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HYPERHOMOCYSTEINEMIA AS A FACTOR CONTRIBUTING TO THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE

Abstract. *Hyperhomocysteinemia is one of the pathogenetic factors of occurrence and progression of non-alcoholic fatty liver disease. Hypomethylation and activation of oxidative stress are biochemical mechanisms of hyperhomocysteinemia steatogene action. Keeping rats on a high fat diet for a long time leads to steatosis which is proved by a significant increase in cholesterol and triglycerides. Homocysteine is a factor of activation of oxidative stress in the formation of non-alcoholic fatty liver disease.This is evidenced by an increased activity of NADPH-oxidase, SOD and levels of carbonyl groups of proteins in liver homogenate in rats.*
Key words: *physical activities of various intensity, hypercholesterolemia, endothelial dysfunction, femoral artery.*

Introduction. Non-alcoholic hepatic steatosis (non-alcoholic fatty liver disease (NAFLD), fatty degeneration of liver) is the primary disease characterized by excessive accumulation of fat (mostly triglycerides) in the liver, where the proportion of hepatocytes, which histologically determine the presence of drops of fat is more than 5 % (histologically).

NAFLD covers 10-40% of the human population and therefore is considered to be one of the most common chronic diseases in the world. Due to the fact that approximately 30% of patients with steatosis develop nonalcoholic steatohepatitis (NASH), which in 10% of cases can be transformed into liver cirrhosis (LC) NAFLD is also one of the most urgent health problems. In developed countries NAFLD is registered in 20-35% of adults, and in in 75% of women who have reached the postmenopausal age [1]. The number of patients with NAFLD is growing. At present, NAFLD is considered to be one of the most frequent causes of rising transaminases in the serum of patients in Europe and the US [2, 3]. Fatty degeneration of liver is more common in individuals with obesity, type 2 diabetes, metabolic syndrome and cardiovascular diseases.

It is believed that one of the causes of NAFLD is hyperhomocysteinemia (HHC), which is a quite widespread phenomenon. For example, in Ukraine HHC is found in 10% of healthy population and up to 43% of patients with cardiovascular disorders [4]. HHC is observed in cardiovascular diseases, in patients with renal failure, psoriasis, osteoporosis, diabetes, liver disease, miscarriages, some neuropsychiatric diseases, defects in development, carcinogenesis, etc., and there is a tendency to a permanent extension of the list [5-7]. A lot of mechanisms of pathogenic action of HHC have been described, among them the main are believed to be inhibition processes of methylation, activation of oxidative stress and protein homocysteinemia that trigger other pathological processes – destabilizing the genome (due to the decrease in DNA methylation), dysregulation of some redox-sensitive genes, reducing the synthesis of hydrogen sulphide, thrombophilia, etc. At the time there are practically no studies about the influence of HHC on the development of liver steatosis. Obviously, the research of the processes of oxidative stress, antioxidant and steatogenesis will allow to study the pro-steatogene effect of HHC.

Therefore the objective of the paper is to study chronic hyperhomocysteinemia due to the development of hepatic steatosis in rats.

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Materials and methods. The study was conducted over 60 days at 40 nonlinear white rats weighing approximately 260 grams, which received standard starch and casein diet with saline (groups 1 and 2) or high (groups 3 and 4) fat. All the animals had free access to drinking water.

Animals of groups 1 and 2 were kept on a standard diet containing 180 g of casein, 660 g of starch and 100 g fat (50 g of lard and 50 g sunflower oil) for 1000 g of dry mixture.

The composition of the diet included a mixture of vitamins, which was prepared in accordance with physiological needs. Water-soluble vitamins were included into the the diet as their mixtures , produced on glucose. Fat-soluble vitamins were added to sunflower oil. Salt mixture and cellulose (20 g to 1000 g of dry food) were also added to the diet [8].

The animals of groups 3 and 4 were kept on a high fat diet in which 180 grams of casein accounted for 310 g of starch and 250 g fat (lard 125 g and 125 g sunflower oil).

In fact, in high fat diet the fat quota was increased up to 50 % of the total calorie content by reducing carbohydrates by 26 %.

The animals were divided into 4 groups, 10 animals in each one.

Group 1 was the control one where the rats were kept on a standard diet;

Group 2 – the rats were kept on a standard diet plus chronic HC thiolactone administering thiolactone on 1% starch solution at the rate of 100 mg / kg intragastric once a day . The control group of rats received intragastric an equivalent amount of 1% starch solution instead of thiolactone during the experiment.

