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The Influence of Fluoride Ions upon Selected Enzymes of Protein Metabolism in Blood Plasma of Rabbits with Hypercholesterolemia

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Abstract Three-month studies were performed on 18 adult rabbits of New Zealand breed divided into three groups, with six animals in each: a control group on standard diet, a cholesterol group receiving 500 mg of cholesterol/100 g of feed per rabbit per 24 h (CH group), and a cholesterol+fluorine group (CH+F group) receiving 500 mg of cholesterol/100 g of feed per rabbit per 24 h and 3 mg of F^-/kg of body weight per 24 h. The conducted studies proved that cholesterol in the applied dosage (500 mg cholesterol per rabbit per 24 h) has an atherogenic action. Fluoride ions administered together with a 500-mg cholesterol atherogenic diet inhibit the atheromatosic changes in the aorta. The concentration of plasma cholesterol was elevated in both study groups when compared to the control group but decreased in the CH+F group when compare to the CH group. The influence of fluoride ions has been examined upon the activity of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase (GLDH) in the plasma in the liver of rabbits in the course of experimental hypercholesterolemia. Increase in the activity of study enzymes has been observed in the blood plasma, which may be due to damage occurring to hepatocytes of the animals examined (a statistically significant increase in the activity of GLDH in the plasma). In the liver, the inhibition of activity for all examined enzymes has been observed in the group of rabbits with hypercholesterolemia, which testifies the disturbances in protein metabolism in examined animals. The addition of sodium fluoride to the diet rich in cholesterol results in "removing the block" on those

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activities, which increase. We suppose that the permeability of the hepatocyte membrane was elevated, so the activities of examined enzymes increased in the plasma ("escape" to plasma). On the one hand, fluoride ions result in probable lesion of hepatocytes membranes; on the other hand, they inhibit the atheromatosic changes in the aorta.

Keywords Fluoride ion · ALT · AST · GLDH · Rabbits · Hypercholesterolemia · Atheromatosis · Histopathology of aorta

Introduction

On the one hand, fluorine influences positively the dentition condition, albeit on the other hand, the compounds containing it have been listed among the most significant endotoxins that appear in natural environment as after-effects of industrial activity of humans. Fluoride ions, after absorption to the blood from the alimentary tract or the lungs, easily penetrate to cells through membranes. Only a portion of it would be expelled from the body in urine. The most substantial amounts of fluorides are gathered in hard tissues (bones, teeth). On the other hand, soft tissues are constantly saturated with them, as continuous inflow of fluorides occurs. The accumulation of fluorides in the body initially leads to moderate symptoms, yet it gradually leads to irreversible fluorosis of bones or degenerative changes in soft tissues. In addition, fluorine and its compounds (e.g., NaF) may generate radical processes and lead to oxidative stress [1–5].

The enzymes of protein metabolism contain, among others: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamate dehydrogenase (GLDH). Those enzymes catalyze transamination reactions, which consist of transferring amine groups from various amino acids to one of three α-keto-acids: pyruvic acidoxaloacetic acid or α-ketoglutaric acid.

Glutamic acid is a product of transamination, which undergoes oxidative deamination. The catalyst for the reaction is GLDH. That enzyme cooperates with both nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Its activity is regulated in an allosteric manner. The allosteric inhibitors of GLDH are guanosine triphosphate and adenosine triphosphate, while the allosteric activators are guanosine diphosphate and adenosine diphosphate [6].

Atherosclerosis, in turn, is a pathological state characterized by a focal concentration in the tunica intima of middle-sized and large arteries of cholesterol, polysaccharides, monocytes, collagenous fibers, and calcium salts. The factors that initiate the process of atherosclerosis include changes in the endothelium of arterial vessels, as well as the penetration of low-density lipoprotein (LDL) to the subendothelial spaces. Among the atherogenic factors, the following are included: chronic hypertensive disease, vasoactive substances, such as catecholamines, free radicals, as well as increased concentration of homocysteine [7–10].

