Morphological Changes in the Kidneys of Rats with Postischemic Acute Renal Failure after Intrarenal Administration of Fetal Mesenchymal Stem Cells from Human Bone Marrow

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Chronic experiments on outbred albino rats were performed to compare the dynamics of histological signs for postischemic renal injury (90-min thermal ischemia) after intraparenchymal injection of cultured fetal MSC from human bone marrow. Functional indexes of the ischemic kidney were predetermined. In the early period after ischemia (day 4), administration of human bone marrow MSC was followed by the increase in blood flow in the microcirculatory bed and decrease in the degree of alteration in renal tubules. An increase in the area of zones with histological signs for normal function of tubules was accompanied by the improvement of biochemical indexes for renal function. In the delayed period, a protective effect of cell therapy was manifested in the prevention of death of renal tubules. Mild calcification of the necrotic tubular epithelium served as a marker of this process. Human bone marrow MSC were labeled with the fluorescent probe Calcein. These cells migrated from the site of injection, spread in the interstitium, and retained viability for 7 days. During this period, some cells were incorporated into the lumen of renal tubules.

Key Words: *renal ischemia; acute renal failure; fetal stem cells; cell therapy*

Extracorporeal blood purification (various types of dialysis therapy) or renal transplantation should be used to prevent the development of irreversible metabolic changes in acute and chronic dysfunction of the kidneys. Alternative methods for the therapy of these disorders were studied in recent years (*e.g.*, cell therapy to increase the regenerative capacity of cells in the injured organ). Previous experiments showed that administration of hematopoietic or bone marrow MSC (BM MSC) contributes to the reduction of dysfunction after renal ischemia of different duration [1,7,8,11,14,15].

Our studies revealed that administration of fetal BM MSC into the renal tissue of rats with chronic and postischemic acute renal failure (ARF) significantly improves the function of the injured kidney and decreases the morality rate of animals from ARF [1].

Here we studied the effect of cell therapy on histological signs of renal function during acute functional deficiency.

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MATERIALS AND METHODS

Experiments were performed on 19 male and female outbred albino rats weighing 340-410 g.

Postischemic ARF was induced by occlusion of the vascular pedicle in the left kidney (90 min) and right nephrectomy.

In series I (10 rats), the suspension of BM MSC (1 million cells in 0.1 ml physiological saline) was punctured into the upper and lower renal segment immediately after blood flow recovery. In series II (control, 5 rats), physiological saline (0.1 ml) was administered into the kidney.

BM MSC were isolated from the bone marrow of tubular bones. Human fetuses (16-18 weeks gestation) were obtained during induced termination of pregnancy. This treatment was performed in licensed institutions (Russian Ministry of Health) in accordance with the legislation of the Russian Federation (order of the Ministry of Health No. 302 of 28.12.1993, supplement No. 3 of 05.04.1994). The cells were cultured as described elsewhere [2].

Experiments were performed with the primary culture of BM MSC (no passage).

For animal treatment, cultured adherent cells were dissociated with 0.25% trypsin-EDTA, washed in DMEM medium, centrifuged, and resuspended in DMEM medium. Viable cells were counted (viability not less than 98%). The suspension was centrifuged at 2000*g* for 5 min and diluted with physiological saline to the concentration required for animal treatment (1 million cells per 0.1 ml).

The animals were examined for 3 months. Renal function was evaluated from biochemical indexes. The kidney was removed at fixed time intervals (after 3-4 days, 2 weeks, and 1, 2, and 3 months). A histological study was conduced by the standard method. Paraffin sections were stained with hematoxylin and eosin.

In series III (4 rats), transplanted cells were visualized by vital staining with the fluorescent probe Calcein-AM. This fluorescent dye easily crosses the lipid membrane and is accumulated in the cell cytoplasm and organelles. Calcein-AM loses the ability to cross the membrane after hydrolysis of the acetoxymethyl group with esterases. These properties exclude the possibility for staining of adjacent cells with the dye, which is released into the intercellular space after deesterification (damage of stained cells). Hence, we can evaluate the integration into the tissue of cells that are stained with a fluorescent probe. For loading with Calcein-AM, these cells were grown on glasses to obtain the monolayer. The cells were washed 3 times with serum-free medium and incubated in serum-free medium with Calcein-AM (5μM) at 37oC for 30 min. The cells were washed 3 times in serum-free medium and dissociated with 0.25% trypsin-EDTA. The suspension was centrifuged in DMEM medium at 2000*g* for 5 min to remove the dissociating agents. The pellet was diluted with physiological saline to the concentration required for animal treatment.

