MORPHOLOGY AND PATHOMORPHOLOGY

Thyroid Modulation of TNF-Dependent Apoptosis and Formation of Chronic Liver Disease in Endogenous Intoxication in Rats

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Dynamic studies on rats with chronic endogenous intoxication showed involvement of the thyroid hormones in modulation of liver injury. Thyroid modulation is realized as more intense induction of TNF apoptosis in the cells and stimulation of microcirculatory disorders at the expense of nitroxide synthase expression. The modulation is quantitative, augmenting liver damage.

Key Words: *TNF-dependent apoptosis; chronic endogenous intoxication; liver; thyroid hormones; hyperthyrosis*

The development of multiple organ failure in chronic patients is one of the pressing problems of modern medicine. One of the components in the pathogenesis of this condition is accumulation of endogenous toxins, damaging the target organs (liver, kidneys, and lungs). Injury to these organs impairs the detoxication and elimination of the toxins and is realized in the form of endogenous intoxication (EI) [3].

Endogenous toxins affect the endocrine organs by involving them in EI pathogenesis. One of these organs is the thyroid. Thyroid effects on target organs, for example, the liver, are realized through numerous receptors to thyroid hormones in liver cells. Many T_3 and T_4 binding sites were detected in the nuclear system of Kupffer's cells, vascular endotheliocytes, and sinusoids [1].

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Thyroid hormones may be involved in the development of cytopathic, vascular, and fibroplastic changes in the liver. However the contribution of each of these factors remains not studied up to the present time. The proportion of cell necrosis and apoptosis in the formation of chronic liver diseases and the involvement of the thyroid hormones in these processes are unclear.

We studied the regularities in thyroid hormone effects on apoptotic processes in the liver in chronic EI.

MATERIALS AND METHODS

The study was carried out on 75 outbred albino rats of both sexes (180-210 g). Selection and keeping of animals, simulation of pathological processes, and sacrifice were carried out in accordance with basic documents of the Ministry of Health of the Russian Federation and WHO recommendations [5].

Chronic EI was simulated in 25 animals using the classical model with the predominant involve-

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ment of the liver. This condition is induced by combined injections of low-dose bacterial LPS and tetrachloromethane [4]. Hyperthyrosis was induced in 25 animals by oral treatment by L-thyroxin (100 mg/kg/day) during 7 days, after which chronic EI (the classical model) was reproduced. The maintenance dose of L-thyroxin was 25 mg/kg every 5 days. Intact rats served as the control. The animals were sacrificed on days 30, 60, and 90 by Nembutal overdosage.

Chronic EI was verified by biochemical values: plasma content of medium molecular weight substances (MMWS) and their oligopeptide fractions, MDA concentration, hepatic and renal acylase activities.

Serum concentrations of thyrotropic hormone (TTH), T_3, and T_4 were measured by EIA using Stat Fax 2100/2600 system (AWARENESS Technology) using Vector-Best kits.

The morphology of hepatic tissue was studied. Histological sections of tissues were stained by hematoxylin and eosin by the standard methods. Connective tissue was detected using Van-Gieson staining.

Apoptosis in liver tissue were detected using monoclonal antibodies (DakoCytomation) to NOS-3, TRAIL, and caspase-3 antigens. The expression of NOS-3 (endothelial nitroxide synthase) has been evaluated because this enzyme generates NO, which, reacting with superoxide anion, can lead to the formation of ONOOH peroxynitrite (apoptosis inductor). TRAIL was selected as the receptor triggering apoptosis induction by reacting with TNF-α. The significance of the TNF-dependent cell death is determined by high concentration of TNF-α in chronic EI [2]. Caspase-3 served as apoptosis marker because this antigen is an enzyme with nuclease activity. The degree of its expression correlates with nucleic acid degradation in the nucleus and cytoplasm.

The results were visualized by indirect immunoperoxidase method with high temperature decamouflage of antigens; positive and negative controls for antigens and negative control for antibodies were used.

Morphological changes were quantitatively evaluated using Image Tool for Windows, v. 3.00 (UTHSCSA) software. Morphometry of immunohistochemical sections was carried out in order to evaluate the volume percent of immunopositive material.

The data were mathematically processed directly from the Microsoft Excel common data matrix using STATGRAPH 5.1 software. The means, their mean quadratic deviations, and representation error were evaluated.

