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COMPARATIVE ANALYSIS OF REGENERATIVE ACTIVITY OF BONE MARROW CELLS AND TOTAL RNA EXTRACTED FROM THEM IN CHRONIC FIBROSING LIVER DISEASE

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Aim: to conduct a comparative assessment of the effectiveness of liver regeneration occurring after induction of chronic fibrosing liver disease (CFLD) using bone marrow mononuclear cells (BMMCs) and total RNA (tRNA) extracted from BMMCs. **Materials and methods.** The study involved 140 Wistar rats. CFLD was modeled in 100 rats, of which 25 died. The surviving 75 rats (CFLD formed by the third month) were divided into 3 groups: Group 1 – control (administered with physiological saline); Group 2 – a single injection of tRNA from BMMCs at a dose of 30 μg/100g body weight; Group 3 – a single injection of BMMCs at a dose of (30–35) × 10^6 cells. The dynamics of regenerative processes in the liver was evaluated based on the animal mortality, dynamics of restoration of biochemical markers (ALAT, ASAT, alkaline phosphatase and total protein) and morphological picture of the liver on the seventh day and after three, six and nine months. The significance of differences in the compared values was determined through Student's t-test for <0.05. **Results.** Mortality in Group 1 was 12%, in Groups 2 and 3 – 4%; In Group 1, ALAT and ASAT were restored to normal values after two months, alkaline phosphatase after 3 months, and total protein remained low for over 4 months. In Groups 2 and 3, all hepatic homeostasis markers returned to the values they were before CFLD modeling faster than in Group 1 (after two months). However, in Group 2, the regeneration rate was higher than in Group 3. It was revealed that normalization of functional liver parameters in all groups were ahead of restoration of the histological structure of the liver. Liver defibrotic processes in Group 2 were activated after 3 months, and in Groups 1 and 3 – after 6 months. The histological structure of the liver was restored in Group 2 after 6 months, and in Groups 1 and 3 after 9 months. **Conclusion.** BMMCs and tRNA extracted from them in biologically effective doses trigger liver regeneration in CFLD. However, regulatory effect from the use of tRNA appears earlier and is more effective.

Keywords: chronic liver failure, cirrhosis, bone marrow mononuclear cells, total RNA, liver regeneration.

Chronic liver failure (CLF) and liver cirrhosis are the result of major alterations in reparative regeneration processes. This creates conditions for chronically supported inflammation and progression of fibrosis [1].

At the current stage in medicine, donor liver transplantation is the only solution to irreversible liver damage in CLF patients [2, 3]. Meanwhile, steadily increasing donor organ shortage, along with continuing increase in the number of patients in need of liver transplantation limits the applicability of this method in all patients with end-stage CLF. Under these circumstances, there is need to continue the search for more accessible and effective CLF treatment methods that are based on induction of the patient's own regenerative liver reserves. The use of bone marrow stem/progenitor cells has become a new promising treatment strategy in CLF and cirrhosis.

At present, there are enough clinical and experimental observations, showing that BMMC-derived hematopoietic and stromal cells have a positive effect on the structure and function of the liver in chronic fibrosing conditions. Moreover, several studies [4–9] have even showed that there could be at least partial regression of already formed cirrhosis during stem/progenitor BMMCs transplantation. However, not all researchers recognize the fibrolytic effect of BMMCs. They even argue that BMMC use might increase fibrosis [10–12].

Finding diametrically opposite results from the use of stem/progenitor BMMCs is apparently a consequence of underestimating a number of factors: type of cells used, their initial bioregulatory potential (allogeneic cells of a healthy donor or autologous cells of a CLF patient), degree of reversibility of existing structural disorders in the liver which is reflected through severity of concomitant immune imbalance in the body, characterized by development of immunodeficiency up to immune paralysis [13, 14]. The absence or short duration of activation of fibrolytic processes in the liver during cell therapy can be caused primarily by the use of autologous BMMCs of a patient in whose body an immune imbalance has already developed, inhibiting the functional activity of BMMCs

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restored by culture and returned to the body [13]; besides, preliminary administration of G-CSF, which is used to restore reduced regulatory activity in the patient's bone marrow cells [15], also has a temporary effect, since the patient's BMMCs continue to remain under the influence of a complex of immunopathological factors paralyzing their activity. Preexisting immunopathological restructuring in a CLF patient seems to also have a paralyzing effect when using allogeneic BMMCs.

In the last decade, people started associating achievement of the regenerative potential of BMMCs with the recently discovered class of numerous protein – non-coding RNAs that are found in these cells: with the participation of microRNA molecules, long non-coding RNAs, short interfering RNAs, short nuclear RNAs, etc. [16–25], which served as the basis for extraction of total RNA from BMMCs and its use for induction of regenerative processes in the bone marrow itself when it is damaged [26–28].

