The influence of diet supplementation with methionine on the pathomorphological changes of rabbit organs in experimental atherosclerosis

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Abstract

Introduction: The aim of this study was to examine the influence of methionine on the development of pathomorphological changes in the organs of rabbits in experimental atherosclerosis.

Material and methods: The experiment was carried out on three groups of 6 New Zealand rabbits that, for three months, were administered standard chow (C), cholesterol (CH), or cholesterol with methionine (CH + M) (doses: 0.5 g of cholesterol/rabbit/24 hours, 70 mg methionine/kg b.w./24 hours). Plasma lipids were monitored monthly. At autopsy, blood was collected for biochemical analyses and aorta, kidney, liver and heart were collected for histopathological tests. The pathomorphological changes in the organs were assessed with slides made by the normal paraffin method, stained with haematoxylin and eosin and by the freezing microtome method stained with Sudan III for detecting neutral fats. The activity of the most important antioxidative enzymes (SOD, total and both its isoenzymes, GPX) was indicated in the liver. The concentrations of 7-ketocholesterol and MDA were determined in the aorta.

Results: Presence of atheromatous plaque was noticed in the aorta, in the arterial vessels of the heart and of kidneys in both groups. The atherogenic changes in the CH + M group were of lesser intensity than in the CH group. In the CH + M group, steatosis of hepatocytes was of lesser intensity than in the CH group. There was focal steatosis in the tubule epithelium cells noticed only in kidneys of the CH group. Concentration of triglycerides and LDL cholesterol in the study groups was statistically increased when compared with the control group. Statistically an increase in the HDL cholesterol concentration was noted in the group CH + M after the 1st and 3rd month. There were no differences in the concentration of MDA in groups CH and CH + M in the aorta. A decrease in 7-ketocholesterol in the aorta in the CH + M group was noted in comparison to the CH group. The administration of methionine with cholesterol changed the activity of the studied enzymes.

Conclusions: It was found that methionine administered together with cholesterol atherogenic diet inhibited the atherogenic processes as well as fatty degeneration in livers and kidneys.

Key words: atherosclerosis, rabbit, methionine, pathomorphological examinations, lipids, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (PGX).

Introduction

Peroxidation processes play an important role in the development of atherosclerosis and its consequences, such as ischaemic heart disease and myocardial infarction [1]. Atherosclerosis is characterized by a group of regressive and progressive changes taking place in the intima and media tunica of arterial vessels. This is a complicated process, which includes endothelial cell dysfunction, accumulation of lipids, smooth muscle cell (SMC) proliferation and migration, recruitment of inflammatory cells, formation of connective tissue and thrombus [2]. The most important risk factors for atherosclerosis are: high serum LDL and low HDL cholesterol levels, hypertension, diabetes, haemodynamic factors, smoking, inflammatory mediators, low physical activity, homocysteine, toxins, viruses, male gender and genetic background [3-5].

The oxidation theory of atheromatosis development has led to a number of studies assessing the role of antioxidants (β-carotene, vitamins E and C as well as methionine, cysteine, glutathione, lycopene, ubiquinone, melatonin, flavonoids, and selenium) in the prevention of atheromatosis [6-12].

Methionine (L-α-amino-γ-methylo-thiobutyric acid) is a unipolar exogenous amino acid. It plays a significant role in numerous metabolic processes. Methionine residues can act as endogenous antioxidants in proteins [13, 14]. Antioxidant effects of L-methionine lead to protection against membrane damage, reduction of lipid peroxidation and to restoration of changes in the glutathione system [15, 16]. Moreover, methionine has also been reported to reduce liver and kidney damage in rats [17]. The indirect antioxidant action of methionine might be relevant for the preventive rich diets and methionine under conditions of inflammation and oxidative stress [18]. It protects the liver as a lipotropic factor. It regulates lipid concentrations in the blood and tissues. It is used in the treatment of chronic hepatic diseases: cirrhosis, fatty degeneration, hepatotoxic drug and alcohol poisoning [10, 15-18].