Due to the continued increase in the incidence of atherosclerosis, as well as a high mortality rate of that disease, there is an intensified need to carry out multidirectional research concerning that disease. The available literature contains relatively scarce information concerning the influence exercised by fluorine upon atherosclerosis. Thus, an interesting issue to examine is the influence of fluorine compounds upon the course of atherosclerosis.

Recent studies have shown that liver transaminases are associated with components of the metabolic syndrome including central obesity, type 2 diabetes, dyslipidemia, and high blood pressure, but their direct influence on coronary atherosclerosis has not been investigated before [11]. We conducted our study to elevate value of liver transaminases and GLDH in experimental atherosclerosis.

The aim of the study was to assess the influence changes in protein metabolism enzymes (AST, ALT, GLDH) caused by fluoride ions, upon experimental atherosclerosis. It could be helpful in therapeutic methods.

Materials and Methods

Studies were performed on 18 adult rabbits of New Zealand breed, with initial body mass of 3,000±50 g and were conducted following the experimental protocol approved by the Committee for Research and Animal Ethics in the Silesian Medical University. Rabbits were housed in separate cages, in a temperature-controlled room (22–25°C) with a 12:12-h light/dark cycle and free access to water and feed (ad libitum). Before the experiment started, the rabbits went through a 2-week adaptation period to the experimental conditions. The rabbits have been randomly divided into three groups, with six animals in each:

1. Control group on standard diet (control group)
2. Cholesterol group (CH group) receiving 500 mg of cholesterol/100 g of feed per rabbit per 24 h
3. Cholesterol+fluorine group (CH+F group) receiving 500 mg of cholesterol/100 g of feed per rabbit per 24 h and 3 mg of F^-/kg of body weight per 24 h. The dose of fluorine administered to the rabbits in the CH+F group was roughly equivalent to that applied in the study by Kaur et al. [12]

Every month, in sterile manner, a single sample of 6 ml of blood was taken from the marginal auricular vein. After 3 months, the rabbits were put to sleep by means of administering a 20% solution of ethyl carbamate, applying the dose of 2.5 g/kg of body mass.

Blood and liver have been taken to be examined. Plasma cholesterol was estimated using the kit manufactured by EMAPOL. The activity of the following enzymes has been determined in blood plasma: AST, ALT, and GLDH by kinetic methods [13]. The results have been provided in international units, calculated per 1 l of plasma (IU/l). In the liver, the activity of the above-quoted enzymes has been determined as calculated for protein (IU/g of protein), which have been determined using the Lowry method [14]. As it was described previously, we conducted the histopathological study too [15].

Aortas collected for the pathomorphological study were preserved in an aqueous solution of formalin. The pathomorphological changes in rabbits' internal organs were assessed on the basis of preparations prepared in the ordinary paraffin technique, stained with hematoxylin and eosin (H–E). The presence of fats in the atheromatous lamellae of aortas was confirmed by the preparations obtained on a freezing microtome, stained with Sudan III [16]. Microphotographs were taken with the Docuval microscope equipped in the photo device by Carl Zeiss Jen.

The results obtained have undergone statistical analysis, using the software STATISTICA PL. Statistical methods included mean and standard error of mean (SEM). Analysis of variance and Mann–Whitney U test were used for the comparisons of groups. A value of $p<0.05$ was considered to be significant.

Results

Histopathological Assessment

Macroscopic Assessment The presence of creamy atheromatous plaque was noticed in the area of the arch and abdominal part of aortas in the studied groups. In the CH group, the focuses of changes were large and covered the entire circumference, while in the CH+F group, they were of much lesser intensification.

Microscopic Assessment

Aorta In the CH group, there was a focal hyperplasia of tunica interna in the form of a large atheromatous plaque. There were also changes in the tunica media of animals from this group in the form of a focal proliferation of macrophages in the proximity of membrana elastica interna. The atherogenic changes in aortas of the CH+F group were of much lesser intensity than in the CH group. There were numerous foam-like cells in the atheromatous plaque, and fat was also noticed in intercellular spaces. The light microscopy picture of the aortas is shown Fig. 1a–c.

Biochemical Results

The results have been presented on seven figures.

