Effect of Triterpene Derivatives on the Total Hepatocyte Count in the Liver of Rats with Toxic Hepatitis

D. E. Semenov*, N. A. Zhukova, E. P. Bessergeneva, I. V. Sorokina, D. S. Baev, B.M. Glukhov*, G. I. Nepomnyaschikh*, and T. G. Tolstikova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 6, pp. 837-840, June, 2012
Original article submitted March 7, 2012.

We studied hepatoprotective activity of betulonic acid and its alaninamide on the model of combined CCl_4- and ethanol-induced toxic liver damage in rats. The test substances, especially betulonic acid alaninamide, considerably reduced the elevated biochemical parameters in animals with toxic liver damage. Betulonic acid alaninamide also stimulated reparative processes in the liver (activated hepatocyte proliferation). Heptral (reference drug) produced no appreciable effects on the reparative processes. Our findings suggest that betulin derivatives exhibit pronounced protective properties.

Key Words: *toxic hepatitis; triterpene derivatives; heptral; biochemical markers; hepatocyte count*

Toxic damage to the liver is still an urgent problem due to increasing use of drugs and foodstuffs contaminated with toxicants of anthropogenic origin. Pronounced hepatotoxicity of antitumor drugs (*e.g.* anthracycline antibiotics, cyclophosphamide, *etc.*) is also an important aspect of the problem of drug-induced liver damages. [2,3,5]. The liver is functionally interposed between the site of absorption and the systemic circulation and is a major site of metabolism and elimination of foreign substances [13]. An alternative effect of toxins on microsomal system of the liver with the formation of free radicals underlies the pathogenesis of toxic hepatitis and leads to pronounced functional and structural changes in hepatocytes [1].

Natural antioxidants can counteract the adverse effects of toxic agents by neutralizing free radicals and correcting the inflammatory response. Natural lupine-type triterpene derivatives exhibit these properties; these substances have a wide spectrum of pharmacological activities (antitumor, antiviral, bacteriostatic, antioxidant, hepatoprotective, and anti-inflammatory) and the corresponding raw material is easily available [8-12].

Here we studied changes in biochemical parameters of the blood and hepatocyte count in rats with combined CCl_4- and ethanol-induced liver injury receiving betulin derivatives (betulonic acid and its alaninimide) and reference pharmacopeial preparation heptral (ademothionine).

MATERIALS AND METHODS

Experiments were performed on albino female rats (n=75; mean body weight 178.1±5.6 g) obtained from the vivarium of Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Medical Sciences, and maintained under standard vivarium conditions with usual food and water supply. The experiments were performed with strict adherence to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasburg, 1986).

Toxic liver injury was modeled by administration of CCl_4 in a dose of 0.1 ml/kg in vegetable oil (0.3 ml/100 g) 3 times in combination with free access to

N. N. Vorozhtsov Institute of Organic Chemistry, Siberian Division of the Russian Academy of Sciences; *Research Institute of Regional Pathology and Pathomorphology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** pathol@soramn.ru. D. E. Semenov

5% ethanol solution as the only source of fluid. The animals were divided into 5 groups (15 rats per group) and received CCl_4+ethanol (group 1, negative control), heptral in a dose of 6 mg/kg (group 2, reference group), betulonic acid (BA, group 3), or betulonic acid alaninamide (BAA) in a dose of 50 mg/kg in water-Tween solution (group 4). The test substances were administered daily through a gastric tube. Intact animals served as the positive control (group 5). Treatment with BA, BAA, and heptral (ademethionine, lyophilic preparation) during weeks 2-6 of the experiment was followed by a 2-week recovery period (weeks 7 and 8). The animals were decapitated in 3, 6, and 8 weeks. Activity of ALT, AST, alkaline phosphatase (AP), and lactate dehydrogenase (LDH) and the concentrations of total protein, direct bilirubin, and glucose in the serum were measured [6].

Alkaline dissociation of the liver was performed for evaluation of the relative content of multinuclear hepatocytes among the total hepatocyte population and the relative content of connective tissue component. The cell suspension was stained for 30 min with 1% orsein in 70% acetic acid and adjusted to a concentration of 5 mg/kg with distilled water. For each tissue sample, hepatocyte nuclei were counted in 6 successively filled Goryaev chambers. The number of hepatocyte nuclei per 1 mg tissue (n), absolute number of hepatocytes in the liver (M), and index of binuclear hepatocytes (ratio of binuclear to total hepatocyte count) were calculated.

