Regeneration of Red Bone Marrow in Rat Lower Jaw after Transplantation of Mesenchymal Stem Cells into the Site of Injury

I. V. Maiborodin, V. A. Matveeva, I. S. Kolesnikov, M. N. Drovosekov, M. S. Toder, A. I. Shevela

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 4, pp. 215-221, December, 2011
Original article submitted February 21, 2011

Regeneration processes in rat mandibular bone after transplantation of a suspension of autologous BM MSC in culture medium were studied by methods of light microscopy and X-ray densitometry. It was found that the structures of red BM in the callus after transplantation of autologous BM MSC formed earlier than in natural reparation. The formation of cavities containing BM determines lower tissue density at the site of injury after transplantation of autologous BM MSC on weeks 4 and 5 of observation than during spontaneous healing. These changes progressed throughout the observation period and attested to accelerated bone tissue reparation.

Key Words: *autologous mesenchymal stem cells of bone marrow origin; regeneration of the bone tissue; red bone marrow*

Despite new achievements of traumatology and orthopedics, complete restoration of the bone and cartilaginous tissues is associated with serious problems, because extensive defects cannot heal spontaneously. The use of SC provides the possibility of controlling the reparation processes in biology and medicine and gives hope for successful conservative treatment of injuries and traumas that cannot be cured by modern surgical methods [1,3,4].

Red BM contains progenitor cells, MSC capable of differentiation into osteogenic and chondrogenic lineages. This allows using these cells for promoting regeneration of the bone tissue [1,5,6,8].

Transplantation of MSC leads to acceleration of bone regeneration and increase in the density of the bone tissue in comparison with natural reparation [2,9,10,14].

Center of New Medical Technologies, Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences, Novosibirsk, Russia. ***Address for correspondence:*** imai@mail.ru. I. V. Maiborodin

Here we studied regeneration processes in rat mandibular bone at different terms after transplantation of a suspension of autologous BM MSC in culture medium.

MATERIALS AND METHODS

Experiments were carried out on 6-month-old male Wag rats weighing 180-200 g. All manipulations were performed under general ether anesthesia in strict adherence to Regulations for Conducting Animal Experiments. At least 6 animals were used at each stage of the experiment.

Bone tissue defect was modeled as described previously [11]. The rats were anesthetized with ether, the skin was treated with ethanol, and a 1.5-2.0-cm cut was made along the lower edge of the lower jaw under sterile conditions. The masseter muscle was bluntly separated with a raspatory and the bone surface was exposed at the site lower jaw angle. A round hole (2 mm in diameter) was drilled in the lower jaw angle bone (the defect was not connected with the oral ca-

0007-4888/12/1524-0528

vity). The operation wound was sutured layer-by-layer with vicryl.

The animals were divided into 2 groups depending on the course of regeneration of the bone defect: rats with natural course of the reparative process (group 1, n=58) and animals receiving transplantation of autologous BM MSC in 100 μl culture medium (10^6 cell/ml) into the artificial defect of the mandibular bone (group 2, n=58).

Autologous BM MSC were isolated from the epiphyses of the femoral bones. The suspension was placed into plastic flasks (Nunk). Non-adherent cells were removed 48 h after BM explantation. Adherent cells were cultured in α-MEM with 10% FCS (Biolot) at 37°C in a CO_2 incubator at 5% CO_2 and saturated humidity. The medium was replaced every 3 days. After attaining confluence, the culture was subcultured at a seeding density of 1000-5000 cell/cm^2 using standard versen and trypsin solutions.

Light and fluorescent microscopy and cytological methods showed that cultured rat BM cells:

- were $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$;
- adhered to culture plastic *in vitro*;
- retained fibroblast-like morphology throughout the period of culturing;
- survived several passages;
- formed colonies of fibroblast-like cells, when seeded at a low density;
- differentiated into bone tissue cells in the presence of lineage-specific factors.

Cells of passages 2 and 3 were used in the experiments.

However, physical, morphological, and phenotypic characteristics cannot be the criteria for specific identification of autologous BM MSC. The capacity of induced *in vitro* differentiation into osteogenic and chondrogenic lineages is the only critical requirement for choosing SC population.

Autologous BM MSC can differentiate into bone tissue cells and the conditions of this differentiation can be easily reproduced, therefore this differentiation is routinely used for *in vitro* characterization of SC cultures and is a typical default differentiation pathway for the majority of autologous BM MSC in culture. Osteogenic differentiation was induced by adding 0.1 μM desoxymethasone, 50 μM ascorbic acid, and 10 μM β-glycerophosphate (Sigma).

