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EFFECT OF TRANSDERMAL IMMUNOMODULATION ON LIVER REGENERATION

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Introduction. The use of immunomodulators to regulate reparative processes in affected organs and tissues remains a pressing issue. Of greatest interest is liver regeneration after extended hepatic resection (EHR) in donors in right lobe living related donor liver transplantation. We propose a transdermal therapeutic system (TTS) with an immunomodulator to enhance the natural process of liver tissue regeneration. **Objective:** to study the effect of transdermal administration of immunomodulator sodium aminodihydrophthalazinedione on early recovery processes in the liver after EHR in in vivo experiments. **Materials and methods.** Sodium aminodihydrophthalazinedione was used as an active substance in TTS in the form of powder for preparation of intramuscular injection solution (Galavit®, SELVIM LLC). An experimental EHR model was performed on 22 male Wistar rats weighing 350–380 g. After HER, all animals were divided into two groups. Group 1 (n = 10) consisted of untreated animals. In group 2 (n = 12), TTS was applied immediately after liver resection. The experiment lasted for 48 hours; the TTS was changed once after 24 hours from the beginning of application. **Results.** In either group, there was no significant difference in the weight of liver remnant gain and in biochemical blood parameters at 48 hours after EHR. Assessment of the mitotic index (MI) of hepatocytes 48 hours after EHR revealed a significant increase in MI in both groups in comparison with the baseline (before liver resection) equal to 0.14 ± 0.07‰. The MI in group 1 and group 2 animals was 12.70 ± 4.9‰ and 17.43 ± 4.90‰, respectively ($p \leq 0.05$). **Conclusion.** Studies on the regenerative activity of sodium aminodihydrophthalazinedione TTS on an experimental EHR model in rats showed that this drug form had a pronounced stimulating effect on the mitotic activity of liver cells.

Keywords: transdermal therapeutic system, sodium aminodihydrophthalazinedione, immunomodulator, extended liver resection, mitotic index.

INTRODUCTION

The immune system, whose entire elements are actively involved in restoring the structure and function of damaged tissue cells, play an important role in regulation of regenerative processes in the body. This raises the question about the feasibility of using immunomodulators (IM) to influence reparative processes in affected organs and tissues [1, 2]. Of greatest interest is the problem of accelerated recovery of the liver after extended resection in cancer patients, as well as in donors in right lobe living related liver transplantation [3]. The extended hepatic resection (EHR) model is usually used in experimental studies. This type of surgery belongs to the critical injury class, because it removes 60% or more of the total mass of the organ, and often there are clinical manifestations of acute liver failure in the postoperative period [3].

There have been studies confirming the positive effect of a single injection of various immunomodulators on liver tissue repair [1, 4]. It should be noted that the question of duration of IM effect in a single injection remains open.

It is known that mitotic activity of hepatocytes is reduced in the first day after the operation but increases already by the second day [5]. Maximum mitotic and functional activity of hepatocytes is observed between day 2 and day 5 after the resection [5, 6]. It can be assumed that the use of prolonged drug form in the form of a transdermal therapeutic system will enhance the natural process of liver tissue regeneration by maintaining a constant concentration of IM in the blood for the required period.

The authors developed a transdermal therapeutic system containing a synthetic low molecular weight drug substance sodium aminodihydrophthalazinedione (Galavit®) [7]. In vivo experiments showed that the use of Galavit® transdermal therapeutic system (TTS) provides bioavailability of the immunomodulator, equal to the bioavailability of intramuscular injection of the drug substance at the same dose. This significantly reduces the maximum blood levels of the drug, but the retention time of sodium aminodihydrophthalazinedione in the body increases by more than 10 times, which may contribute to prolonged drug effect [8].

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In view of the above, the **objective** of the study was to investigate the effect of transdermal administration of immunomodulator aminodihydrophthalazinedione on the early stage of recovery in the liver in the experimental EHR model.

MATERIALS AND METHODS

Sodium aminodihydrophthalazinedione was used as an active substance in TTS in the form of a powder for preparing a solution for intramuscular injection (trade name Galavit®, Selvim LLC).

Excipients and materials approved for medical purposes were used in the manufacture of laboratory samples of Galavit® TTS.

The experimental animals were 22 male Wistar rats weighing 350–380 g, in which the EHR model was reproduced. Before EHR modeling, the operated rats were anesthetized by inhaled diethyl ether. Then, observing the rules of aseptics and antiseptics, the abdominal cavity was opened, the liver was exposed to the wound, and ligatures were sequentially applied to the bases of the medial, left lateral, and right upper lobes of the liver, after which ~70% of the total liver mass was removed. The operation was always performed in the morning hours (between 10 am and 12 noon), when the daily rhythm of mitotic activity of the liver cells was minimal.