The data were processed by methods of parametric statistics using Microsoft Excel software. The differences were significant at $p<0.05$.

RESULTS

In group 1 animals (negative control), serum activities of ALT, AST, AP, and LDH increased by 94, 141, 83 и 134%, respectively, in comparison with those in group 5 rats (positive control) in 3 weeks of the experiment (Table 1). In 6 weeks, AST and AP activities increased in comparison with the previous term and activities of all enzymes were elevated in comparison with the positive control (group 5). The concentration of hepatocyte nuclei and their absolute count decreased by 70%, binucleated hepatocytes constituted 0.87% of the whole hepatocyte population (Table 2), which indicates low level of regeneration (binucleated hepatocytes in positive control constituted 14.5%). In 6 weeks, the concentration of hepatocyte nuclei, their absolute number, and the relative content of binucleated hepatocytes did not change significantly. This indicates the development of severe progressive toxic damage of the liver in all animals.

In animals treated with heptral (group 2), activities of AST and ALT decreased by 52 and 32%, respectively, in comparison with negative control (group 1). These changes in enzyme activities attest to some alleviation of the cytolysis syndrome [13]. However, activities of AP and LDH and the content of direct bilirubin remained at the level of negative control. After 6 weeks, activities of AST and AP were increased by 204 and 74%, respectively, in comparison with the values observed at the previous term. The levels of ALT, LDH, total protein, glucose, and bilirubin little changed (Table 1). In 3 and 6 weeks, the qualitative parameters of hepatocytes little differed from those in negative control (Table 2). By week 7, animal mortality in this group was 80% and by week 8 all animals died. Administration of heptral had no significant positive effect on the course of the pathological process in comparison with the groups receiving BA and BAA. Low activity of heptral in the experiment can be related to both the species specificity and the administration route (*per os*).

After 3-week course of BA (group 3), ALT and AST activities decreased by 44 and 35%, respectively, in comparison with the negative control, but AP and LDH activities remained high, which indicated persistent cholestasis [6] (Table 1). After 6 weeks, activities of ALT, AST, and AP decreased by 19, 29, and 33%, respectively, in comparison with the negative control group (Table 1). In parallel, the concentration of hepatocyte nuclei and the absolute number of hepatocytes after 3 weeks increased by 72 and 36%, respectively, in comparison with the negative control group. After 6 weeks, the absolute number of hepatocytes considerably increased (by 59%) against the background of minor changes in nucleus concentration (Table 2). The index of binuclear hepatocytes after 3 and 6 weeks was 1.5 and 0.7% respectively (Table 2). These findings suggest that BA administered during a long time partially corrected cholestasis and stimulated regeneratory activity of the liver.

The most pronounced positive dynamics of enzyme activity was observed in rats receiving BAA (group 4). After 3 weeks of the experiment, ALT and AST activities decreased by 42% in comparison with those in negative control. AP and LDH activities were considerably lower than in experimental groups 1, 2, and 3, but surpassed the corresponding values in the intact control group (group 5) by 11 and 103%, respectively (Table 1). After 6 weeks, AST, ALT, and AP activities decreased by on average 69% and only LDH activity remained elevated by 6% (Table 1). It should be noted that activities of ALT, AST, and AP and direct bilirubin content after 3- and 6-week BAA treatment were considerably lower than in animals receiving heptral (group 2, Table 1). The concentration of nuclei and absolute number of hepatocytes were considerably increased (by 7 and 54%, respectively) only

TABLE 1. Effect of Triterpene Derivatives on Biochemical Parameters of the Blood in Rats with Toxic Hepatitis (*M*±*m*)