Osteogenic differentiation was determined by two markers: activity of alkaline phosphatase and mineralization of extracellular matrix with calcium ions. Cytochemically, alkaline phosphatase was detected using nitroblue tetrasolium in the presence of 5-bromo-4-chloro-3-indolyl phosphate as the substrate. Accumulation of calcium in extracellular matrix was recorded by alizarin red staining.

The animals were sacrificed 1, 2, 3, 4, and 5 weeks after surgery. The fragment of the lower jaw with artificial defect was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for at least 24 h.

For X-ray densitometry, fixed and prepared fragments of the lower jaw without skin and subcutaneous fat were used. Bone tissue density was evaluated using a radiovisograph (Russia, 2004) equipped with appropriate software; the ratio of the bone density at the site of injury to that on the contralateral side was calculated and expressed in arbitrary units).

The data were processed using MS Excell software; the arithmetic mean and standard error of the mean were calculated. The differences were significant at $p \leq 0.05$.

Fragments of the lower jaw were decalcinated in Biodek R (Bio Optica Milano) for 1 day, dehydrated in ascending ethanol concentrations, clarified in xylene, and embedded in paraffin. Paraffin sections (5-7 μ) were stained with hematoxylin and eosin after Romanovskii and examined under an Axioimager M1 light microscope (Zeiss).

The areas of red BM structures on sections prepared parallel to the mandibular bone surface were measured using a square test system superposed on the monitor with the images from the digital camera of the microscope (objective ×5, the area of the test square 16,900 μ^2, square side 130 μ). Three to five measurements were performed for each section.

RESULTS

In the course of spontaneous regeneration, the artificial defect in the lower jaw bone was filled with blood, fragments of loose connective tissue and granulations were somewhere seen 1 week after injury. The initial stage of bone formation in the defect (formation of young bone and cartilage islets among granulations) was observed. At the same time, nonviable bone tissue fragments most likely formed during defect modeling (scobs) were present in the holes. These fragments were surrounded by macrophages and multinuclear cells, probably osteoclasts or foreign body giant cells formed for lysis of large dead tissue fragments.

Two weeks after bone defect modeling, the hole was completely closed with young bone tissue with a great number of plethoric blood vessels at the edge of the defect. Cartilage tissue was often seen among newly formed structures, especially in the center of the formed defect.

Three weeks after defect modeling, the hole was completely filled with the newly formed bone tissue. Only remained large vessels and chaotically arranged bone trabeculae (callus) indicated the site of injury. Sometimes the bone in the defect did not differ from

the surrounding tissue, but callus structures allowed tracing the site of intervention (Fig. 1, *a*, *b*).

After 4 weeks of spontaneous healing, the site of surgery could be detected in most cases only by callus traces. By this moment, completely formed cavities with BM were detected in the majority of animals. In one case, the hole was presented by a cavity filled with red BM containing many plethoric blood vessels (Table 1, Fig. 1, *c, d*).

After 5 weeks of spontaneous healing, the hole was completely filled with the bone tissue with callus traces.

In animals receiving autologous BM MSC suspension in culture medium, the defect was filled with blood and typical granulations were seen between the blood clot and defect edge 1 week after surgery. The initial stage of bone formation in the defect (formation of young bone and cartilage tissue islets and granulations) was observed, *i.e.* the state of tissues in the defect at this term corresponded to that in the control, except greater number of blood vessels in structures filling the defect, including granulations.

On weeks 2 after transplantation of autologous BM MSC, the mandibular bone defect was completely filled with young bone tissue and cartilage tissue (numerous merged islets) with numerous thin-walled plethoric vessels. Active formation of BM structures was seen, *i.e.* we observed sharp acceleration of processes leading to rapid formation or regeneration of hemopoietic structure, BM, in the callus (Table 1, Fig. 1, *e, f*).

Three weeks after transplantation of autologous BM MSC suspension in culture medium, the bone defect was completely filled with merging islets of young bone tissue with red BM structures. Small fragments of the connective tissue of different types with large thin-walled blood vessels and large multinuclear cells, presumably megakaryocytes (the sign of BM formation) were seen (Table 1, Fig. 1, *g*, *h*).