Vital renal sections were stained for the transmembrane potential ($\Delta\psi$) using tetramethylrhodamine ethyl ester (TMRE). The accumulation of this compound depends on $\Delta\psi$. This treatment served as a criterion for normal cell function. Kidney sections were obtained at the site of MSC injection and incubated in the nutrient medium of 200 nM TMRE at 25°C for 10 min.

Fluorescence of kidney sections was measured under a LSM510 confocal microscope (Carl Zeiss) using special software. Calcein was visualized with a 488-nm excitation laser. Fluorescence was estimated at 505-530 nm. TMRE was visualized with a 543-nm excitation laser. Fluorescence was estimated at a wavelength of more than 560 nm.

The results were analyzed by Statistica 6.0 software.

RESULTS

All untreated rats (n=5) died from ARF on day 2-4 after 90-min thermal ischemia of the kidney. None of the rats (n=10) died from ARF after administration of fetal BM MSC. Renal function in survived rats was restored after 1-2 months. The exception was tubular reabsorption of sodium, which returned to normal after 2 weeks (Table 1).

We examined the kidneys of control rats, which died from ARF after 90-min thermal ischemia of the kidney. Fig. 1, *a* shows that the cortex includes regions of complete destruction of renal structures with different area.

Morphological changes in the glomeruli were not found beyond these regions. Proximal convoluted tubules were layered with the cubic epithelium of severe hydropic degeneration. Granular degeneration was revealed in a small number of epithelial cells. Epithelial cells were enlarged. The lumen of tubules was narrowed. A wide tubular lumen was typical of the Henle's loop. The cells were flattened and had hyperchromic nuclei. In distal convoluted tubules, the cytoplasm of most epithelial cells was vacuolized due to large-drop and small-drop hydropic degeneration or focal colliquation necrosis of cells (Fig. 1, *b*). Nuclei were replaced toward the apical region of cells. Collecting tubules were widened. The lumen was filled with the eosinophilic content. The interstitium was

TABLE 1. Functional Recovery of the Kidney after 90-min Thermal Ischemia and Intraparenchymal Injection of BM MSC

Parameter	Normal	Period of study				
		4 days	2 weeks	1 month	2 months	3 months
Blood creatinine, mmol/liter	46±2	266±64*	135±8*	60±3*	55±2	40
Blood urea, mol/liter	4.4±0.1	14.0±2.1*	10.4±1.1*	4.6±0.5	3.6±0.1	3.1
Creatinine clearance, ml/min/kg	3.32±0.12	0.84±0.11*	1.93±0.09*	2.60±0.18*	3.39±0.05	5.22
Sodium reabsorption, %	99.64±0.05	97.06±0.88*	99.72±0.02	99.32±0.04	99.56±0.01	99.79

Note. *$p<0.05$ compared to normal.

infiltrated with polymorphonuclear leukocytes. These histological signs reflect the development of severe acute tubular necrosis.

Heterogeneous changes in the renal cortex were revealed on day 4 after intraparenchymal injection of fetal BM MSC. Focal destruction of renal structures was not found. Hydropic degeneration of the tubular epithelium was less pronounced than in the control. In some zones, proximal tubules of the nephron were layered with swollen epithelial cells. The cells were characterized by granular degeneration, narrowed lumen, and presence of basal nuclei (Fig. 2, *a*). These histological signs probably reflect the increased functional activity of renal structures. A study of renal glomeruli revealed the signs of severe plethora in capillary loops and widening of the Schumlyansky—Bowman's capsule (Fig. 2, *b*), which serve as an indirect criterion for the increase in glomerular filtration. Severe plethora of peritubular capillaries was also found in this period (Fig. 2, *c*).

Similar changes were revealed 2 weeks after ischemia and intraparenchymal injection of BM MSC. The renal parenchyma was heterogeneous. Normal regions of high functional activity of renal tubules alternated with zones of small-drop or large-drop hydropic degeneration of the tubular epithelium. The glomeruli and peritubular capillaries were characterized by severe plethora.