Group 3 – the rats were kept on a high fat diet plus injection of 1% starch solution as in group 1of animals.

Group 4 – the rats were kept on a high fat diet plus HC thiolactone injection as in group 2 of animals.

The animals were examined daily and the amount of food eaten by them was calculated. Every week the animals were weighed.

At the end of the experiment the animals were withdrawn from the study by decapitation under thiopental anesthesia in accordance with the European Convention for the protection of experimental animals 86/609 EEC and the Law of Ukraine № 3447-IV of 21.02.2006 "On protection of animals from cruelty." We also found biometric parameters, such as mass of the animals, body length, relative and absolute weight of the liver.

Biochemical studies. We found the activity of ALT in the serum samples (EC 2.6.1.2), AST (EC 2.6.1.1), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), very low density lipoprotein cholesterol (VLDL). All these parameters were determined at Beskman Coulter AU analyzer 480 OLYMPUS.

To evaluate the intensity of oxidative stress, we measured the activity of NADPH-oxidase enzymes (EC 1.6.3.1), the content of malondialdehyde (MDA) and protein carbonyl groups, as well as values of antioxidant - glutathione peroxidase enzyme activity, (EC 1.11.1.9), thioredoxin reductase (EC 1.6.4.5) and reduced glutathione and superoxide dismutase (SOD) (EC 1.15.1.1) in homogenate of the liver, MDA in the homogenate and in the serum.

The level of overall HC in the serum was determined by ELISA set AXIS®.

In liver lipid extract we determined the total content of phospholipids with fero thiocyanate reagent. Malondialdehyde in the serum and liver homogenate was determined by a uniform method with thiobarbituric acid. Carbonyl groups of proteins were determined by the reaction with 2,4-dinitrophenylhydrazine. Determination of superoxide dismutase in homogenate was performed by means of quercetin by using a set of "SOD-test" production NTPK "X-Analysis" (Republic of Belarus). Reduced glutathione was determined by a standardized method with reagent of Elman. Glutathione peroxidase activity was determined by the concentration of reduced glutathione in the presence of hydrogen peroxide.

The statistical significance of differences between groups was calculated by the nonparametric method of White as the distribution of values in groups mostly did not

meet the normal law. The resulting values in the experimental animals are shown in the tables as M ± m, where M - arithmetic mean, m - the average deviation from the mean.

Results and discussion. It was established (Table. 1) that during the experiment in group 4

Table 1

Values of weight in experimental animals

Values	Groups of animals (explained in the text)			
	1	2	3	4
Rats' weight before the experiment, g	261±15	263±19	262±20	265±32
Rats' weight after the experiment, g	360,5±22,6	374,0±28,8	380,2±34,8	400,5±33,5*
The increase in body weight, g	99,5±15,5	111,0±18,8	118,2±21,8	135,5±21,4*
Absolute liver weight, g	9,40±0,61	11,37±1,49*	11,76±1,5*	13,83±1,27*#&
Relative liver weight,%	2,90±0,25	3,05±0,30	3,10±0,26	3,46±0,26*#&

Note: *(*) - statistical significance of differences relative to group 1 exceeds 95%.*
(#) - Statistical significance of differences relative to group 2 exceeds 95%.
(&) - Statistical significance of differences relative to group 3 exceeds 95%.

of rats which received a high fat diet in combination with the additional introduction of HC thiolactone the body weight increased reliably compared with the control group. Perhaps this is due to inhibition of thyroid function observed at HHC. Some, but not statistically reliable increase in body weight was also observed in rats of group 2. We noticed a reliable increase in absolute liver weight in all experimental groups relatively to absolute liver weight in intact animals and between the liver mass of group 4 animals relatively to absolute liver weight of animals in groups 2 and 3. However, due to the increased body weight, the relative weight of the liver only was reliably increased in the rats of group 4.

Changes of biochemical parameters (including lipid metabolism) in experimental animals are shown in Table 2. It was established that introduction of HC thiolactone to rats at a dose of 100 mg / kg for 2 months led to a pronounced HHC. For instance, HC content in the blood plasma of rats in group 2 increased by more than 1.5 times compared to the control group. It is noted that in rats of group 4 HC rate is statistically higher (almost by twice) as compared with both group 1 and group 2 and 3, which indicates the potential effect of high fat diet on HC level.