Four and five weeks after transplantation of autologous BM MSC suspension, the bone defect in most cases was filled with callus with completely formed red BM structures.

Statistical processing of densitometry results revealed no significant differences in tissue density in the focus of injury between the groups with natural spontaneous healing and transplantation of autologous BM MSC at all terms of the study. However, tissue density in the groups with spontaneous healing and with transplantation of autologous BM MSC was significantly lower than in normal bone during week 2 by 11 and 10.7%, respectively, and after 5 weeks by 9.5 and 16.8%, respectively, *i.e.* tissue density at the site of injury in these groups did not exceed the value observed 1 week after surgery (Table 2, Fig. 2, *a-d*).

Moreover, tissue density at the site of injury after application of autologous BM MSC was somewhat lower that in spontaneous healing during week 4 and especially during week 5 (Table 2, Fig. 2, *a-d*).

In the groups with transplantation of autologous BM MSC, the state of tissues (after 1 week) in the focus of injury did not practically differ from that in the control, except greater number of blood vessels in structures filling the hole, including granulations.

Two weeks after transplantation of autologous BM MSC we observed active formation of red BM, *i.e.* sharp acceleration of processes leading to rapid formation or regeneration of hemopoietic structure, BM, in the callus.

TABLE 1. Appearance and Development of Red BM during Regeneration of Bone Defect in the Lower Jaw after Transplantation of Autologous BM MSC

Time after surgery, weeks	Reparative process					
	natural course			transplantation of autologous BM MSC		
	rats with restored red BM		cavity with restored red BM, mm^2 ($M\pm m$)	rats with restored red BM		cavity with restored red BM, mm^2 ($M\pm m$)
	abs.	%		abs.	%	
1	-	-	-*	-	-	-*
2	-	-	-*	8	66.7	0.203±0.013*+
3	3	25	0.225±0.035*	12	100	0.287±0.032
4	11	91.7	0.269±0.024	11	91.7	0.3400±0.0316
5	10	100	0.323±0.040	10	100	0.355±0.037

Note. $p\leq0.05$ in comparison with: *intact bone (0.339±0.040 mm^2), +natural course of the reparative process.

