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THE ROLE OF APOPTOTIC BONE MARROW CELLS IN ACTIVATION OF LIVER REGENERATION

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Objective: using an adoptive transfer model to study the cellular mechanisms involved in the formation of the initial stage of liver regeneration during intraperitoneal injection of a healthy recipient with apoptotic bone marrow-derived mononuclear cells (BM-MNCs) from a donor after extended liver resection. **Materials and methods.** Male Wistar rats (n = 40) were used to create a model of adoptive transfer of apoptotic BM-MNCs (a-BM-MNCs) taken from the donor after extended liver resection to a healthy recipient. During the experiments, the animals were divided into five groups. Four experimental groups with intraperitoneal injection of the same doses to the recipient: freshly isolated BM-MNCs (group 1); BM-MNCs subjected to apoptosis for 48 hours by storage at t = 4–6 °C in phosphate-buffered saline (PBS) (group 2) or in a Custodiol HTK solution (group 3). In group 4, the animals were injected with PBS after storing BM-MNCs in it. The control animals were animals injected with saline (group 5). For selection of effective modes of apoptosis induction, BM-MNCs stained with 7AAD after incubation in solutions were analyzed by flow cytometry. Targeted transfer of regenerative signals to the recipient was assessed by the mitotic activity of hepatocytes in the liver and tubular epithelium in the kidneys, as well as by the intensity of microstructural changes in the liver 24, 48 and 72 hours after injection of the studied material. **Results.** BMC incubation in PBS and HTK for 48 hours at t = 4–6 °C provides the most effective accumulation of a-BM-MNCs in early apoptosis. It was shown that a-BM-MNCs retain the ability to target-focused transmission of regulatory signals to the liver supported by autophagy process during adoptive transfer. It was established that a-BM-MNCs (groups 2 and 3) in comparison to native BM-MNCs (group 1) at adoptive transfer increased the regenerative potential of the liver due to pronounced increase in the activity of autophagy processes and directed infiltration of immunomodulatory mononuclear cells in the liver. **Conclusion.** a-BM-MNCs create a stronger basis for development and implementation of a targeted and effective regeneration program by enhancing autophagy processes and immunomodulatory effect on mononuclear cells, which are regenerative signal carriers.

Keywords: apoptosis, autophagy, bone marrow cells, liver, resection, regeneration.

INTRODUCTION

Organ and tissue damage through autoregulation mechanisms involves evolutionarily developed regeneration processes in the body. Targeted transport of regenerative signals to the damaged tissues is facilitated by mononuclear immunoregulatory blood cells and primarily by lymphocytes [1–3]. Hematopoietic and bone marrow-derived mesenchymal stem/stromal cells (BM-MNCs) also have a high regenerative potential. However, the effect of clinical application of BM-MNCs turned out to be not so pronounced and not always reproducible [4, 5], which made researchers turn to the study of how the regenerative processes of BM-MNCs are activated in order to increase their regulatory role.

The initial opinion about the influence of the processes of transdifferentiation or fusion of bone marrow-derived stem/progenitor cells with differentiated cells on the regeneration of damaged tissue/organ was not confirmed [6–9]. Activation of regenerative processes under the influence of BM-MNCs, as well as the supernatant obtained after their cultivation, was attributed to the action of paracrine factors secreted by these cells [10–13].

Further study of alternative mechanisms of induction of regenerative processes by bone marrow cells [14], allowed Thum et al. to put forward a hypothesis [15], which states that cells producing paracrine factors in the state of apoptosis, whose content in the BM-MNCs pool varies from 5 to 25%, are responsible for enhancement of regenerative processes during therapy with these cells.

At first glance, the opinion that dying apoptotic cells can increase the survival of other cells seems absurd, but at the same time, such a view contains an evolutionary justification and rationalism used by living organisms to

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maintain their vital activity [16–19] and protect against the development of pathological conditions [20–22].