All animals after EHR were divided into two groups. The first group (n = 10) consisted of animals with EHR without treatment.

In the second group (n = 12), immediately after liver resection, Galavit® TTS (10 cm^2) was applied in the back area of the rats to the skin areas with previously removed hair. Each TTS contained 40 mg of the drug substance. The experiment lasted for 48 hours with a single change of TTS 24 hours after the start of application.

To assess the dynamics of liver weight recovery in each operated animal, the removed part of the liver was weighed on Ohaus Explorer (Switzerland) electronic scale immediately after EHR, which was taken as 70% of the total liver weight. Then the initial mass of the residual liver was calculated for each animal based on these data. Then, after 48 hours, the remaining portion of the liver was excised, its weight was measured, and the values obtained were compared with the calculated initial mass of the residual liver for the particular animal.

In addition, the following biochemical blood parameters were determined: total protein, albumin, urea, creatinine and hepatic cytolysis enzymes: alanine aminotransferase (ALT), asparagine aminotransferase (AST) and alkaline phosphatase (ALP). For this purpose, the tail tip of the rat was incised under ether anesthesia, 28–32 μL blood was pipetted and applied to Reflotron™ test strips, which were immediately placed in a Reflotron™ biochemical analyzer (Roche, Switzerland). Blood from intact animals (n = 4) was used as a control.

Efficiency of stimulating effect of transdermal immunomodulation on liver regeneration processes after EHR was evaluated by mitotic (proliferative) activity of hepatocytes in the resected liver remnant. For this purpose, histological preparations of the resected liver were prepared, then the mitotic index (MI) – the number of mitotically dividing cells per 1000 analyzed cells – was calculated. For each specimen on the histological section of liver tissue stained with hematoxylin and eosin under 400× magnification (Leica LMLS microscope), we determined the number of mitotic figures and the average (total) number of cells. The formula used was:

$$MI = \frac{M}{N} \times 1000,$$

where *M* is the sum of dividing cells, *N* is the total number of cells analyzed. Mitotic index was expressed in ppm.

The significance of difference between the studied indicators in the compared groups was assessed using the parametric Student's t-test.

RESULTS AND DISCUSSIONS

It is known that after EHR, the process of organ recovery up to its initial mass occurs through proliferation and polyploidization of hepatocytes. It was noted that distinct signs of increased proliferative activity of the liver cells after EHR appear only 48 hours after this operation [9]. Considering the above, we evaluated the effect of percutaneous immunomodulator injection on stimulation of regeneration of the remaining part of the resected liver at 48 hours from the start of TTS.

The degree of recovery of rat liver weight in groups 1 and 2, 48 hours after EHR can be judged from the calculated results presented in Table 1.

As can be seen from Table 1, the increase in the liver remnant weight in the experimental animals, 48 hours

Table 1

Changes in rat liver weight 48 hours after extended resection

Animal group	Taken (~70%), g	Remnant (calculated, ~30%), g	Liver weight (calculated), g	Remnant (after 48 hours), g	Weight gain of liver remnant, g	Weight gain of liver, %
Group 1 (EHR; n = 10)	7.77 ± 0.754	3.33 ± 0.32	11.10 ± 1.06	8.08 ± 1.26	4.75 ± 1.17	43.06 ± 10.49
Group 2 (EHR + TTS; n = 12)	7.16 ± 1.05	3.07 ± 0.45	10.24 ± 1.50	7.00 ± 0.96	3.93 ± 0.79	39.03 ± 9.30

after resection, was 43 ± 10%; while in the group of animals with EHR and Galavit® TTS, it was 39 ± 9% for the same period. There was no significant difference in the weight of the liver remnant gain.

The effect of immunomodulator on restoration of liver homeostasis in rats at 48 hours after EHR was estimated by comparing the calculated blood biochemical indicators in the groups. Results of blood tests are presented in Table 2.

As can be seen from Table 2, biochemical blood parameters in both groups were significantly higher than in the intact animal group.

Analysis of results of the two experimental groups showed that such biochemical blood parameters as total protein (50.5 ± 2.9 g/L in group 2 and 52.3 ± 3.6 g/L in group 1) and albumin (24.1 ± 2.8 g/L and 24.9 ± 2.6 g/L respectively), in both groups, 48 hours after EHR, were practically the same.