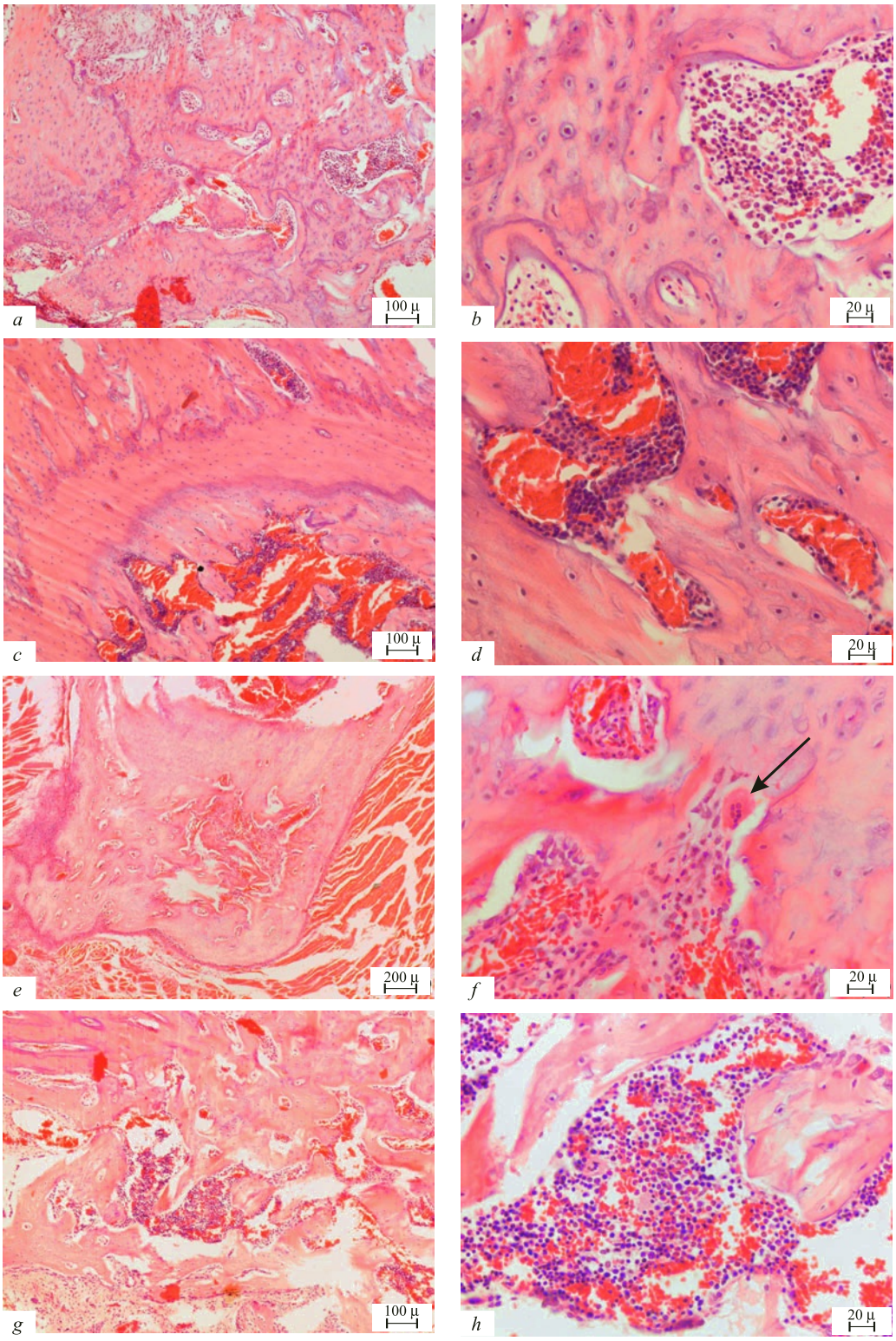

Fig. 1. Focus of injury in rat lower jaw after transplantation of BM MSC suspension in culture medium. Hematoxylin and eosin staining. a) natural course of reparative regeneration 3 weeks after surgery: defect is filled with young bone tissue containing red BM. *b*) fragment of Fig. 1, *a*: start of formation of cavities filled with red BM in young bone tissue; *c*) natural course of reparative regeneration 4 weeks after surgery: callus with clear-cut borders and a large cavity filled with BM at the site of artificial bone defect; *d*) fragment of Fig. 1, *c*: BM structures and wide blood vessels in the callus; *e*) results of BM MSC transplantation 2 weeks after surgery: defect filled with young bone tissue containing formed BM structures; *f*) fragment of Fig. 1, *e*: BM cytogram. Large multinuclear cells, megakaryocytes (arrow) are somewhere seen. *g*) bone defect in the lower jaw 3 weeks after surgery and transplantation of autologous BM MSC: BM development in the callus in the center of damage focus; *h*) fragment of Fig. 1, *g*: completely formed BM structures.

At later terms, fusion of bone tissue islets leading to the formation of the callus and restoration of red BM were observed.

According to published data, MSC cells form BM stroma, while hemopoietic SC participate in the regeneration of red BM. That is why no BM formation is observed after transplantation of only hemopoietic cells in different parts of the body [12]. However, recent studies demonstrated the possibility of transdifferentiation of hemopoietic BM cells into tissue-specific SC and vice versa [13].

In experiments on rodents, transplantation of MSC suspension with demineralized bone matrix into a hollow bone marrow cavity of a tubular bone led to the formation of trabeculae and hemopoiesis-maintaining stroma [7]. Transplantation of a hydroxyapatite–collagen gel composite (with MSC) for replacement of the middle third of the femur in rabbits apart from the formation of new bone tissue with gradual biodegradation of the artificial material was followed by the appearance of BM structures [2].

Since in our experiments we used BM MSC, we cannot exclude the presence of a minor admixture of hemopoietic cells. The presence of these cell elements can explain rapid and early regeneration of hemopoietic structure, red BM. The possibility of transdifferentiation of autologous BM MSC into hemopoietic SC should also be taken into account [13].

Densitometry of bone defect in the lower jaw in spontaneous healing and after transplantation of autologous BM MSC 2 and 5 weeks significantly differed from that of normal bone. Moreover, 4 and 5 weeks after surgery the bone density in the defect after transplantation of autologous BM MSC was smaller than in natural reparative process.

Taking into account published data [2,9,10,14], acceleration of regeneration of the damaged bone and, hence, its higher density in comparison with the densitometry data of spontaneously healed bone could be expected.

