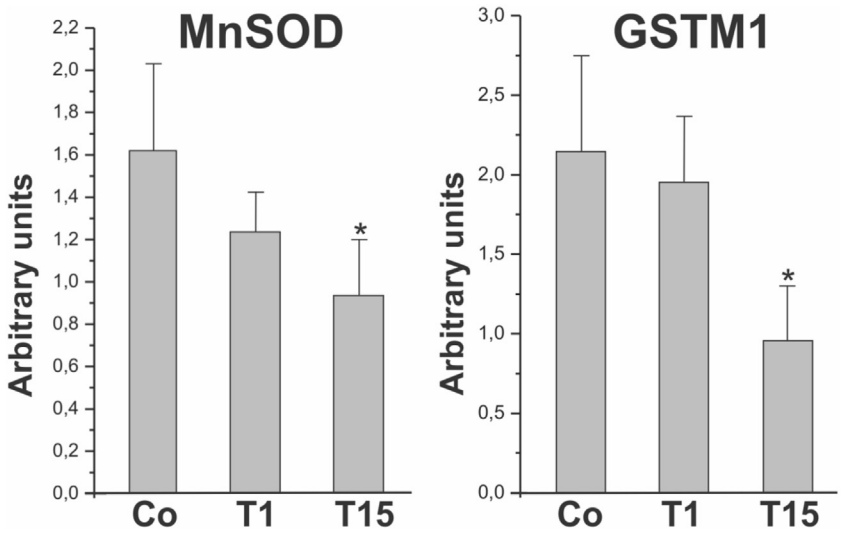

Fig. 4. Analysis of mRNA expression by semi-quantitative reverse transcriptase - PCR. Changes in mRNA are expressed as normalized densitometric units relative to β-actin mRNA. Values are the mean ± SEM. $P < 0.05$ indicates statistical significance. (*) significant difference from the control group (0.03 and 0.01 respectively); Co = control; T1 = 1 mg/kg/day; T15 = 15 mg/kg/day; n = 4.

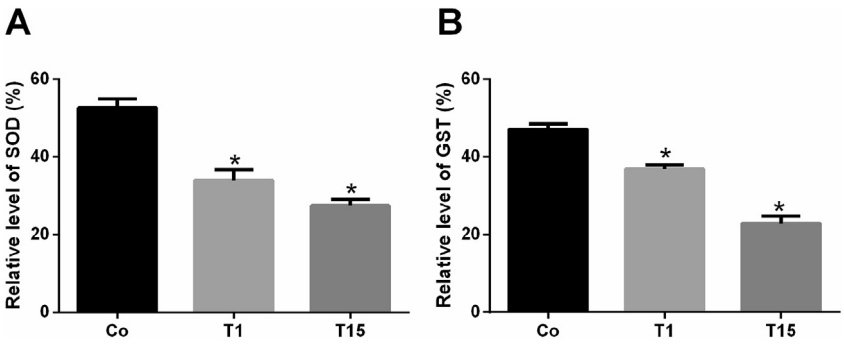

Fig. 5. Fluorometric quantification for SOD (A) and GST (B) was reduced in exposed groups when compared to control animals (Co). Co = control; T1 = 1 mg/kg/day; T15 = 15 mg/kg/day; n = 5. (*) significant difference from the control group. $P < 0.0001$ for both enzymes.

antioxidant enzymes [2].

In accordance, we showed that the mRNA levels of *Mn-SOD* and *GSTM1* diminished in the liver of rats as a response to fluoride treatment, corroborating the early supposition. Besides, immunolocalization of SOD-1 and GST-1 protein also showed decrease after fluoride exposition. Basha and Sujitha [38] attributed this decrease to direct action of fluoride on the enzyme rather than to increased generation of free radicals. This imbalance could cause tissue injury.

Corroborating this idea is the fact that chronic exposure to sodium fluoride produced hepatocyte and parenchymal damage. The extent of changes increased along with increasing doses of sodium fluoride. These events have been related by other authors [2,3] and confirm hepatotoxicity caused by fluoride intake.