It is known that it is the apoptotic cells that release growth-stimulating signals in the form of nanovesicles [23], lipids in exosomes [24], microRNA and proteins [25], which not only accelerate repair processes in the body, but also have immunomodulatory effects, blocking inflammatory reactions, which, being an integral part of any damage, prevent regeneration.

To date, the hypothesis that apoptotic bone marrow-derived cells play a determining regulatory role in regenerative processes has been proven [26] and has been repeatedly confirmed by preclinical studies on cell suspensions and in experiments on animals with simulation of various pathological processes [27].

Meanwhile, early changes in damaged organs arising in response to induction effect of apoptotic bone marrow-derived mononuclear cells (a-BM-MNCs) regulating regenerative processes more efficiently have not been the subject of special studies.

The aim of this study is to investigate the cellular mechanisms of formation of the early stage of regenerative processes in the liver during intraperitoneal injection of healthy recipient with a-BM-MNCs of donor after extended liver resection (ELR) on an adoptive transfer model.

MATERIALS AND METHODS

The work was performed on male Wistar rats weighing 250–300 g (n = 40). The ability of a-BM-MNCs to regulate and target regenerative signals to the damaged organ (liver) tissue was studied by adoptive transfer method [1]. For this purpose, created in the donor was an experimental ELR model -70–75%, which is known to be accompanied by activation of hypertrophic regeneration mechanisms with pronounced mitotic activity in the remaining part of the organ [28]. Rats with ELR constituted the donor group (n = 15). Bone marrow was harvested from donor rats 12 hours after liver resection (the specified interval is necessary for appearance of morphogenetically active cells in bone marrow) and a mononuclear (hematopoietic) fraction of BM-MNCs was obtained for subsequent single intraperitoneal administration of these cells to intact (unoperated) recipient rats (n = 20) in 4 experimental groups that differed by apoptosis activation method.

Freshly isolated unsorted donor BM-MNCs at a dose of 3.0–3.5 × 10^7 cells per rat (group 1, n = 5), as well as a-BM-MNCs at the same dose activated by incubation were used, in PBS (group 2, n = 5) or in preserving ion-balanced Custodiol HTK solution (group 3, n = 5) at temperature t = 4–6 °C for 48 hours.

In group 4 we used conditioned medium – PBS – after storing BM-MNCs in it for 48 hours (n = 5). Intact rats injected with 1 mL of saline served as the control group (group 5, n = 5).

To select the modes and timing of apoptosis activation of BM-MNCs, we performed a comparative study of the dynamics of reversible and irreversible BM-MNC apoptosis during incubation in PBS by Paneco (Russia) and in preservation solution Custodiol (NTK-histidine-tryptophan-ketoglutarate solution) by Dr. Franz Köhler Chemie GmbH (Germany) at different temperatures (t = 18–22 °C and t = 4–6 °C) and different incubation times (6, 18, 24, 48, and 72 hours).

Under the indicated storage times and temperature regimes, BM-MNCs retain viability and maintain their structural and functional homeostasis due to an evolutionarily developed mechanism – autophagy – adaptive structural, functional and energy restructuring of their own metabolic reserves, which is accompanied by a gradual development of early reversible and then late irreversible cell apoptosis.

FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, USA) was used to detect early apoptosis and late apoptosis/necrosis of BM-MNCs during cell incubation for the indicated periods. Cells were suspended in 100 μL of Annexin V Binding Buffer at 1 × 10^7 cells/mL and mixed with 5 μL of FITC-conjugated Annexin V and 7-AAD (dye 7 aminoactomycin D penetrates only non-viable cell nuclei and intercalates into the DNA double helix). Positively stained, i.e. non-viable mononuclear cells in the BM-MNCs pool used in 7-AAD studies should be no more than 7–10% [29]. After incubation at room temperature for 15 min in the dark, 400 μL of Annexin V Binding Buffer was added to the mixtures. The stained cells were then analyzed using a Beckman Coulter Cytomics FC 500 flow cytometer with appropriate settings.