The observed decrease in bone density in the focus of injury 2 and 5 weeks after surgery in both groups can be explained by the development of BM. Active bone development and increase in its density observed during the first week was followed by the formation red BM in cavities and bone density decreased. After transplantation of autologous BM MSC, the formation of BM was more rapid and pronounced and therefore, bone density at the site of injury decreased more markedly and became even lower that during spontaneous healing. However, possible impairment of strength characteristics of the regenerating bone caused by the presence of large cavities filled with BM should be taken into account.

The peculiarities of regeneration of the bone defect in rat lower jaw after transplantation of autologous BM MSC consist in earlier appearance of BM structures in the forming callus. These changes progressed throughout the observation period and attested to accelerated development of reparative processes.

Thus, transplantation of autologous BM MSC promoted the development of red BM structures in the callus in comparison with natural course of regeneration. The formation of cavities filled with BM determined lower density of tissues in the site of injury after transplantation (on weeks 4 and 5) of autologous BM MSC in comparison with spontaneous healing. These changes attest to acceleration of reparative processes, but the strength characteristics of the regenerating bone can be impaired due to the presence of large cavities filled with BM.

The study was supported by the program of Presidium of the Russian Academy of Sciences “Fundamental Sciences for Medicine” (project No. 5.81 “Development of Technologies for Application of Biodegradable Polyhydroxyalkanoate Polymers as the

TABLE 2. Bone Density in the Defect of the Lower Jaw in Comparison with Intact Adjacent Tissues during Regeneration after Transplantation of Autologous BM MSC ($S\pm\sigma$)

Time after surgery, weeks	Reparative process		Differences in defect densities (autologous BM MSC–control)
	natural course	transplantation of autologous BM MSC	
1	0.885±0.081	0.928±0.044	0.043±0.102
2	0.901±0.035*	0.903±0.046*	0.002±0.042
3	0.901±0.061	0.96±0.086	0.059±0.067
4	0.944±0.057	0.932±0.052	-0.012±0.055
5	0.913±0.042*	0.856±0.028*	-0.058±0.015

Note. *$p\leq0.05$ in comparison with intact contralateral bone.