The area of regions with morphological signs for increased functional activity of the tubular epithelium significantly decreased 1 month after ischemia and administration of BM MSC. Hydropic degeneration of the epithelium persisted in the majority of tubules. Dead epithelial cells were calcified in some tubules (Fig. 3). Moderate plethora of peritubular capillaries was also observed in these specimens.

Histological signs of acute tubular necrosis became less pronounced after 2 months. Small-drop or large-drop hydropic degeneration of the epithelium was revealed in many tubules. Degenerative decalcification of necrotic epithelial cells was found in some tubules. The interstitium was characterized by moderate sclerosis. Plethora of capillary loops in the glomeruli and peritubular capillaries was less significant.

The degree of pathological changes was least pronounced 3 months after ischemia. A large part

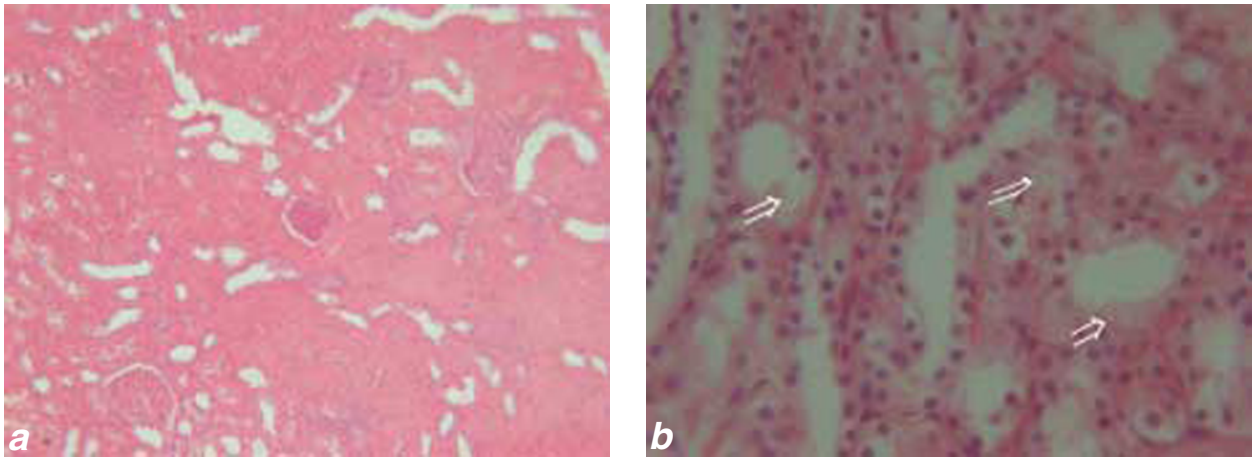

Fig. 1. Changes in the kidney of rats on day 2 after 90-min thermal ischemia (hematoxylin and eosin staining). Zone of destruction of renal tubules (×200, *a*); severe hydropic degeneration of the epithelium in renal tubules and focal colliquation necrosis of epithelial cells (arrows, ×400, *b*).

