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APOPTOTIC BONE MARROW-DERIVED MONONUCLEAR CELLS ACCELERATE LIVER REGENERATION AFTER EXTENDED RESECTION

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Objective: to compare the efficiency of regenerative processes in the liver using apoptotic bone marrow-derived mononuclear cells (BMMCs) and intact BMMCs from healthy animals on an extended liver resection (ELR) model. **Materials and methods.** Male Wistar rats (n = 77) with an ELR model (70–75%) were divided into 3 groups: group 1 (control with a single intraperitoneal injection of saline), group 2 (single intraperitoneal injection of unsorted intact BMMCs at a dose of 30–35 × 10^6, and group 3 (single intraperitoneal injection of apoptotic BMMCs at the same dose). Restoration of biochemical parameters of liver function and mass, as well as the emerging microstructural changes in hepatocytes in histological preparations, were monitored by assessing hepatocyte mitotic activity (MA) during the first 7–10 days after ELR. **Results.** It was found that in groups 2 and 3, as compared with group 1, there was no death after ELR modeling, and that the biochemical parameters of liver function normalized more rapidly (at days 10–14). Hepatocyte MA in group 3 sharply increased as early as on day 1, and mitotic index (MI) averaged 14‰, reaching 20.9‰ in some experiments; MI in the control group remained at the baseline by this time, while in group 2, MI was only 3.2‰. In group 3, liver mass recovered more rapidly after ELR to baseline values already at days 8–10, whereas the recovery was at day 12–14 and day 17–20 in group 2 and group 1, respectively. It was suggested that the more pronounced increase in the efficiency of regenerative processes in the liver after ELR in group 3 after using apoptotic BMMCs was due to the release from these cells of a large spectrum of formed paracrine factors, including various classes of RNA molecules involved in the regeneration process. **Conclusion.** Apoptotic BMMNCs have a more effective adaptive and regulatory potential than intact BMMCs because reorganizations are rapidly formed in the damaged liver cells, providing an early and more powerful activation of the targeted regenerative program.

Keywords: apoptotic bone marrow cells, regeneration, liver resection.

INTRODUCTION

It is known that damage to the liver and to other organs, triggers adaptive processes in them, which, in turn, activates evolutionarily programmed reparative regeneration mechanisms. Meanwhile, under severe chronic or extensive acute liver injury (under conditions when a significant mass of cells perishes), the remaining cells are forced to perform their functions with an increased load, which exceeds the evolutionarily programmed norm of cellular energy expenditure for these processes.

Under the created conditions, due to the developing energy deficit in liver cells, activation of the reparative regeneration mechanisms is sharply inhibited, which is believed to be a consequence of insufficient efficiency of adaptation processes, energetically supporting the regenerative process.

According to modern concepts, adaptive restructuring of metabolism in tissues begins with the development of cell autophagy processes in them [1–3]. Therefore, the initially reduced level of cell autophagy activity in an organ predetermines the low regenerative potential of the remaining cells and insufficient efficiency of regenerative processes [4–6]. To activate regenerative processes in the damaged organs, it was proposed to use hematopoietic and stromal cells derived from the bone marrow, which are known to have the highest regenerative potential in the body. However, the experience in clinical application of bone marrow-derived cells (BMDCs) turned out to be not so convincing and not always reproducible [7, 8], which forced researchers to start directly studying the mechanisms of induction of regenerative processes caused by BMDCs to increase the therapeutic efficiency of their use. A hypothesis on the determining regulatory role of apoptotic BMDCs producing paracrine factors in the state of apoptosis has been put forward [9]. The validity of this hypothesis has been subsequently proven [10] and repeatedly confirmed [11, 12]. By now, it has been established that it is the apoptotic cells that release

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growth-stimulating signals in the form of nanovesicles [13], lipids [14], exosomes, various miRNAs, proteins [15] and other components called secretomes into the body. The result is not only an acceleration of regenerative processes in the body, but also an increase in their efficiency through immunomodulatory effects and blocking of inflammatory reactions [16, 17].