The ability to enhance targeted (liver) transport of regenerative signals by a-BM-MNCs was assessed by the severity of proliferative activity of hepatocytes in the liver compared to the renal tubular epithelium (control of targeted exposure). The severity of mononuclear cell infiltration of these organs (by macrophages and lymphocytes) in intact recipient rats was also evaluated 24, 36, 48 and 72 hours after administration of donor freshly isolated BM-MNCs and apoptotic BM-MNCs.

The livers and kidneys of recipient rats were dissected on the indicated dates, and histological preparations, stained with hematoxylin and eosin, were prepared from them. Using a Leica DML5 microscope (Germany), we performed histological analysis of the preparations and determined in 30 fields of view the number of mitotically dividing cells, and then calculated the mitotic index (MI) in ppm (‰).

The significance of the differences in the studied indicators in the compared groups was assessed using the parametric Student's t-test at $p < 0.05$.

RESULTS AND DISCUSSION

The results of studies of the effect of the compositions of incubation solutions, the timing and temperature regimes of incubation of BM-MNCs in them on activation of early (reversible) and irreversible apoptosis/necrosis of cells are demonstrated in Figs. 1 and 2. Fig. 1 shows that at room temperature (t = 18–22 °C) of incubation of BM-MNCs in HTK and PBS solutions, the cells in the state of late apoptosis prevail after 18 and 24 hours, exceeding acceptable levels [29].

Meanwhile, storage of BM-MNCs in the same solutions at t = 4–6 °C inhibited the development of late apoptosis in cells and increased during storage (by 48 and 72 hours) the BM-MNCs content in the state of early (reversible) apoptosis, which was significantly more pronounced after 48 and 72 hours for cells in HTK – 44.8 ± 10.9% and 51.84 ± 12.2% solutions versus 29.5 ± 7.1% and 38.6 ± 10.8% in PBS solution (Fig. 2). Since after 48 hours of BM-MNCs storage in HTK and PBS solutions, they showed the highest content of cells in the state of early reversible apoptosis, while the content of BM-MNCs in the state of late apoptosis did not exceed 7–10%, ($p < 0.02$), we studied the regulatory potential of these cells in different groups of experiments when modeling adopting transfer.

There were no significant changes in the mitotic activity of hepatocytes in the liver and tubular epithelium cells in the kidneys (control of tissue-specificity of adoptive

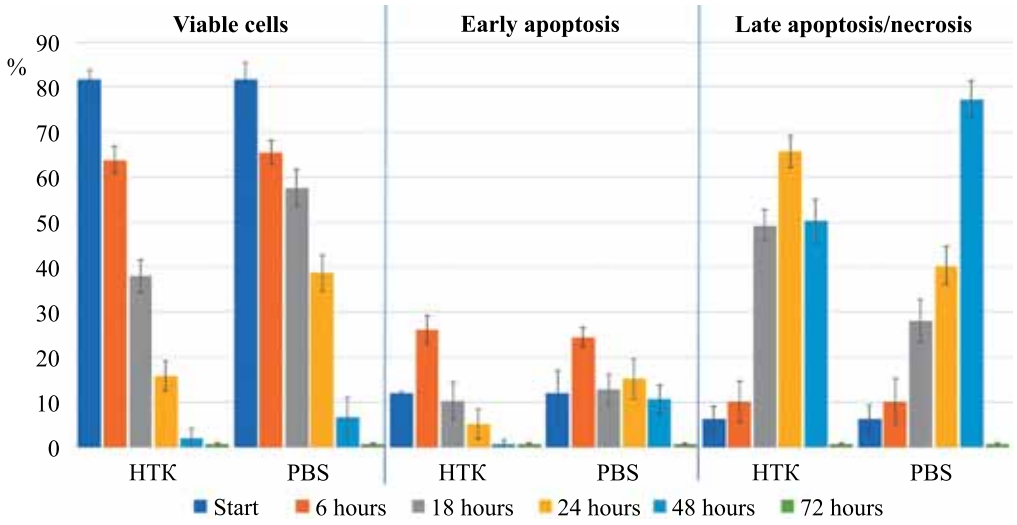

Fig. 1. Dynamics of changes in the content of viable cells, cells in a state of early apoptosis and late apoptosis/necrosis during BMC incubation in PBS and Custodiol (NTC) solutions at t = 18–22 °C

