

Experimental Xenotransplantation of Antlerogenic Cells into Mandibular Bone Lesions in Rabbits: Two-year Follow-up

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Abstract. Different types of cells require activation, and take part in annual, dynamic growth of deer antlers. Stem cells play the most important role in this process. This report shows the results of a two-year long observation of xenogenic implant of antlerogenic stem cells (cell line MIC-1). The cells were derived from growing antler of a deer (*Cervus elaphus*), seeded onto Spongostan[®] and placed in postoperative lesions of mandibular bones of 15 experimental rabbits. The healing process observed in the implantation sites in all rabbits was normal, and no local inflammatory response was ever observed. Histological and immunohistochemical evaluations were performed after 1, 2, 6, 12 and 24 months, and confirmed the participation of xenogenic cells in the regeneration processes, as well as a lack of rejection of the implants. The deficiencies in the bones were replaced by newly formed, thick fibrous bone tissue that underwent mineralization and was later remodelled into lamellar bone. The results of the experiment with rabbits allow us to believe that antlerogenic cells could be used in reconstruction of bone tissues in other species as well.

Healthy bones possess the ability to autoregenerate. However, large deficiencies in the bony structure, as a result of trauma, congenital deformities or after extensive oncological surgery, often require surgical reconstruction (1). None of the currently used methods is free from certain disadvantages. The most suitable material for reconstruction is autologous, living bone tissue, usually taken from the iliac crest. The limited amount of bone tissue in children, additional surgical procedures

performed in order to acquire the material, and the risk of implant rejection are the main drawbacks of this method. An infection and inflammatory reaction may appear after using titanium reconstruction plates and distraction osteogenesis in the reconstruction of large defects of mandibular bone (2). It was observed that mesenchymal stem cells (MSCs) used during distraction osteogenesis of the mandibular bone speed up the formation of the new bone (3). MSCs are also used in conjunction with organic or synthetic carriers and bone morphogenetic proteins (BMPs) (4, 5). MSCs can be acquired from bone marrow, fatty tissue, periosteum, and cambium of a tooth (6). Significant proliferative potential, multipotency, low immunogenicity, and no tendency for neoplastic transformations are their major advantages (7). Thank to these properties, MSCs have already found their place in reconstruction of various bone lesions in humans, for example: skull cap, maxilla or in treatment of osteogenesis imperfecta (8-11).

Deer antlers are built from well-vascularized and -nerved osteo-cartilaginous tissue (12). Their growth is dependent on activation of MSCs of pedicle periosteum and growing antler, and later their intensive proliferation and differentiation (13-15). Due to the recurring cycles of regeneration, the antlers present a unique model for research of the processes regulating simultaneous regeneration of different types of tissues. The factors participating in angiogenesis and the growth of extremities are also involved in the regeneration of deer antlers: epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), parathyroid hormone-related peptide (PTHrP) and Wnt signalling pathway (16-19). The cells that were isolated by our team from the apical part of growing antler (antlerogenic stem cells, AC) quickly amplify *in vitro*, and when applied on a proper scaffold to damaged tissues may take part in their regeneration (15, 20).

The goal of this study was a long-term observation of processes ongoing in the lesion in mandibular bones of rabbits filled with AC seeded onto Spongostan[®].

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Materials and Methods

Experimental animals. Fifteen (ten in the experimental group, five in the control group) healthy, 8-month-old, California White female rabbits, weighting about 4 kg, were used in this study. All animals were kept under standard conditions, in separate cages with an unlimited supply of water and balanced rabbit food. The experimental protocols were prepared and accepted by the First Local Ethical Committee for Animal Research in Wrocław (decision nr 41/2006).

Implantation material. MIC-1 stable line of antlerogenic stem cells (ACs), designated as DSM ACC2854 and deposited in 2007 in the German Resource Centre for Biological Material (DSMZ), was used for implantation. Proliferating cells were isolated from mechanically fragmented distal and lateral parts of growing deer antler. The cells were cultured under standard conditions, passaged, washed and suspended in Eagle's minimal essential medium (EMEM) without serum (Cambrex, BioScience, Verviers, Belgium) at a density of 2×10^6 cells/ml (15). Spongostan® (Johnson&Johnson, Warsaw, Poland) – a commonly used gelatine-based haemostatic sponge – was used as a scaffold for implanting the MIC-1 cells. Spongostan® is gaining popularity in tissue engineering as a scaffold for various cells and tissues (20, 21). Additionally, collagen-based carriers can be easily adapted to the lesion shapes and sizes (22, 23). Our previous research has shown that Spongostan® is easily resorbed, and all residual material is quickly removed by foreign body giant cells (FBGC). This accounts for proper reconstruction and revascularization of the bone (21, 24).