As results from Fig. 2, the activity of AST in the plasma in the CH group, in comparison with the control, increased in the first month already and later in the second and third month. The activity of AST in the CH group, which received fluoride ions at the same time (CH+F), increased by 32% in the first month, while in the third month by 75%.

The activity of ALT in the blood plasma (Fig. 3) appears to be different. In the CH group, the activity of ALT increased slightly (6%) only in the third month of the experiment, while the activity of ALT in the CH+F group increased by 14% in the third month.

The activity of GLDH (Fig. 4) in the blood plasma in the CH group did not undergo substantial changes, in comparison with the control. On the other hand, in the CH+F group, it increased substantially already by 39% in the first month of the experiment. In the second and third months of the study, the examined activity of GLDH increased by 114% and 76%, respectively (statistically significant results).

Of interest are the results concerning the activity of the three enzymes (AST, ALT, GLDH) in the liver (Figs. 5, 6, 7). In the CH group, namely, the activity of all the three enzymes was inhibited (statistically significant results). Under the influence of fluorine (CH+F), the level of those activities increased, in comparison with the CH group, by 29% (ALT), 9% (AST), and 92% (GLDH). In respect to the control, however, those outcomes were lower (not significantly statistically), respectively, by 29% (ALT), 10% (AST), and 17% (GLDH).

The concentration of the plasma cholesterol in the CH group was statistically elevated after 1, 2, and 3 months. In the CH+F group, cholesterol concentration was decreased after each month (Fig. 8).

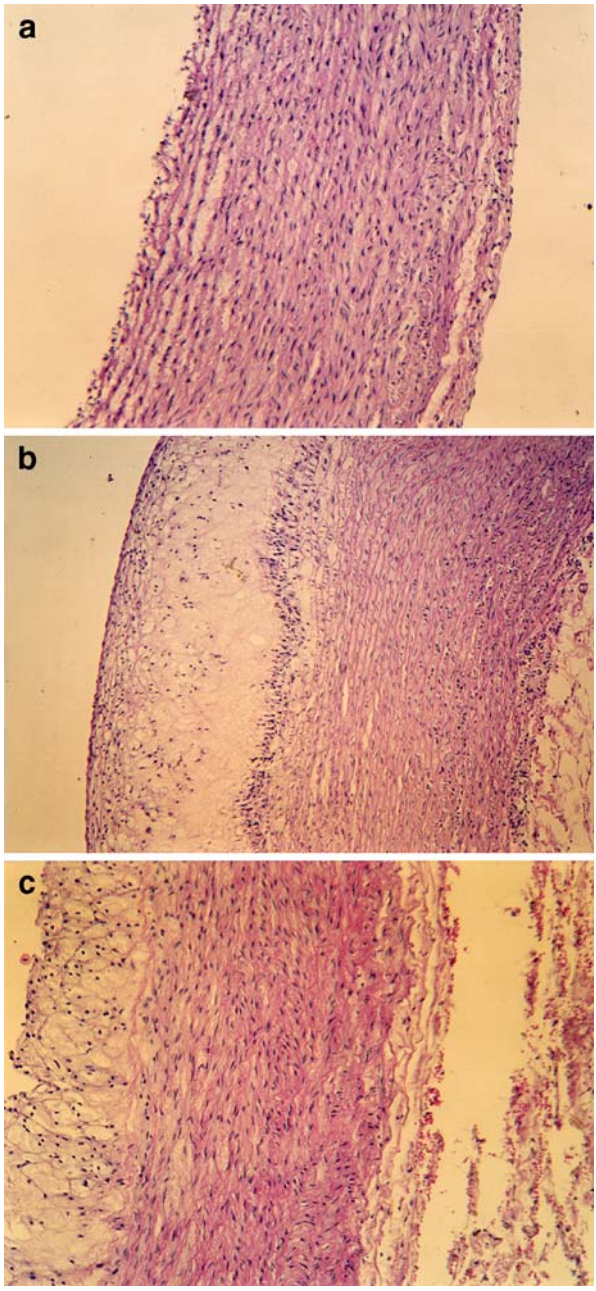

Fig. 1 **a** Control group. Aorta. Normal pattern. H–E, ×180. **b** CH group. Aorta. Atheromatous plaque. Foam cells in the intima. H–E, ×130. **c** CH+ F group. Aorta. Atheromatous plaque. Foam cells in the intima. H–E, ×150