Existing ideas about the mechanisms of induction of regenerative processes suggest that in order to produce an effective regenerative response in cells in a damaged organ, the impact force of the adaptive stress signal should be high enough to enhance the severity of autophagy process and even reach the state of reversible apoptosis by cells, but should not exceed the evolutionarily programmed response, i.e. be physiological. Apoptotic BMDCs, which, as it has already been shown [11], support renewal and replenishment of the blood and immune system cell composition in the body, obviously possess such properties – adequate and physiological adaptogen.

The objective of this study is to investigate the possibility of increasing the regenerative activity of liver cells after extended resection by using apoptotic BMMCs of a healthy donor as an adaptive and regulatory stress signal.

MATERIALS AND METHODS

Work was carried out on male Wistar rats weighing 250–300 g (n = 77). The animals were kept in the vivarium at 18–20 °C on a mixed diet with free access to water. Experiments on animals were performed in the morning hours at room temperature (t = 22–24 °C), which excluded the influence of diurnal fluctuations in MA of liver cells. Relative humidity was 50–65%, lighting cycle was 12 hours; room air volume was changed ten times per hour. The animals were fed with standard compound feed for laboratory animals (microbiological status corresponded to GOST R 51849-2001 "Veterinary and sanitary standards and quality requirements for nonproductive animals"). Filtered tap water ad libitum was delivered in standard drinking bottles (microbiological status of water corresponded to SanPiN 2.1.4.1074-01 "Hygienic requirements for water quality in centralized drinking water supply systems"). Experiments and all manipulations with animals were performed according to the rules adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), Strasbourg, 1986).

The ability of apoptotic BMMCs to amplify regenerative signals and ensure their targeted delivery to the damaged liver tissue was studied in an ELR model (70–75%) in rats, which is accompanied by activation of hypertrophic regeneration mechanisms with marked hepatocyte MA in the remaining part of the organ [18].

Before the ELR modeling, the operated rats (n = 65) were anesthetized by diethyl ether inhalation, then the abdominal cavity was opened in compliance with aseptic and antiseptic rules, the liver was removed into the wound and ligatures were sequentially applied to the bases of medial, left lateral and right upper lobe of the liver, after which they were removed (70–75% of total liver mass). Surgery and subsequent studies were always performed between 10 and 12 hours, when the diurnal rhythm of MA in liver cells was minimal. In the early postoperative period, the operated animals always developed a clinical picture of acute liver failure.

To prove the possibility of enhancing the regenerative activity of cells in the damaged liver using apoptotic BMMCs, healthy rats without ELR were used as donors (n = 12). An unsorted mononuclear (hematopoietic) cell fraction was obtained from the bone marrow of these rats for subsequent administration to ELR rats at a dose of $30–35 \times 10^6$ cells. All animals after ELR were divided into 3 groups: Group 1 (control, n = 25, rats were injected once intraperitoneally with 1.0–1.5 ml of saline), Group 2 (n = 25, experimental, 1.5 ml of $30–35 \times 10^6$ freshly isolated intact BMMCs were injected intraperitoneally 3–5 hours after ELR modeling), and Group 3 (n = 25, experimental, 1.5 ml of $30–35 \times 10^6$ apoptotic BMMCs were injected intraperitoneally 3–5 hours after ELR modeling). Apoptotic BMMCs were obtained by incubating freshly isolated BMMCs in Custodiol ion-balanced preservative solution (Bretschneider's HTK-solution) at a temperature of 4–6 °C for 48 hours, because according to our studies [19], under the specified storage regimes, the content of apoptotic BMMCs – (cell secretomes) in the state of early reversible apoptosis was significantly expressed and reached 44.8 ± 0.9%, while the content of apoptotic BMMCs in the state of late irreversible apoptosis in the cell pool did not exceed 2–8% ($p < 0.02$).

