

Histopathological liver changes in natrium fluoride administration to a population of mice NMRI type

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ABSTRACT

It has been revealed that excessive fluoride intake on long-term is associated with toxic effects and can damage a variety of organs and tissues in the human body, for example, the liver tissue. The molecular mechanisms of fluoride-induced hepato-toxicity are not well understood. The study wants to offer up-to-date insights concerning the effects of natrium fluoride on hepatic tissues when this substance is administrated to a population of mice. The study was conducted on NMRI mice descending from the pregnant females treated with 0.25 mg and 0.5 mg natrium fluoride by daily gavage, from the beginning of pregnancy until the lactation was ceased. Then, the mice, males and females, were divided in six groups, three groups descending from the pregnant females treated with 0.5 mg natrium fluoride (Groups 1A, 1B and 1C) and three groups from the pregnant females with 0.25 mg natrium fluoride (Groups 2D, 2E and 2F). From the moment the lactation is finished until adulthood, the animals received the following treatments: homeopathic (a CH7 solution of natrium fluoride – Groups A and D), allopathic-homeopathic (0.25 mg‰ natrium fluoride administered like drinking water ad libitum and CH7 solution of natrium fluoride – Group E; 0.5 mg‰ natrium fluoride administered like drinking water ad libitum and CH7 solution of natrium fluoride – Group B), and allopathic administration of natrium fluoride (0.25 mg‰ natrium fluoride like drinking water ad libitum – Group F; 0.5 mg‰ natrium fluoride like drinking water ad libitum – Group C). When the males reached adulthood, the administration of natrium fluoride was stopped and, by randomization, they were selected for euthanasia. The euthanasia was realized by cervical dislocation. The testes for the histopathological examination were preserved in a 10% formalin solution. The preparation of samples for optical microscopy was realized with Hematoxylin-Eosin staining. The results indicate that natrium fluoride administered in different doses, even in homeopathic dose or in allopathic-homeopathic dose, determined centrolobular vacuolar dystrophy of hepatic tissue, cariomegalia, granulovacuolar dystrophy, centrolobular hyperemia centrolobular degenerescences of hepatocytes and discreet centrolobular apoptosis.

Key words: natrium fluoride, histopathology, mice, hepatic tissue

INTRODUCTION

The administration of fluoride during human growth and development has raised numerous questions about the effects on various organs. Because the sodium fluoride is administered from birth to adulthood in humans, for 18 years, some of the studies aim to demonstrate the effect of sodium fluoride following long-term oral administration. The exposure to sodium fluoride revealed various modifications on the liver of animals or humans. Recent studies found that the exposure at fluoride doses of 1,5 mg/kg induces the apoptosis and the increase of intracellular calcium concentration. In addition, the presence and the level of PI3K-Akt signal the liver injury caused by fluorosis (1). Several studies reveal that drinking water fluoride levels over 2.0 mg/L can cause liver damage and kidney dysfunctions in children and that the dental fluorosis was independent of damage to the liver but not the kidney. Further studies on the mechanisms and significance underlying damage to the liver without dental fluorosis in the exposed children are warranted (2).

In some cases of administration of isoflurane or halothane in anesthesia for cesarean section, the serum fluoride concentrations in the mother after anesthesia were not significantly above preanesthetic levels and there was no biochemical evidence of tissues toxicity- in this study, the renal tissue. In all neonates' fluoride ion concentrations in the first voided urine sample were less than 7 $\mu\text{mol/l}$, a value well below that associated with nephrotoxicity (3).

The effect of oral administration of fluoride may vary depending on the dose, duration of exposure and species, and include anemia and leukopenia (4). For sodium fluoride, the doses administered are in a very large range of values, up to 120 mg, as it has been shown by a study about a single case of acute fluoride poisoning in a man. Following the ingestion of 120 mg of sodium fluoride, multiple reactions like tetany, multiple episodes of ventricular fibrillation and an esophageal stricture were observed (5).