Given that CLF, especially its terminal phase, proceeds amidst immune imbalance and inhibition of regulatory functions of the patient's BMMCs, which are indispensable participants in the regenerative process [14], as well as evidence that the used BMMCs (autologous and allogeneic) quickly lose their induction effect on reparative processes in the body, we have proposed that the total RNA extracted from the BMMCs of a healthy donor, will, in CLF, act as a biochemical regulator of regenerative processes in liver cells more quickly, more independently, and therefore more effectively than BMMCs.

Purpose of this work: To study ways of boosting the efficiency of liver regeneration in chronic toxic (fibrosing) liver disease, by comparatively evaluating the regulatory effect of BMMCs and total RNA from BMMCs on these processes.

MATERIALS AND METHODS

All studies using laboratory animals were carried out in strict compliance with the laws of the Russian Federation (in accordance with the Laboratory Practice Rules approved by the Ministry of Health of Russia through order No. 708 dated August 23, 2010, in accordance with standard GOST R ISO 10993-2-2009 "Medical devices. Biological evaluation of medical devices. Part 2. Animal welfare requirements") and in compliance with bioethical principles approved by the European Convention for the Protection of Vertebrate Animals (2005).

The work involved 140 male Wistar rats weighing 250–350 g. Chronic fibrosing liver disease (CFLD) was modeled in 100 of them; the remaining rats were used to obtain bone marrow mononuclear cells (BMMCs) and to extract total RNA (tRNA) from them. The animal model for CFLD was created through chronic CCl4 inoculation of animals in combination with Freund's incomplete adjuvant for 42 days according to the scheme. Of the 100 animals induced with CFLD, 25 died and by the end of the injection, 75 survived. All the surviving rats after CFLD modeling were divided into three groups: Group 1 – control (n = 25) with a single injection of physiological saline seven days after the end of injection; Group 2 – experimental (n = 25), where tRNA from the BMMCs of a healthy animal was intraperitoneally administered only once at a dose of 30 μg/100 g body weight seven days after CFLD modeling; Group 3 – experimental (n = 25), where BMMCs were intraperitoneally administered only once at a dose of (30–35) × 10^6 cells per rat seven days after CFLD modeling, which is comparable to the dose of cells used for tRNA extraction and single use. tRNA was extracted from BMMCs by a method developed by biotechnology company Evrogen (Russia) using the ExtractRNA reagent. This allowed to obtain about 148.5 ± 22.3 μg of tRNA from every 35.0 × 10^6 extracted cells.

The effectiveness of the stimulating effect of tRNA and BMMCs on reparative regeneration processes taking place in the liver after CFLD had been administered in the animals was evaluated based on animal mortality in the three groups, as well on the dynamics of restoration of hepatic homeostasis in the animal's body, and on the dynamics of elimination of structural (fibrosing) disorders in the liver tissue on the seventh day, and also after three, six and nine months. The regeneration dynamics of hepatic homeostasis in the body was evaluated by measuring the total protein and hepatic cytolysis enzymes in the blood serum: alanine aminotransferase (ALAT), aspartic aminotransferase (ASAT) and alkaline phosphatase (ALP).

Differences in the structure of liver tissue in the control and experimental groups were studied at the same time based on the severity of fibrolytic processes in the connective tissue and on the number of young newly formed hepatocytes in the hepatic lobule. To this purpose, the liver was excised within specified time frame, histological preparations were prepared from it, the sections were then stained with hematoxylin and eosin, while the connective tissue was treated with Masson trichrome stain.

The significance of differences in the markers for the compared groups was evaluated using the parametric Student's t-test for $p < 0.05$.

RESULTS AND DISCUSSION

The effectiveness of the regulatory effect of tRNA and BMMCs on regenerative processes occurring in the liver after CFLD modeling was evaluated primarily based on the animal mortality. It was found that in control Group 1, three of the 25 rats died in the first two months, which is 12%; in experimental Groups 2 and 3, the mortality rate was 4% (one rat in each group) within the entire observation period. Through a comparative

study of the restoration dynamics of biochemical markers of the functional state of the liver in the three studied groups, we were able to establish severe alterations in the markers immediately after inoculation and a gradual regeneration in all the groups. Meanwhile, the restoration rate of markers was different for each group (tables 1, 2, 3). In control Group 1 (table 1), the serum ASAT and ALAT values remained at a significantly higher level for two months than the initial values, ALP for 3 months, while the total protein content remained significantly reduced for 4 months. In Group 2 (with single tRNA injection), serum ASAT and ALAT values were restored after 1 month, serum ALP after two months, while the serum total protein content did not significantly differ from the initial values after two months (table 2). In Group 3, after a single injection of BMMCs, all the markers studied remained significantly altered from their baseline values within two months after CFLD modeling. However, when ASAT, ALAT, ALP, and total protein values were compared with each other two months after CFLD modeling, no significant differences were found in Groups 2 and 3.