Methionine is used for the synthesis of glutathione. Glutathione protects cells against harmful activity of toxins. In a reduced form, thanks to the free thiol group, it reduces peroxides, mainly hydrogen peroxide, in a reaction catalysed by glutathione peroxidase [10, 15, 16, 18].

However, some studies conducted on methionine show that high intake of it in situations of vitamin deficiencies (vitamins B_6, B_{12}, folic acid, vitamins E and C) may increase the risk of atherosclerosis development [15].

The greatest controversy is caused by methionine as characterized by antioxidative action. It participates in lipid transformations, preventing their deposition in blood vessels. It has both lipotropic and anti-inflammatory action. These facts prove its anti-atherogenic action. It was shown in clinical studies that its deficiency leads to fatty degeneration of the liver [19].

The aim of this study was to examine the influence of methionine on the development of pathomorphological changes in the inner organs of rabbits in experimental atherosclerosis.

Material and methods

The study was conducted on 18 male rabbits of the New Zealand breed. The animals were divided into 3 groups of 6 animals: a control group C and 2 study groups (CH and CH + M). Animals in the control group C were fed standard rabbit ration. Animals from the study groups were put on an atherosclerotic diet and received 0.5 g of cholesterol/rabbit/24 h. Additionally, animals from the study group CH + M were supplemented with methionine in the amount of 70 mg/kg b.w./24 h. The experimental dose of methionine was calculated proportionally according to the optimal dose of it for humans.

After 3 months of the experiment blood was collected for biochemical analyses and at autopsy the heart, kidney, liver and aorta were collected for histopathological tests. Plasma lipids were monitored monthly. The pathomorphological changes in the organs were assessed with slides made by the normal paraffin method, stained with haematoxylin and eosin (H-E), as well as by the freezing microtome, stained with Sudan III for detecting neutral fats [20].

The concentration of LDL cholesterol in plasma was determined by an enzymatic method using the BioMerieux kit (France) and the concentration of HDL cholesterol and triglycerides (TG) was assessed by the Alpha Diagnostics kit (Germany).

The activity of superoxide dismutase (SOD) and its isoenzymes was determined in liver homogenates, according to Oyanagui [21]. The activity of glutathione peroxidase (GPX) was determined in liver homogenates, according to the Paglia method [22]. The concentration of malondialdehyde (MDA) was determined in aorta homogenates, utilizing its reaction with thiobarbituric acid, according to Ohkawa [23]. The concentration of 7-ketocholesterol (7-ketoCH) was determined in the aorta using high pressure liquid chromatography (HPLC) (KNAUER, Berlin, Germany) by Brown [24].

The results were analysed statistically with Statistica PL software. The Mann Whitney U test was used to compare differences between particular groups. Additionally, Friedman's ANOVA test was used for evaluation of the change

of biochemical parameters (values after the 1st, 2nd and 3rd month) in every group. Statistical significance was determined at $P \leq 0.05$.

The study was accepted by the Regional Ethical Commission of Animal Experiments in Katowice, Poland.

Results

Biochemical examination

The results are presented in three tables. Table I shows changes in the lipid concentrations in plasma of rabbits of groups CH and CH+M in the first 3 months. The concentration of triglycerides (TG) in the study groups was statistically increased when compared with the control group; P=0.004. There was also a statistical increase in the concentration of LDL cholesterol in both cholesterol groups (P=0.004) after 1 and 2 months when compared to the control group. Administration of methionine along with cholesterol caused a downward tendency in the LDL concentration (about 23%) in the CH group after 3 months. A statistical increase in the HDL cholesterol concentration was noted in the group CH + M after 1 and 3 months.

Table II shows changes in the MDA and 7-ketoCH in the aorta of rabbits of study groups after the 3rd month. There were no differences in the concentration of MDA in groups CH and CH+M vs. the control group. The concentration of 7-ketoCH in the aorta of the CH group increased vs. the control group. In the CH+M group the concentration of 7-ketoCH decreased about 19.5% vs. the CH group (without statistical significance).