For some substances, the detection of drugs or other types of poison in various tissues and organs may be sufficient to prove exposure (6). Tolerable Upper Intake Level for sodium fluoride is not yet defined (7). Because of the daily fluoride intake in fluoridated areas estimated to 0,20 mg/kg/day for adults and 0.11 mg/kg/day for infants, the reasonable maximum exposure (RME) estimates were above the upper tolerable intake limit (8,9). For sodium fluoride not only the presence of substance is necessary in order to

diagnose exposure, but also to detect, for example, histopathological alterations of tissues. The physiologists, pathologists and hepatologists studied hepatic stellate cells over 130 years and they reached the conclusion that the cells we mentioned have an important role in hepatic injury and fibrosis. Those alterations could be appreciated with refinement methods of cell isolation and characterization (10).

The signals inducing apoptosis are very varied and the same signals can induce differentiation and proliferation in other situations. However, some pathways appear to be of particular significance in the control of cell death; recent analysis of the apoptosis induced through the cell-surface Fas receptor has been especially important for immunology (11).

The administration of sodium fluoride has less effect on the tissues when blackberry and quercetin are administered because of the synergistic effects of flavonoids on all antioxidant and histological parameters (12).

When administered, fluoride showed significant increased oxidative damage of hepatocytes; consistent with the increased MDA levels and decreased levels of ascorbate in tissue and free radical scavenging enzymes like catalase, superoxide dismutase, and glutathione peroxidase (13,14). The exposure of a cell culture to sodium fluoride shows a loss of mitochondrial membrane potential ($\Delta\psi$) (8).

The structural unit of the liver, the hepatic lobule, is modeled on the blood flow within the liver and is commonly used for descriptive pathology and morphological diagnoses (15).

The present study aims to investigate the possible histopathological changes in the liver tissue of mice, when fluoride is administered in three doses: allopathic dose (0.25 mg% sodium fluoride and 0.5 mg% sodium fluoride, administered ad libitum like drinking water), homeopathic dose (CH7) and allopathic - homeopathic dose.

MATERIALS AND METHODS

Animals and treatment

The study was performed on NMRI-type mice. All researches were conducted in accordance with the European Directive 86/609/EEC/24.11.1986, the European Convention on the Protection of Vertebrate Animals (2005) and the Romanian Government Ordinance No. 37/2002 regarding the protection of animals used for experimental and other scientific purposes (8,16).

The administration is realized in three different doses: homeopathic, allopathic and allopathic-homeopathic. The substances administrated are different doses of natrium fluoride: 0.25 mg‰ natrium fluoride, 0.5 mg‰ natrium fluoride, and CH7 homeopathic solutions.

The animals for the experiments are descendants from two kinds of maternal parents: pregnant females (mothers) treated with 0.25 mg natrium fluoride and pregnant females (mothers) treated with 0.5 mg natrium fluoride, daily administered by gavage, during pregnancy and lactation.

The experimental animals were classified in six groups, males and females, compared with males and females control.

The descendants were divided into Groups 1A, 1B, 1C, 2D, 2E, 2F, males and females. Groups 1A, 1B, 1C are descendants from the mothers with 0.5 mg natrium fluoride allopathic administration and Groups 2D, 2E, 2F are descendants from the mothers with 0.25 mg natrium fluoride allopathic administration.

- Groups 1A and 2D – Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of a natrium fluoride solution;
- Groups 1B and 2E – Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of a natrium fluoride solution with allopathic administration of 0.5 mg‰ natrium fluoride ad libitum (Group 1B), respectively of a CH7 dilution of a natrium fluoride solution with allopathic administration of 0.25 mg‰ natrium fluoride ad libitum (Group 2E), males and females;
- Groups 1C and 2F – Allopathic administration of 0.5 mg‰ natrium fluoride ad libitum (Group 1C), respectively allopathic administration of 0.25 mg‰ natrium fluoride ad libitum (Group 2F), males and females.