Cytoplasm degeneration, empty nuclear spaces, nuclear pyknosis, karyorrhexis, and karyorrhexis followed by karyolysis were observed. These events characterize cell death process [39]. Karyorrhexis is a sort of destructive fragmentation of the nucleus proceeded by pyknosis and is followed by karyolysis. In turn, karyolysis is the complete dissolution of the chromatin matter of a dying cell. Therefore, we make evident the induction of cell death.

Once we demonstrated that there was no apoptosis, what kind of cell death fluoride have been induce? Microinfiltrations composed of mononuclear cells point out an inflammatory response implicated with fluoride exposition [3,40]. This is suggestive of cell death by necrosis, but the levels of ALT did not confirm this mechanism. Another possible mechanism is autophagic cell death, which is characterized by nuclear pyknosis, cellular atrophy, and cytoplasmic vacuolization due to macroautophagy [27,41].

One of the first studies that addressed this question in mammalian cells reported that cells exhibit type-II cell death morphology when lysosome–autophagosome fusion is inhibited, suggesting that autophagy may be cytoprotective. The accumulation of autophagic vacuoles triggers a pre-lethal program, which can be suppressed by mitochondrial membrane permeability inhibitors, or caspase antagonists. Autophagic vacuoles accumulation precedes $\Delta\Psi m$ loss, which marks imminent cell death and apoptosis [42].

Therefore, our data let us state that (1) fluoride presented genotoxic activity, (2) hepatic cell death process (apoptosis) did not occur like a consequence of this damage and (3) hepatic mitochondrial swelling did not occur, (4) DNA damage probably has relationship with oxidative stress in consequence of increase on -SH groups and (5) tissue damage was evident showing cell death and inflammatory processes. We hypothesized that autophagy has been involved in these event.

The exact mechanisms for occurrences pointed by our study are not known but it seems pertinent to carry out well controlled

