INFLUENCE OF FLUORIDE ON RABBIT ORGAN MORPHOLOGY IN AHEROMATOSIS

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SUMMARY: A three-month study was conducted on the influence of fluoride on the development of pathomorphological changes in the inner organs of New Zealand male rabbits in experimental atherosclerosis. Five groups of six rabbits were used: a control group and four study groups. Animals in study groups I and II were fed a cholesterol-supplemented diet providing 0.5 g cholesterol/rabbit/day. Additionally, animals in study group II were supplemented with 3 mg F/kg bw/day in their drinking water. Animals in study group III were fed a higher cholesterol-supplemented diet providing 2.0 g of cholesterol/rabbit/day. Animals in study group IV were fed the same high-cholesterol diet and were also given 3 mg F/kg bw/day) in their drinking water. Plasma lipids were monitored monthly, and after three months the experiment was terminated. At autopsy, blood was collected for biochemical analyses, and the heart, kidney, liver, and aorta were examined and collected for histopathological tests. Pathomorphological changes in these organs were assessed with slides made by the normal paraffin method stained with hematoxilin and eosin, and also by the freezing microtome method stained with Sudan III for detecting neutral fats. At 3 mg/kg bw/day, fluoride did not have a significant preventive influence from the high-cholesterol diet on the atherogenesis processes in the aorta and heart arterial vessels or on the regressive changes (steatosis) occurring in the liver and kidneys. However, this daily dosage of fluoride was found to inhibit the atherogenesis processes caused by 0.5 g of cholesterol/rabbit/day to occur in the aorta and arterial vessels in the heart as well as the regressive changes (fatty degeneration) in the liver and kidneys.

Keywords: Aorta; Atheromatosis; Cholesterol in rabbits; Fluoride and atheromatosis; Heart; Histopathology; Kidney; Liver; Rabbits and cholesterol.

INTRODUCTION

Atherosclerosis is a very common ailment of the cardiovascular system, especially in highly developed countries. It is characterized by regressive and progressive changes in the intima and tunica of arterial vessels. 1 Cholesterol is a well-documented pathogenic factor in atherosclerosis. Recent studies indicate that oxidative processes play an important role in the pathogenesis of atherosclerosis. Oxidative free radicals oxidize low density lipoproteins (LDL) that then damage blood vessel endothelium. These free radicals also affect lamella adhesion, smooth muscle cell proliferation, immunological responses, and the influx of macrophages into inner tunica. And evidently this is how favorable conditions for atherosclerosis are created.2-5 Changes usually occur in the aorta and coronary arteries and later in brain and kidney arteries.6,7

Enzymes are the first line of defense against oxidative free radicals: superoxide dismutase, glutathione peroxidase, and catalase. Moreover, vitamins A, E, and C,
as well as methionine, cysteine, and co-enzyme Q, help protect the organism from free radical oxidation. Recent studies show that fluoride (F) ions play a role in the free-radical processes as well as in the metabolism of lipids. F may decrease lipid levels, increase them, or even have no effect on them.

F ions inhibit lipid peroxidation as well as the development of oxidatively-modified LDL (oxy-LDL) and therefore prevent its entrapment by scavenger receptors found in macrophages and monocytes. In this way, F decreases the accumulation of cholesterol and its esters in the above-mentioned cells. The role F therefore plays in lipid metabolism and free radical processes suggests that it may be an important link in the atherosclerosis process.

The aim of this study was to assess the influence of alimentary administration of F on the morphology of the aorta, liver, kidney, and heart organs of rabbits in experimental atherosclerosis produced by a cholesterol (CH) supplemented diet.

