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PECULIARITIES OF OXIDATIVE MODIFICATION OF PROTEINS IN CHILDREN SUFFERING FROM CHRONIC HEPATITIS C

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The results of research of oxidative modification of proteins in 94 children suffering from chronic hepatitis C (CHC) are produced here. It has been established that in children suffering from CHC intensive oxidation of proteins takes place which is accompanied with high-sensitivity of proteins in blood serum to oxidation and with accumulation of proteins' oxidated forms in blood serum. Degree of proteins' oxidation depends on activity of inflammatory process in liver and on duration of disease.
Key words: children, chronic hepatitis C, oxidative stress, oxidative modification of proteins.

Introduction

It is known that contamination with hepatitis C virus leads to development of serious liver disorders, such as CHC, cirrhosis and hepatocellular carcinoma. Today in Ukraine, it is practically impossible to evaluate real scale of prevalence of this infection in children, because clinically apparent forms of acute hepatitis C are registered since 2003 and CHC — only since 2009.

Molecular mechanisms of liver damages caused by HCV are being studied by many researchers for over 20 years. It is proved that oxidative stress is a major factor in development and progressing of liver disorders associated with HCV [1]. The level of oxidative system activation is higher in CHC cases than in cases of hepatitis B, this is caused by virus factor and peculiarities of infectious process when HCV is present. It is proved that, unlike HBV, HCV can directly induce oxidative stress in hepatocytes [10,14]. Besides, HCV reduces expression of hepcidin gen and stimulates inflow of iron from the gastrointestinal tract and leads to its accumulation in liver with further activation of oxidative damage of hepatocytes [8,9,13].

The influence of free radicals on proteins of various types causes difficult modifications in structure of protein's molecule and correspondingly changes its physic-chemical and biological features [15]. Formation of carbonyl groups due to oxidation of amino acids is the most prevailing type of damaged proteins [15]. Oxidized proteins are relatively stable and they are early factors of oxidative stress [2,15].

Researches of oxidized modifications of proteins (OMP) in patients suffering from viral hepatitis are rare and only adults were examined. According to literature sources, there is dependence of OMP expressiveness on activity and severity of the disease [3,4,7,12].

***The aim* of the research** is to study peculiarities of OMP as the early marker of oxidative stress in children with CHC in order to broaden knowledge about mechanisms of progressing of this disease in childhood.

Methods and materials of the research

94 children suffering from CHC and 9 children with cirrhosis of HCV-etiology were examined. The children were patients of gastroenterology department in State Institution «IROG of NAMS of Ukraine»» and of infectious department in NSHC «OHMATDYT»». For diagnosis verification according to the existing protocols, all patients underwent clinical, laboratory and instrumental trials. Etiology of viral hepatitis and stage of infectious process were established with the use of enzyme multipliedimmunoassay and amplification method with polymerase chain reaction. All children with CHC underwent complete blood count and biochemical blood assay. Expressiveness of cytological syndrome, being a component of liver inflammation activity, was evaluated according to the level of alanine-aminotransferase (ALT): minimal activity characterized by increase of ALT up to two norms, while low activity — up to 5 norms, moderate activity — by increase of ALT up to 9 norms and high activity — increase of ALT over 9 norms [6]. Cirrhosis in children was diagnosed according to results of clinical, laboratory and instrumental examinations (splenomegaly with or without

Table 1

Amount of 2.4 DNPH in blood serum before and after initiation in groups of examined children, nmole/mg of proteins, M (95% CI)

2.4 DNPH	CHC, (n=94)	Control group, (n=13)	Cirrhosis, (n=9)
nADNPH -before initialization -after initialization - % of growth	 1.7 (1.6-1.8) * ** 2.9 (2.8-3.1) * 91.4 (76.5-106.3) * **	 1.0 (0.9-1.0) 1.4 (1.2-1.5) 39.3 (34.3-44.3)	 1.3 (1.0-1.5) *** 2.9 (2.4-3.4) *** 151.4 (89.8–213.0) ***
nKDNPH -before initialization -after initialization -% of growth	 1.6 (1.5-1.7) * 2.7 (2.6-2.9) * 90.7 (75.5-105.9) * **	 1.1 (1.0-1.2) 1.5 (1.3-1.7) 33.0 (25.2-40.7)	 1.2 (1.0–1.45) 2.8 (2.1-3.4) *** 145.9 (76.0–215.9) ***
mADNPH -before initialization -after initialization - % of growth	1.0 (0.9-1.1) * 1.9 (1.7-2.0) * 103.5 (88.9-118.0) * **	 0.7 (0.7-0.8) 1.0 (0.8-1.1) 33.1 (20.3-46.0)	 0.8 (0.6-0.9) 2.0 (1.5-2.4) *** 155.9(122.5–189.3) ***
mKDNPH -before initialization -after initialization -% of growth	 0.24 (0.2-0.3) 0.51 (0.4-0.6) * 148.4 (116.9-179.8) *	 0.16 (0.09-0.2) 0.19 (0.12-0.3) 28.4 (14.9-41.9)	 0.13 (0.07-0.19) 0.44 (0.2–0.6) *** 200.3 (132.5-268.0) ***