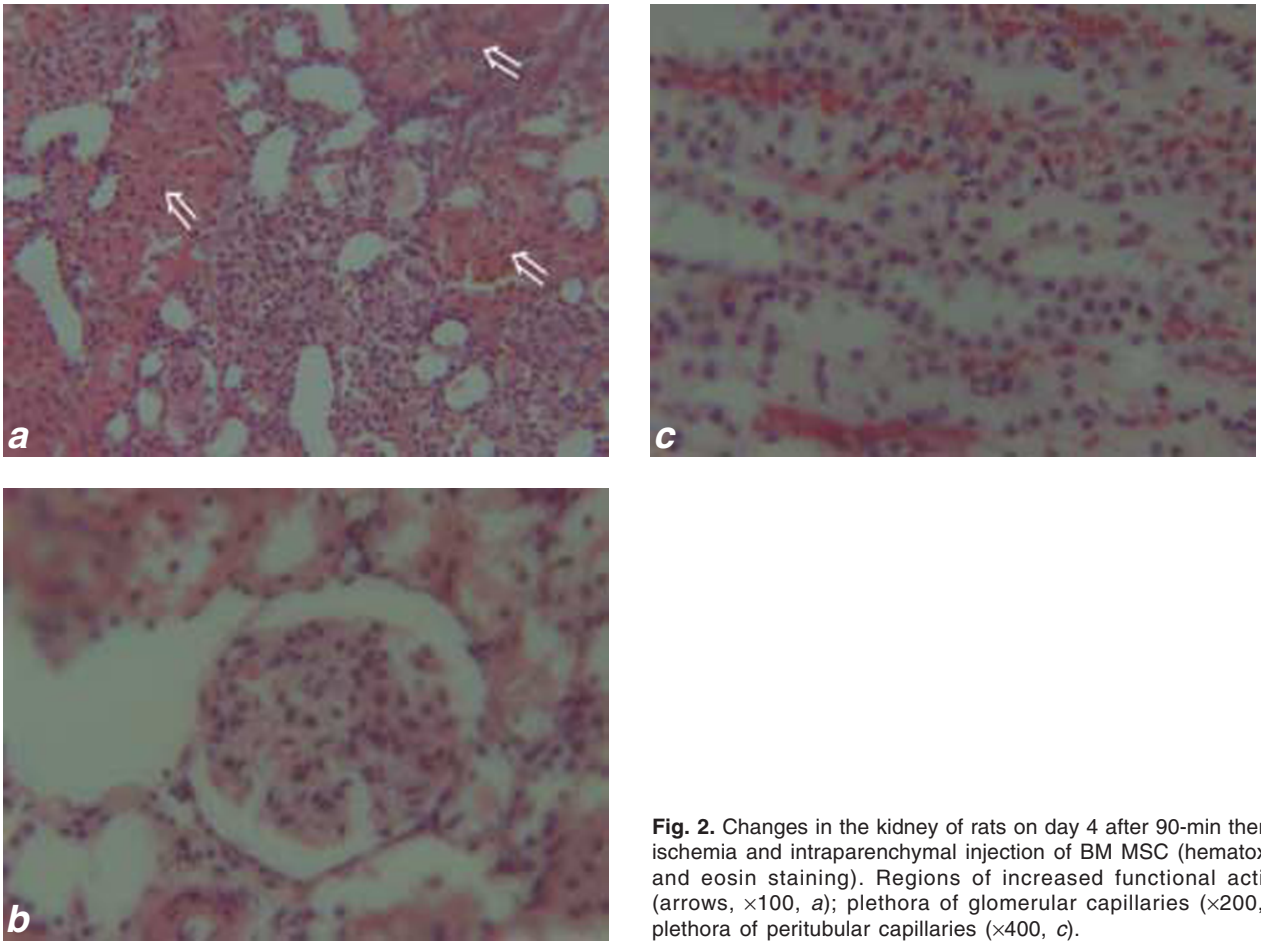

Fig. 2. Changes in the kidney of rats on day 4 after 90-min thermal ischemia and intraparenchymal injection of BM MSC (hematoxylin and eosin staining). Regions of increased functional activity (arrows, ×100, *a*); plethora of glomerular capillaries (×200, *b*); plethora of peritubular capillaries (×400, *c*).

of the parenchyma was presented by histologically normal glomeruli and tubules with the moderately vacuolized epithelium. The consequences of ischemia were small subcapsular focal sclerosis, sclerosis and hyalinosis of some tubules, moderate sclerosis of the interstitium, and calcification of some renal tubules (Fig. 4).

We studied whether a therapeutic effect of BM MSC in postischemic ARF is associated with the incorporation of transplanted cells into renal structures or results from the paracrine action of these cells. In some experiments, BM MSC were loaded with a fluorescent dye Calcein and injected into the kidney. Kidney sections were examined 1 day after treatment. Fluorescent BM MSC were found at the site of injection and adjacent regions (Fig. 5, *a*).

Labeled cells were revealed only in the interstitial space of renal tissue at a long distance from the site of treatment. However, these cells were absent in the tubular epithelium (Fig. 5, *a*). Double staining of vital renal sections with the mitochondrial dye TMRE and Calcein-AM showed that a considerable number of cells retain the mitochondrial transmembrane potential (Fig. 5, *b*). Therefore, the majority of transplanted cells were intact and functionally active. However, a lot of Calcein-stained cells were not stained with TMRE. Hence, these cells were dead and included the mitochondria of reduced functionality.