Based on the results obtained from comparative study of dynamics of restoration of biochemical markers cha-

Table 1

Dynamics of changes in serum total protein and liver cell cytolysis enzymes (ALAT, ASAT and ALP) for Group 1 rats after CFLD modeling and administration of physiological saline (n = 25)

Timing after CFLD modeling	Group 1 (control group), n = 25			
	ASAT	ALAT	ALP	Total protein
Baseline	58 ± 8	40 ± 6	240 ± 24	98 ± 20
7 days	282 ± 31*	320 ± 13*	1322 ± 21*	21 ± 16*
1 month	196 ± 22*	168 ± 21*	909 ± 31*	26 ± 13*
2 months	98 ± 15*	96 ± 14*	532 ± 26*	42 ± 15*
3 months	72 ± 14	62 ± 8	426 ± 25*	48 ± 11*
4 months	62 ± 10	48 ± 7	250 ± 24	52 ± 6*
6 months	64 ± 15	44 ± 8	240 ± 20	65 ± 7

Note. * – $p < 0.05$ compared with baseline.

Table 2

Dynamics of changes in serum total protein and liver cell cytolysis enzymes (ALAT, ASAT and ALP) for Group 2 rats after CFLD modeling and administration of total RNA (n = 25)

Timing after CFLD modeling	Experimental Group 2 (tRNA), n = 25			
	ASAT	ALAT	ALP	Total protein
Baseline	58 ± 8	40 ± 6	240 ± 24	98 ± 20
7 days	303 ± 20*	278 ± 17*	1187 ± 56*	24 ± 10*
1 month	93 ± 15*	88 ± 10*	532 ± 28*	56 ± 10*
2 months	68 ± 19	68 ± 15	295 ± 14*	67 ± 14
3 months	65 ± 11	62 ± 6	275 ± 15	72 ± 9
4 months	62 ± 8	58 ± 12	247 ± 11	86 ± 7
6 months	66 ± 7	44 ± 6	230 ± 14	92 ± 12

Note. * – $p < 0.05$ compared with baseline.

Table 3

Dynamics of changes in serum total protein and liver cell cytolysis enzymes (ALAT, ASAT and ALP) for Group 3 rats after CFLD modeling and BMMCs injection (n = 25)

Timing after CFLD modeling	Experimental Group 3 (BMMCs), n = 25			
	ASAT	ALAT	ALP	Total protein
Baseline	58 ± 8	40 ± 6	240 ± 24	98 ± 20
7 days	350 ± 10*	262 ± 27*	1205 ± 47*	36 ± 16*
1 month	97 ± 16*	89 ± 16*	570 ± 35*	55 ± 10*
2 months	72 ± 15*	70 ± 13*	310 ± 18*	61 ± 8*
3 months	64 ± 12	61 ± 10	274 ± 18	68 ± 12
4 months	59 ± 12	59 ± 6	252 ± 19	78 ± 8
6 months	62 ± 9	46 ± 11	242 ± 12	88 ± 10

Note. * – $p < 0.05$ compared with baseline.

racterizing the functional state of the liver after CFLD modeling and the use of tRNA and BMMCs, we can conclude that both tRNA and BMMCs accelerates restoration of hepatic homeostasis in the body. However, the effect of tRNA was more pronounced.

Studies have shown that both in the clinic (in CLF) and in experiment (in CFLD modeling), restoration of liver biochemical markers under the influence of the therapy applied is usually not accompanied by liver regeneration at the histological level, especially in hepatic cirrhosis [29, 30]. Meanwhile, available data on the fibrolytic effect of hematopoietic and stromal stem/progenitor BMMCs in hepatic cirrhosis [4–9] compelled us to comparatively study the effectiveness of tRNA and BMMCs contained in biologically effective doses not only on biochemical but also on histology markers of the liver in CFLD modeling.

A study of the dynamics of development of morphological changes in rat liver after CFLD modeling showed that cirrhosis had formed in the liver in all the three study groups by the third month of observations. However, for these groups, the intensity of defibrotic processes in liver tissue differed with increasing observation time. One week after being injected in the pericentral zones of the liver, pronounced necrotic and dystrophic changes in hepatocytes, as well as alterations in the lobular structures of hepatic parenchyma were detected in all rats (Fig. 1, a, b).

Three months after inoculation and infusion of saline, a clear disruption in the trabecular patterns of the hepatic tissue and formation of pseudo-lobules were detected in the liver of Group 1 rats (control group); hepatocytes were without pronounced dystrophic changes; mild cell infiltration was also observed (Fig. 2, a, b).