Note: * — p<0.05 between CHC and control, ** — between CHC and cirrhosis, *** — p<0.05 between cirrhosis and control

manifestation of hypersplenism, portal hypertension, disorder of liver synthetic ability).

The average age of the patients was 11.6±4.3 years. 61.1% of them were boys and 38.9% — girls (p>0.05). In most children (68.1) 1st genotype of HCV was diagnosed, in 5 children (5.3%) — 2nd genotype and in 25 examined children (26.3%) — 3rd genotype. Duration of the disease was less than 3 years in 31.3% (n=23) of patients. 51.6% (n=47) children were contaminated for more than 3 years. The duration of the disease in other children was not established. The average duration of the disease in children with established way of contamination was over 6 years (M., 77.2 months; 95% CI, 65.9–89.9 months). Most examined children (n=58, 61.1%) had minimal and low activity of disease. 3 children had moderate and 1 child — high activity of inflammatory process in liver.

The OMP stage was established by the method of E.E. Dubinina [2]. The essence of the method consists in forming (due to oxidation) of aldehyde and ketonic groups of amino-acid residues of proteins interacting with 2.4 dinitrophenylhydrazone (2.4 DNPH). The formed derivatives of 2.4 DNPH are registered with spectrophotometer Spekol-11 when the length of the waves is 356 nm, 370 nm, 430 nm, 530 nm. The author of the method established that on wave length 356 nm neutral aldehydedinitrophenylhydrazones (nADNPH) were registered, on the wave length 370 nm — neutral ketondinitrophenylhydrazones (nKDNPH), on the wave length 430 nm — main aldehydedinitrophenylhydrazones (mADNPH), on the wave length 530 nm — main ketondinitrophenylhydrazones (mKDNPH). The investigation of spontaneous OMP (output layer 2.4-DNPH) and induced OMP (accumulation of 2.4-DNPH after OMP stimulation with Phenton medium FeSO4 та H_2O_2) was carried out. Contents of 2.4-DNPH (measured in nmoles) in 1 mg of proteins were calculated with the use of molar extinction coefficient (21000 M- cm-) for 2.4-DNPH derivatives. The amount of 2.4-DNPH before and after initiation was evaluated in order to establish degree of tissue damage in conditions of oxidative stress and reserve-and-adaptive resources (RAR) of organism [2]. Organism's RAR to resist oxidative stress was calculated according to formula (spontaneous OMPx100/induced OMP). Control group consisted of 13 healthy children.

Statistical data processing was done with the use of programs Statistica 6.1 and Excel 7.0. General statistics analysis included calculation of the average (M), standard quadratic deviation (SD), standard error (SE), confidential interval (95% CI). Interconnection for nominal variables was calculated with the use of χ^2 criterion. The results considered credible when p<0.05.

Results of researches and their discussion

Investigation of OMP in children allowed to establish that oxidation of proteins takes place in ill children, as well as in healthy children. The main OMP

Table 2

Amount of 2.4 DNPH in blood serum in subgroups of patients suffering from chronic hepatitis C depending on activity of inflammatory process nmole/mg of proteins, M (95% CI)