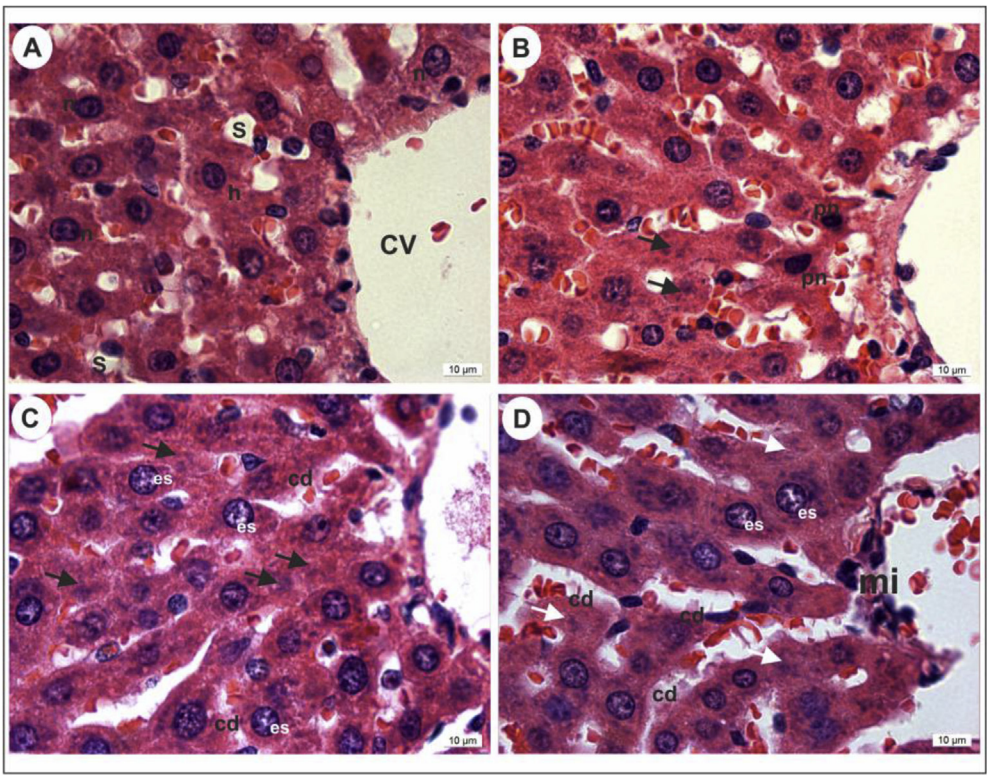

Fig. 6. Histological sections of male Wistar rat liver after fluoride exposition submitted to HE staining (immersion objective). A – control group; B - T1 group; C - T10 group; D – T15 group; (n = 5). h - hepatocyte; n - nucleous; s - sinusoid vessels; pn - picknotic nucleous; es - empty nuclear spaces; cd - cytoplasm degeneration; mi - microinfiltration; black arrow - karyorrhexis; white arrow - karyorrhexis followed by karyolysis.

chronic studies that measure various endpoints to reach a conclusion about fluoride toxicity.

5. Conclusion

In summary, our results showed that continuous intake of fluoride induced genotoxic effects, although DNA damage did not induce apoptosis or mitochondrial swelling. It depleted antioxidant enzyme production, which demonstrates induction of oxidative stress. Probably through this event, morphological alteration occurred in the liver, corroborating the literature. Altogether, our studies provide consistent evidence of the genotoxic potential of long term fluoride exposition and the necessity of further research to establish safety doses that prevent dental caries but which ensures the maintenance of homeostasis of the organism even after long term exposure.

Acknowledgements

The authors thank to Fundação Hermínio Ometto (FHO-UNIARARAS AEN 26.01.01.48) and PIBIC/CNPq (128925/2009-4; 109275/2010-1) for financial support, to Prof. Dr. Mary Anne Heidi Dolder for English text revision and to Profa. Dra. Jussara de Sousa Ribeiro Bettini for pathological evaluation.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.cbi.2017.01.005.

References

[1] L. Ko, K.M. Thiessen, A critique of recent economic evaluations of community water fluoridation, Int. J. Occup. Environ. Health 21 (2015) 91–120.
[2] O. Barbier, L. Arreola-Mendoza, L.M. Del Razo, Molecular mechanisms of fluoride toxicity, Chem. Biol. Interact. 188 (2010) 319–333.
[3] N.J. Trivedi, M.H. Verma, R.J. Chinoy, Amelioration by black tea of sodium fluoride-induced effects on DNA, RNA and protein contents of liver and kidney and on serum transaminase activities in swiss albino mice, Fluoride 41 (2008) 61–66.