Histopathologic assessment of liver

When mice reached adulthood, they were selected by randomization for euthanasia. For the histopathological examination, the animals were killed by cervical dislocation, the testes from every specimen selected for the examination were taken and introduced in a 10% formalin solution. The preparation of samples for optical microscopy was realized with Hematoxylin-Eosin staining (17).

RESULTS

The classification of experimental groups and the doses administrated for every group are highlighted in table 1.

Histopathological assessment of liver (table 2)

Microscopic evaluation

We observed binucleate hepatocytes, which is common in mice liver and discrete hyperemia to the control group. When the substance is administrated, we can observe that vacuolar dystrophy is common in all groups, but apoptosis, apoptotic corpse and necrosis are more common for the allopathic doses. We can observe that at the macroscopic examination, the liver is dystrophic and discolored.

For group 1A, descending from pregnant females with 0,5 mg natrium fluoride daily administration by gavage, and, after birth until adulthood, with homeopathic dose (CH7), we discovered that cariomegalia, anisocytosis, vacuolar dystrophy and granular dystrophy are present. (figure 1)

For group 1B, descending from pregnant females with 0,5 mg natrium fluoride daily administration by gavage, and after birth until adulthood with homeo-

Table 1 - The classification of experimental groups by dosage

| No | Groups (males and female) | Dose of administered natrium fluoride |
|----|---------------------------|---|
| 1 | Control group | None |
| 2 | Group 1A | Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of natrium fluoride solution |
| 3 | Group 1B | Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of natrium fluoride solution with allopathic administration of 0,5 mg‰ natrium fluoride ad libitum |
| 4 | Group 1C | Allopathic administration of 0,5 mg‰ natrium fluoride solution ad libitum |
| 5 | Group 2D | Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of natrium fluoride solution |
| 6 | Group 2E | Homeopathic administration by gavage with one channel micropipette of a CH7 dilution of natrium fluoride solution with allopathic administration of mg‰ natrium fluoride ad libitum |
| 7 | Group 2F | Allopathic administration of 0,5 mg ‰ natrium fluoride solution ad libitum |

Table 2 - Pathological aspects of liver tissue revealed at the histopathological examination for every group

| No | Groups (males and female) | Pathological aspects |
|----|---------------------------|--|
| 1 | Control group | Discrete hyperemia, binucleate hepatocytes (normal), cariomegalia, without mythosis |
| 2 | Group 1A | Cariomegalia, anisocitosis, vacuolar dystrophy, granular dystrophy |
| 3 | Group 1B | Diffuse vacuolar dystrophy, discrete hyperemia, cariomegalia with big cell nuclei |
| 4 | Group 1C | Mononucleate and binucleate hepatocytes, cariomegalia, numerous cytoplasmic granules, apoptosis, apoptotic corpse, necrosis. Macroscopically: dystrophic liver, discoloration |
| 5 | Group 2D | Apoptosis, vacuolar dystrophy, apoptotic corpse, cariomegalia |
| 6 | Group 2E | Vacuolar dystrophy, anisokariosys, apoptosis Macroscopically: dystrophic liver |
| 7 | Group 2F | Vacuolar dystrophy, centrilobular apoptosis, anisokariosys |

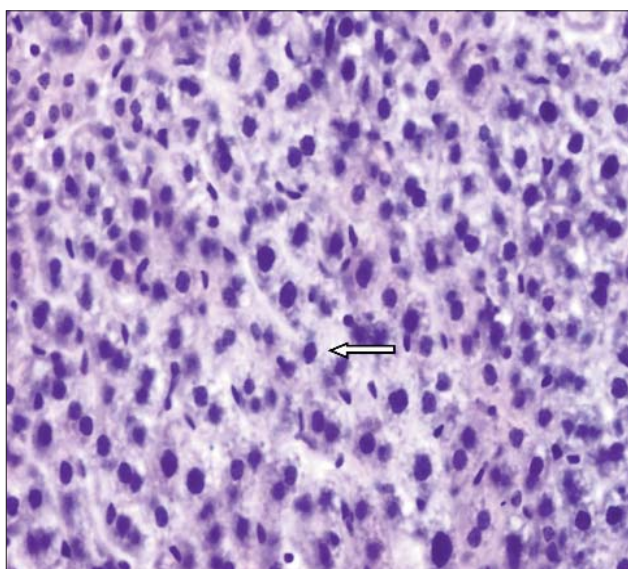


Figure 1 - Granulo-vacuolare dystrophy (H- E staining 400 x). Uninucleate and binucleate hepatic cells with granules and vacuolization; the cells nuclei remains stable, are not removed by granules or vacuolization