MATERIALS AND METHODS
The study was performed on 30 male New Zealand rabbits with an initial body weight of 3000g±50g. The animals were provided by the Central Experimental Animal Quarters of the Silesian Medical School in Katowice, Poland. They were divided into five equal groups of six as follows. Animals in the control group (C) were fed standard rabbit ration. Animals in the study Group I (0.5 g CH) were on an atherosclerosis diet and received 0.5 g of cholesterol/rabbit/24 hr, while animals in study Group II (0.5 g CH+F) were fed the same cholesterol-supplemented diet but were also given 3 mg F/kg bw/24 hr in their drinking water by receiving 9 mg F (=19.9 mg NaF) in 100 mL distilled water every morning and afterward being given distilled water without F ad libitum until the next morning. Animals in study groups III (2.0 g CH) and IV (2.0 g CH+F) were fed a cholesterol-supplemented diet providing 2.0 g cholesterol/rabbit/24 hr. Additionally, as in study Group II, animals in study Group IV were also supplemented with 3 mg F/kg bw/24 hr in their drinking water. The experiment lasted 3 months with the animals being kept in wooden cages. Once a month, blood was collected for biochemical analyses from the marginal vein of the ear. The concentration of LDL cholesterol in plasma was determined by an enzymatic method using a BioMerieux kit (France) while the level of HDL cholesterol and triglycerides was assessed with an Alpha Diagnostics kit (Germany).

Results were analyzed statistically with Statistica PL software. The U Mann Whitney test was used to compare differences between particular groups. Statistical significance was restricted at p≤0.05.

Methodology of the histopathological study
During postmortem examination the following were collected for histopathological tests: aorta, liver, heart, and kidneys. The organs were preserved in aqueous formaldehyde for pathomorphological assessment, and changes were assessed on the basis of paraffin preparations stained with hematoxilin and eosin (H-E). The presence of fats in the atheromatous plaques of the aortas and vessels
as well as in the organ tissues was confirmed by examination of preparations obtained on a freezing microtome and stained with Sudan III for neutral fats. Microphotographs were taken with the Docuval microscope equipped with a Carl Zeiss Jena photo device.

This study was approved by the Bioethical Committee for Animal Testing of the Silesian Medical University.

RESULTS

Figures 1–3 show changes in the lipid concentrations in plasma of the rabbits after three months in the four groups on a cholesterol diet: Group I (0.5 g CH); Group II (0.5 g CH+F); Group III (2.0 g CH) and Group IV (2.0 g CH+F). The concentration of triglycerides increased significantly in all the study groups compared with the control group; p<0.01 (Figure 1).

Moreover, there was also a significant increase in the concentration of LDL cholesterol in these groups compared with the control group; p=0.004 (Figure 2).

Administration of F along with 0.5 g cholesterol/kg bw/day (Group II) caused a downward tendency in the LDL concentration when compared to Group I without...
F. No statistically significant change in the HDL cholesterol concentration was observed in these two groups. In Group IV with the higher level of cholesterol and F (2.0 g CH+F) there was a statistically significant increase in HDL after the third month when compared to group III (2.0 g CH) without F and to the control group (Figure 3).

**Histopathological findings**

**Macroscopic assessment:**

The presence of creamy atheromatous plaque was noticed in the arch area and abdominal part of aortas in studied groups. In groups I, III, and IV the foci of changes were large and covered the entire circumference, while in Group II (0.5 g CH+F) they were much less intense. There was a color change—xanthochromia—in the liver of all the study groups. However, there was no macroscopic change in the kidney or heart.