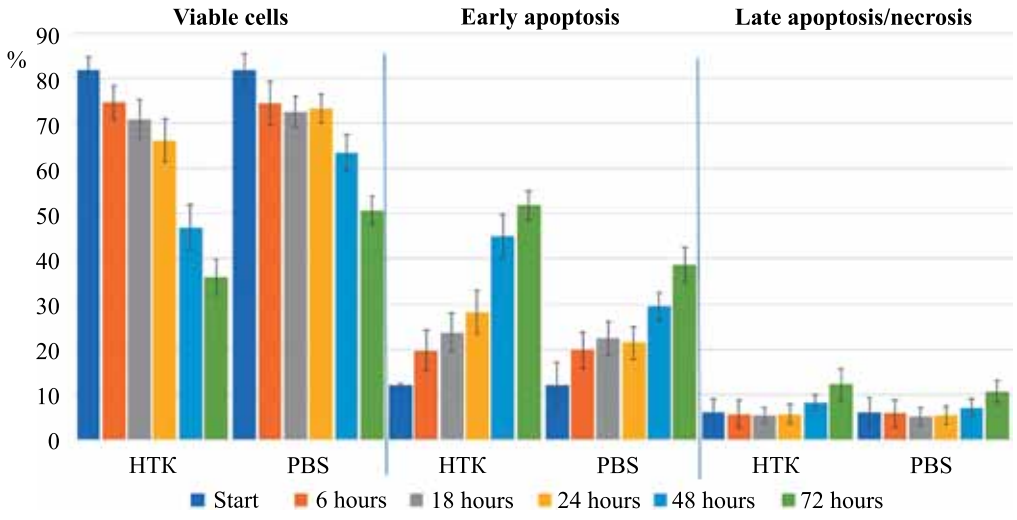

Fig. 2. Dynamics of changes in the content of viable cells, cells in a state of early apoptosis and late apoptosis/necrosis during BMC incubation in PBS and Custodiol (NTC) solutions at t = 4–6 °C

transfer) in all groups and at all time points studied after cell administration to the recipient.

The values of mitotic index (MI) in the liver and kidneys at 36, 48 and 72 hours in groups 1–4 did not exceed 0.02 ± 0.01‰ (0–2 mitoses per 30 fields of view) and did not differ from the initial values, as in group 5. Fig. 3 (a, b, c, d) shows histological picture of the liver tissue of recipient rats at different time points after administration of a-BM-MNCs incubated in Custodiol solution (group 3). One can see from Fig. 3 that the expected rise in mitotic activity of hepatocytes in the livers of recipient rats using apoptotically induced donor material after ELR (at different stages after its administration) was not detected.

At the same time, it is known that after introduction of ELR-activated donor spleen lymphocytes, the mitotic activity of not only hepatocytes but also Kupffer cells in the intact recipient's body significantly increases [1]. In this connection, we expected a similar effect for recipient liver hepatocytes and with the injection of donor a-BM-MNCs after ELR.

Meanwhile, based on the current concepts of regeneration, the result we obtained (absence of mitoses) is not surprising. In the early stages after exposure to damage, directed mobilization of the energy and metabolic reserves of cells with the development of their autophagy, which predetermines the efficiency of subsequent regenerative process, occurs in the surviving cells of the damaged organ to trigger the regenerative process [30–33].

Earlier, we also showed by morphocytometry that in the rat liver tissue at the earliest stages (up to 48 hours) after ELR, there is mobilization increase in cell density, decrease in liver weight, decrease in the areas of hepatocytes, their nuclei and cytoplasm [34], and these facts confirm the important induction role of autophagy and early reversible apoptosis in triggering an an effective regenerative process.