pathic- allopathic dose (CH7 + 0.5‰ in drinking water ad libitum), we found diffuse vacuolar dystrophy, discrete hyperemia, cariomegalia with big cell nuclei. (figure 2 a, b)

For group 1C, descending from pregnant females with 0,5 mg natrium fluoride daily administration by gavage and after birth until adulthood with allopathic dose (0.5‰ in drinking water ad libitum), the morphological aspects consist in mononucleate and binucleate hepatocytes, cariomegalia, numerous cytoplasmic granules, apoptosis, apoptotic corpse, necrosis. Macroscopically: dystrophic liver, discoloration. (figure 3 a,b,c)

For group 2D, descending from pregnant females with 0,25 mg natrium fluoride daily administration by gavage and, after birth until adulthood, with homeopathic dose (CH7) the microscopy revealed apoptosis, vacuolar dystrophy, apoptotic corpse and cariomegalia. (figure 4)

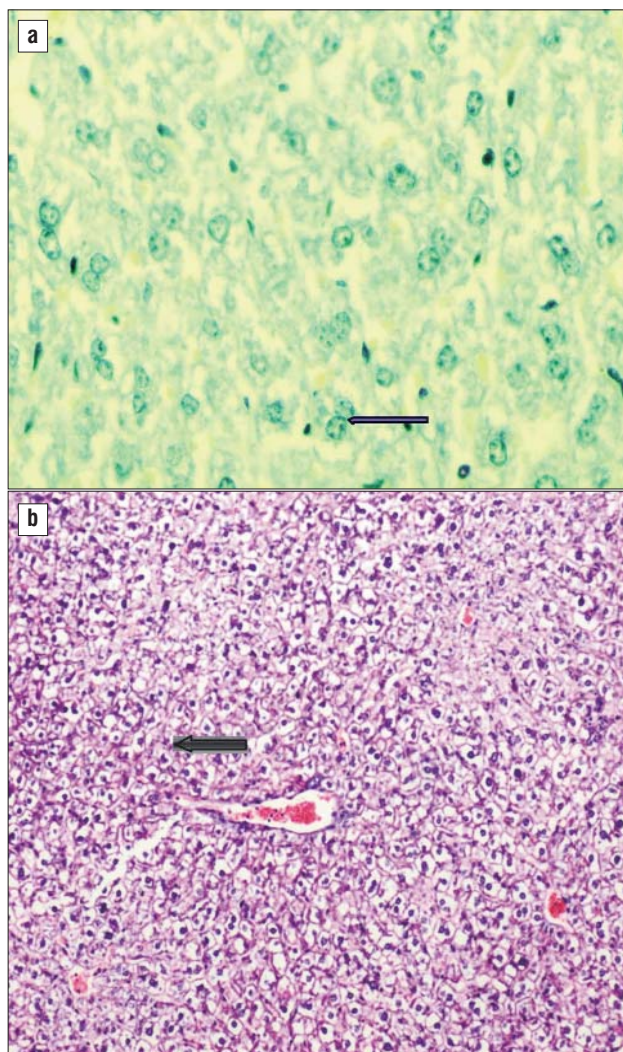


Figure 2 - (a) Cells with euromatic nuclei, isolated cariomegalia; binucleate hepatic cells (arrow) (HE 100X). (b) Vacuolar dystrophy and apoptotic corpse. Vacuoles does not change the position of the cells nuclei (H- E staining 100X).