Group	Experimental weeks	Total protein, g/liter	Direct bilirubin, μmol/liter	Glucose, mmol/liter	ALT, U/liter	AST, U/liter	AP, U/liter	LDH, U/liter
Group 1 (CCl_4)	3	87.5±12.2*	21.3±10.9	5.6±0.3	216.6±41.3**	400.4±151.3*	719.8±232.7*	1460.3±184**
	6	97.02±5.9**	13.97±6.0	5.02±0.9*	202.35±41.0**	508.27±85.2**	1355.97±465.2**	1336.5±101.6**
	8	98.32±8.1**	2.75±1.2**	6.88±0.3*	221.97±22.6**	296.93±29.9**	940.23±105.3**	1845.3±259.7**
Group 2 (heptral)	3	109.5±11.8^{+}	28.7±6.9	6.4±0.5	148.1±10.1^{+}	192,1±13.0^{+}	672.8±47.4	1440.1±381.6
	6	72.4±10.3^{++}	29.6±9.1^{++}	6.1±0.6^{+}	182.0±29.6	584.3±153.0	1170.0±185.2	994.4±128.6^{++}
Group 3 (BA)	3	98.2±12.5	18.6±4.2	6.1±0.4	120.4±15.5^{++}	258.4±47.4	1227.5±129.9^{++}	1491.0±135.3
	6	84.0±6.6^{++}	21.9±1.4^{++}	7.00±0.27^{++}	164.5±45.7	363.1±84.5^{++}	906.6±301.7	1191.6±145.3
	8	77.9±5.6^{++}	5.6±1.5	3.8±0.3^{++}	127.8±16.0	244.2±25.1^{++}	519.8±49.5^{+}	727.7±167.2
Group 4 (BAA)	3	82.0±8.4	11.6±2.4	6.0±0.3	128.3±11.7^{++}	233.8±27.4	435.0±49.3^{+}	1266.1±57.4
	6	85.5±7.4^{+}	20.7±1.5^{+}	8.5±0.5^{++}	78.9±5.5^{++}	164.9±8.4^{++}	317.6±26.7^{++}	1414.0±68.4
	8	103.6±7.9	5.8±1.8^{++}	5.2±0.4^{++}	107.7±15.4^{++}	217.7±20.7^{++}	285.5±32.2^{++}	583.3±113.3^{++}
Group 5 (intact control)	8	64.8±2.2	8.90±3.04	6.2±0.4	111.4±11.7	165.9±27.2	393.2±83.0	623.5±230.9

Note. Here and in Table 2: *$p \le 0.05$,**$p \le 0.01$ in comparison wit intact control; $^{+}p \le 0.05$, $^{++}p \le 0.01$ in comparison wit negative control.

TABLE 2. Effect of Triterpene Derivatives on Population Dynamics of Hepatocytes against the Background of Toxic Hepatitis (*M*±*m*)

Group	Experimental weeks	Number of hepatocyte nuclei per 1 mg tissue, $\times 10^3$	Absolute count of hepatocyte nuclei, $\times 10^6$	Absolute count of hepatocytes, $\times 10^6$	Count of mononuclear cells, $\times 10^6$	Count of binuclear cells, $\times 10^6$
Group 1 (CCl_4)	3	35.14±0.7**	398.37±32.1**	394.89±33.5**	391.46±34.8	3.44±1.5
	6	64.73±3.6**	486.18±94.3**	466.05±89.8**	445.92±85.6	20.13±6.9
	8	44.82±7.6**	595.07±116.9**	584.04±115.9**	573.0±115.0	11.03±3.7
Group 2 (heptral)	3	36.45±1.3	326.04±27.2^{+}	320,19±25.4^{+}	314.33±23.7	5.86±2.40
	6	56.29±4.30^{++}	566.49±73.90	562.68±73.00	558.87±72.20	3.81±2.00
Group 3 (BA)	3	60.46±10.30^{++}	543.46±80.70^{++}	535.11±77.50^{++}	526.76±74.30	8.35±3.60
	6	67.44±7.50	744.96±84.40^{++}	739.79±84.50^{++}	734.62±84.7	5.17±2.70
	8	54.91±14.70	431.41±106.70	397.79±102.90	364.17±99.93	33.62±9.78
Group 4 (BAA)	3	43.04±3.40^{++}	404.67±38.10	400.35±38.89	396.03±39.70	4.32±1.80
	6	66.56±4.90	742.15±81.70^{++}	719.09±77.40^{++}	696.02±73.80	23.07±8.10
	8	81.39±6.40^{++}	745.24±54.50^{+}	695.46±49.20	645.68±44.70	49.78±8.00
Group 5 (intact control)	8	115.73±4.70	1252.75±49.30	1093.90±45.70	935.04±42.70	158.85±6.00

after 6 weeks (Table 2). The population dynamics of binucleated hepatocytes in 3 and 6 weeks little differed from that in negative control (Table 2). Hence, BAA maximally alleviated both cholestasis and cytolysis symptoms and stimulated regeneratory potential only under conditions of long-term administration.