Surgical procedures. A single dose of penicillin (50,000 IU/kg) was administered to the rabbits 30 minutes before the surgery. All surgical procedures were carried out in deep anesthesia (20 mg ketamine/kg and 3 mg xylazine/kg). The right side of each rabbit's head was shaved and the exposed skin was cleaned with Octenisept (Schulke & Meyr GmbH, Norderstedt, Germany). An approx. 2 cm long incision was then made in order to separate the skin and underlying soft tissues in the area of the angle of the mandible. The bone with the periosteum was drilled to the marrow cavity with a steel surgical drill of 1 mm diameter. In the experimental group, a piece of Spongostan® soaked with the suspension of MIC-1 cells was placed in the drilled bone defect (about 0.5 cm in diameter). In the control group, the mandible defect was left unfilled. The defect sites were covered with the skin flaps. Small bleeding vessels were closed with electrocoagulation. Postoperative wounds were single-layer stitched with Mersilene 4.0 (Ethicon, Edinburgh, Scotland), washed with Octenisept and left uncovered. The sutures were removed at the eighth day. The general and local condition of the animals was closely monitored throughout the experiment. At 1, 2, 6, 12 and 24 months after the implantation, two rabbits from the experimental group and one from the control group were injected with 1-2 ml/kg of Morbital (Biovet, Puławy, Poland), and their mandibles were removed for further inspection.

Radiological evaluation. Removed mandibular bones were x-rayed (Tur D 800-4, Dresden, Germany) to assess radiological density of the implantation sites and to confirm the regeneration process. The mandibles were x-rayed before preparing them for histological evaluation.

Histology and immunohistochemistry. The bones were fixed in 10% buffered formalin solution (Chempur, Piekary Śląskie, Poland) following a weekly softening in 10% buffered EDTA solution (PPH Standard, Lublin, Poland). The bones underwent a demineralization process in a mixture of sodium citrate and formic acid for 2 weeks. After demineralization, the bones were dehydrated and later soaked in paraffin. Blocks were cut into 5 µm sections, decalcified for 72 hours in Shandon TBD-2 Decalcifier and stained with hematoxylin and eosin (H&E). Immunohistochemical reactions were carried out on material taken from experimental and control rabbits (after 1 and 2 months). The expression of osteogenesis markers was assessed using polyclonal anti-BMP4 antibody (diluted 1:400; Abcam, Cambridge, UK) and monoclonal anti-osteopontin antibody (diluted 1:100; Novocastra, Wetzlar, Germany). Immunological response to xenogenic implant was estimated by examining the expression of markers typical for T lymphocytes (CD3, diluted 1:50; Dako, Glostrup, Denmark), B lymphocytes (CD20, diluted 1:50; Dako) and macrophages (CD68, diluted 1:50; Dako). All sections were incubated with the primary antibodies at room temperature for 1 hour. Additionally, antigen determinants of osteopontin were exposed by boiling sections in 10 mM citrate buffer (pH 6.5 by Dako) at 98°C for 15 minutes. The reactions were visualized with biotinylated secondary antibody, peroxidase-conjugated streptavidin and 3,3' diaminobenzidine (LSAB2 System HRP; Dako). Negative control was performed for all reactions, where the specific antibody was replaced with Primary Negative Control (Dako). The samples were observed under light microscopy using an Olympus BX41 (Olympus, Tokyo, Japan).

Electron microscopy. Material collected from experimental and control rabbits (after 1 and 2 months) for electron microscopy was demineralized in 10% buffered solution EDTA, fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M; pH 8.4), dehydrated and embedded in Epon 812. Ultrathin sections were observed using a JEOL JEM-1011B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan).

Results

Clinical observations. The experiment allowed long-term observation of healing processes and tolerance to the MIC-1 implants. All the animals tolerated the surgical procedures well. None of the 15 rabbits presented any significant health problems during 2 years of observation. Postoperative wounds healed properly *per primam intentionem*. At the implantation sites of AC seeded on Spongostan®, we observed a regenerative process, without inflammatory responses or implant rejection. In the control rabbits, a slow self-healing process was apparent.

Radiological studies. In the radiological studies, we initially observed thickening of compact and lamellar bone tissue on the border of the lesion, making the 1- and 2-month-old implant clearly visible and distinguishable from the surrounding tissue. The middle part was filled with a structure of lower radiological density (Figure 1a). In the long-term observation, after 6-12 months, the implantation sites had blended with the surrounding bone tissue, together

with ongoing remodeling of the bone. It was no longer possible to see the implantation site on the x-rays. On the rim of the control defects, a slow process of autoregeneration during first two months was apparent. In radiological evaluation ACs result in a formation of bony tissue very similar to the host's own.