RESULTS

Simulation of chronic EI led to an increase in the concentrations of MMWS and their oligopeptide fractions on day 30 of experiment; the parameters were 2.69 ($p<0.05$) and 2.32 times ($p<0.05$) higher than in the control, respectively (Table 1). Later (by day 90 of experiment) the concentrations of

TABLE 1. Biochemical Values of EI Severity in Rats with a Model Induced by Chronic LPS and Tetrachloromethane Treatment (*M*±*m*)

Parameter	Control	Day of experiment		
		30	60	90
No hyperthyrosis				
MMWS, arb. units	0.16±0.02	0.43±0.05*	0.49±0.05*	0.51±0.06*
Oligopeptides, mg/liter	115.5±13.8	268.5±29.8*	289.4±30.5*	291.0±36.4*
MDA, mmol/g lipids	5.33±0.43	11.40±0.94*	10.53±1.25*	11.08±1.35*
Liver acylase, μcat/g protein	27.91±1.93	9.06±0.87*	7.58±0.73*	5.97±0.68*
Kidney acylase, μcat/g protein	35.61±2.90	18.21±1.78*	13.61±1.45*	9.61±1.12*
Hyperthyrosis				
MMWS, arb. units	0.17±0.02	0.55±0.01*+	0.63±0.01*+	0.66±0.02+
Oligopeptides, mg/liter	280.35±7.08+	303.81±11.7	296.67±9.23	315.11±1.7
MDA, mmol/g lipids	8.10±0.71+	13.21±1.01*+	11.93±0.91*+	11.71±0.8
Liver acylase, μcat/g protein	24.97±1.03	8.53±0.09*+	6.31±0.09*+	4.57±0.09*+
Kidney acylase, μcat/g protein	29.51±1.21+	22.31±1.17*	11.71±0.78*	10.13±0.11*

Note. Here and in Tables 2, 3: $p<0.05$ *vs.* *initial status, +parameter during the same period in animals without hyperthyrosis.

biochemical substrates of EI increased even more: 3.18 and 2.53 times, respectively, in comparison with the control ($p<0.01$).

Biochemical markers of chronic EI, simulated under conditions of hyperthyrosis, indicated augmenting intoxication. On day 90 of experiment the concentration of MMWS was 1.29 times higher than in animals with EI without hormonal imbalance and 4.13 times higher than in control rats ($p<0.01$). Similar changes were recorded for oligopeptide fraction and MDA. The concentrations of liver and kidney acylases were reduced significantly in experimental animals in comparison with intact rats and animals with chronic EI without hyperthyrosis ($p<0.05$) This indicates that hyperthyrosis aggravated the course of chronic EI.

Measurements of thyroid hormones in the systemic bloodflow indicated that chronic EI was associated with an increase in the pool of virtually all hormones with the peak on day 60 (Table 2).

These changes in the hormonal profile indicate hyperthyroid status in chronic EI. The levels of thyroid hormones were even higher in the hyperthyrosis group.

Study of hepatic tissues in animals with chronic EI on day 30 of experiment showed the development of changes characteristic of chronic intoxication of the liver with manifest hepatofibrosis. Moderately pronounced sclerosis of the periportal tracts, lymphohistiocytic infiltration of new connective tissue were seen.

Centrolobular hepatocytes were in a state of vacuolar degeneration, just few of them with nuclear caryopyknosis and caryolysis. Hepatic cells at the periphery of the lobule were less damaged. This was paralleled by involvement of the small veins and venules (fibrous connective tissue growth in vascular walls, endothelial proliferation with capillarization of the sinusoids). Vascular walls and Disse's space were moderately edematous.

On day 60 of experiment the portal tracts and bile capillaries were still dilated, their walls were—μhickened, in some cases with biliary clots in the lumen. Accumulations of macrophageal cells were hypertrophic and contained brown pigment granules in the cytoplasm.

Part of hepatocytes near the triads exhibited signs of granular and vacuolar degeneration; these cells formed foci. Hepatocyte nuclei were pyknotic and partially lyzed.

Study of hepatic tissue on day 90 of experiment showed violation of the hepatic lobular structure (incomplete bulky structure in some places). The triads were surrounded by well-developed connective tissue with slight lymphohistiocytic infiltration.

Study of liver tissue in animals with chronic EI concomitant with hyperthyrosis showed a more severe course of the disease in comparison with the classical model. It manifested by earlier development of balloon degeneration, progress of sclerotic injuries, and loss of fragments of bulky structure as early as by day 60 of experiment.

Immunohistochemical study of TRAIL expression on day 30 of experiment showed accumulation of this antigen in liver cells; by the histotopographic distribution its level was the highest in the periportal zones. A similar picture was observed on day 60. By day 90 of intoxication the expression of TRAIL was verified over the entire porto-central area (Fig. 1, *a*).