For group 2E, descending from pregnant females with 0,25 mg natrium fluoride daily administration by gavage, and, after birth until adulthood, with homeopathic - allopathic dose (CH7 + 0.25‰ in drinking water

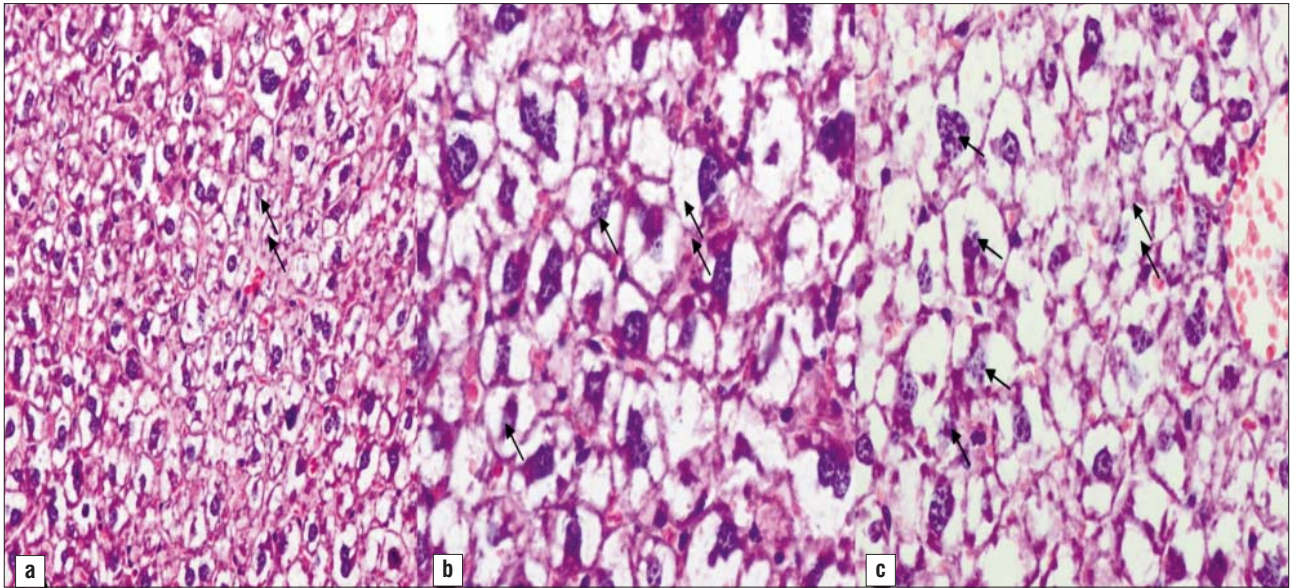


Figure 3 - (a) - liver apoptosis (H-E staining 200X), (b) - liver apoptosis (apoptotic corpse) (arrow) (H-E staining 400X), (c) - liver apoptosis (apoptotic corpse) (arrow) (H-E staining 400X)

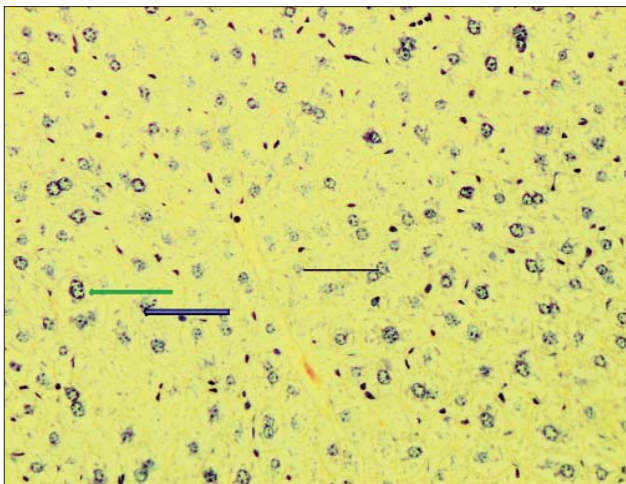


Figure 4 - Unineucleate and binucleate hepatic cells with intracytoplasmic granules and vacuolisation (arrow). Some of cells have excentric nuclei, cariomegalia and regenerative processes (binucleate cells) (H-E staining 200X)