The activity of mitochondrial isoenzyme Mn-SOD in the liver of the CH group decreased vs. the control group (statistical significance, P=0.004), whereas activity of cytoplasmic isoenzyme ZnCu-SOD in this group statistically increased when compared with the control (P=0.004). There were no statistically significant differences in the activity of GPX between study groups. In the CH + M group activities of ZnCu-SOD and PXG increased by about 5, 17% respectively vs. the CH group (Table III).

Histopathological assessment

Macroscopic assessment

Presence of creamy atheromatous plaque was noted in the area of the arch and abdominal part of the aortae in the study groups. In the CH group,

Table I. Concentrations of lipids in plasma of rabbits being on the 0,5 g% cholesterol diet of the CH + M group when compared to the C and CH groups

	Group	C mean [mmol/l]	CH mean [mmol/l]	CH + M mean [mmol/l]	P CH/C	P CH + M/C	P CH + M/CH
TG	0 month	0.66	0.52	0.67			
(plasma)	after 1 month	0.82	1.16	1.78	–	–	–
	after 2 months	0.76	1.45	1.58	–	–	–
	after 3 months	0.68	2.08	2.09	<0.01↑	<0.05↑	–
LDL	0 month	0.74	1.07	1.10			
(plasma)	after 1 month	0.70	13.97	18.80	0.004↑	0.004↑	–
	after 2 months	0.49	29.71	21.95	0.004↑	0.004↑	–
	after 3 months	0.67	42.35	32.55	0.004↑	0.004↑	↓(0.078)
HDL	0 month	0.94	0.95	0.96			
(plasma)	after 1 month	0.79	0.98	1.25	–	<0.05↑	–
	after 2 months	0.83	0.97	0.80	–	–	–
	after 3 months	0.75	0.99	1.77	–	<0.05↑	–
LDL/HDL	0 month	0.78	1.13	1.08			
(plasma)	after 1 month	0.87	14.63	14.69	0.004↑	0.004↑	–
	after 2 months	0.75	35.86	43.46	0.004↑	0.004↑	–
	after 3 months	0.88	53.69	21.48	0.004↑	0.004↑	0.05↓

↑ *Statistically significant increase when compared to the C or CH group*
↓ *Statistically significant decrease when compared to the C or CH group*
– No statistically significant change

Table II. Concentration of 7-ketoCH and MDA in aorta

	Groups					
	C – control group					
	Mean	Median	SD	SEM		
7-ketoCH [µg/g tissue]	4.16	4.27	2.10	0.86	–	–
MDA [µmol/g tissue]	1.33	1.45	0.72	0.30	–	–
	CH group					
	Mean	Median	SD	SEM	P CH/C	
7-ketoCH [µg/g tissue]	8.20	6.72	5.52	2.25	0.150	–
MDA [µmol/g tissue]	1.06	1.02	0.21	0.09	0.749	–
	CH + M group					
	Mean	Median	SD	SEM	P CH/C	P CH + M/CH
7-ketoCH [µg/g tissue]	6.61	5.06	4.89	2.00	0.423	0.262
MDA [µmol/g tissue]	1.27	1.37	0.46	0.19	0.631	0.262

Table III. Activity antioxidant enzymes in liver

	Groups					
	C – control group					
	Mean	Median	SD	SEM		
SOD [NU/mg protein]	6.38	6.38	0.30	0.12	–	–
Mn-SOD [NU/mg]	2.14	2.10	0.47	0.19	–	–
ZnCu-SOD [NU/mg]	4.23	4.38	0.39	0.16	–	–
POX [IU/mg]	2.86	3.25	1.16	0.47	–	–
	CH group					
	Mean	Median	SD	SEM	P (CH/C)	
SOD [NU/mg]	5.72	5.80	0.23	0.10	0.004	–
Mn-SOD [NU/mg]	0.55	0.52	0.38	0.16	0.004	–
ZnCu-SOD [NU/mg]	5.17	5.25	0.46	0.19	0.004	–
POX [IU/mg]	3.79	3.85	2.06	0.84	0.423	–
	CH + M group					
	Mean	Median	SD	SEM	P (CH/C)	P (CH + M/CH)
SOD [NU/mg]	5.95	5.90	1.23	0.50	0.200	0.423
Mn-SOD [NU/mg]	0.54	0.38	0.60	0.24	0.006	0.631
ZnCu-SOD [NU/mg]	5.42	5.36	1.52	0.62	0.200	0.631
POX [IU/mg]	4.42	4.37	1.85	0.76	0.200	0.631

the foci changes were large and covered the entire circumference, while the lesion in the CH + M group was much less intensified. There was a colour change – xanthochromia – noted in the liver of the study groups. There were no macroscopic changes in the kidneys or hearts of these rabbits.