In experiments on the model of adoptive transfer, we obtained only indirect evidence that apoptotic BM-MNCs, in contrast to freshly isolated BM-MNCs, further enhance the autophagy process of liver cells at early

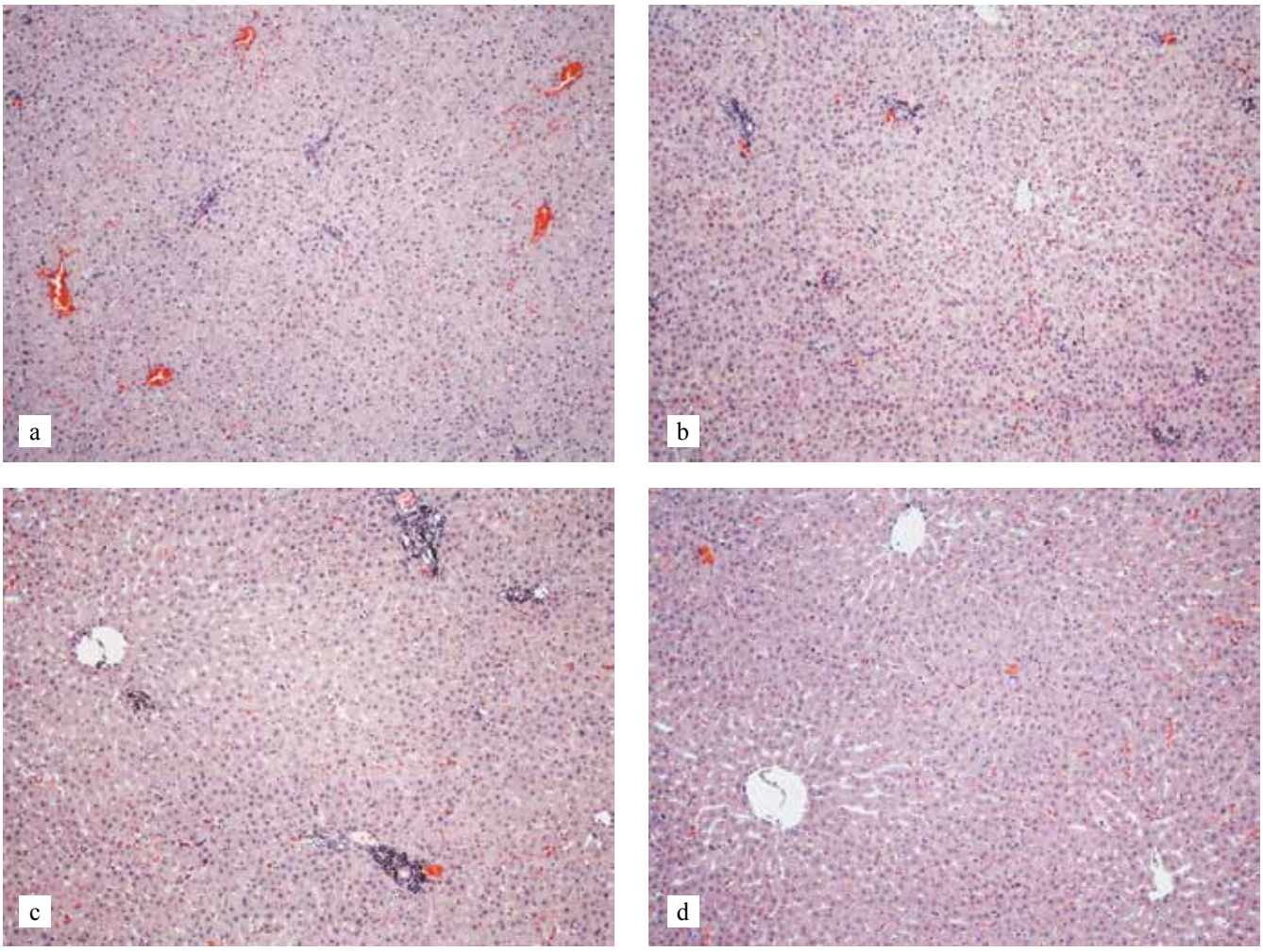

Fig. 3. Histological structure of liver tissue at different periods after a-BM-MNC injection (group 3); H&E stain, 100× magnification. a, 24 hours; b, 36 hours; c, 48 hours; d, 72 hours. No mitotic activity of hepatocytes was observed at all periods of observation