**Microscopic assessment:**

**Aorta.** In contrast to the condition of the aorta in the control group C (Figure 4A), the study groups I (0.5 g CH) and III (2.0 g CH) exhibit a focal hyperplasia of tunica interna in the form of a large atheromatous plaque. There were also changes in the tunica media of animals in this group in the form of a focal proliferation of macrophages in the proximity of membrana elastica interna. There were numerous foam-like cells in the atheromatous plaque, and fat was also noticed in intercellular spaces. There were also changes in the tunica media of animals from this group in the form of a focal thickening with no signs of fibrosis as well as a focal proliferation of macrophages in the proximity of membrana elastica interna. There were also calcification foci in interna membrana (in 2 animals) and focal losses of elastica lamella. The atherogenic changes in aortas of Group II (0.5 g CH+F) (Figure 4C) were much less intense than in Group I (0.5g CH) (Figure 4B). On the other hand, the morphology of aortas of rabbits in Group IV (2.0 g CH+F) (Figure 4E) was similar to that of animals in Group III (2.0 g CH) (Figure 4D).
Unlike the liver of the control group C (Figure 5A), there was a distinct steatosis of hepatocytes in the liver of all study groups. It was intensified around the central veins of the liver lobules (Figures 5B, 5C, 5D, and 5E). In Group II (0.5g CH+F), the steatosis of hepatocytes was of lesser intensity (Figure 5C). Steatosis of hepatocytes in Group IV (2.0 g CH+F) was similar to that in Group III (2.0 g CH) (Figures 5E and 5D, respectively).
Kidney. In contrast to the kidneys of the control group C (Figure 6A), there was a focal steatosis in the tubule epithelium cells noticed only in kidneys of the 0.5 g CH and 2.0 g CH groups I and III (Figures 6B and 6D, respectively). Also observed in the kidneys of Group III were areas of local parenchyma fibrosis. There were also foci of renal tubule cell steatosis in Group IV (2.0 g CH+F) (Figure 6E), but no regressive changes were seen in kidneys of the 0.5 g CH+F Group II (Figure 6C).
Heart. Differing from the appearance of the arterial vessels of the control group C (Figure 7A), there was a focal proliferation and steatosis of membrana interna (atheromatous plaque) in the arterial vessels of the heart in all groups (Figures 7B, 7C, 7D, and 7E). In the 0.5 g CH Group I the above-described changes were intense and applied to arterial vessels of different size (Figure 7B), whereas in the 0.5 g CH+F Group II smaller atheromatous plaques were present (Figure 7C). In Group III (2.0 g CH) atheromatous plaques were large and covered the entire
circumference of the vessel (Figure 7D). However, atheromatous plaques in arterial vessels of the heart were not so numerous and slightly smaller in the Group IV (2.0 g CH+F) (Figure 7E).

Figure 7. Heart.

Figure 7A. Control group (C). Heart. Normal pattern. H-E. 280×.

Figure 7B. Group I (0.5g CH). Heart. Atheromatous plaque in arteriole. H-E. 150×.

Figure 7C. Group II (0.5g CH + F). Heart. Atheromatous plaque in arteriole. H-E. 280×.

Figure 7D. Group III (2.0g CH). Heart. Atheromatous plaque in arteriole. H-E. 200×.

Figure 7E. Group IV (2.0g CH + F). Heart. Atheromatous plaque in arteriole. H-E. 140×.

The presence of fatty deposits in the atheromatosis laminas as well as in the renal tubule cells and hepatocytes was confirmed by histochemical studies. Fats were stained with Sudan III into yellow-orange color.
Our study shows that an increased serum lipid level is not only an atherogenic factor, but it also leads to pathomorphological changes in the internal rabbit organs in the form of steatosis of both the renal tubule epithelium and hepatocytes in liver. The unquestionable regressive changes in the parenchymatous cells in liver and kidney disturb the regular functioning of an organism, including normal cholesterol metabolism. Administration of ionic fluoride (F) with the higher cholesterol diet (Group IV) did not significantly influence the development of atherosclerosis or regressive changes in organs. The morphological picture of the rabbit organs in the study groups correlated with the biochemical assays results for these animals.