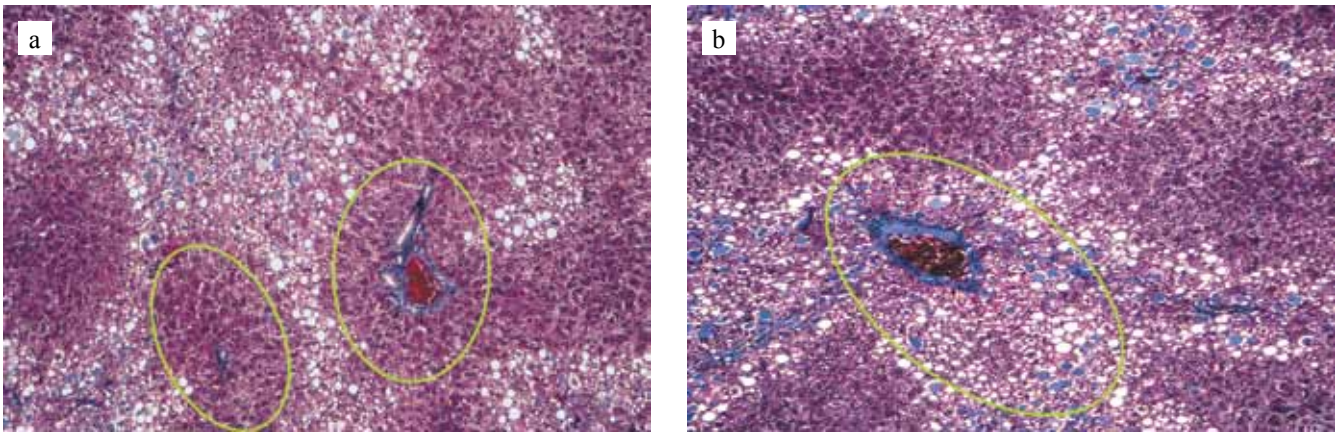

Fig. 1. Histological structure of the rat liver in 1 week after completion of CFLD modeling: a – periportal zone with the minimally expressed degenerative changes of hepatocytes; b – pericentral zone with pronounced necrotic and dystrophic changes of hepatocytes (fat and protein dystrophy); the beginning of connective tissue septa formation (marked by an oval). Masson staining. ×100

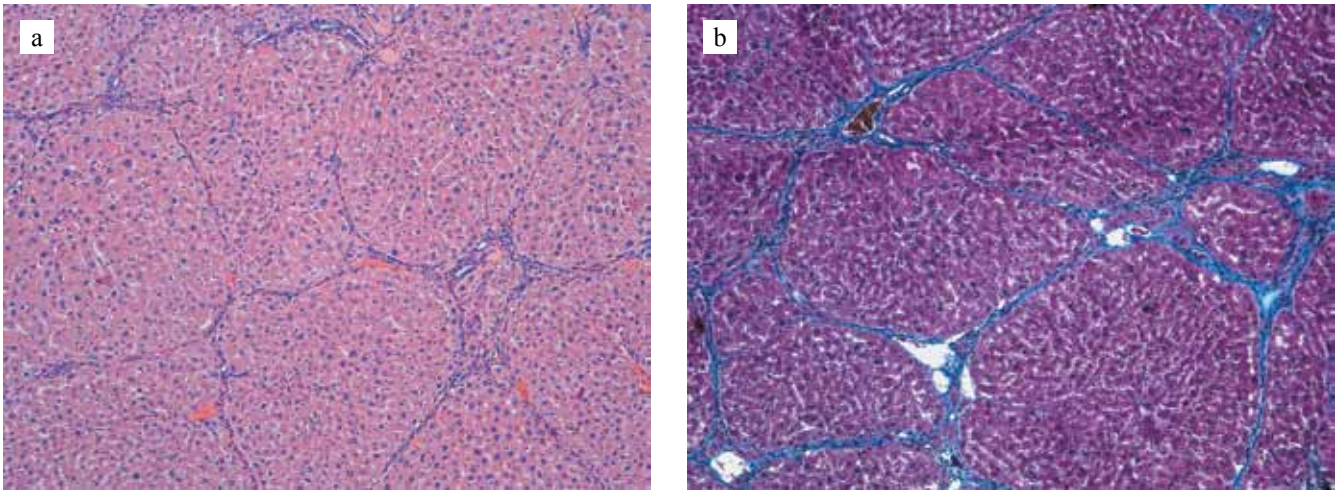

Fig. 2. Histological structure of the rat liver in 3 months after completion of CFLD modeling and administration of saline solution. Numerous false lobules (nodes) of parenchyma bounded by septa. The formed liver cirrhosis: a – hematoxilin-eosin staining; b – Masson staining. ×100

Thus, already by the third month after the end of chronic inoculation in the liver of Group 1 rats (control group), the histological picture of stage 3–4 cirrhosis was formed according to the classification by Desmet et al., 1994 [31]. Similar results of structural disorders in the liver after three months were obtained in Group 3 rats (experimental group) injected with BMMCs (Fig. 3).