Microscopic assessment

Aorta. Unlike the aorta of the control group C (Figure 1), there was a focal hyperplasia of the tunica intima in the form of atheromatous plaque in the aorta of the study groups (Figures 2, 3). The atherogenic changes in aortae of the CH + M group

(Figure 3) were of lesser intensity than in the CH group (Figure 2). There were numerous foam-like cells in the atheromatous plaque, and fat was also noted in intercellular spaces.

Liver. In contrast to the liver of the control group (Figure 4), there was distinct steatosis of hepatocytes in the livers of the CH and CH + M groups. It was intensified around the central veins of hepatic lobule (Figure 5). In the CH + M group, the steatosis of hepatocytes was of lesser intensity (Figure 6).

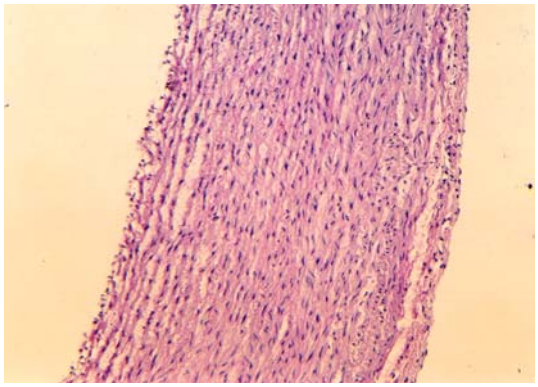

Figure 1. Control group C. Aorta. Normal pattern. H-E staining. Mag. 180×

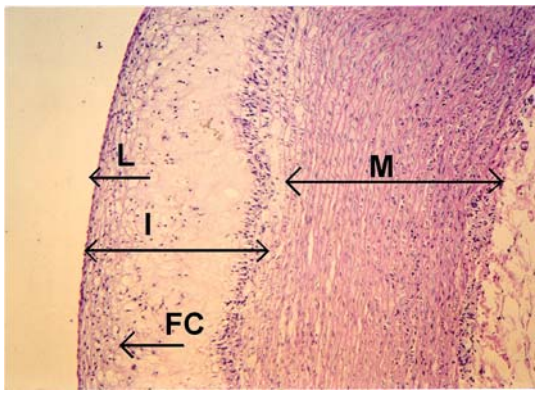

Figure 2. Study group CH. Aorta. Big atheromatous plaque. Foam cells in the tunica intima. H-E staining. Mag. 130×
L – lumen, I – tunica intima, M – tunica media, FC – foam cells

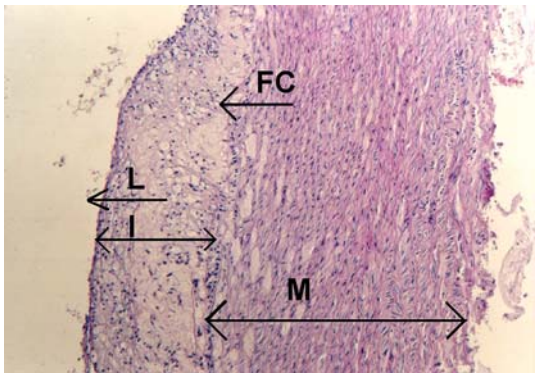

Figure 3. Study group CH + M. Aorta. Atheromatous plaque. Foam cells in the tunica intima. H-E staining. Mag. 140×
L – lumen, I – tunica intima, M – tunica media, FC – foam cells