The dynamics of restoration of hepatic homeostasis in rats after injection of ELR and BMMCs (intact and apoptotic) were studied using standard methods on biochemistry analyzer Arik-test (Germany) according to the content of total protein and bilirubin in the blood serum in the early postoperative period (within 14 days). We also measured the activity of hepatic cytolysis enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). We evaluated the rate of overcoming the critical mass of the liver residue and its recovery to baseline values after ELR within 20–22 days. For this purpose, we weighed the resected part of the liver in each operated animal immediately after ELR, which was taken as 70% of the total liver mass, and then the initial liver mass was calculated for each animal on the basis of the weighing results. Then, the remaining liver was explanted at each study period, its mass was determined by weighing, and the values obtained were compared with the calculated initial liver mass for the given animal.

The degree of severity of ELR induction effect on regenerative processes in the liver during modeling of critical injury (group 1), as well as changes in the nature

of regenerative processes in the liver after ELR against the background of introduction of intact BMMCs (group 2) and apoptotic BMMCs (group 3) were determined quantitatively by degree of changes in the microstructural state of liver cells in histological preparations. For this purpose, in groups 1, 2 and 3, we studied hepatocyte MA in the liver remnant at 24, 36, 48 and 72 hours, as well as on day 5, 7 and 10 after ELR. The liver was dissected on these time points and histological specimens stained with hematoxylin and eosin. We performed histological analysis of the preparations (Leica DM microscope, Germany), and measured the number of dividing hepatocytes in 30 fields of view, followed by calculation of MI in ppm (‰).

Significance of difference between the studied parameters in the compared groups was assessed using the parametric Student's t-test at $p < 0.05$.

RESULTS

A comparative study of the recovery of liver homeostasis in the 3 groups showed that in the control group 1, 5 out of 25 rats died within the first 5 days after liver resection (20% mortality). In experimental groups 2 and 3 (administration of intact BMMCs (n = 25) and apoptotic BMMCs (n = 15), respectively), there were no dead animals during the entire follow-up period. The absence of mortality in these groups was accompanied by a higher rate of recovery of hepatic homeostasis in the body, which was expressed in an early normalization of total protein and cytolytic enzymes (ALT, AST and ALP) in the blood serum compared with group 1.

Cytolysis indicators in groups 2 and 3 rats, as in group 1, increased during the first 3 days, but then in group 3, they stabilized more sharply than in group 2 and recovered by day 10–14. The reduced level of total protein after ELR in these groups also recovered by the end of the follow-up period (14 days), while in the control group, normalization of all studied parameters did not occur by the end of the observation period. The higher rate of recovery of hepatic homeostasis in groups 2 and 3 compared with the control can be down to the higher activity of restorative and proliferative processes in the cells of the remaining part of the liver, which was due to the introduction of intact BMMCs and apoptotic BMMCs. Indeed, a comparative study of hepatocyte MA in the liver after ELR in all three studied groups revealed a rapid increase in hepatocyte MA in comparison with the initial level (MI was 0.2–0.3‰ before ELR). At the same time, the rate of development and the severity of the rise of MA in groups 2 and 3 were higher than in the control group (Fig. 1).

So, in group 1 (control), MI reached a maximum of 5.378‰ (36 mitoses per 6,653 cells) at 48 hours after ELR. In group 2, MI at 48 hours was 6.11‰ (60 mitoses per 8,448 cells) with a maximum of 10.5‰ at 72 hours (93 mitoses per 8,858 cells). In group 3, MI reached a maximum as early as 24 hours after ELR modeling. On average, it was 14‰ (135 mitoses per 9,762 cells), but in some experiments, MI reached 20.9‰. Thus, it follows from the maximum MI values in the three studied groups that apoptotic BMMCs increase hepatocyte MA almost 1.5-fold in comparison with intact BMMCs administration and almost 3-fold increase in hepatocyte MA in comparison with the control. It is important to note that apoptotic BMMCs not only intensify, but also accelerate the realization of their regulatory effect (as early as 24 hours after ELR modeling) compared to the control and the use of freshly isolated BMMCs. This fact may be due to the fact that immediately after introduction of apoptotic BMMCs into the body, they facilitate delivery and production of a complex of numerous growth-stimulating signal molecules and regulatory factors already formed in apoptotic cells [20], which provide early intensive activation of regenerative and proliferative processes in the lesion site. The maximal high and early activation of hepatocyte MA in liver tissue after ELR modeling and application of apoptotic BMMCs is also demonstrated in Fig. 2.