TABLE 2. Changes in the Thyroid Hormone Profile in the Peripheral Blood of Rats with Chronic EI ($M\pm m$)

Parameter	Control	Day of experiment		
		30	60	90
No hyperthyrosis				
TTH, nmol/liter	1.53±0.32	1.56±0.6	1.01±0.3*	0.95±0.11*
Total T_3, nmol/liter	10.2±0.9	10.84±0.70	12.45±1.10	8.84±0.90
Free T_3, pmol/liter	7.34±0.70	6.94±0.90	7.04±0.70	5.74±0.90
Total T_4, nmol/liter	65.24±1.90	165.05±11.30*	114.35±10.10*	94.22±6.70*
Free T_4, pmol/liter	15.52±13.60	23.7±0.9*	13.66±0.70	12.63±0.90
Hyperthyrosis				
TTH, nmol/liter	1.10±0.09+	1.86±0.08*+	1.01±0.09	2.51±0.04*+
Total T_3, nmol/liter	12.01±0.91+	12.84±0.70*+	13.43±0.97*+	10.51±0.11*+
Free T_3, pmol/liter	6.52±0.91+	8.57±0.80*+	6.92±0.91	9.21±1.01*+
Total T_4, nmol/liter	84.27±2.11+	129.15±9.30*+	152.9±10.7*+	133.08±9.30*+
Free T_4, pmol/liter	13.26±0.80	21.25±0.89*	13.49±0.90*	13.32±0.89

Study of the histotopographic distribution of receptors to TNF-α in animals with chronic EI concomitant with hyperthyrosis showed accumulation of cells expressing TRAIL in all compartments of the lobules as early as by day 30 of experiment. Later the number of cells involved in presentation of the antigen on cell surface increased (Fig. 1, *c*).

Study of caspase expression in animals with the classical model of chronic intoxication showed an opposite trend in liver tissue. Solitary caspase-po-