stages. It is known that autophagy is also assigned the role of a cellular autonomous defense system, which in the process of degradation of altered cellular proteins, releases receptors, including its own damage-associated molecular patterns (DAMPS), which manifests as increased tissue immunogenicity and more pronounced enhancement of its cell infiltration, which we observed in our work (see below).

We know from the literature about the appearance of pronounced mitotic activity of hepatocytes in the liver of rat recipients after introduction of donor spleen lymphocytes. We attribute the absence of a similar effect in our experiments after administration of BM-MNCs in recipient rats to cell specialization existing in the body. It is expressed in the fact that during formation of the regeneration process, BM-MNCs seem to play the role of accumulator and transducer of regeneration signals, while mature actively migrating informed lymphocytes of the peripheral immune system – lymphocytes from lymph nodes and spleen, but not BM-MNCs – are already the executors of this process [1–4].

Analysis of histological preparations of recipient liver and kidney samples (Fig. 4, a, b, c) allowed us to ascertain the appearance of cellular infiltration, which was most pronounced in the groups with a-BM-MNCs introduction (groups 2 and 3), especially in the group using cells stored in Custodiol (NTC) solution (group 3) in all groups of experiments at 36 and 48 hours only in liver but not in kidneys. The most pronounced cellular infiltration of the liver tissue in group 3 as compared to group 2 indicated both a dose-dependent regulatory effect of a-BM-MNCs and powerful enhancement of autophagy and early apoptosis processes when exposed to a-BM-MNCs.

When a-BM-MNCs incubated in Custodiol solution were injected after 36 and 48 hours, the liver of a healthy recipient showed signs characteristic of an inflammatory process: infiltration of portal tracts and sinusoids with cells as well as diffuse activation of sinusoid cells were observed, indicating, apparently, reticuloendothelial system activation (Fig. 4, a, b). It should also be noted that mononuclear cells (predominantly lymphocytes)