It can be seen (see Fig. 2) that at 24 hours, only in group 3 with apoptotic BMMCs (Fig. 2, d) that early and maximum activation of hepatocyte MA occurred, whereas in experiments with freshly isolated intact BMMCs (see Fig. 2, c), MA was just beginning to intensify. In the control with saline (Fig. 2, b), MA was totally absent at this time point, but maximally intensified only at 48 hours. Moreover, in the liver cells of groups 2 and 3 rats, signs of diffuse fine vacuolar degeneration of hepatocytes appeared 24 hours after ELR modeling, which is known [21] to be a morphological marker of the developing cellular autophagy. At the same time, the greatest expression of hepatic cell autophagy (Fig. 3) was

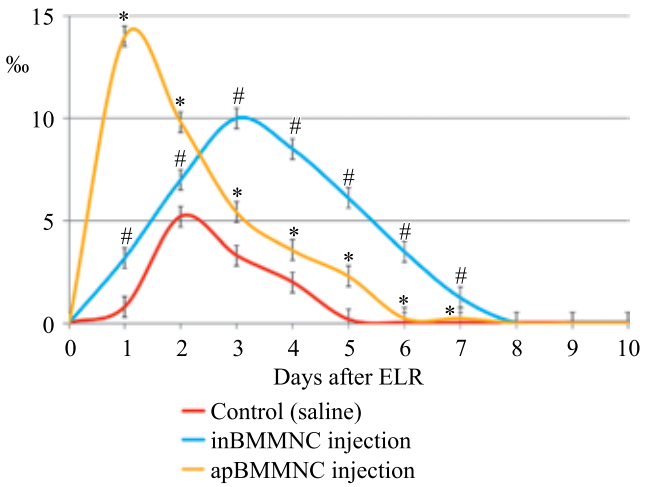

Fig. 1. Changes in mitotic index in hepatocytes in the liver of rats after ELR (‰) in 3 experimental groups. *, $p < 0.05$ compared to control; #, $p < 0.05$ compared to injection of apoptotic BMMCs

observed at 48 hours in group 3 (apoptotic BMMCs). See Fig. 3, d.

Morphological study of the liver confirms a more pronounced ability of apoptotic BMMCs compared to intact BMMCs to stimulate adaptive changes in cells at the initial stages after damage for optimal energy restructuring of metabolism in cells and early start of repair and mitotic processes in the damaged organ [2].

Another confirmation of a more pronounced inductive effect of apoptotic BMMCs on regenerative processes in the liver as compared to intact BMMCs is the dynamics of liver mass recovery in the three studied groups of animals with ELR modeling (Fig. 4).

The most accelerated rate of liver mass recovery (see Fig. 4) was observed in group 3, where apoptotic BMMCs were injected intraperitoneally in a dose of 30–35 × 10^6 cells 3–5 hours after ELR. Liver mass in this group was restored on days 8–10. Injection of intact BMMCs in the same dose in group 2 also accelerated reparative processes in the resected liver remnant, liver mass restoration to the initial values in this group occurred on day 12–14, i.e. the recovery occurred at a slower pace. In group 1 (control), restoration of liver mass after injection of saline occurred on day 17–20. Earlier we showed [22] that intraperitoneal administration of total RNA, obtained from freshly isolated intact BMMCs at 30 μg/100 g mass, also intensified the regenerative activity of rat liver cells after ELR modeling. The regenerative response of the liver to administration of total RNA from intact BMMCs was higher than that to the administration of freshly isolated intact BMMCs. So, when total RNA from intact BMMCs was injected, the MI at 48 hours was 23.4‰, and when intact BMMCs were injected, MI at 48 hours was 6.96‰, and liver mass recovery occurred on days 10–12 and 14–18 respectively. Similarity in the direction and higher efficiency of regulation of recovery processes at administration of total RNA from both freshly isolated intact BMMCs and apoptotic BMMCs as compared to freshly isolated intact BMMCs suggests the following mechanism. In the process of isolation of total