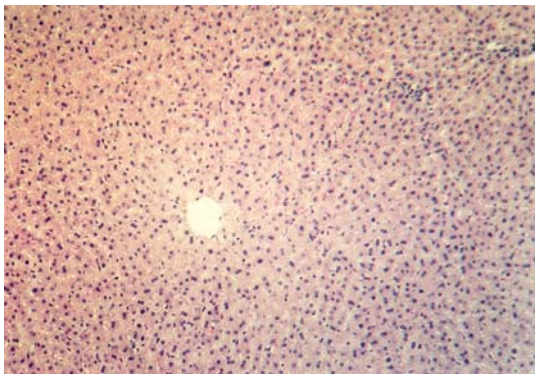

Figure 4. Control group C. Liver. Normal pattern. H-E staining. Mag. 140×

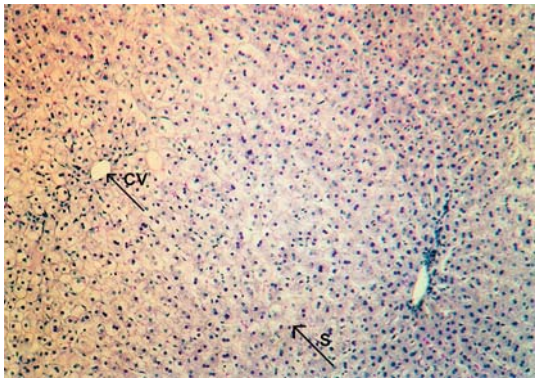

Figure 5. Study group CH. Liver. Intensified steatosis of hepatocytes around the lobule central vein. H-E staining. Mag. 140×
CV – central vein, S – steatosis

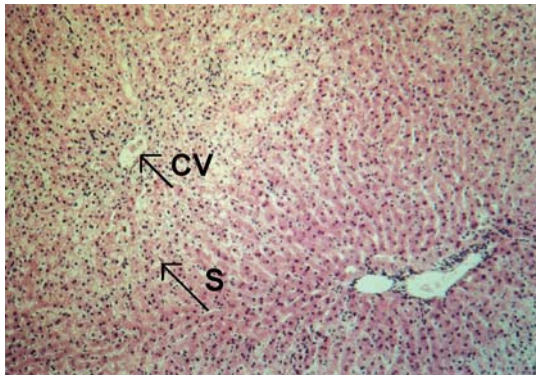

Figure 6. Study group CH + M. Liver. Steatosis of hepatocytes around the central vein. H-E staining. Mag. 140×
CV – central vein, S – steatosis

Kidney. Figure 7 shows the kidney of the control group. There was focal steatosis in the tubule epithelium cells noted only in kidneys of the CH group (Figure 8), while no regressive changes in kidneys of the CH + M group were noted (Figure 9). There were also atheromatous changes in the arterial vessels of kidneys of the CH group (Figure 10). Slight atheromatous changes in the arteries were noted in rabbits of the CH + M group (Figure 11).

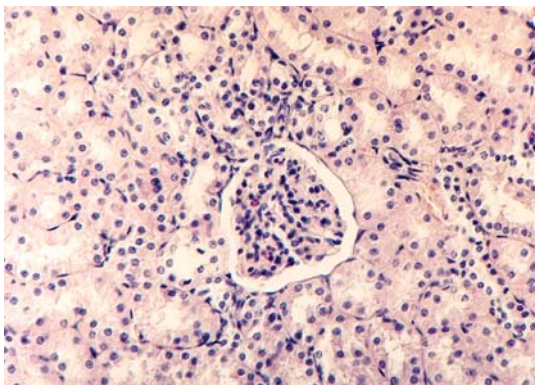

Figure 7. Control group C. Kidney. Normal pattern. H-E staining. Mag. 280×

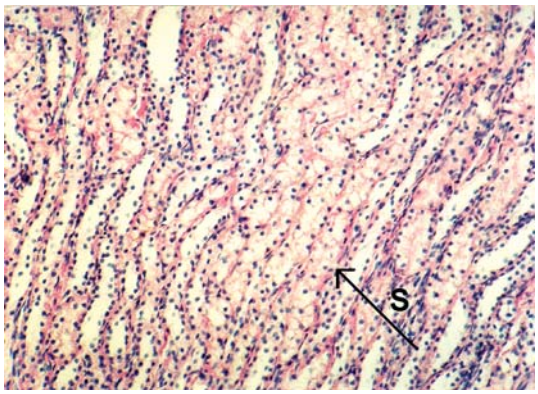

Figure 8. Study group CH. Kidney. Steatosis of tubule epithelium. H-E staining. Mag. 160×
S – steatosis