Discussion

In the experiments carried out, it has been found that in the CH group, the concentration of plasma cholesterol was statistically elevated. Histopathological studies showed that cholesterol in the applied dosage (500 mg per rabbit per 24 h) has an atherogenic action

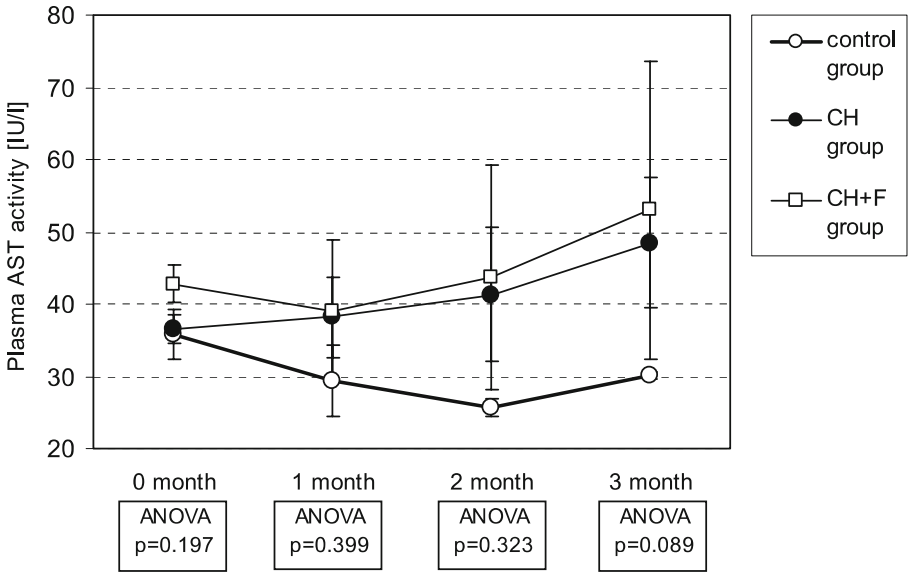

Fig. 2 Activity of plasma aspartate aminotransferase (*AST*) in all groups (mean±SEM)

(atheromatous plaque was observed in the aorta). The administration of fluoride ions inhibits the atheromatosic changes [15].

Enzymes of protein metabolism are indicator enzymes, nonspecific for organs. They are characterized by the fact that in physiological conditions, they demonstrate a slight activity in the plasma, which is a manifestation of continuous mortification of cells. Only under the

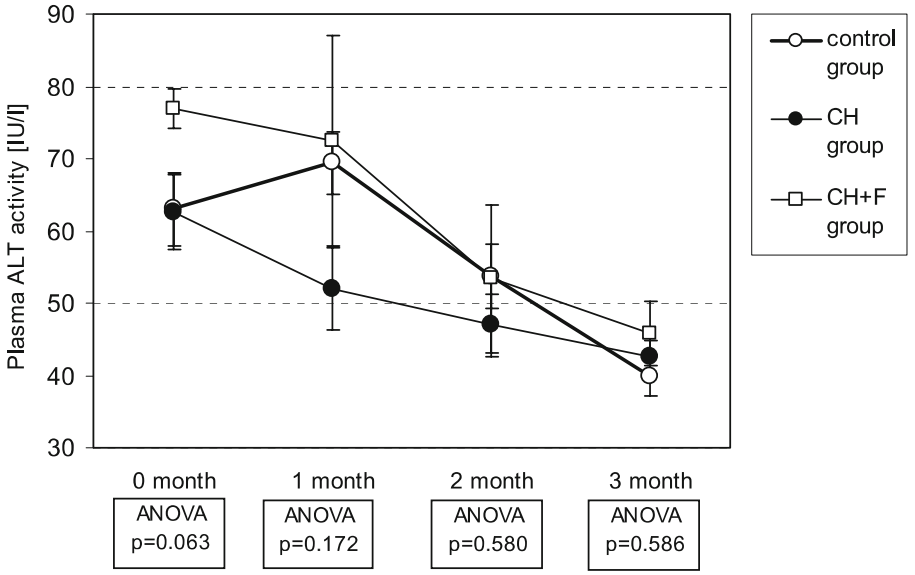

Fig. 3 Activity of plasma alanine aminotransferase (*ALT*) in all groups (mean±SEM)