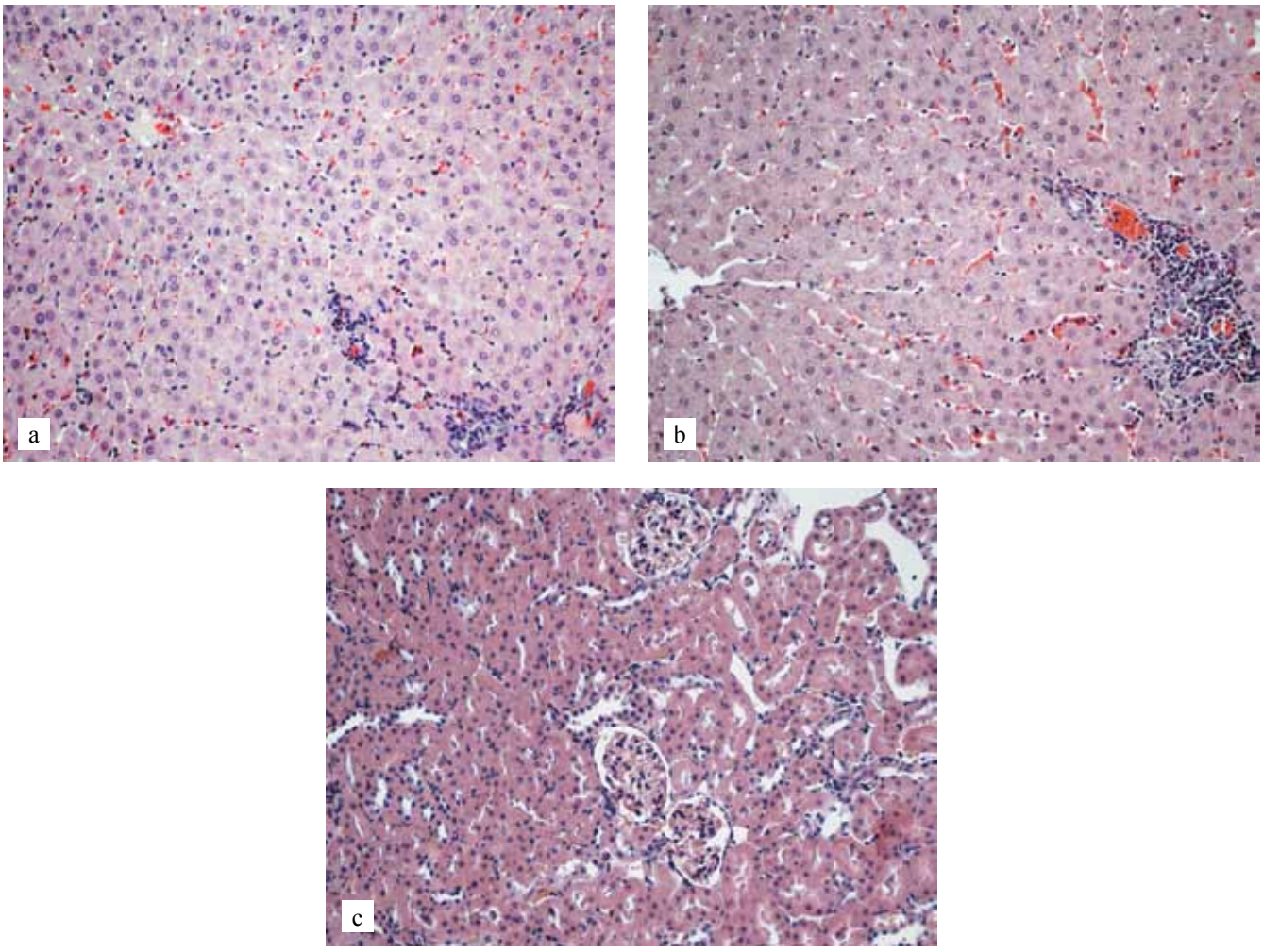

Fig. 4. Histological structure of liver tissue (a, b) and kidney (c) at different periods after a-BM-MNC injection (group 3). a, 36 hours; b, 48 hours; c, 48 hours; H&E stain, 200× magnification. a, b, pronounced infiltration of mononuclear cells in the liver; c, no cellular infiltration in the kidney tissue