It is seen that under the influence of HHC the animals in group 2 had reliably increased levels of total cholesterol (TC), LDL cholesterol, and atherogenic index (AI), indicating the involvementof HHC in atherogenesis. In Group 3 of the rats which received a high fat diet TG rate alone was increased.

In Group 4 rats, most values, except for HDL cholesterol levels were statistically higher than almost all other groups. Attention is drawn to the fact that the content of atherogenic LDL cholesterol and ALT were elevated as compared with group 2 (HHC) and 3 (high fat diet).

The ratio of the AST / ALT remained virtually unchanged in all groups, which can be interpreted as a lack of transition from the pathological process into chronic one.

Changes in indices of oxidative stress and antioxidant are shown in Table 3.

We found a reliable increase in MDA levels in serum and liver homogenate, NADPH-oxidase and protein carbonyl groups in liver homogenate of rats in group 2 compared to group 1. Group 3 animals that were on high fat diet increased reliably the level of all values of oxidative stress compared with group 1 except the levels of carbonyl groups of proteins. Most values of oxidative stress in rats of group 4 were higher compared with the previous groups, indicating that progression of oxidative stress in the liver tissue of animals that are kept on a high fat diet against the background of HHC.

At the same time we noted a significant reduction in the activity of antioxidant enzymes. For instance, studying antioxidant levels we

Table 2.

Biochemical values in the serum of the experimental animals

Values	Groups of animals (explained in the text)			
	1	2	3	4
Homocysteine, micromole/l	5,8±0,43	9,6±1,11*	7,4±1,22	11,3±0,96*#&
TCL, micromole/l	1,25±0,23	1,59±0,16*	1,35±0,22	1,76±0,28*
TG, micromole/l	0,69±0,28	0,56±0,15	0,83±0,25#	1,03±0,25*#
HDL, micromole/l	0,70±0,10	0,75±0,12	0,76±0,14	0,80±0,18
LDL, micromole/l	0,20±0,09	0,58±0,17*	0,23±0,11	0,45±0,25 *&
VLDL, micromole/l	0,32±0,13	0,26±0,08	0,33±0,11	0,46±0,10*#&
IA, s.u.	0,77±0,19	1,20±0,38*	0,81±0,15	1,25±0,42*&
АЛТ, u/l	52,0±9,7	53,28±14,16	48,4±10,8	68,1±20,0 *#&
АСТ, u/l	213,6±53,4	240,5±50,1	194,6±24,8	322,7±138,6*&
AST/ALT	4,21±0,83	4,86±1,25	4,33±1,14	4,60±1,25

Note: *(*) - Statistical significance of differences relative to group 1 exceeds 95%.*
(#) - Statistical significance of differences relative to group 2 exceeds 95%.
(&) - Statistical significance of differences relative to group 3 exceeds 95%.

Table 3.

Values in the system of lipid peroxidation and antioxidant defense in the liver homogenate and serum of rats

Values	Groups of animals (explained in the text)			
	1	2	3	4
liver homogenate				
MDA, micromole/g	0,46±0,03	0,63±0,03*	0,59±0,05*#	0,91±0,08*#&
Thioredoxin reductase nmole/min*mg of protein	5,85±0,43	5.02±0.62	4,55±0,62*	4.23±0.39*
NADPH-oxidase, nmole/min on 1 mg of protein	1,25±0,14	1.47±0.09*	1,52±0,15*	1.59±0.18*
Reduced glutathione, micromole/g of protein	76,2±11,2	55,4±6,4*	68,5±3,8*#	38,4±4,3*#&
Carbonyl groups, nmole/mg of proteins	2,15±0,34	2,82±0,32*	2.45±0.22	3.25±0.33*&
Glutathione peroxidase, nmole/min*mg of protein	12,3±2,6	10,5±1,5	11,2±2,2	8,5±1,0*
SOD, u/mg of protein	1,40±0,18	1,18±0,19	1,05±0,12*	0,96±0,18*
Serum				
MDA, micromole/l	3,24±0,30	4,07±0,36*	3,62±0,41	4,59±0,42*

Note: *(*) - Statistical significance of differences relative to group 1 exceeds 95%.*
(#) - Statistical significance of differences relative to group 2 exceeds 95%.
(&) - Statistical significance of differences relative to group 3 exceeds 95%.

found a reliable decrease in levels of reduced glutathione in the liver homogenate in group 2 animals compared to the control group of animals. The rats kept on high fat diet showed a significant decrease in activity of thioredoxin reductase and SOD in liver homogenate. Animals of group 4 decreased reliably (by twice) the level of reduced glutathione compared with groups 1, 2 and 3. We also noticed a reliable reduction in other values of the antioxidant protection as compared to the control group. The results indicate a decrease in functional abilities of the antioxidant system in the activation of lipid peroxidation.