Table 2

Biochemical blood parameters of rats after EHR

Biochemical blood parameters	ALT, U/I	AST, U/I	GGT, U/I	ALP, U/I	Total protein, g/l	Albumin, g/l	Urine, mmol/l	Creatinine, μmol/l
Intact group (n = 4)								
Mean	**52.6**	108.4		**184.2**	**60.8**	**30.0**	**7.5**	**24.7**
SD	6.7	21.0		87.0	1.5	12.0	0.4	3.3
Group 1 (EHR; n = 10)								
Mean	**407.3**	**657.5**	**6.7**	**559.7**	**52.3**	**24.9**	**8.6**	**49.1**
SD	203.5	225.6	4.9	239.5	3.6	2.6	7.4	28.9
Group 2 (EHR + TTS, n = 12)								
Mean	**470.3**	**830.9**	**10.8**	**568.1**	**50.5**	**24.1**	**12.1**	**62.8**
SD	225.7	334.0	7.1	193.9	2.9	2.8	14.4	54.8

Table 3

Mitotic index of hepatocytes 48 hours after EHR

Sample name / group	No. of histological sample	MI, ‰	MI mean, ‰	SD
Source tissue (n = 5)	2536	0.19	0.14	0.07
	2540	1.54		
	2542	0.14		
	2559	0.11		
	2621	0.05		
Group 1 (EHR; n = 10)	2535	10.21	**12.70**	4.9
	2539	10.80		
	2541	5.38		
	2544	8.37		
	2617	16.4		
	2619	16.8		
	2620	22.08		
	2622	13.26		
	2623	8.82		
	2624	15.13		
Group 2 (EHR + TTS, n = 12)	2537	19.40	**17.43**	4.9
	2545	10.84		
	2548	7.18		
	2626	25.09		
	2636	21.3		
	2637	12.69		
	2638	17.04		
	2639	17.6		
	2640	19.2		
	2614	17.5		
	2616	24.6		
	2617	13.7		

Urea levels were 12.1 ± 14.4 mmol/L in group 2 and 8.6 ± 7.4 mmol/L in group 1, while *creatinine* levels were 62.8 ± 54.8 μmol/L and 49.1 ± 28.9 μmol/L in group 2 and group 1, respectively. No significant difference was found.

The levels of liver cell cytolysis enzymes (ALT, AST, GGT, ALP) were slightly higher on average in group 2 (experimental) (EHR + TTS) – 470.3 ± 225.7 U/L; 830.9 ± 334.0 U/L; 10.8 ± 7.1 U/L; 568.1 ± 193.9 U/L, respectively. In group 1, ALT, AST, GGT, and ALP values were lower on average – 407.3 ± 203.5 U/L; 657.5 ± 225.6 U/L; 6.7 ± 4.9 U/L; 559.7 ± 239.5 U/L, respectively. However, the observed differences in cytolysis enzymes at 48 hours after EHR in the compared groups were not significant.

So, in a comparison of biochemical blood values at 48 hours after EHR, transdermal administration of immunomodulator Galavit® was not found to have any positive effect on restoration of hepatic homeostasis in rats.

The effect of using immunomodulator TTS to accelerate liver regeneration in EHR at 48 hours was found when comparing the results of histological study of hepatocyte proliferation by determining the MI of hepatocytes (see Table 3).

Research on the mitotic activity of liver hepatocytes at 48 hours after EHR, assessed according to the MI, revealed a significant increase in MI in both groups (0.14 ±

0.07‰) when compared to the baseline (before liver resection). As can be seen from Table 3, the MI in group 1 (EHR without treatment) at 48 hours of EHR averaged 12.70 ± 4.9‰, whereas in group 2 (with Galavit® TTS application immediately after EHR), the MI averaged significantly higher – 17.43 ± 4.90‰, ($p \leq 0.05$). Thus, percutaneous administration of immunomodulator Galavit® had a pronounced stimulating effect on the mitotic activity of liver cells.

The found pattern is supported by results from comparative morphological analysis of histological liver tissue preparations, in which higher mitotic activity of hepatocytes after 2 days in group 1 with EHR (Fig. 2) compared with the original tissue (Fig. 1) and more pronounced manifestations of mitotic activity of hepatocytes in group 2 with application of IM TTS (Fig. 3) are determined.

Figs. 1–3 show, as examples, photos of histological samples of rat liver tissue: initial; 48 hours after EHR, 48 hours after EHR and application of IM TTS.

Note that there was no death in all groups of animals throughout the duration of the experiment.