The administration of F, together with the cholesterol diet (0.5g CH/kg bw/day) had an inhibitory effect on the atherogenesis and the development of the regressive changes in organs. Our results indicate that the early atheromatous changes (low cholesterol dose) were accompanied by the preventive activity of F ions on the observed histopathological changes resulting from the cholesterol burden. Recent studies indicate that in the pathogenesis of atheromatosis, oxidative processes play a significant role, especially the oxidation of low-density lipoproteins. Other studies are concerned with the role of F in oxidative stress. For example, de Ferreyra et al. suggested a rise in reduced glutathione levels caused by F with subsequent removal of hydrogen peroxide and oxygen free radicals by glutathione peroxidase, and, in effect inhibition of peroxidation. There are also studies disclosing the activation or inhibition of F activity on lipid peroxidation and the formation of malondialdehyde (MDA). Chlubek et al. has suggested that F at relatively low concentrations stimulates lipid peroxidation, but at high and very high concentrations may act as inhibitor of MDA generation. Findings by Gardner and Fridovich indicate that F ions stabilize dehydratase and block the effect of oxidants. In contrast, a number of studies indicate that generation of ROS and formation of MDA can be directly induced by F. There are also recent reports indicating that F ions can inhibit the activity of enzymes responsible for oxygen free radical metabolism (glutathione peroxidase, superoxide dismutase). According to Chlubek et al., a decrease in SOD activity can be attributed to a direct action of F on the enzyme rather than to increased generation of free radicals induced by F intoxication. Reddy et al. reported finding no changes in lipid peroxide and GSH levels or in GSH-Px, SOD, and CAT (catalase) activities in red blood cells of F-intoxicated rabbits and fluorotic humans.

Recently, Birkner reported that cholesterol added to the diet of male rabbits caused a statistically significant increase of MDA in blood plasma in comparison to the control group. In our Group II rabbits (0.5 g CH+F) the MDA concentration in plasma was considerably lower after three months in comparison to Group I (0.5 g CH). The lower concentration of MDA—an oxidation stress marker—probably prevents dysfunction of vascular epithelia. Also, as Birkner noted, biochemical studies of male rabbits fed a 0.5% cholesterol diet supplemented with F for three months revealed a statistically significant increase of SOD in plasma when compared to the cholesterol control group without F. Thus Birkner’s results
showing a positive effect of F on the rate of oxidation processes are in agreement with the pathomorphological findings in our research.

As already noted, administration of F with cholesterol (0.5g CH+F) caused a downward tendency in the LDL plasma concentration when compared to the 0.5 g CH group. In the biochemical studies, no influence of F on the concentration of triglycerides and HDL cholesterol in plasma was noticed when compared to Group I (0.5 g CH). In our experiments, we observed a statistically significant increase in HDL cholesterol levels in animals of Group IV (2.0g CH+F) compared to Group III (2.0g CH) without F. Other lipid parameters (LDL and triglycerides) did not change significantly in both study groups compared to the control group. However, recent studies indicate that F may play a significant role in atherogenesis through its influence on the lipid profile in plasma. For example, Luoma et al.\textsuperscript{15,16} found that F causes a reduction in the levels very low density lipoproteins (VLDL), LDL cholesterol, and LDL phospholipids in rats, as Singh et al.\textsuperscript{17} had reported earlier. Likewise, Kessabi et al.\textsuperscript{14} record similar findings in sheep.

On the other hand, other studies on the influence of F on the serum level of lipids are ambiguous. An increase in lipid concentration caused by F has been reported in earlier studies,\textsuperscript{13,19} but a later report indicated no influence at all by F on the level of lipids in blood.\textsuperscript{34}

The observed steatosis of hepatocytes in the liver and renal tubule epithelium cells resulting from a cholesterol burden indicates a malfunction in lipid metabolism and lipoprotein metabolism in the liver and kidney.\textsuperscript{35} In animal experiments it has also been shown that F causes regressive changes in the liver.\textsuperscript{36}

The results we have observed indicate that although the influence of F on lipid management and free radical processes is controversial, lipid disorders are known to be a major risk factor in atherosclerosis. Lipid metabolism (lipogenesis or lipolysis) in the organism may be affected by F due to repression of the activity of a number of enzymes for lipid transformation: some nonspecific esterases such as lipase.\textsuperscript{37}

In our studies we have seen that regulation of the metabolism of cholesterol in the liver was disturbed. Fluoride ions administered together with a lower cholesterol atherogenic diet inhibited the atheromatous changes in the aorta and arterial vessels as well as the regressive changes in the liver and kidneys.

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