Meanwhile, distinct signs of septal fibrolysis of the formed pseudo-lobules were detected at the third month in Group 2 rats (experimental group) – against the background of cirrhosis that formed. These rats were at the

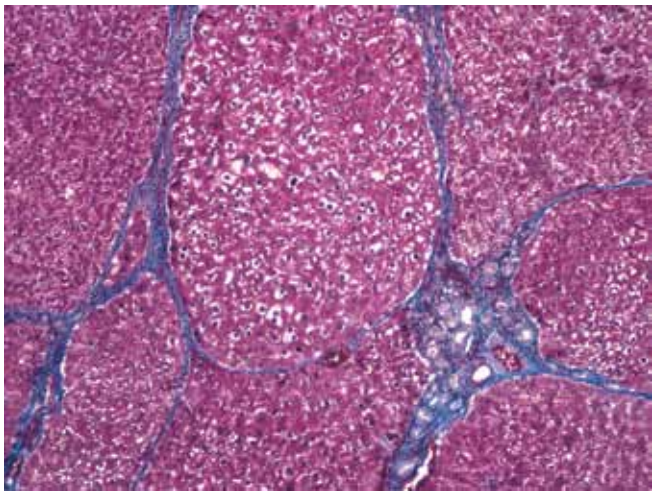

Fig. 3. Histological structure of the rat liver in 3 months after completion of CFLD modeling and administration of unsorted BMMC. The formed false lobules (nodes) of parenchyma restricted by septa . The formed liver cirrhosis. Masson staining, ×100

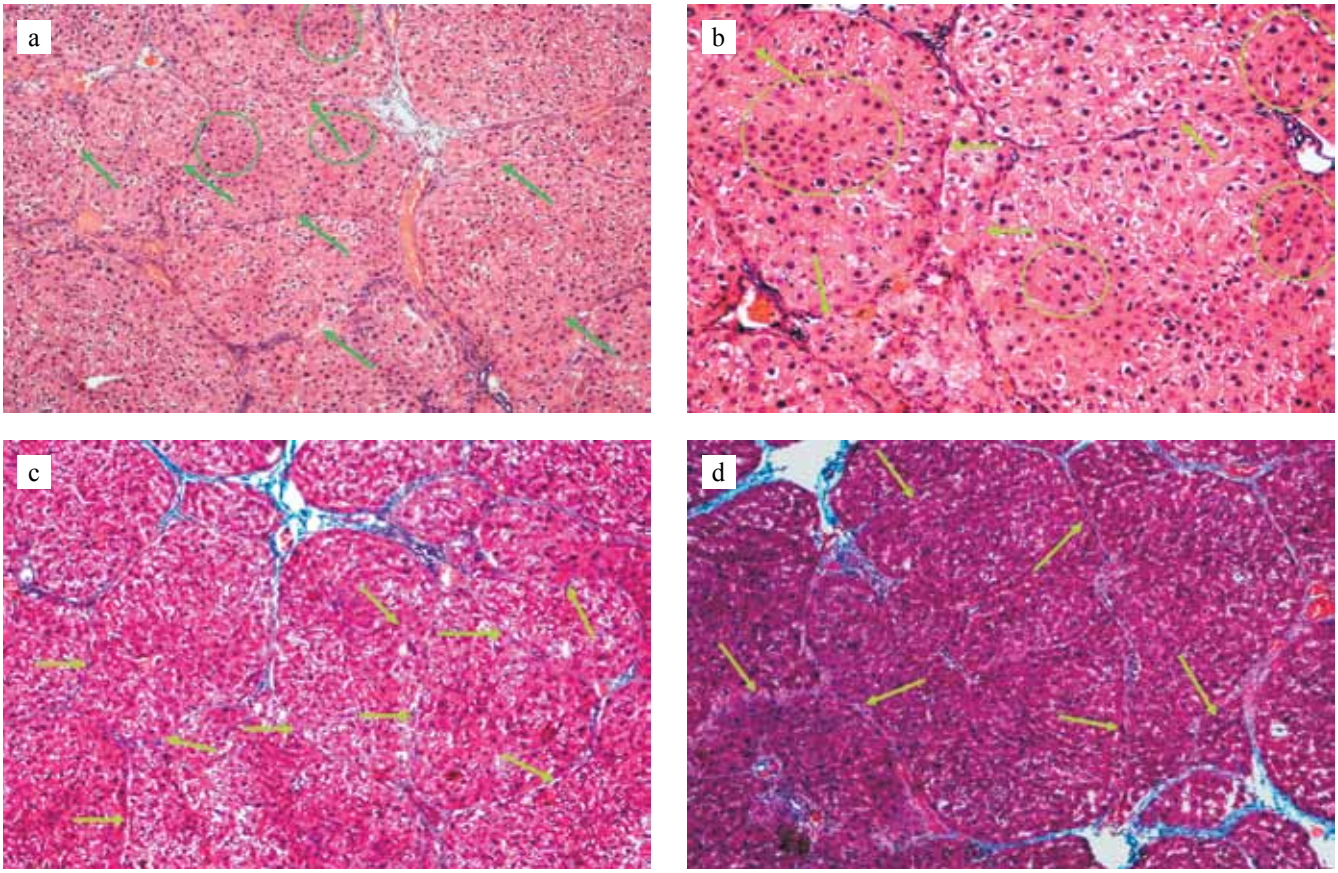

Fig. 4. Histological structure of the rat liver in 3 months after completion of CFLD modeling and infusion of the tRNA from BMMC at a dose of 30 мкг/100 g of animal weight. The septal lysis of false lobules and appearance of significant areas of young hepatocytes. The arrows indicate the zones of lysed septa; the areas of accumulation of young hepatocytes are circled by an oval; a and b – hematoxilin and eosin staining; c and d – Masson staining; a, c – ×100; b, d – ×200