Comparison of the values in hepatic steatosis is presented in Table 4.

Table 4.

Markers of hepatic steatosis in rats' liver homogenate

Values	Groups of animals (explained in the text)			
	1	2	3	4
liver homogenate				
Phospholipids, u.opt.g.	0,176±0,008	0,146±0,020*	0,160±0,025	0,137±0,019*
Triglycerides, mmol / g	20,2±3,1	29,2±4,9*	34,6±4,2*	72,2±8,1*#&
Cholesterol, mmol / g	7,14 ±0,64	8,66±0,93*	8,31±0,82*	9,45±0,94*

Note: *(*) - Statistical significance of differences relative to group 1 exceeds 95%.*
(#) - Statistical significance of differences relative to group 2 exceeds 95%.
(&) - Statistical significance of differences relative to group 1 exceeds 95%.

The animals of groups 2 and 3 showed a reliable increase in levels of triglycerides and cholesterol in the liver homogenate compared with animals of the control group, indicating the formation of hepatic steatosis. In group 4 of rats we noted a significant increase in all values of steatosis compared to almost all previous groups. However it should be noted, that the level of phospholipids was significantly reduced in the rats of groups 2 and 4 compared with the control group, indicating the severity of the hypomethylation process.

Conclusions. HHC is one of the pathogenetic factors of occurrence and progression of NAFLD. Hypomethylation and activation of oxidative stress are biochemical mechanisms of HHC steatogene action.

Keeping rats on a high fat diet for a long time leads to steatosis which is proved by a significant increase in cholesterol and triglycerides.

Homocysteine is a factor of activation of oxidative stress in the formation of NAFLD.This is evidenced by an increased activity of NADPH-oxidase, SOD and levels of carbonyl groups of proteins in liver homogenate in rats.

Prospects for further research. Thus, hyperhomocysteinemia accelerates the development of NAFLD significantly and knowledge of this fact will make a contribution to the development of methods to prevent accelerated development of NAFLD.

References:

1. Звенигородская Л.А. Атеросклероз и органы пищеварения. – М.: Медпрактика, 2011.- 312 с.

2. Хворостинка В.Н. Особенности нарушения микро- и макроэлементарного спектра сыворотки крови прижировой дистрофии печени у больных СД 2 типа /В.Н. Хворостинка, О.В. Лахно, О.И. Цивенко // Междун. эндокрин. ж. – 2007. – № 3 (9). – С. 23-26.

3. Драпкина О.М. Патогенез, лечение и эпидемиология НАЖБП – что нового? Эпидемиология НАЖБП в России. / О.М. Драпкина, В.И. Смирин, В.Т. Ивашкин // РМЖ. – 2011. – № 28. – С. 1717-1721.

4. Андрушко І.І. Рівні гомоцистеїну, цистеїну та аргініну у практично здорових осіб: вікові та статеві особливості / І.І. Андрушко // Укр. Кардіологічний журнал. – 2008. – № 5. – С. 89-95.

5. Синдром гіпергомоцистеїнемії: причини виникнення, способи профілактики та лікування /М.Б. Луцюк, Н.В. Заічко, Г.С. Григор'єва [та ін.] // Рациональная фармакотерапия. – 2013. – Т. 29, № 4. – С. 55-60.

6. Hyperhomocysteinemia and Neurologic Disorders: a Review/R.Ansari, A.Mahta, E.Mallack, J.J.Luo // J. Clin. Neurol. – 2014. – Vol. 10, № 4. – P. 281–288.

7. Faloon W. AS WE SEE IT. Newly Identified Risks Of Excess Homocysteine / W. Faloon // Life Extension Magazine. – 2015. – № 5. [Електронний ресурс http://www.lifeextension.com/Magazine/2015/5/Newly-Identified-Risks-Of-Excess-Homocysteine/Page-01].

8. Экспериментальная витаминология : Справ. руководство / Юрий Михайлович Островский . – Минск : Наука и техника, 1979 . – 552 с.