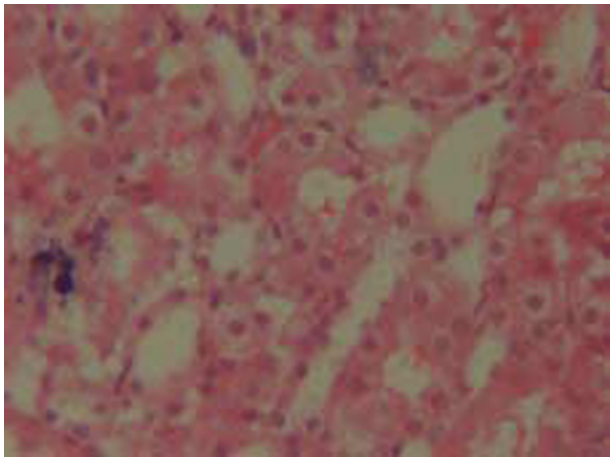

Fig. 3. Changes in the kidney of rats 1 month after 90-min thermal ischemia and injection of BM MSC: calcification of some epithelial cells; and hydropic degeneration of the epithelium in renal tubules (hematoxylin and eosin staining, ×400).

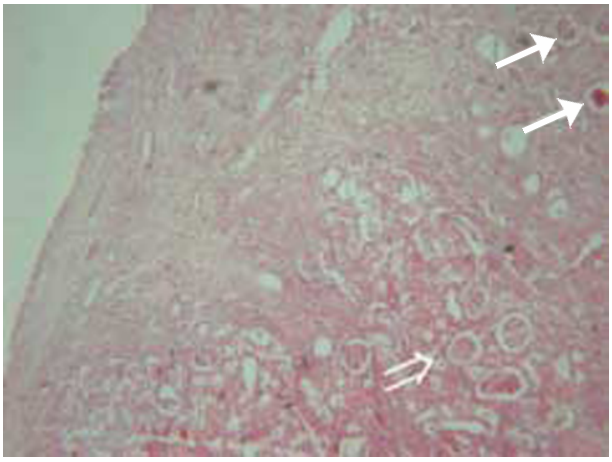

Fig. 4. Changes in the kidney of rats 3 months after 90-min thermal ischemia: sclerosis and hyalinosis of some glomeruli (thin arrows) and moderate subcapsular sclerosis. The main part of the parenchyma is presented by relatively intact renal tissue (double arrow, hematoxylin and eosin staining, ×50).

The cells lining renal tubules were not stained with Calcein. These data suggest that BM MSC were not incorporated into the epithelial layer of damaged tubules (at least, during the early period after treatment). The substitution of dead cells for transplanted cells does not play an important role in the improvement of renal function after prolonged ischemia. A therapeutic effect is mediated by the paracrine mechanism.

Calcein-labeled BM MSC were revealed in the kidney on day 7 after transplantation. However, these cells were diffusely distributed in the interstitium. The cells were not arranged in groups at the site of injection. The number of cells per section during this period was much lower than on day 1 (Fig. 6, *a*). BM MSC migrated over the renal parenchyma from the site of injection. This conclusion was derived from the examination of serial kidney sections.

TMRE staining showed that the mitochondrial transmembrane potential of renal tubules is strongly heterogeneous (Fig. 6, *a*). It was probably associated with exposure to ischemia. The mitochondrial potential was relatively low in the majority of Calcein-stained cells. However, the potential was comparable to that of most tubules.

The majority of Calcein-stained cells were revealed in the interstitium. At the same time, these cells were also present in tubular structures (Fig. 6, *b*). These features reflect the two possible consequences of MSC treatment. First, labeled MSC can cross the membrane of renal tubules and play a role in structural recovery. And second, the formation of intercellular contacts between donor cells and host cells (up to cell fusion) is accompanied by transfer of the intracellular content, including the fluorescent probe. The possibility of this transfer was demonstrated in *in vitro* experiments with coculturing of MSC and cardiomyocytes [12].