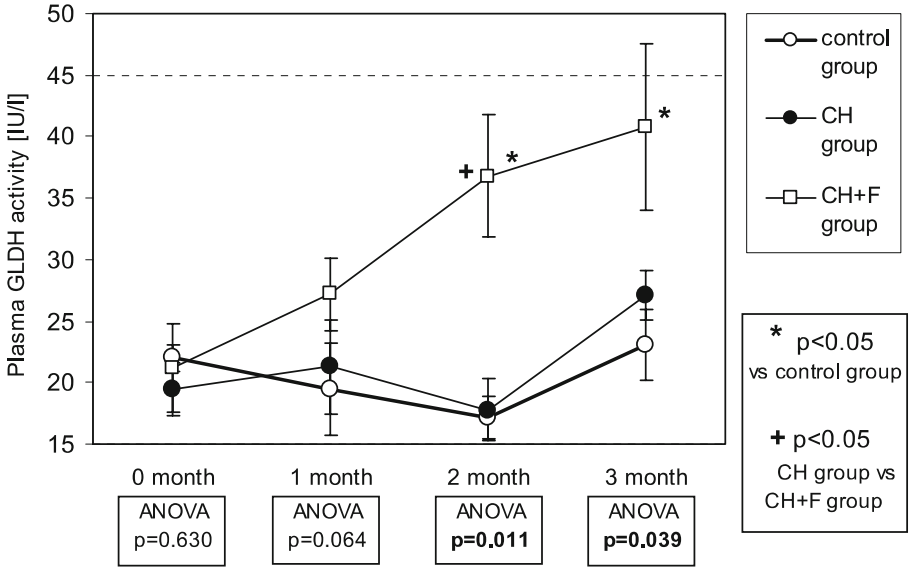

Fig. 4 Activity of plasma glutamate dehydrogenase (*GLDH*) in all groups (mean±SEM)

condition of organ injury does the increase in their activity in the plasma testify about the degree of lesion and is in proportion to it. When ALT, AST, and GLDH appear to have high values in the blood plasma, this testifies about a lesion of the liver. The most specific enzyme for the injury of that organ is GLDH, which is a mitochondrial enzyme.

The activity of AST in the CH group increased in the course of the first and second months of the experiment, whereas the activity of ALT decreased. The activity of GLDH has not changed in that group, in comparison with the control. It results from the research done by other authors that those enzymes (ALT and AST) behave in different fashions in

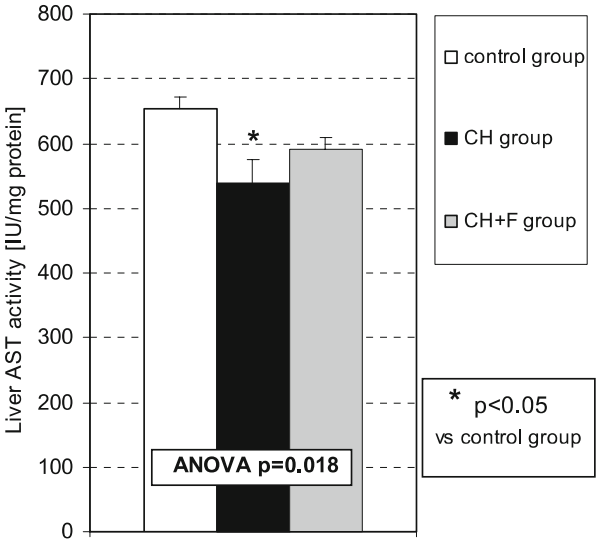

Fig. 5 Activity of liver aspartate aminotransferase (*AST*) in all groups (mean±SEM)