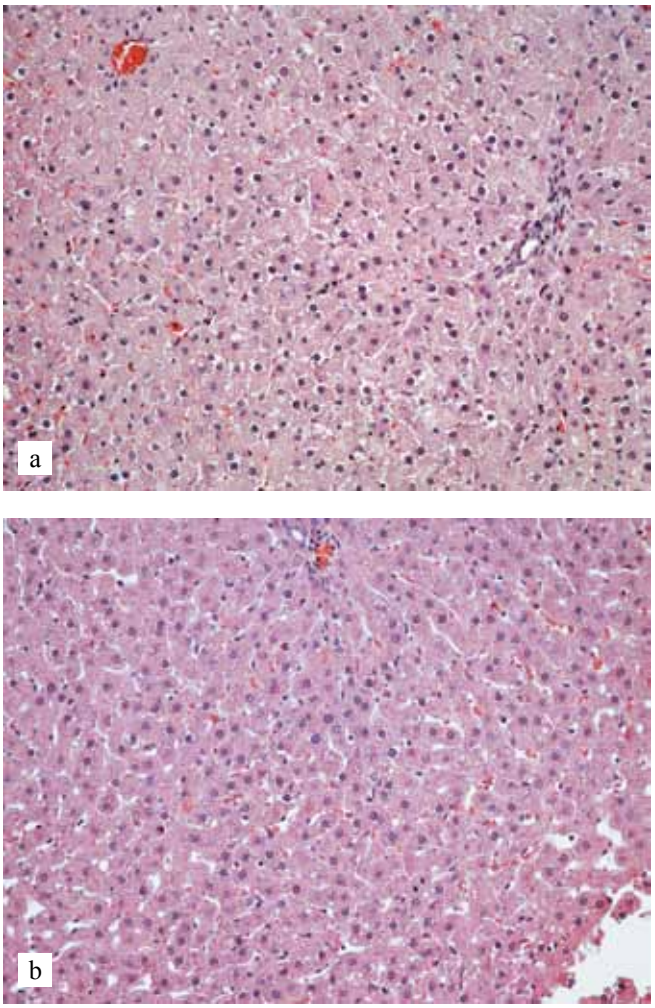

Fig. 5. Histological structure of liver tissue (a, b) at different periods after a-BM-MNC injection (group 3). a, 24 hours; b, 72 hours; H&E stain, 200× magnification. a, b, there is practically no infiltration of mononuclear cells in the liver

predominated among the inflammation cells, abundantly filling the lumen of sinusoids. In 24 and 72 hours after administration of a-BM-MNCs stored in ion-balanced Custodiol solution, there were no effects in the livers of recipients determined at the light-optical level, and the histological pattern of the liver parenchyma, in general, did not differ from that of the intact animal (Fig. 5, a, b), which in our opinion is evidence of the pulse nature of the regulatory effect of a-BM-MNCs on regenerative processes. The absence of cellular infiltration in the recipient kidneys at all time points studied after BM-MNC injection confirms the targeted (tissue-specific) nature of the regulatory effect of freshly isolated BM-MNCs and apoptotic BM-MNCs.

From earlier studies [35], it is known that apoptosis-induced mononuclear cells have not proinflammatory but immunomodulatory effects, releasing immune mediators into the blood directly or indirectly through activation of macrophages and dendritic cells. Administration of apoptotic cells has also been shown to attenuate inflammatory responses after their use, including in the liver in fulminant hepatitis [22], by enhancing the production of anti-inflammatory cytokines (TGF-β, IL-10) by macrophages and inhibiting proinflammatory cytokine production (TGF-β, TNF-α) in the body [20, 21, 36]. In addition, the existence of a relationship between progressive proinflammatory IL-1β/TNF-α-dependent liver damage [37, 38] and reduced ability of liver cells to autophagy, as well as the existence of the possibility of increasing the efficiency of the regenerative process in the liver due to medication activation of autophagy processes in it [30, 31, 39, 40] have all been proven.

CONCLUSION

From the results obtained, the following conclusions are made:

- Incubation of BM-MNCs in PBS and in ion-balanced Custodiol (NTC) solution for 48 hours at t = 4–6 °C optimizes accumulation of a-BM-MNCs in an early apoptosis state;
- Apoptosis-induced and intact BM-MNCs in the adoptive transfer model at early stages after administration do not induce mitotic activity in liver cells;
- in the adoptive transfer model, a-BM-MNCs increase the regenerative potential of liver cells by enhancing the processes of autophagy and directed infiltration of immunomodulatory (mononuclear) cells – carriers of regenerative signals;
- a-BM-MNCs retain the ability for targeted (tissue-specific) transmission of regulatory signals supported in the body by the autophagy process.

All of the above gives us grounds to believe that a-BM-MNCs, unlike intact BM-MNCs, create a stronger foundation in the body for the development and implementation of a targeted and efficient regeneration program. Particularly, after ELR, a-BM-MNCs contribute to targeted and more powerful activation of autophagy process in the liver (due to their stronger regulatory stress-induced potential), a universal mechanism for regulating adaptation processes. By enhancing autophagy, apoptotic bone marrow-derived mononuclear cells exert a more pronounced immunomodulatory effect on immunoregulatory cells, promoting their production of anti-inflammatory cytokines and formation of an effective regenerative response in the damaged liver.

The authors declare no conflict of interest.

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