So, the study of the mitotic activity of hepatocytes after EHR in the groups of animals investigated showed that EHR induces proliferative activity in hepatocytes, and that transdermal administration of immunomodulator Galavit® after EHR has a stimulating effect on proliferation of hepatocytes in the resected liver. Increased proliferative activity of hepatocytes is accompanied by adaptive restructuring of the metabolism of these cells, which seems to have predetermined the absence of a positive effect of the immunomodulator TTS on biochemical indicators of hepatic homeostasis at 48 hours after EHR.

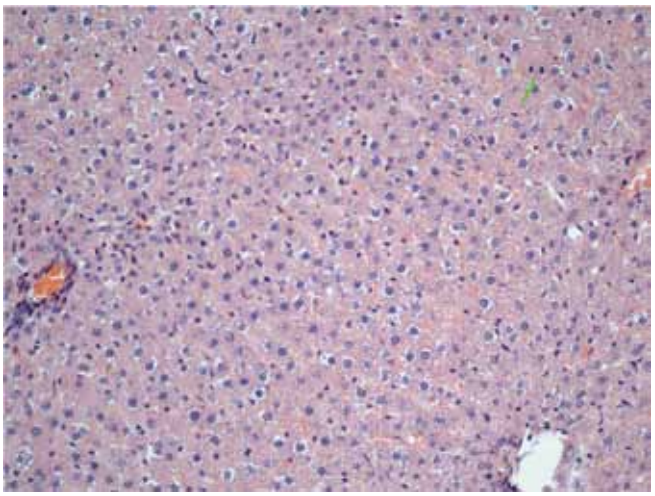

Fig. 1. Histological picture of the original rat liver tissue. Single figures of mitoses in the parenchyma (indicated by arrow). H&E staining. 200×

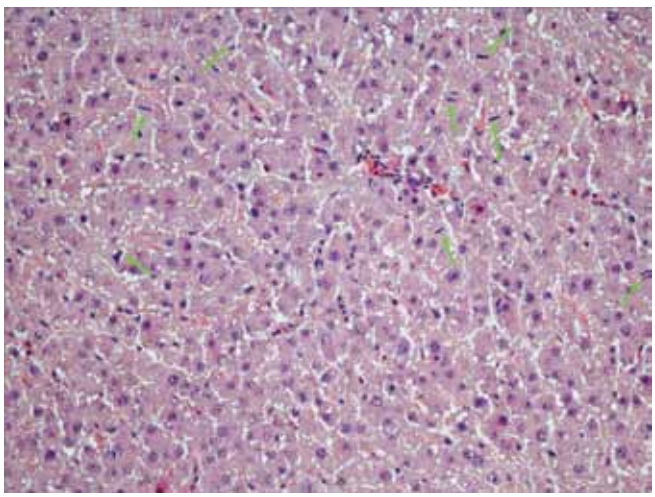

Fig. 2. Mitotic activity of hepatocytes 48 hours after EHR. Arrows indicate hepatocytes in the mitosis stage. H&E staining. 200×

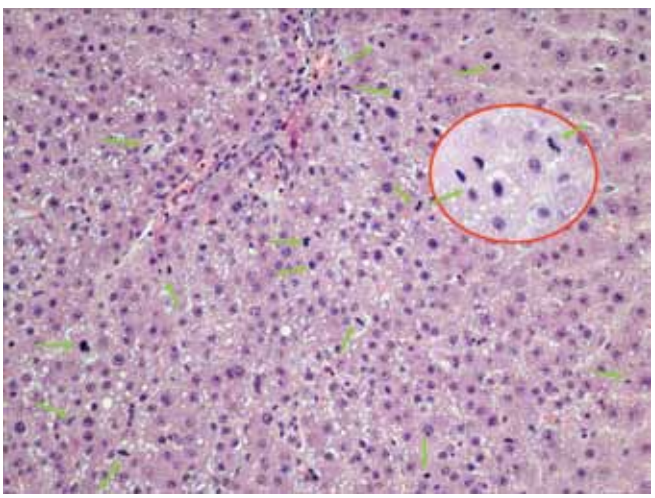

Fig. 3. Mitotic activity of hepatocytes 48 hours after EHR and application of the immunomodulator TTS. Multiple mitosis figures in the field of view (indicated by arrows). H&E staining. 200× (400× in the selected area)

CONCLUSION

The conducted studies on the regenerative activity of sodium aminodihydrophthalazinedione transdermal therapeutic system on an experimental EHR model of rats showed the prospects for percutaneous administration of this immunomodulator, as well as the need to further study its effect on liver repair processes at different periods of TTS application.

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The authors declare no conflict of interest.

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