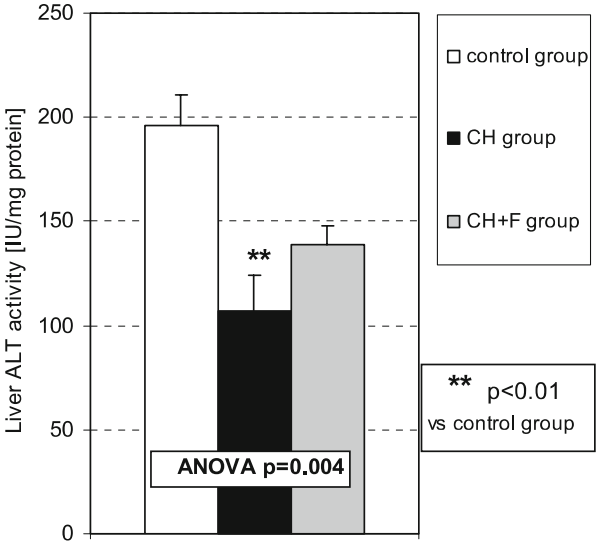

Fig. 6 Activity of liver alanine aminotransferase (*ALT*) in all groups (mean±SEM)

hypercholesterolemia. Arafa [17] proved that in hypercholesterolemia in rats, the activity of both of those enzymes increases in the blood serum. In turn, in the patients with hypercholesterolemia after treatment with atorvastatin administered in doses of 20 mg daily, the activity of ALT and AST decreased [18]. On the other hand, Garcia-Mediavilla et al. [19] demonstrated that in rats with hypercholesterolemia, the activity of ALT and AST in the blood serum has been enhanced.

Adibi et al. [11] reported that an elevated ALT/AST ratio in women predicts coronary atherosclerosis independently of the metabolic syndrome and serum C-reactive protein concentration and should warrant further diagnostic therapeutic interventions. Dessein et al.

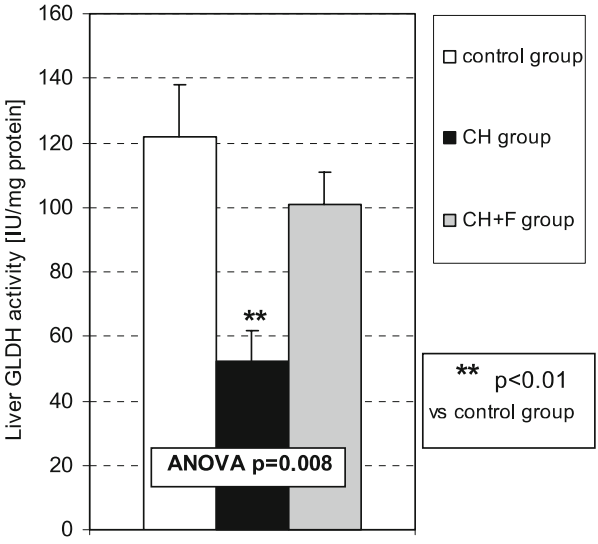

Fig. 7 Activity of liver glutamate dehydrogenase (*GLDH*) in all groups (mean±SEM)