end of inoculation administered once with tRNA from BMMCs. Besides, significant areas of young newly formed hepatocytes were detected in the structure of pseudo-lobules (Fig. 4, a, b, c, d). From our results, it can be argued that tRNA inhibits liver fibrogenesis by the third month unlike in the control Group 1 and experimental Group 3, where fibrotic processes remain clearly pronounced. However, at the third month, pseudo-lobules and alterations in the trabecular patterns of the liver remain in experimental Group 2, as well as in the control group (Group 1) and experimental group (Group 3).

In continuing the dynamic study of morphological changes in the liver of rats in the control and experimental groups, we noted that after six months, spontaneous lysis at the septa of the pseudo-lobules and a zone of young hepatocytes appear in the liver of rats in Group 1 (control group) and experimental Group 3 (Fig. 5, a, b, c, d).

At the same time, the histological pattern of the liver of rats in experimental Group 2 was fully restored (Fig. 6, a, b, c) by the sixth month after tRNA administration: the structure of hepatic lobules and their trabecular patterns were restored, the connective tissue septa were completely lysed in the liver tissue and no other structural alterations were detected.

Based on results obtained from comparative examination of liver tissue in the three groups, we conclude that tRNA from BMMCs accelerates the regeneration of liver tissue structure after chronic toxic fibrosing effects on it. We also state that rat liver tissue has a huge plasticity potential, since it retains the ability for spontaneous lysis of the fibrous tissue for a long time. At the same time, injection of BMMCs exerted its regulatory effect only on the functional markers of the liver. It almost did not affect the rate of restoration of structural alterations in it because defibrotic processes in the liver were activated at the same time as in the control group. Our results are fully consistent with the observations of clinicians on the use of BMMCs in chronic alcoholic liver cirrhosis [32]. By the ninth month after chronic inoculation of rats, the liver tissue structure in the control group (Group 1) and experimental Group 3 was normalized, although in the liver of Group 1 rats, sections of connective tissue septa were preserved in separate fields of view (Fig. 7, a and b).

CONCLUSION

Based on our study of the dynamics of liver regeneration after CFLD modeling and use of BMMCs and tRNA from BMMCs, we make the following conclusions.