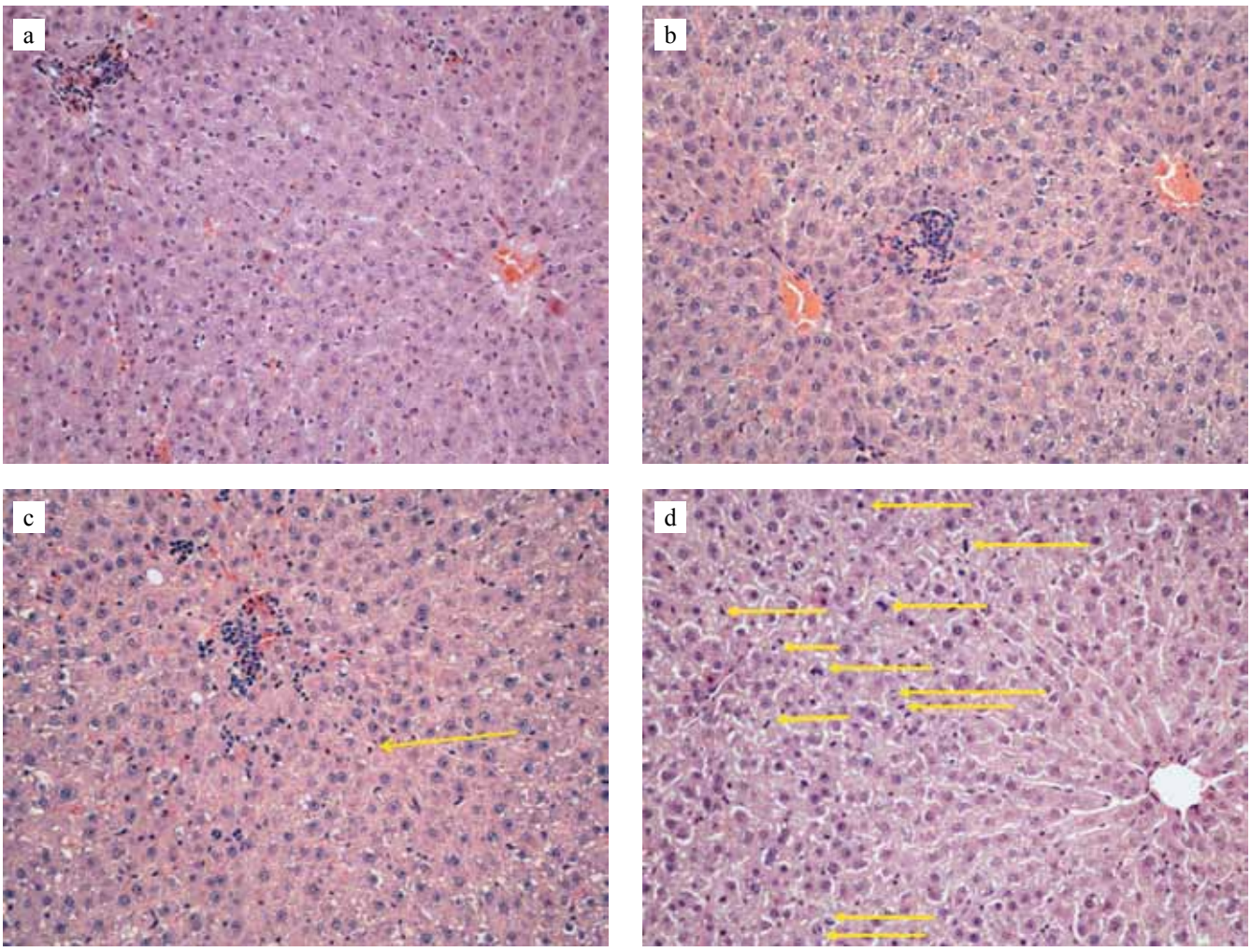

Fig. 2. Hepatocyte mitotic activity in rat liver before and 24 hours after ELR and injection of BMMCs: a, baseline (before ELR); b, group 1 (control), saline injection; c, group 2, injection of intact BMMCs; d, group 3, injection of apoptotic BMMCs. Arrows indicate hepatocytes at different stages of mitosis. H&E stain, 200× magnification

RNA from intact BMMCs, these cells are also exposed to apoptosis. Cell apoptosis is known to promote the ability of such cells to produce numerous and diverse paracrine factors, including various types of RNA and microRNA [9, 15]. The accumulating paracrine factors of apoptotic cells additionally exert a powerful stress-regulatory effect on repair processes in the damaged organs.