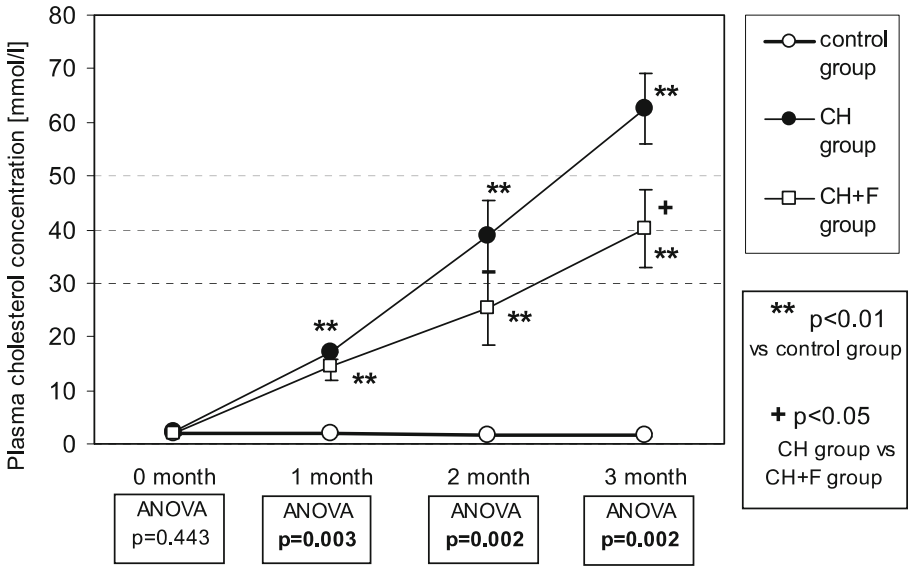

Fig. 8 Plasma cholesterol concentration (mmol/l) in all groups (mean±SEM)

[20] described that serum aminotransferases concentrations may be strongly associated with insulin resistance and atherosclerosis in patients with rheumatoid arthritis.

In the CH+F group, the cholesterol concentration was decreased. That phenomenon can be explained in the aspect of inhibition of reductase 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) caused by sodium fluoride, while the reductase is responsible for biosynthesis of endogenous cholesterol [21, 22]. März and Wieland [23] reported that treatment with HMG-CoA reductase inhibitors has proven the most successful strategy to reduce the concentration of LDL in the circulatory system. These compounds lower LDL cholesterol by inhibiting the mevalonate pathway in the liver, which in turn depletes the regulatory pool of cholesterol (study in vitro).

More characteristic changes in the activity of both aminotrasferases and GLDH have been found in the CH and CH+F groups. It has been demonstrated that the activity of ALT at the beginning of the experiment increases violently and is an early indicator of liver lesion, whereas AST is a late indicator of a lesion occurring to that organ, as the activity of that enzyme appears to increase the most towards the end of the experiment namely, in the third month.

However, the most characteristic changes have been manifested in the CH+F group by the GLDH enzyme, which occurred to be a good indicator of liver lesions. After the second and third month of the experiment, the activity of that enzyme increased in a statistically significant manner. It seems thus that sodium fluoride intensifies changes in the liver, which manifest in the lesion of hepatocyte membranes. That has also been confirmed by the research reported by Guo et al. [24], who—in the cultures of rat hepatocytes with an incubation time of 24 h with various concentrations of sodium fluoride—found the activity of AST and ALT to have been higher, with statistical significance, than that in the control. Those authors indicate that fluoride induces toxic changes in hepatocytes, which manifest in damage to their membranes and the membranes of their organella. Likewise, Wang et al. [25] demonstrated an increase in the activity of AST in human supernatants of hepatocytes incubated in vitro for the period of 12 h with sodium fluoride. Those authors additionally

found selenium to be an antagonist of hepatocyte apoptosis, induced by the fluoride. Previously, we found that GLDH was elevated significantly in the rat's liver after a single intraperitoneal injection of 35 mg NaF/kg of body mass [26].

In addition, the influence of inorganic fluoride has been examined, after the patients had been exposed to sevoflurane; it has been found that after application of the latter, the activity of ALT and AST in patients' serum increased [27].

Extremely interesting are the results of enzyme examination in the livers of rabbits with experimental hypercholesterolemia. In the CH group, the activity of all examined enzymes (ALT, AST, and GLDH) in the liver decreased, with statistical significance. The addition of sodium fluoride to the diets results in "removing the block" on those activities, which increase. It is difficult to explain that phenomenon. We suppose that permeability of the hepatocyte membrane (low endogenous cholesterol in the membrane or influence of fluoride on the membrane) was elevated, so the activities of examined enzymes increased in the plasma ("escape" to plasma). It is necessary to conduct some additional studies.

When summing up, one should state that the addition of sodium fluoride to the diet rich in cholesterol would, on the one hand, result in a probable lesion of hepatocyte membranes and, on the other hand, inhibits the atheromatosic changes in the aorta.

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