During the recovery period (weeks 7 and 8), ALT and LDH in group 1 animals remained considerably elevated in comparison with the parameters during week 6 of the experiment (Table 1). The increase in the concentration of nuclei and the absolute count of hepatocytes and binucleated cells (Table 2) probably attested to activation of the natural regeneration mechanisms.

In animal receiving BAA (group 4), bilirubin content and activities of AP, LDH, and ALT returned to normal, while in animals receiving BA (group 3) only direct bilirubin concentration normalized (Table 1). Triterpenoids induced an increase in binuclear hepatocyte population: the index of binucleated hepatocytes in groups 3 (BA) and 4 (BAA) was 8.4 and 7.1%, respectively, whereas in the group of negative control it was only 1.8% (Table 2). The increase in the relative content of binucleated hepatocytes induced by long-term treatment with triterpenoids (BA and BAA) can be regarded as a peculiarity of their stimulating effect. The increase in nucleus concentration and the absolute number of hepatocytes against the background of BAA treatment in comparison with the negative control and BA treatment indicates stimulation of the regeneratory potential of the liver. It should be noted that BA treatment increased only the number of hepatocyte nuclei and the number of binucleated cells (by 23 and 204%, respectively) in comparison with the corresponding parameters in the negative control group during week 8 of the experiment. The absolute number of hepatocytes and their nuclei and the number of mononuclear hepatocytes decreased by 30% on average in comparison with negative control (group 1) by the end of the experiment. Hence, BA and BAA produced a stimulatory influence on different components of the regeneratory reaction, which could be determined by chemical structure of these compounds [8,9].

Hence, BAA in long-term intragastric administration most effectively alleviated symptoms of cholestasis and cytolysis and stimulated regeneratory potential of the liver, thus exhibiting the properties of a highly effective hepatoprotector. BA in intragastric administration only partially reduced the signs of cholestasis, improved population dynamics of hepatocytes, and had no effect on the proliferative reserve of the liver during the recovery period. Intragastric administration of heptral had no effect on activity of cytolysis enzymes and the state of hepatocyte pool in rats with modeled toxic injury of the liver.

REFERENCES

1. V. V. Kosarev and S. A. Babanov, *Consilium Medicum. Gastroenterology*, No. 2, 29-30 (2009).
2. E. L. Lyshnikova, O. P. Molodykh, L. M. Nepomnyashchikh, *et al., Byull. Eksp. Biol.*, **151**, No. 6, 699-704 (2011).
3. O. P. Molodykh, E. L. Lyshnikova, M. G. Klinnikova, L. M. Nepomnyashchikh, *Byull. Eksp. Biol.*, **141**, No. 5, 579-585 (2006).
4. L. M. Nepomnyashchikh, E. L. Lushnikova, and D. E. Semenov, *Regeneratory and Plastic Insufficiency of the Heart. Morphological Bases and Molecular Mechanisms* [in Russian], Moscow (2003).
5. L. M. Nepomnyashchikh, O. P. Molodykh, E. L. Lyshnikova, and Yu. A. Sorokina, *Byull. Eksp. Biol. Med.*, **149**, No. 1, 113-120 (2010).
6. T. N. Popova, T. I. Rakhmanova, and S. S. Popov, *Medical Enzymology. A Manual* [in Russian], Voronezh (2008).
7. L. A. Semenova, L. M. Nepomnyashchikh, and D. E. Semenov, *Morphology of Plastic Insufficiency of Cardiomyocytes* [in Russian], Novosibirsk (1985).
8. I. V. Sorokina, N. A. Zhukova, T. G. Tolstikova, *et al., Vopr. Biol. Med. Farm. Khimii*, No. 1, 29-31 (2006).
9. I. V. Sorokina, T. G. Tolstikova, N. A. Zhukova, *et al., Dokl. Akad. Nauk*, **399**, 274-277 (2004).
10. I. V. Sorokina, T. G. Tolstikova, N. A. Zhukova, *et al., Byull. Eksp. Biol*, **141**, No. 1, 69-72 (2006).
11. T. G. Tolstikova, I. V. Sorokina, G. A. Tolstikov, *et al., Bioorgan. Khimiya*, **32**, No. 1, 42-55 (2006).
12. T. G. Tolstikova, I. V. Sorokina, G. A. Tolstikov, *et al., Bioorgan. Khimiya*, **32**, No. 3, 291-307 (2006).
13. S. Russmann, G. A. Kullak-Ublick, and I. Grattagliano, *Curr. Med. Chem.*, **16**, No. 23, 3041-3053 (2009).