The results obtained, in our opinion, suggest that apoptotic BMMCs, introduced into the body against the background of ELR, act as an adequate evolutionarily engineered adaptogen within a non-specific adaptation syndrome of cellular systems. This adaptogen is designed to incorporate and optimize the survival reserves of cells in the damaged organ, by abrupt and accelerated switching of evolutionarily programmed mechanisms of cell death (such as autophagy and reversible apoptosis) to cell proliferation. The possibility of existence of such switching mechanisms in cells in a co-activated state has been discussed [23, 24].

CONCLUSION

In the early stages after ELR modeling, apoptotic BMMCs and freshly isolated intact BMMCs enhance mitotic activity in liver cells in comparison with the control (administration of saline). However, the severity of enhancement of the activation effect on liver cell proliferation when using apoptotic BMMCs is significantly higher than when using intact BMMCs. With intraperitoneal injection of apoptotic BMMCs, MI reaches its maximum values at 24 hours, whereas with intact BMMCs – only at 72 hours.

Early vacuolization of hepatocyte cytoplasm in the liver after ELR modeling, which is a morphological marker of cell autophagy and reflects activation of the evolutionarily programmed process of their adaptation to damage, develops in all groups of experiments at 24 hours, but more intensively at 48 hours. Early vacuolization was more pronounced in group 3 (apoptotic BMMCs) than in group 2 (intact BMMCs) and group 1