The dynamics of histological changes in renal tissue was compared in ARF rats of the BM MSC group and animals with normal renal function. In-

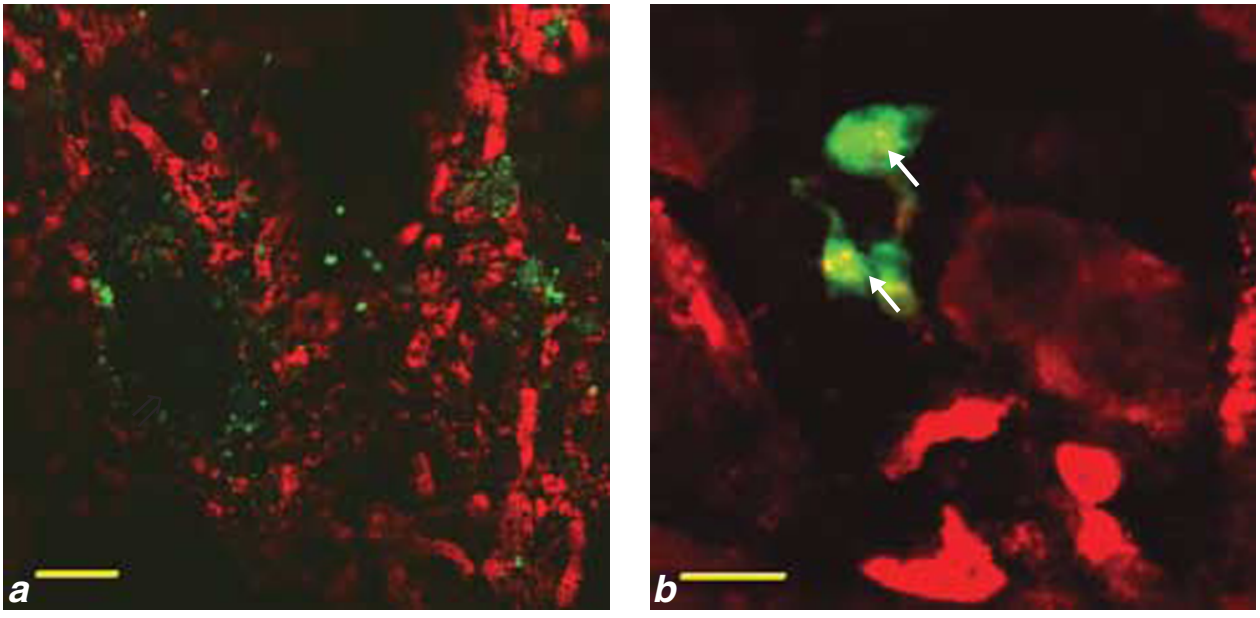

Fig. 5. Fluorescence microscopy of vital renal sections on day 1 after 90-min thermal ischemia and intraparenchymal injection of Calcein-labeled BM MSC. Additional staining of sections with the fluorescent probe TMRE. Distribution of exogenous cells (green fluorescence) over the renal interstitium between renal tubules (red fluorescence; arrow, site of injection; *a*). Staining of viable BM MSC with two dyes produces a yellow color (arrows, *b*).