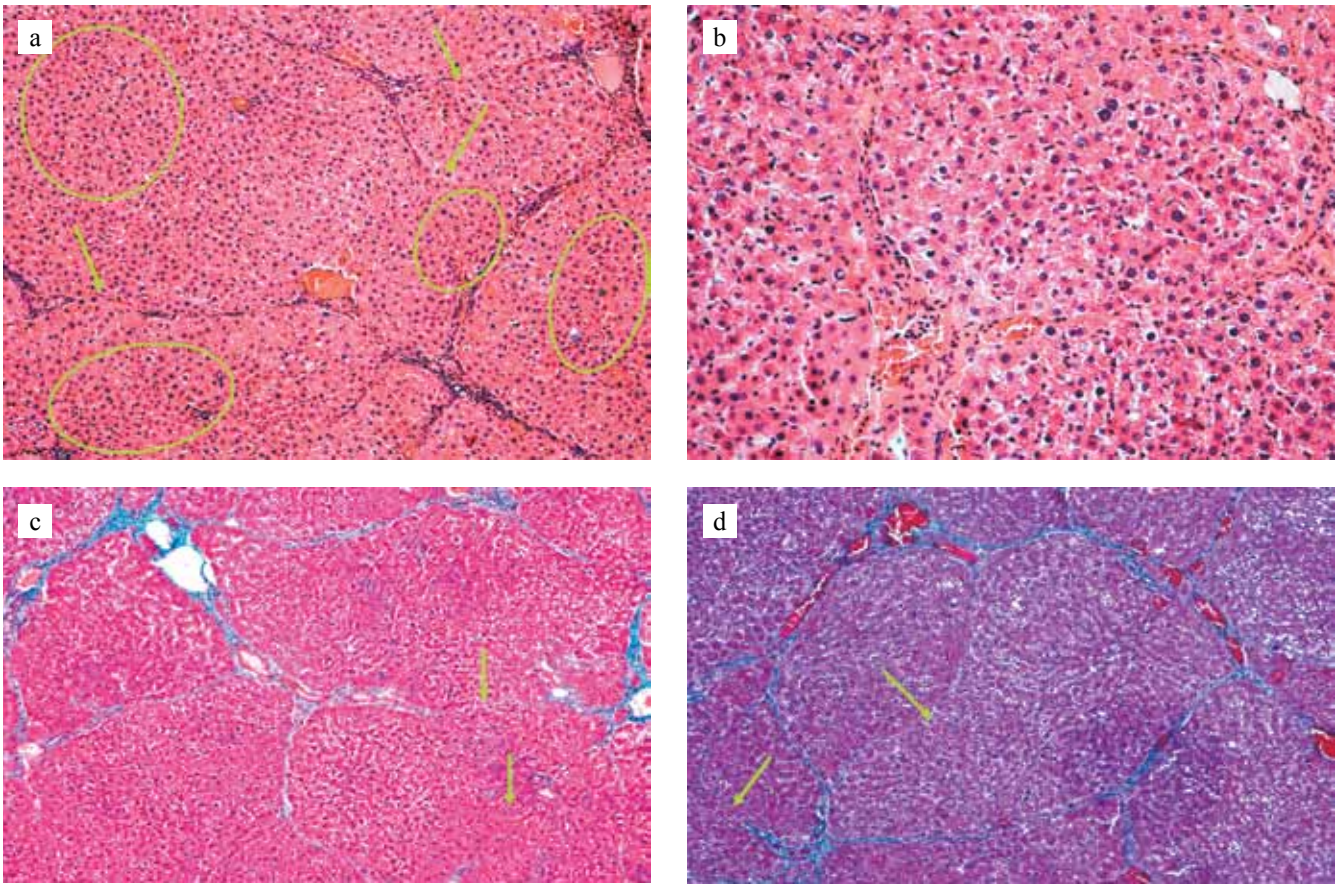

Fig. 5. Histological structure of the rat liver in 6 months. after completion of CFLD modeling and infusion of saline (a, c) and BMMC (b, d). The arrows indicate areas of septal fibrolysis of false lobules; it is seen also the areas of young (newly formed) hepatocytes (marked by an oval); a and b – hematoxilin and eosin staining; c and d – Masson staining; a, c, d – ×100, b – ×200