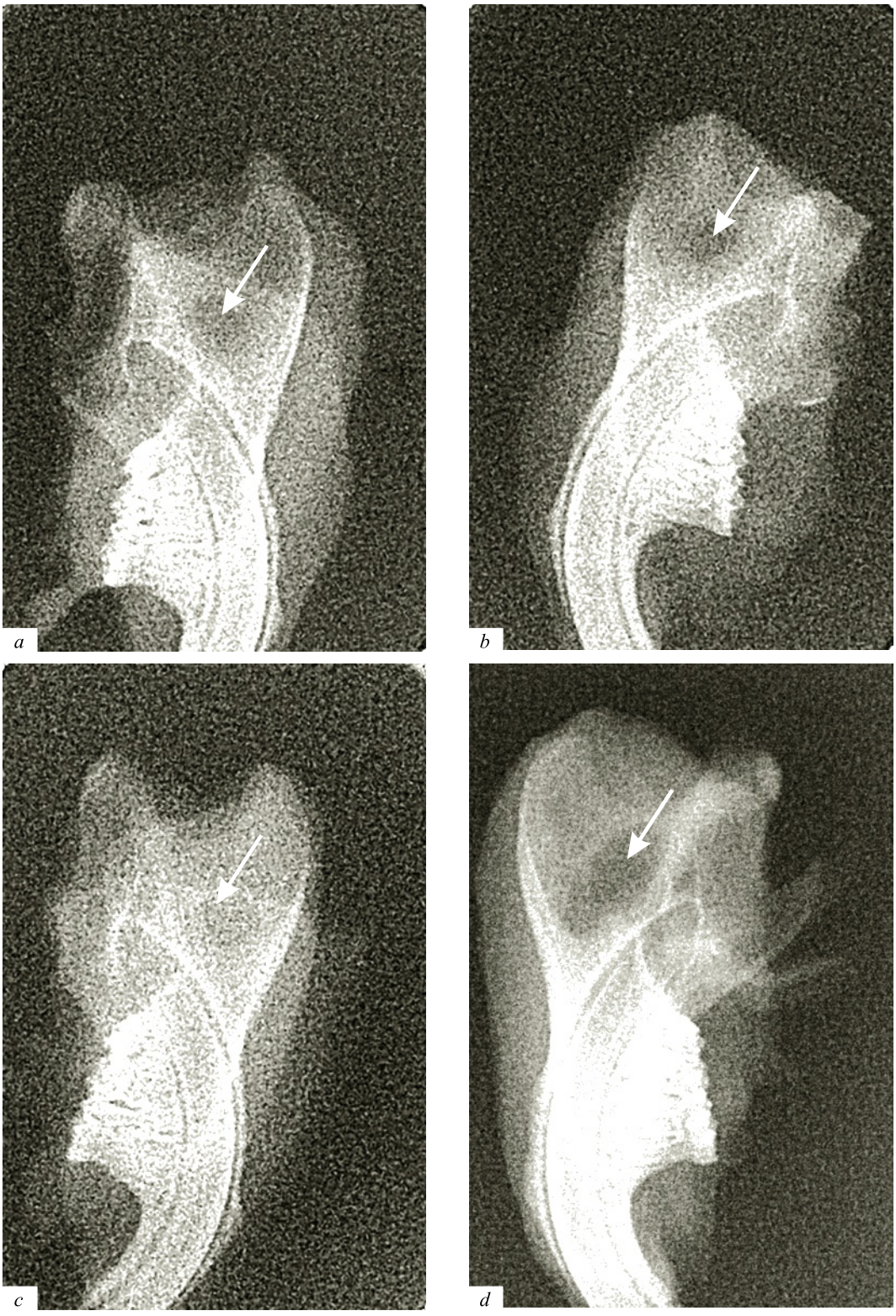

Fig. 2. Radiovisiographic study of bone defect in the lower jaw after transplantation of autologous BM MSC (arrows show the artificial defect). a) natural course of reparative regeneration 4 weeks after surgery: artificial hole is still seen; *b*) healing of the defect in the lower jaw 4 weeks after surgery and transplantation of autologous BM MSC: artificial hole is still seen, tissue density in the defect practically corresponds to that during spontaneous healing; *c*) natural course of reparative regeneration 5 weeks after surgery: artificial hole is still seen, tissue density in the defect approximates that of adjacent tissues; *d*) regeneration of the defect in the lower jaw 5 weeks after surgery and transplantation of autologous BM MSC: artificial hole is still seen, tissue density in the defect is lower than during natural reparation and than in the same group at the previous term; cavity in the bone is wider.

Matrix for Delivery of Stem Cells in the Body").

REFERENCES

1. D. Chanda, S. Kumar, and S. Ponnazhagan, *J. Cell. Biochem*., **111**, No. 2, 249-257 (2010).
2. S. H.Chang , K. Y. Tung, Y. J. Wang, *et al., Plast Reconstr Surg*., **125**, No. 5, 1393-1402 (2010).
3. G. A. Clines, *Curr. Opin. Organ. Transplant*., **15**, No. 1, 73-78 (2010).
4. J. Galle, A. Bader, P. Hepp, *et al., Curr. Med. Chem*., **17**, No. 21, 2274-2291 (2010).
5. C. Goepfert, A. Slobodianski, A. F. Schilling, *et al., Adv. Biochem. Eng. Biotechnol.,* **123**, 163-200 (2010).
6. T. Goldschlager, G. Jenkin, P. Ghosh, *et al., Curr. Stem Cell. Res. Ther*., **5**, No. 4, 345-355 (2010).
7. O. Gurevitch, B. G. Kurkalli, T. Prigozhina, *et al., Stem Cells*, **21**, No. 5, 588-597 (2003).
8. D. Hong, H. X. Chen, R. Ge, and J. C. Li, *Anat. Rec. (Hoboken)*, **293**, No. 3, 531-537 (2010).
9. I. Kallai, G.H. van Lenthe, D. Ruffoni, *et al., J. Biomech*., **43**, No. 12, 2315-2320 (2010).
10. S. Kumar, C. Wan, G. Ramaswamy, *et al., Mol. Ther.*, **18**, No. 5, 1026-1034 (2010).
11. I. Maiborodin, A. Shevela, T. Perrin, *et al., Surgical Science*, **1**, No. 1, 1-6 (2010).
12. H. M. Patt and M. A. Maloney, *Exp. Hematol.*, **3**, No. 2, 135-148 (1975).
13. M. Z. Ratajczak, M. Kucia, R. Reca, *et al., Leukemia*, **18**, No. 1, 29-40 (2004).
14. Z. Y. Zhang, S. H. Teoh, M. S. Chong, *et al., Biomaterials*, **31**, No. 4, 608-620 (2002).