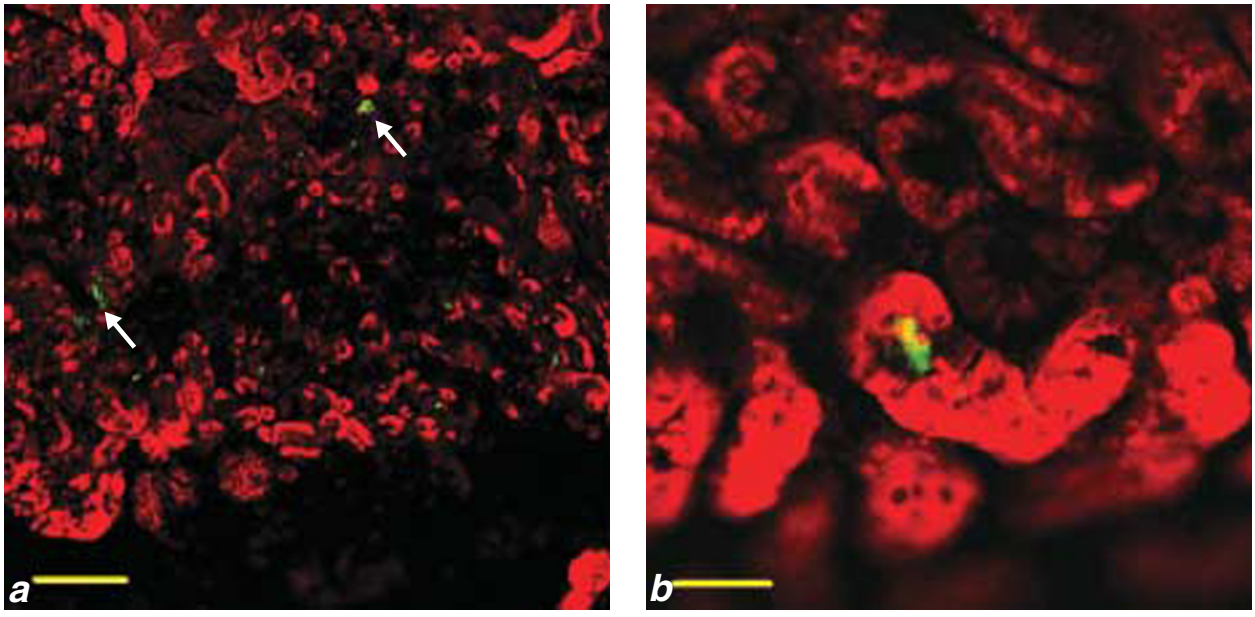

Fig. 6. Fluorescence microscopy of vital renal sections on day 7 after 90-min thermal ischemia and intraparenchymal injection of Calcein-labeled BM MSC. Additional staining of sections with the fluorescent probe TMRE. Diffuse distribution of exogenous cells (green fluorescence, arrows) over the renal interstitium between renal tubules (red fluorescence, *a*); Calcein-stained cell inside the renal tubule (*b*).

jection of BM MSC during the early period after ischemia (day 4) contributes to the reduction of alterative changes in renal tubules (focal destruction) and appearance of zones with relatively intact structures of high functional activity. The observed changes were accompanied by an increase in blood flow in the microcirculatory bed (plethora of glomerular and peritubular capillaries). These effects of BM MSC probably determine the survival of animals. In the delayed period, a protective effect of cell therapy was manifested in the prevention of death of renal tubules. This conclusion was derived from mild calcification of the necrotic tubular epithelium.

Injection of BM MSC provides rapid recovery of reabsorption in the tubular epithelium (over 2 weeks) as compared to filtration in the renal glomerulus (up to 2 months). Histological signs of damage to the tubular epithelium persisted for up to 3 months (despite good functional indexes).

There is no general agreement concerning the mechanisms of functional improvement and reduction of histological signs for renal injury after ischemia. Only few authors believe that exogenous BM MSC transdifferentiate into epithelial cells of renal tubules and substitute for damaged structures of the kidney [8,11]. Several investigators reported that phenotypic changes in transplanted cells is a rare event, which probably results from the nonspecificity of markers and genomic fusion of bone marrow cells and renal cells [3,7,9,13]. Studying the heterogeneous cell population (but not clonal cultures) contributes to the misinterpretation of data, which is related to the presence of stem cells with different specificity.

Most authors believe that transplanted BM MSC have a paracrine effect. There are two possible mechanisms for this effect. First, BM MSC produce a direct stimulatory effect on regeneration of the intact epithelial layer in renal tubules. This action is associated with the release of growth factors and cytokines and reduction of the inflammatory response and/or systemic immunomodulation [7,11, 14,15]. It was hypothesized that exogenous BM MSC have a modulatory effect on the microenvironment due to the induction of dedifferentiation and proliferation of survived tubular epithelial cells [6]. Many epithelial cells gain properties of MSC, restore the integrity of de-epithelialized basal membrane, and undergo subsequent redifferentiation into epithelial cells [10]. And second, exogenous BM MSC may stimulate resident progenitor cells in the kidney. After proliferation and differentiation into mature epithelial cells or other types of cells, they have a stimulatory effect on regeneration of the intact epithelial layer in renal tubules [6,9].

The cells similar to resident progenitor cells were revealed in the outer medulla and renal papilla (in the interstitium between renal tubules). Renal ischemia is followed by proliferation of these cells. Subcapsular injection of these cells is accompanied by their incorporation into damaged structures [4,5].

Our results indicate that administration of human fetal BM MSC into the kidney has a therapeutic effect during postischemic ARF. This effect

is mediated by the paracrine mechanism. The mechanism of action of BM MSC requires further investigations.

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