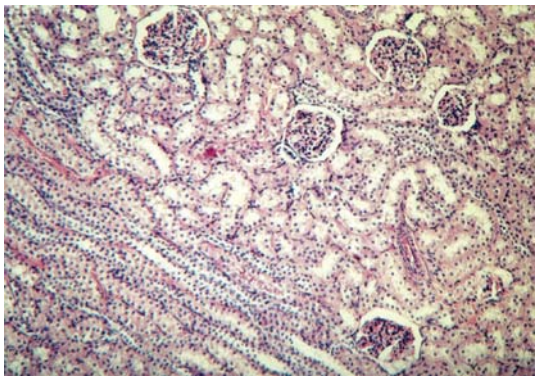

Figure 9. Study group CH + M. Kidney. Normal pattern. H-E staining. Mag. 140×

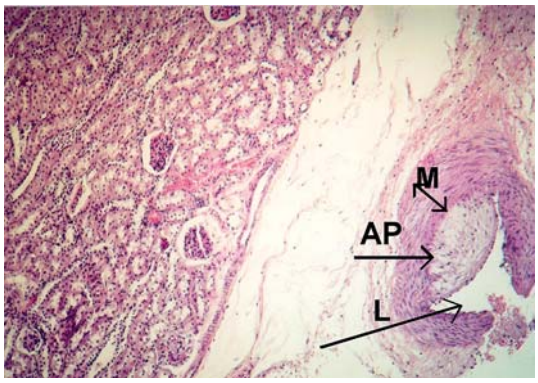

Figure 10. Study group CH. Kidney. Atheromatous plaque in the artery. H-E staining. Mag. 130×
L – lumen of the artery, AP – atheromatous plaque, M – tunica media

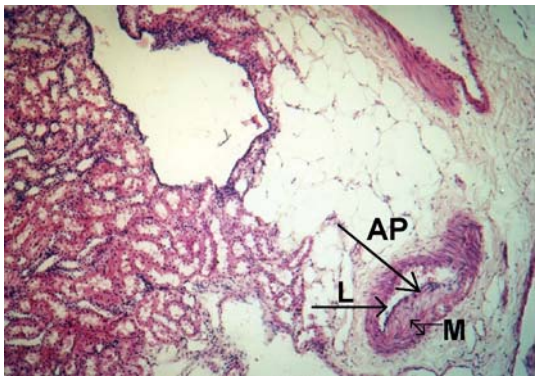

Figure 11. Study group CH + M. Kidney. Atheromatous plaque in the artery. H-E staining. Mag. 150×
L – lumen of the artery, AP – atheromatous plaque, M – tunica media