2,4 DNPH	ALT>40 (n=61)	ALT<40 (n=33)	control group (n=13)
nADNPH -before initialization -after initialization - % of growth	 1.8 (1.7–1.9) * *** 3.0 (2.8-3.2) * 86.6 (68.2-105.2) *	 1.5 (1.3-1.6) ** 2.6 (2.4–3.9) ** 102.9 (74.7–125.6) **	 1.0 (0.9-1.1) 1.4 (1.2-1.5) 39.3 (34.3-44.3)
nKDNPH -before initialization -after initialization -% of growth	 1.7 (1.5-1.8) * *** 2.8 (2.6-2.9) * 84.9 (66.8-102.9) *	 1.4 (1.2-1.6) 2.5 (2.2-2.7) ** 92.4 (71.0-113.8) **	 1.1 (1.0-1.2) 1.5 (1.3-1.7) 33.0 (25.2-40.7)
mADNPH -before initialization -after initialization - % of growth	 1.1 (0.9-1.2) * 2.0 (1.8–2.1) * 96.9 (80.2–113.7) *	 0.9 (0.8–1.0) 1.7 (1.5–1.8) ** 109.4 (83.8-134.9) **	 0.7 (0.7-0.8) 0.9 (0.8-1.1) 33.1 (20.3-46.0)
mKDNPH -before initialization -after initialization -% of growth	 0.3 (0.2-0.3) 0.5 (0.4–0.6) * 136.1 (108.3-163.9) *	 0.2 (0.1-0.2) 0.4 (0.3-0.5) ** 141.0 (95.5-186.3) **	 0.2 (0.09-0.2) 0.2 (0.12-0.3) 28.4 (14.9-41.9)

Note: * — p<0,05 between the group with active hepatitis and control, ** — between the group with inactive hepatitis and control, ***— p<0.05 between the group with active hepatitis and inactive hepatitis

products in examined groups of children were nADNPH and nKDNPH, while content of mADNPH and mKDNPH was significantly lower in both ill and healthy children. Moderate activation of free-radical-processes was a part of general adaptive mechanism aimed at supporting of cellular homeostasis.

The amount of 2.4 DNPH in blood serum of examined children didn't depend on the age (r=0.18). The amount of OMP products was equal in boys and girls.

In patients with CHC, unlike healthy children, the OMP processes were more intensive. Probably, higher levels of oxidized proteins in blood serum before and after initialization indicated this. Only in point of mKDNPH amount before initialization (spontaneous OMP) patients suffering from CHC didn't change from control group (table 1).

At the same time, the amount of nKDNPH, mADNPH, mKDNPH in blood serum of patients with cirrhosis was practically the same as in the control group, and only amount of nADNPH was different in these groups.

Induction of OMP processes *in vitro* with generating of active oxygen forms by Phenton medium led to increase of 2.4 DNPH amount in both healthy and ill children (table 1). But in healthy children these changes were insignificant, unlike children suffering from CHC and cirrhosis. And in 3 healthy children the amount of mKDNPH decreased which was a sign of increased resistance of blood serum proteins to oxi-

Table 3

Index of reserve-and-adaptive resources of organism in the groups of examined children (%)

Group	For nADNPH	For nKDNPH	For mADNPH	For mKDNPH
CHC, duration<36 months (n=23)	65.6 (58.4–72.7)	65.7 (57.5–74.0)	59.3 (52.7–66.0) ***	60.9 (54.1–67.6) * ***
CHC, duration>36 months (n=48)	56.3 (51.4–61.1) **	56.9 (51.9–61.9) **	53.3 (48.4–58.1) **	42.8 (37.3–48.2) * **
Control group (n=13)	72.1 (69.4–74.7)	77.5 (71.5–80.6)	77.5 (69.4–85.6)	80.8 (71.6–90.0)
Cirrhosis (n=9)	43.8 (34.4–53.3)	46.0 (34.5-57.2)	40.5 (35.2–45.7)	31.7 (22.5-40.8)

Note: * — p<0.05 between the group with duration of the disease >36 months and the group with duration of the disease <36 months; ** — p<0,05 between the group with duration of the disease >36 months and control; *** — p<0.05 between the group with the duration of the disease <36 months and control.

dants. As it is shown in table 1, induction of OMP processes led to increase of oxidized proteins by 30% in healthy children, at the same time, the amount of OMP products doubled in patients suffering from CHC, and in patients suffering from cirrhosis they increased 2.5 times. It should be noted that in patients suffering from CHC and cirrhosis the most significant changes concerned mKDNPH. Before initialization, this index was practically equal in all groups. But after initialization amount of mKDNPH increased by 150% in children suffering from CHC, in children suffering from cirrhosis it increased by almost 200%, while in healthy children — only by 28%. It should be noted that the presence of KDNPH after spontaneous OMP shows degree of oxidative destruction of protein, and increase of their amount after initiation of protein oxidation process (inductive OMP) indicates emaciation of reserve-adaptive abilities of organism to resist oxidative stress.