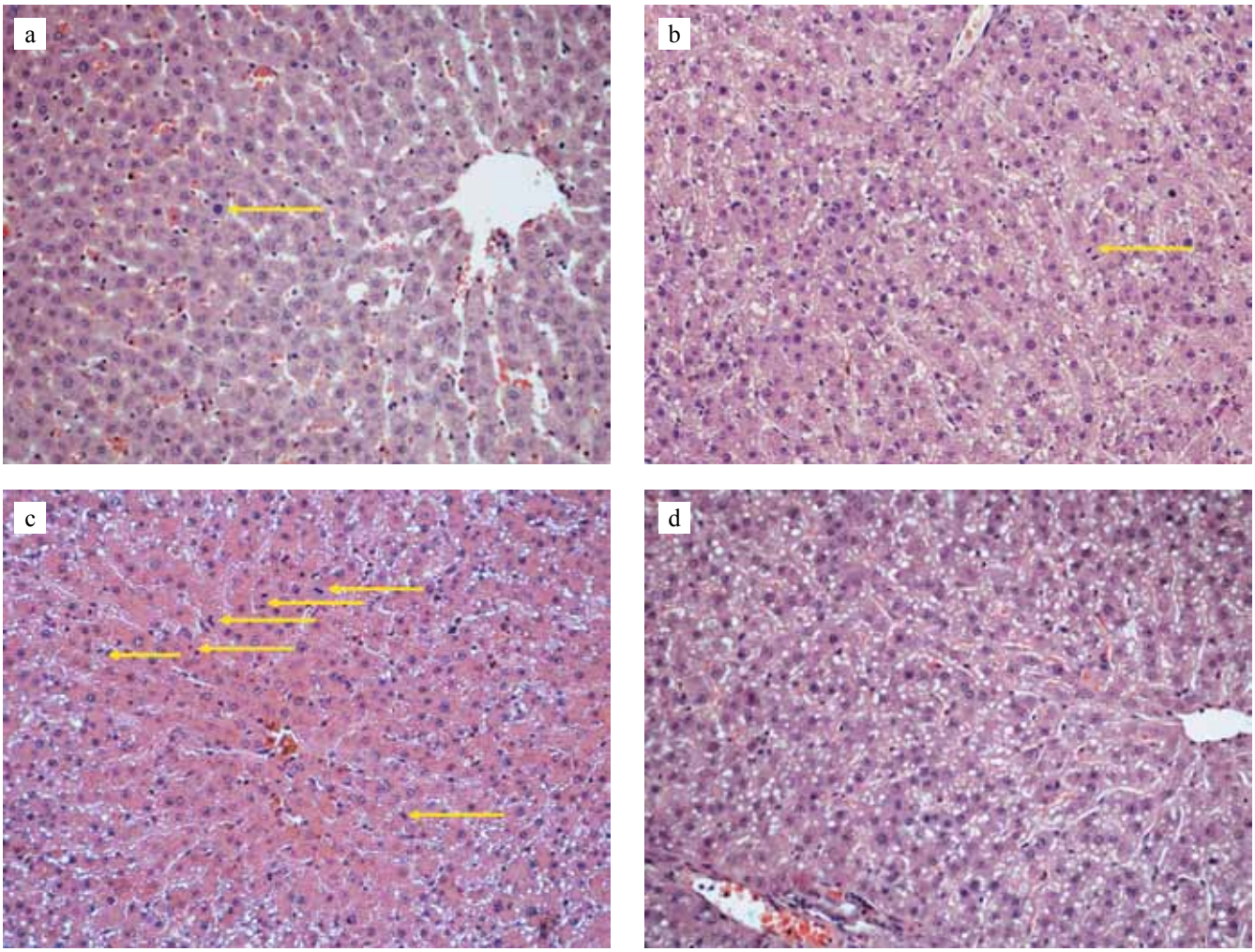

Fig. 3. Histological structure of the rat liver before and 48 hours after ELR modeling and BMMNC injection. Changes in hepatocyte morphology: a, baseline (before ELR), b, group 1 (control), saline injection; c, group 2, injection of intact BMMCs; d, group 3, injection of apoptotic BMMCs. Arrows indicate hepatocytes at different stages of mitosis. H&E stain, 200× magnification

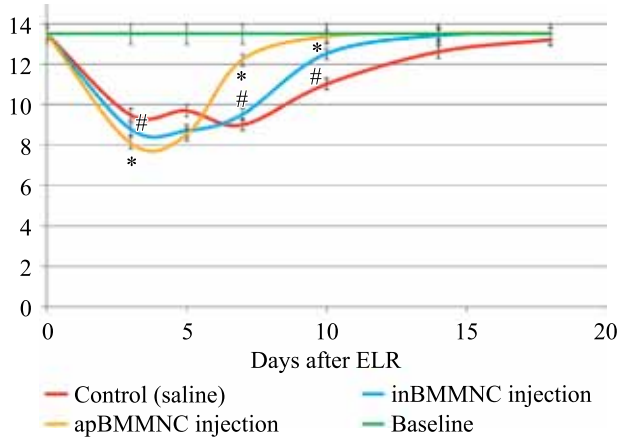

Fig. 4. Recovery of rat liver mass after extended resection in 3 experimental groups: with saline injection, with injection of intact BMMCs and with injection of apoptotic BMMCs (in grams). *, $p < 0.05$ compared to control; #, $p < 0.05$ compared to injection of apoptotic BMMCs

(saline), indicating the higher regulatory capabilities of apoptotic BMMCs.

Apoptotic and intact BMMCs provide targeted transmission of regenerative signals to the resected liver and accelerate its regenerative process. However, the rate of acceleration of reparative processes and the timing of restoration of the liver mass to its initial values for apoptotic BMMCs were higher (day 8–10) than for intact BMMCs (day 12–14) and saline (day 18–20).

All of the above suggests that apoptotic BMMCs, due to their acquisition of a more powerful regulatory stress-induced potential, unlike intact BMMCs, have more pronounced adaptive and regulatory properties that create a stronger foundation in the body for implementation of a targeted and more effective regeneration program. Higher intensity of the adaptive effect of apoptotic BMMCs due to the release of numerous and diverse paracrine factors (including various types of RNA) promotes early and more effective activation of autophagy processes in liver cells after ELR. This induces distinct increase in regenerative activity and increases the rate of recovery of resected liver mass to initial values.

The authors declare no conflict of interest.

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