[4] A. de, L. Leite, J.F. Santiago, F.M. Levy, A.G. Maria, M. da, S. Fernandes, D.M.F. Salvadori, et al., Absence of DNA damage in multiple organs (blood, liver, kidney, thyroid gland and urinary bladder) after acute fluoride exposure in rats, Hum. Exp. Toxicol. 26 (2007) 435–440.
[5] J.X. Zhan, Xiu-An Wang, Min Xu, Zi-Rong Li, Toxic effects of fluoride on kidney function and histological structure in young pigs, Fluoride 39 (2006) 22–26.
[6] M. Jothiramajayam, S. Sinha, M. Ghosh, A. Nag, A. Jana, A. Mukherjee, Sodium fluoride promotes apoptosis by generation of reactive oxygen species in human lymphocytes, J. Toxicol. Environ. Health. A. 77 (2014) 1269–1280.
[7] S.F. Nabavi, A.H. Moghaddam, S. Eslami, S.M. Nabavi, Protective effects of curcumin against sodium fluoride-induced toxicity in rat kidneys, Biol. Trace Elem. Res. 145 (2012) 369–374.
[8] G.H. Song, F.B. Huang, J.P. Gao, M.L. Liu, W.B. Pang, W. bin Li, et al., Effects of fluoride on DNA damage and caspase-mediated apoptosis in the liver of rats, Biol. Trace Elem. Res. 166 (2015) 173–182.
[9] B. Fan, Y. Yu, Y. Zhang, PI3K-Akt1 expression and its significance in liver tissues with chronic fluorosis, Int. J. Clin. Exp. Pathol. 8 (2015) 1226–1236.
[10] R. Raina, N.A. Baba, P.K. Verma, M. Sultana, M. Singh, Hepatotoxicity induced by subchronic exposure of fluoride and chlorpyrifos in wistar rats: mitigating effect of ascorbic acid, Biol. Trace Elem. Res. 166 (2015) 157–162.
[11] N. Yan, Y. Liu, S. Liu, S. Cao, F. Wang, Z. Wang, et al., Fluoride-induced neuron apoptosis and expressions of inflammatory factors by activating microglia in rat brain, Mol. Neurobiol. (2015) 1–12.
[12] S. Bonassi, A. Znaor, M. Ceppi, C. Lando, W.P. Chang, N. Holland, et al., An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans, Carcinogenesis 28 (2006) 625–631.
[13] O.G. Mekenyan, P.I. Petkov, S.V. Kotov, S. Stoeva, V.B. Kamenska, S.D. Dimitrov, et al., Investigating the relationship between in vitro–in vivo genotoxicity: derivation of mechanistic QSAR models for in vivo liver genotoxicity and in vivo bone marrow micronucleus formation which encompass metabolism, Chem. Res. Toxicol. 25 (2012) 277–296.