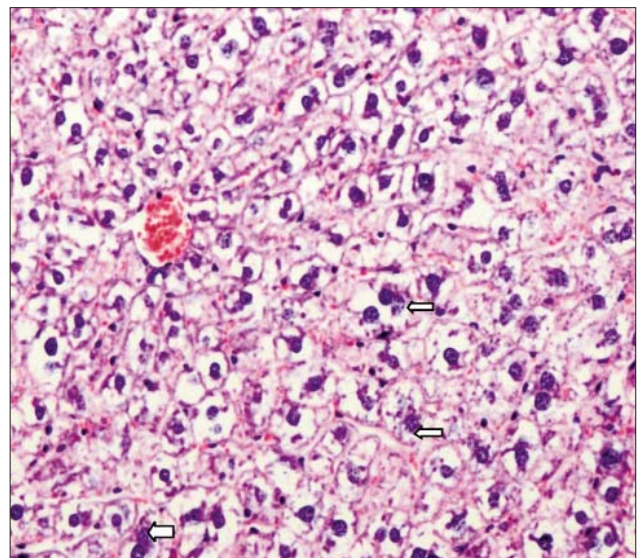


Figure 5 - Vacuolar dystrophy and apoptotic corpse (arrow) (HE 100X)

ad libitum), we found vacuolar dystrophy, anisokariosys, and apoptosis. Macroscopically: dystrophic liver. (figure 5)

For group 2F, descending from pregnant females with 0,25 mg natrium fluoride daily administration by gavage, and, after birth until adulthood, with allopathic dose (0.25‰ in drinking water ad libitum), we found vacuolar dystrophy, centrilobular apoptosis, and anisokariosys. (figure 6)

DISCUSSION

Previous studies have shown that fluoride impaired liver function and produced histopathological changes (18,19,20,21). The present study aims to assess the effect of natrium fluoride administration, at different dosages on histopathological changes of the liver during all childhood of mice NMRI type until adulthood.

The control group had liver tissue characterised by the presence of the binucleated cells or cariomegalia

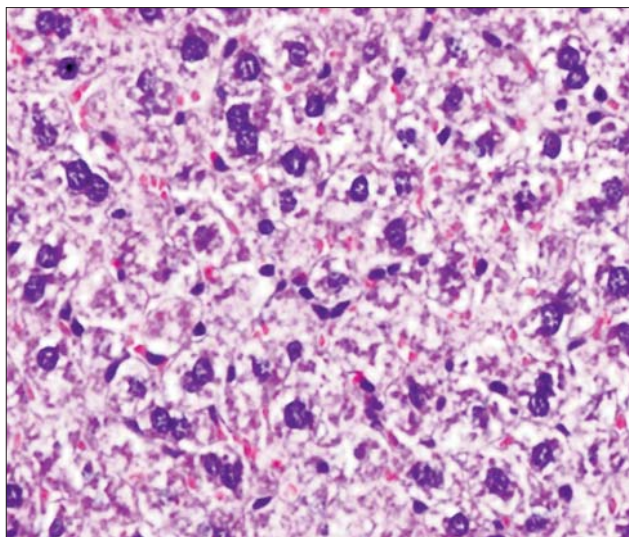


Figure 6 - Apoptosis and vacuolar dystrophy. Apoptotic corpse. (H-E staining 400X)

without mitosis. However, the experimental group, exposed to sodium fluoride, demonstrated the presence of these liver changes, characterized by the diffuse aspects.

The histopathological aspect of the liver is different depending on every dose administered.

The present study revealed that homeopathic doses have minimum influence on the hepatic cells, including cariomegalia and vacuolisation and minimum apoptotic cells.

The allopathic - homeopathic dose is augmented by the hyperemia and centrilobular necrosis; apoptosis is also present. Anisokariosis is present for the groups with allopathic doses and allopathic - homeopathic doses.