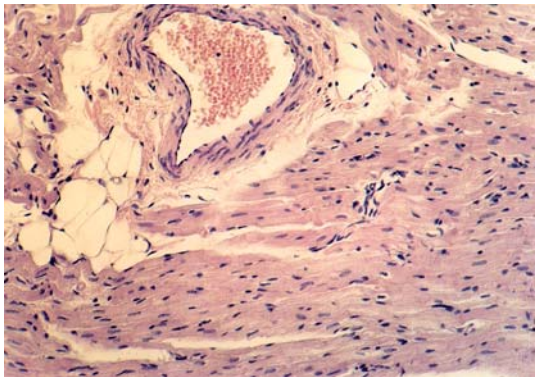

Figure 12. Control group C. Heart. Normal pattern. H-E staining. Mag. 280×

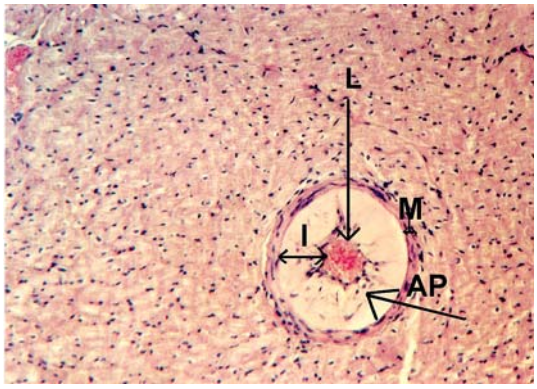

Figure 13. Study group CH. Heart. Atheromatous plaque in arteriole. H-E staining. Mag. 180×
L – lumen of the arteriole, AP – atheromatous plaque, I – tunica intima, M – tunica media

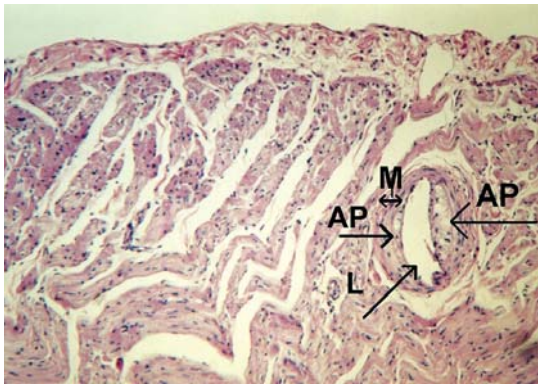

Figure 14. Study group CH + M. Heart. Proliferation of the internal membrane in arteriole. H-E staining. Mag. 150×
L – lumen of the arteriole, AP – atheromatous plaque, I – tunica intima, M – tunica media

Heart. Differing from the appearance of the arterial vessels of the control group C (Figure 12), there was focal proliferation and steatosis of the tunica intima (atheromatous plaque) in the arterial vessels of the heart in both study groups. In the CH group changes were more intense and applied to arterial vessels of different size (Figure 13), while only small atheromatous plaques were present in the CH + M group (Figure 14).

Discussion

The conducted studies proved that cholesterol in the applied dosage (0.5 g/rabbit/24 h) has an atherogenic action. Atheromatous plaque was observed in the aorta and in arterial vessels of the heart and in renal arteries. In the aortae the thickness of the lamellae was approximately equal to the thickness of their tunica intima. Our study shows that cholesterol also caused fatty degeneration of the liver and focal fatty degeneration of collecting tubule epithelium in the kidney. These facts correlate with the existing results of experimental studies, confirming the usefulness of cholesterol in inducing experimental atherosclerosis and the results of clinical studies indicating that a diet high in fat leads to fatty degeneration of the liver [25-27].

Some studies have shown that dietary effects of high fat in animal models induced atherosclerosis and liver steatosis [9, 10, 12, 27]. A statistically significant increase of lipid parameters shown in biochemical studies (LDL cholesterol, triglycerides) in our research in the CH group correlates with morphological changes obtained in histopathological studies. In the examined group CH + M, LDL cholesterol concentration decrease was found in the serum compared to the CH group after 3 months. In our experiment, we also observed a statistically significant increase of the HDL cholesterol concentration in the CH + M group after the 1st and 3rd month. HDL cholesterol is called 'good cholesterol' because HDL cholesterol prevents atherosclerosis by extracting cholesterol from the artery walls and transferring it to the liver. Thus, the statistically higher concentration of HDL cholesterol and lower concentration of LDL in the CH + M group could have influenced the inhibition of the atherogenic processes.