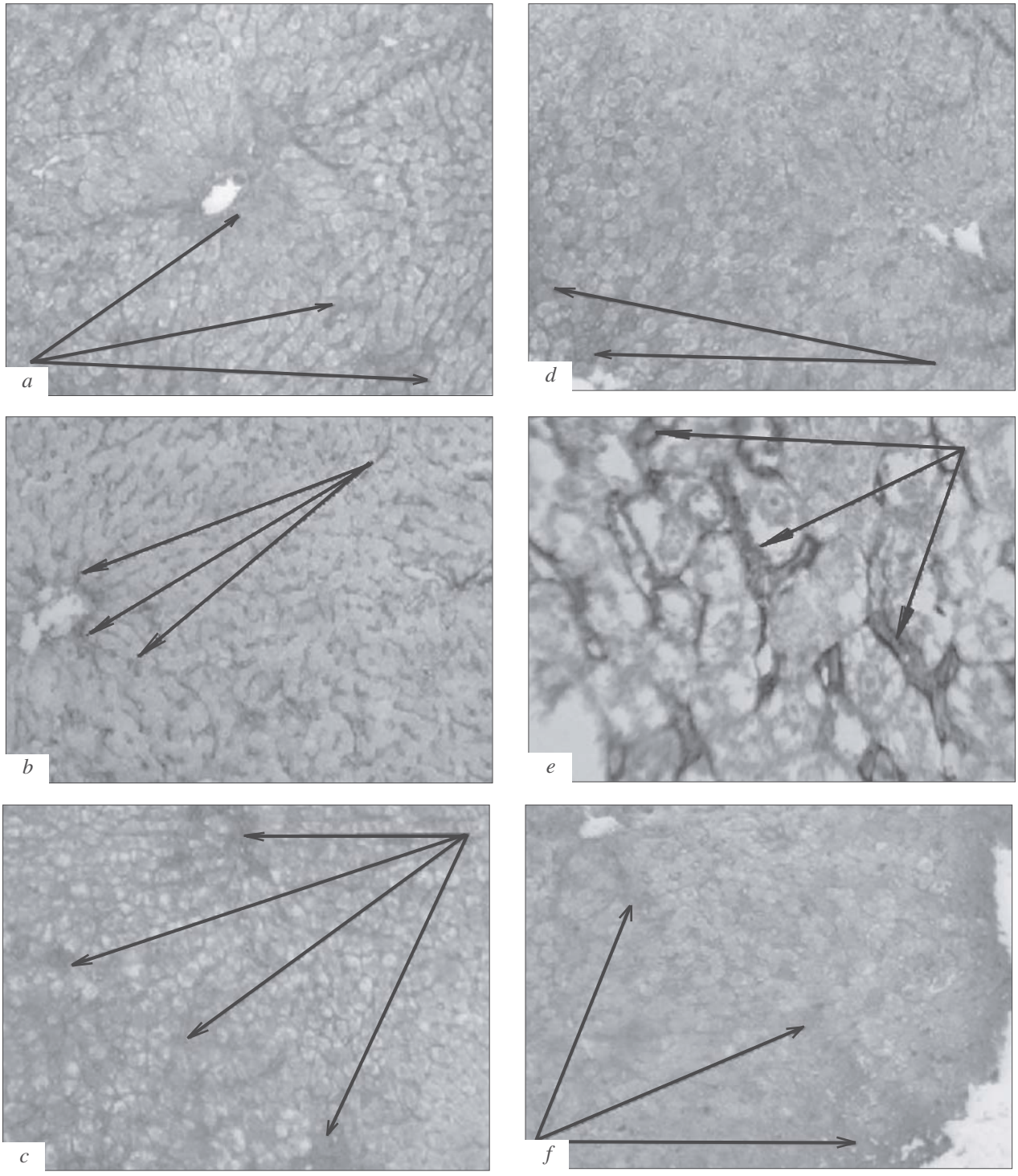

Fig. 1. The liver in chronic EI. Immunoperoxidase method. *a*) day 90. Antibodies to TRAIL; *b*) day 30. Antibodies to NOS-3; *c*) hyperthyrosis, day 60. Antibodies to TRAIL; *d*) day 60. Antibodies to caspase-3; *e*) hyperthyrosis, day 90. Antibodies to NOS-3; *f*) hyperthyrosis, day 60. Antibodies to caspase-3. ×70 (*a-d*, *f*), ×280 (*e*).

TABLE 3. Percent Area of Immunopositive Material in Rats with Chronic EI (*M*±*m*)

Parameter	Control	Day of experiment		
		30	60	90
No hyperthyrosis				
TRAIL	2.6±0.9	15.6±3.0*	12.7±3.9*	17.8±2.6*
NOS-3	6.1±1.3	12.5±5.8*	19.4±6.8*	17.6±7.5*
Caspase-3	1.6±0.4	7.7±6.3*	21.7±1.5*	23.0±4.9*
Hyperthyrosis				
TRAIL	14.2±3.7	16.5±6.2	17.8±1.6*	18.8±2.4*
NOS-3	8.9±5.1	16.2±5.7	20.1±4.5*	22.3±7.7*
Caspase-3	14.5±3.3	19.4±5.5	23.0±2.9*	24.1±4.3*

sitive cells in the central veins were detected on day 30. By day 60 the cells expressing caspase were detected in the center of the lobules, demonstrating a trend to dissemination in the centro-portal direction (Fig. 1, *d*). This process eventuated in involvement of the overwhelming majority of hepatocytes in expression of caspase by day 90 of chronic EI.

Studies of liver tissue from animals with hyperthyrosis showed the same trend, which was more pronounced: caspase accumulation in the cells of the pericentral zones as early as by day 30 of the process and progress of this process to the periportal areas by day 90 of experiment (Fig. 1, *f*).

By day 30 of experiment the expression of NOS-3 in animals with the classical model was detected in endotheliocytes of the portal tracts and in the sinusoids of the periportal regions. The number of cells expressing NOS-3 increased during later periods of experiment. Evaluation of the predominant vector of involvement of the new cells also indicated the porto-central direction (Fig. 1, *b*).

Similar time course was observed in animals with hyperthyrosis. The difference consisted in more pronounced expression and greater number of cells verified as NOS-positive (Fig. 1, *e*).

Morphometric analysis showed significant differences between the percent areas occupied by immunopositive structures in tissue at different terms of experiment in animals with the classical model (Table 3). The results indicate that in chronic EI liver disease forms at the expense of fibroplastic, vascular, and cytopathic components. An obligatory component of chronic EI is hyperthyroid status, characterized by high concentrations of all thyroid hormones in the systemic bloodflow.

The microcirculatory system is involved in chronic EI as its vascular component. These changes are verified by increasing expression of endothelial nitroxide synthase. Thyroid modulation manifests by a sharp increase of NOS-3 expression. It remains unclear whether hyperexpression of nitroxide synthase is a reaction to injury or a result of the direct effects of thyroid hormones on the endothelium and the cause of microcirculatory disorders.

An obligatory cytopathic component of liver disease is activation of TNF-dependent apoptosis. Comparative analysis of thyroid effect (on the model with concomitant hyperthyrosis) showed intensification of this mechanism of cell death by thyroid hormones. Histotopographic analysis of the morphological picture of TNF-dependent apoptosis showed that EI effect manifested by hyperexpression of TRAIL and NOS-3 in liver cells in the porto-central direction. This fact can be interpreted as induction of programmed cell death in the periportal areas (the most close to EI source). On the other hand, accumulation of caspase in the cells is characterized by initial lesions in the pericentral areas.

Hence, we can speak about direct thyroid modulating effect on the course of chronic EI and formation of liver disease. This modulating effect is quantitative, aggravating the course of EI and the respective lesions of hepatic tissue.

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