OMP analysis in control group showed amount of nADNPH and nKDNPH in blood serum before OMP initialization didn't exceed 1.4 nmole/mg, while in almost one third of patients (n=26, 27.7%) suffering from CHC this index was more than 2 nmole/mg. Detailed analysis showed that all children with considerable amount of 2.4-DNPH in blood serum before initialization had active inflammatory process in liver. Two subgroups were formed in order to establish relationship between OMP index and activity of inflammatory process in liver. The first one included patients with active hepatitis (ALT>40 mmole/l) and the second group included patients with inactive hepatitis (ALT<40 mmole/l). The results are presented in table 2. It is established that in children suffering from CHC and with active hepatitis the amount of nADNPH and nKDNPH increased in comparison to inactive hepatitis and healthy children. But even inactive hepatitis (with regard to the amount of transaminase) was accompanied with increase of 2.4 DNPH amount in blood serum in comparison to healthy children. These results proved once more time that absence of biochemical activity does not exclude active inflammatory process in liver tissue.

Activity of inflammatory process in liver didn't influence on amount of mADNPH and mKDNPH in blood serum before initiation (spontaneous OMP) which was indicated by absence of difference in amount of such OMP products in subgroups of ill children.

After OMP initiation in patients suffering from CHC, regardless of inflammatory process activity, the amount of formed 2.4 DNPH increased on all wave lengths, and, as it was mentioned above, mKDNPH formed most actively.

It should be noted that the level of transaminases increased in all patients with cirrhosis (ALT>40), but the amount of nADNPH in blood serum was considerably lower than in patients suffering from CHC (M, 1.3 nmole/mg of proteins; 95% CI, 1.0–1.5 nmole/mg of proteins; M, 1.7 nmole/mg of proteins; 95% CI, 1.6–1.8 nmole/mg of proteins correspondingly, $p<0.05$) and we believe that it can indicate disorder of liver synthetic function caused by durable oxidative stress on precellular stage.

Thus, CHC in children is associated with hypersensitivity of blood serum protein to oxidation and with accumulation of protein's oxidized forms in blood serum.

Increase after initiation of mKDNPH amount in blood serum of patients suffering from CHC does not depend on activity of inflammatory process in liver.

Taking into account that any chronic disease reduces compensatory and adaptive abilities of organism, we calculated RAR index in examined children which shows the ability of organism to resist oxidative stress [5].

RAR index over 70% (the average index figured out from the sum of indices on all wave lengths) was registered in most children from control group (9; 69%) and in 11 children (12%) with CHC and was not registered at all in children with cirrhosis. RAR level within 50%–69% was in 2 (15%) children from control group, in 47 children (50%) with CHC, and in 2 children (22%) with cirrhosis. RAR level below 50% was registered in 36 children (38%) with CHC and in 7 children (78%) with cirrhosis, and was not registered in control group. According to results of the investigation it was established that RAR index reduced with the growth of period of disease ($r=-0.36$; $p<0.05$). The data about RAR of examined patients are presented in table 3.

According to average RAR index which was calculated for nADNPH and nKDNPH, patients suffering from CHC with short period of disease didn't differ from healthy children. Calculation of RAR index for mADNPH and mKDNPH showed that it reduced in patient suffering from cirrhosis unlike patients from control group. It should be noted that RAR index most significantly reduced when it was calculated for mKDNPH in patients suffering from CHC over 3 years and in children with cirrhosis. As it is shown in the table 3, children with cirrhosis had the lowest RAR index — the index was lower than in

children from control group and in children suffering from CHC for less than 3 years.

In 11 children (23%) with duration of the disease over 36 months RAR index for all 2.4 DNPH was <50%. Detailed analysis showed that all these children had been ill for more than 6 years and according to ultrasonic investigation of liver with the use of Doppler velocimetry, these children had signs of fibrosis (sclerosis of Glisson's capsule, diphasic blood flow in hepatic vein, increase of echo-producing structures in liver tissue according to radiofrequency scanning). We believe that calculating of RAR-index in patients suffering from CHC can be one of the evaluation criteria for pathological process in liver.

Conclusions

Thus, CHC in children is associated with oxidative stress, which is proved by OMP activation. Expressiveness of this process is higher in children with active chronic hepatitis and with longer period of the disease. Reduce of OMP products before initiation in children with cirrhosis, unlike patients suffering from CHC, indicates more significant structure damages of blood serum proteins. Calculation of RAR index can be one of the evaluation criteria for CHC progressing in children.

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