[14] D.L. Conine, M. Yum, R.C. Martz, G.K. Stookey, J.C. Muhler, R.B. Forney, Toxicity of sodium pentafluorostannite, a new anticariogenic agent. I. Comparison of the acute toxicity of sodium pentafluorostannite, sodium fluoride, and stannous chloride in mice and/or rats, Toxicol. Appl. Pharmacol. 33 (1975) 21–26.
[15] G.M. Whitford, N.L. Birdsong-Whitford, C. Finidori, Acute oral toxicity of sodium fluoride and monofluorophosphate separately or in combination in rats, Caries Res. 24 (1990) 121–126.
[16] E.L. Maistro, The *in vivo* rodent micronucleus test, in: L. María Sierra, G. Isabel (Eds.), Genotoxicity and DNA Repair: a Practical Approach, Methods in Pharmacology and Toxicology, Springer, New York, 2014, pp. 103–113.
[17] K. Kuriyama, R. Yokoi, K. Kobayashi, S. Suda, M. Hayashi, S. Ozawa, et al., A time-course characterization of male reproductive toxicity in rats treated with methyl methanesulphonate (MMS), J. Toxicol. Sci. 30 (2005) 91–102.
[18] S.A. Mansour, A.H. Mossa, T.M. Heikal, Cytogenetics and hormonal alteration in rats exposed to recommended safe doses of spinosad and malathion insecticides, Int. J. Agric. Biol. 10 (2008) 9–14.
[19] T. Santos, G. Cancian, D.N.R. Neodini, D.R.S. Mano, C. Capucho, F.S. Predes, et al., Toxicological evaluation of ametryn effects in Wistar rats, Exp. Toxicol. Pathol. 67 (2015) 525–532.
[20] Y. Tao, X. Yan, T. Zhou, L. Shen, Z. Liu, C. Liu, Fuzheng Huayu recipe alleviates hepatic fibrosis via inhibiting TNF-α induced hepatocyte apoptosis, BMC Complement, Altern. Med. 14 (2014) 449.
[21] Y. Cai, Z. Zou, L. Liu, S. Chen, Y. Chen, Z. Lin, et al., Bone marrow-derived mesenchymal stem cells inhibits hepatocyte apoptosis after acute liver injury, Int. J. Clin. Exp. Pathol. 8 (2015) 107–116.
[22] A.C. Santos, S.A. Uyemura, J.L. Lopes, J.N. Bazon, F.E. Mingatto, C. Curti, Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria, Free Radic. Biol. Med. 24 (1998) 1455–1461.
[23] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–766.
[24] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[25] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[26] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 52 (1978) 302–310.
[27] F.D. Campos-Pereira, C.A. Oliveira, A.A. Pigoso, E.C.M. Silva-Zacarin, R. Barbieri, E.F. Spatti, et al., Early cytotoxic and genotoxic effects of atrazine on Wistar rat liver: a morphological, immunohistochemical, biochemical, and molecular study, Ecotoxicol. Environ. Saf. 78 (2012) 170–177.
[28] E.C. Jensen, Quantitative analysis of histological staining and fluorescence using ImageJ, Anat. Rec. 296 (2013) 378–381.
[29] M.A. Wallig, C.M. Chan, N.A. Gillett, Specific Methods for Detection and Quantification of Apoptosis in Tissue Sections, in: Apoptosis Methods Pharmacol. Toxicol, Humana Press, New Jersey, n.d, 2002, pp. 59–76.
[30] S. Pal, C. Sarkar, Protective effect of resveratrol on fluoride induced alteration in protein and nucleic acid metabolism, DNA damage and biogenic amines in rat brain, Environ. Toxicol. Pharmacol. 38 (2014) 684–699.
[31] K. Erciyas, R. Sarikaya, Genotoxic evaluation of sodium fluoride in the somatic mutation and recombination test (SMART), Food Chem. Toxicol. 47 (2009) 2860–2862.
[32] M.C. da Silva Faria, N.A.G. dos Santos, M.A. Carvalho Rodrigues, J.L. Rodrigues, F. Barbosa Junior, A.C. dos Santos, Effect of diabetes on biodistribution, nephrotoxicity and antitumor activity of cisplatin in mice, Chem. Biol. Interact. 229 (2015) 119–131.
[33] J.S. Kim, J.-H. Wang, J.J. Lemasters, Mitochondrial permeability transition in rat hepatocytes after anoxia/reoxygenation: role of Ca2+-dependent mitochondrial formation of reactive oxygen species, Gastrointest. Liver Physiol. 302 (2012) G723–G731.
[34] L. Shao, L. Huang, S. Yan, J. Jin, S. Ren, Cordycepin induces apoptosis in human liver cancer HepG2 cells through extrinsic and intrinsic signaling pathways, Oncol. Lett. 12 (2) (2016) 995–1000.
[35] D. Kowalczyk-Pachel, M. Iciek, K. Wydra, E. Nowak, M. Górny, M. Filip, et al., Cysteine metabolism and oxidative processes in the rat liver and kidney after acute and repeated cocaine treatment, PLoS One 11 (2016).
[36] H. Milnerowicz, M. Ściskalska, M. Dul, Pro-inflammatory effects of metals in persons and animals exposed to tobacco smoke, J. Trace Elem. Med. Biol. 29 (2015) 1–10.
[37] D.F. Mello, E.S. de Oliveira, R.C. Vieira, E. Simoes, R. Trevisan, A.L. Dafre, et al., Cellular and transcriptional responses of *Crassostrea gigas* hemocytes exposed *in vitro* to brevetoxin (PbTx-2), Mar. Drugs 10 (2012) 583–597.
[38] M.P. Basha, N.S. Sujitha, Chronic fluoride toxicity and myocardial damage: antioxidant offered protection in second generation rats, Toxicol. Int. 18 (2011) 99–104.
[39] M.A.K. Abdelhalim, B.M. Jarrar, S. Lanone, J. Boczkowski, L. Yu, L.-Y. Yung, et al., Gold nanoparticles induced cloudy swelling to hydropic degeneration, cytoplasmic hyaline vacuolation, polymorphism, binucleation, karyopyknosis, karyolysis, karyorrhexis and necrosis in the liver, Lipids Health Dis. 10 (2011) 166.
[40] B. Stawiarska-Pięta, B. Bielec, K. Birkner, E. Birkner, The influence of vitamin E and methionine on the activity of enzymes and the morphological picture of liver of rats intoxicated with sodium fluoride, Food Chem. Toxicol. 50 (2012) 972–978.
[41] X.-M. Yin, W.-X. Ding, W. Gao, Autophagy in the liver, Hepatology 47 (2008) 1773–1785.
[42] K. Moreau, S. Luo, D.C. Rubinsztein, Cytoprotective roles for autophagy, Curr. Opin. Cell Biol. 22 (2010) 206–211.