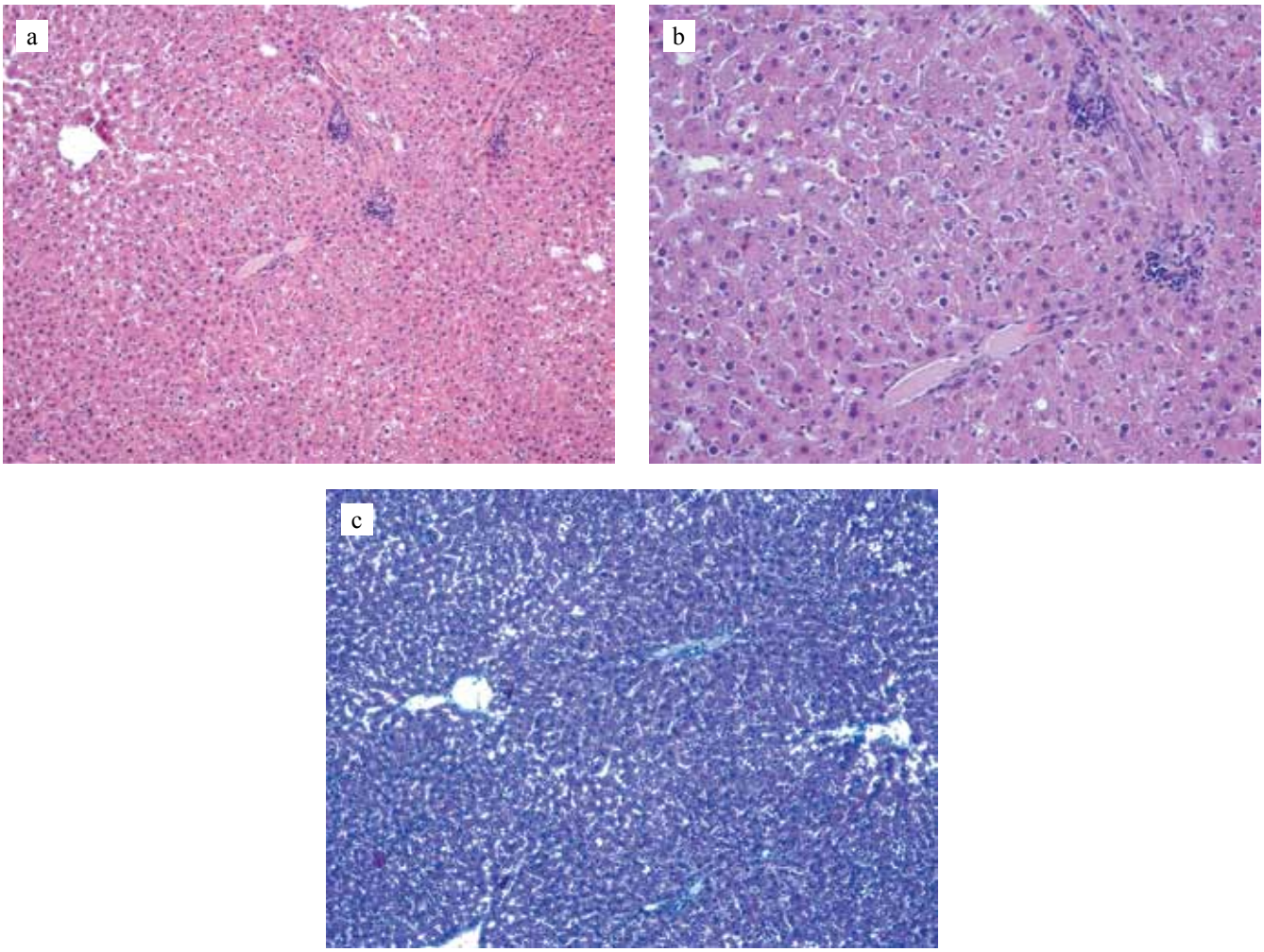

Fig. 6. Histological structure of the rat liver in 6 months. after completion of CFLD modeling and infusion of the total RNA from BMMC. The restoration of liver tissue structure. The absence of pronounced morphological signs of parenchymal fibrosis: a and b – hematoxilin and eosin staining; c – Masson staining; a and c – ×100; b – ×200

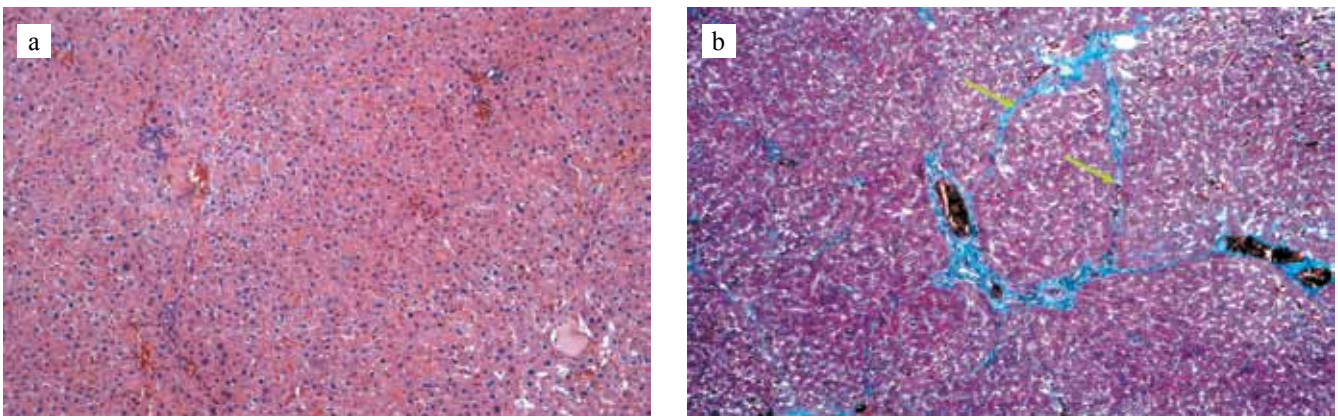

Fig. 7. The Histological structure of the rat liver in 9 months. after completion of CFLD and infusion of saline (control). The arrows indicate the remaining areas of the connective tissue septa; a – hematoxilin and eosin staining; b – Masson staining. ×100

– The liver of initially healthy rats has an extremely high plasticity potential since despite chronic injection through combined use of CCl4 and Freund's incomplete adjuvant, fibrotic processes can be reproduced in the liver within a period not exceeding six months. Six months after inoculation based on the proposed scheme, spontaneous lysis of fibrous tissue in the liver begins and progresses in time.
– Administration of BMMCs of healthy rats helps to accelerate restoration of the functional (biochemical) marker of damaged liver and does not affect the rate of liver regeneration, since disintegration of fi-

brous tissue began and zones of young hepatocytes appeared 6 months after inoculation, as in the control group.

– Administration of tRNA extracted from the BMMCs of healthy rats accelerates regenerative processes in a damaged liver. This manifests as early restoration of the functional and morphological markers of liver function unlike in the control group. After one administration of tRNA, the fibrous septa of the pseudo-lobules start disintegrating and numerous zones of young (newly formed) hepatocytes form – after 3 months; Complete liver regeneration occurs by the 6th month after tRNA administration. Regeneration of the liver tissue structure in control rats and rats injected with BMMCs occurs only by the ninth month.
– tRNA extracted from the BMMCs of a healthy donor is recommended as an alternative tool for exerting a biotechnological effect on reparative regeneration of the organ under the proposed experimental CFLD model.

The authors declare no conflict of interest.

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