Apoptosis is typical for liver disease. It could occur at different levels, depending on various factors that produce hepatocyte injuries (at organite level or membrane). The affected liver depends on the presence of quantities of apoptotic cells. The apoptotic cells and the necrotic cells characterized two important aspects of cells death. Apoptosis is the first manifestation of the affected cell that is going to die, and various biochemical mechanisms are involved. Also, the apoptotic cells have particular histopathological aspects and the tissue examination reveals information about the health level of the liver.

In the present study, the most important pathological liver changes following the natrium fluoride allopathic administration were apoptosis, the presence of apoptotic corps or centrilobular necrosis. These results are consistent with data reported by Wang et al. in which 40-day exposure to sodium fluoride of rabbits

produced hepatocyte necrosis and vacuolar degeneration (22). Other data regarding natrium fluoride administration revealed apoptosis present in other tissues, like the oral mucosal cells (was (13.63 ± 1.81) % in fluoride group, and (12.76 ± 1.67) % in hepatocytes, higher than those in control group (23). Also, the mean values of relative liver and kidney weights at a 100 and 150 ppm natrium fluoride in a population of mice were significantly lower than those in the control group. The mean values of BUN, creatinine, and GPT at the 150 ppm natrium fluoride dose for the experimental groups were significantly higher than those in the control (24). Other cells where apoptosis produced by natrium fluoride is detected are the hippocampal neurons. At a 40 to 80 mg/l of fluoride significantly increased level of lactate dehydrogenase is induced, the presence of intracellular reactive oxygen species, and a percentage of apoptosis (25). In skeletal tissue the fluoride treatment inhibits osteoblasts proliferation and a significant increase of osteoblast apoptosis was observed (after 24 and 72 hours treatment, at the lowest dose of sodium fluoride - 0.5 mg/l). The osteoblast apoptosis was further increased in response to higher doses (26). Apoptosis produced by natrium fluoride is studied at the level of hippocampal neurons. At 40 and 80 mg/l of fluoride the neuronal apoptosis is significantly increased (25). The administration of 10 to 100 $\mu\text{g}/\text{kg}$ body weight sodium fluoride on male Wistar rats aging 75 days and with a DNA study after 2 hours of the administration of substance, a genotoxic effect in multiple organs has not been revealed (27). Changes are also present at the macroscopic level: alterations of the liver, such as dystrophy and discoloration, both in males and females. The pathophysiological mechanisms revealed death receptor mediated cascade, lysosomal permeabilisation, mitochondrial dysfunction (28), as well as antiapoptotic mechanisms.

The study of nano-sized tin colloid can be used to determine the detection in different tissues of agents-like tin fluoride, sodium fluoride, poloxamer-188 and polyvinylpyrrolidone (PVP), mixed and labeled with $(^{99\text{m}}\text{Tc})$ (29). In addition, flow-cytometry could be a technique for the quantitative analysis of apoptosis (apoptotic cells as subdiploid peak") (30-34).

CONCLUSIONS

The study revealed that at different doses of natrium fluoride, the morphological aspect of liver in male and in female mice is modified. These modifications are vacuolar dystrophy and granular dystrophy, cariomegalia with big cell nuclei, mononucleate and

binucleate hepatocytes, cariomegalia, numerous cytoplasmic granules, apoptosis, apoptotic corpse, necrosis. Macroscopically: dystrophic liver, discoloration. The significant modifications are present at the allopathic dose. The presence of the apoptotic cells and apoptotic corps revealed the hepatotoxicity of natrium fluoride. In some cases, necrosis is present and indicates the next stage of cells damages. Necrosis accompanies apoptosis and indicates the level of hepatotoxicity of natrium fluoride, especially at the allopathic doses. Interestingly, the homeopathic dose also indicates a level of hepato-toxicity (with vacuolar dystrophy and granular dystrophy). Further studies are necessary regarding the changes at cellular level, and at the level of cytoplasmic organelles and cell nuclei (DNA studies).

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