An elevated level of lipids in the serum and an oxidative modification of LDL facilitates their accumulation in tissues and in the tunica intima of the aortae. In numerous studies significant participation of free radicals was shown, including those originating from unsaturated fatty acids and active compounds formed as a result of reactive oxygen species (ROS) action on polyunsaturated fatty acids, such as malonic dialdehyde (MDA) and 4-hydroxynonenal (4-HNE) on the formation and development of atheromatous plaque [10]. In our biochemical studies no influence of methionine on the concentration of MDA has been noted when compared to the control and CH groups, while we observed an increase of the concentration of 7-ketoCH in the aorta of the CH group in comparison to the control group. Recently, much attention has been devoted to the participation of products of lipid peroxidation (MDA and oxysterols) in the modification of properties of cell membranes. They are relevant to atherosclerosis development. From among those products, the most negative effects are made by oxysterols oxidised in the 7th position, mostly cholesterol-7-hydroperoxide. Cholesterol-7-hydroperoxide was recently shown to be a primary cytotoxin in oxidized LDL and was found in human atherosclerotic lesions [28, 29].

Our research shows that there was a rise in the concentration of 7-ketoCH (marker of oxidative

stress), which is evidence of increase of lipid peroxidation in rabbit aortae as far as the CH group and its diet are concerned. In the CH + M group the concentration of 7-ketoCH decreased by about 19.5% (without statistical significance). Probably methionine inhibits lipid peroxidation in the aorta.

Harmful action of ROS is diminished by anti-oxidants: enzymes (glutathione peroxidase, peroxidative dismutase and catalase) and vitamins – β-carotene, vitamins E and C and selenium [30-36]. Also L-methionine possesses anti-oxidative action. Oxidative stress is a causative factor of endothelial dysfunction. It plays an important role in the pathology of vascular diseases such as atherosclerosis, diabetes and myocardial infarction. L-methionine counteracts oxidative processes by inactivating ROS through the reaction with reactive oxygen compounds. It regulates the concentration of lipids in the blood and tissues, taking part in the breakdown of fats. L-methionine prevents their deposition in the aortae. This is a theoretical basis of its anti-atherogenic action [37].

The studies that were carried out confirmed the disadvantageous effect of the administered dose of cholesterol upon the anti-oxidative system in the liver of rabbits. The activity of Mn-SOD decreased with statistical significance in comparison to the control group. On the other hand the activity of cytoplasmic isoenzyme ZnCu-SOD statistically increased in the CH group and CH + M group when compared with the control. It indicates activation of the antioxidative mechanism. Activities of ZnCu-SOD and PGX were higher in the CH + M group (5, 17% respectively) vs. the CH group, so the role of methionine is probably beneficial. Thus, our biochemical results show a positive effect of methionine on the rabbits of oxidation processes. They demonstrate a correlation with the pathomorphological changes in rabbit liver in our research.

However, the dose used in the experiment showed a beneficial impact of methionine on the studied rabbit organs in experimental atherosclerosis because it reduces the size of atheromatous plaque in the aorta and also cardiac and renal arteries. Its beneficial action was less intensive fatty degeneration seen in the liver and there is a lack of fatty degeneration changes in the kidney. The obtained results of pathomorphological studies correlate with the results of biochemical examinations of the lipid profile. Methionine's beneficial impact on metabolic processes in the liver comes from its lipotropic action which protects the liver. Such action was shown in the experimental animals. In animals on a low protein and high fat diet there mare fatty degeneration changes in hepatocytes. This is so because the deficiency of proteins with lipotropic action containing methionine causes the impairment of fat secretion by hepatocytes, which leads to their excessive accumulation in the cytoplasm and fatty degeneration. It was also shown that there is a relation between hepatonecrosis development and sulphuric amino acid deficiency: cysteine, methionine and also choline [37, 38].

Recent studies also indicate that methionine supplementation did not augment oxidative stress, atherosclerotic changes in the aorta and hepatotoxicity induced by high cholesterol in mice and in rats [39, 40].

In conclusion, it was found that the dose of methionine used in the experiment inhibited the atherogenesis processes as well as the regressive changes in livers and kidneys. The biochemical results show a positive effect of methionine on oxidation processes in rabbit organs (liver and aorta) and demonstrate a correlation with the pathomorphological changes. It could be expected that a diet enriched with methionine can (through the reduction of oxysterols) restore the lipid profile or bring about the regression of the atherosclerotic change.

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