Histological evaluation. After the first month, on the inner rim of the lesion implanted with scaffold loaded with AC, newly forming trabeculae with layers of active osteoblasts on top of them were observed (Figure 1b). The periosteum formed a thick layer with a large number of active osteogenic cells (Figure 1c). It was clearly visible and heavy mineralized, forming a border between secondary bone and mesenchymal tissue. The center of the lesion was filled with a mesenchymal-like tissue (Figure 1d). In the better vascularized areas the mesenchymal cells differentiated into osteoblasts, which produced osseomucoid (apparent as small, dark vesicles) (Figure 1e). Dark, finely flocculent material was apparent in areas of early crystallization and mineralization of the vesicles. Peripherally, the extracellular substance was already mineralized and osteoblasts changed into osteocytes (Figure 1f). At the implantation sites, no remnants of Spongostan[®] were observed and the whole scaffold appeared to have been absorbed.

After 2 months, along the ramus of the mandible, on the outer side of the periosteum, numerous collagen fibers, adipocytes and striated muscle tissue were found (Figure 2a). In certain areas between mandibular periosteum and adjacent layer of muscles, groups of implanted cells (undifferentiated and proliferating) with accompanying collagen fibers were visible (Figure 2a). Sporadically, implanted cells were isolated with a thin layer of connective tissue capsula (Figure 2b). The outer layer of periosteum was built from fibrous, compact connective tissue; the inner layer consisted of numerous osteogenic cells and blood vessels (Figure 2a). At the regeneration sites, newly formed periosteum was 2-3 times thicker than the animal's own periosteum. Underneath, numerous, chaotically spread osteoblasts were visible. In deeper layers of bone, lamellae with osteocytes typically located in bone cavities were found. The new bone was thick and fibrous, with irregularly shaped lamellae. It contained blood vessels, and marrow cavity filled with red marrow with a large number of adipocytes (Figure 2c). In the control rabbits, mandibular defects were filled with bony tissue covered by thin periosteum (Figure 2d).

The dynamics of changes taking place in the postoperative lesion after 6, 12 and 24 months slowed down noticeably. The periosteum between 6 and 24 months was visible as a thin, uniform line, surrounding the mandible from the outside. Subsequent images of the implantation sites and host's own bone tissue became more and more alike. Gradually, the fibrous bone between 12th and 24th month

became a normal, mature, lamellar bone. This bone contained osteons with two or three systemic lamellae surrounding the blood vessels (Figure 3a-c). The bone marrow cavity was filled with red bone marrow. Histological images of defects in the control group after 6, 12 and 24 months were similar to those from the AC-treated group.

Immunohistochemistry. The immunohistochemical reactions showed localized expression of BMP-4 protein inside 1- and 2-month-old implantation sites. BMP-4 and osteopontin were found in the osteoblasts from the periosteal side and newly formed bone (Figure 4a, c). The lack of any inflammatory response was confirmed with negative reactions for CD3, CD20 and CD68 (Figure 4e, f). In control defects, BMP-4-positive cells were present in autoregenerative layer of bone and expression of osteopontin was not observed (Figure 4b, d).

Electron microscopy. One and two months after the implantation of AC, the electromicrographs showed the formation of thick fibrous bone tissue. During regeneration, numerous bundles of collagen fibers were formed, with accompanying early mineralization and crystallization of matrix vesicles. Additionally, in the dense network of the collagen fibers, newly formed osteocytes with visible cytoplasmatic extensions were found (Figure 5).

Discussion

Autologous implants, usually obtained from the iliac or rib bone, were commonly used for over 40 years (25). However, even after using autologous grafts, problems such as infections, resorption or pseudoarthritis are still possible (26, 27). In comparison to the reimplantation of bone fragments, microvascularized grafts from the iliac bone, forearm, scapula or tibia provide conditions for faster reconstruction without inflammatory reactions (28, 29). The greatest drawbacks of this method are incomplete convalescence and significant pain, especially in children and elderly patients (27, 30). Using allogenic bone to reconstruct mandibular lesion also has certain limitations, such as limited availability or risk of rejection (31).

The search for alternative methods of reconstructing bone deficiencies continues. One of the possible solutions is based on using bone substitutes, such as hydroxyapatite/ β -tricalcium phosphate (HA/TCP), bovine bone mineral (Bio-Oss) or polyglycolic co-lactic acid (PGLA), since all of these integrate well with host tissues (4, 5). However, these scaffolding materials have weak osteoinductive properties and are used mainly as carriers of various growth factors or cells (1). BMP-2 protein has strong osteoinductive capabilities, but when not administered properly, it may cause excessive growth or bone resorption (22, 32). *In vitro* cultured osteoblasts derived from mandibular periosteum or

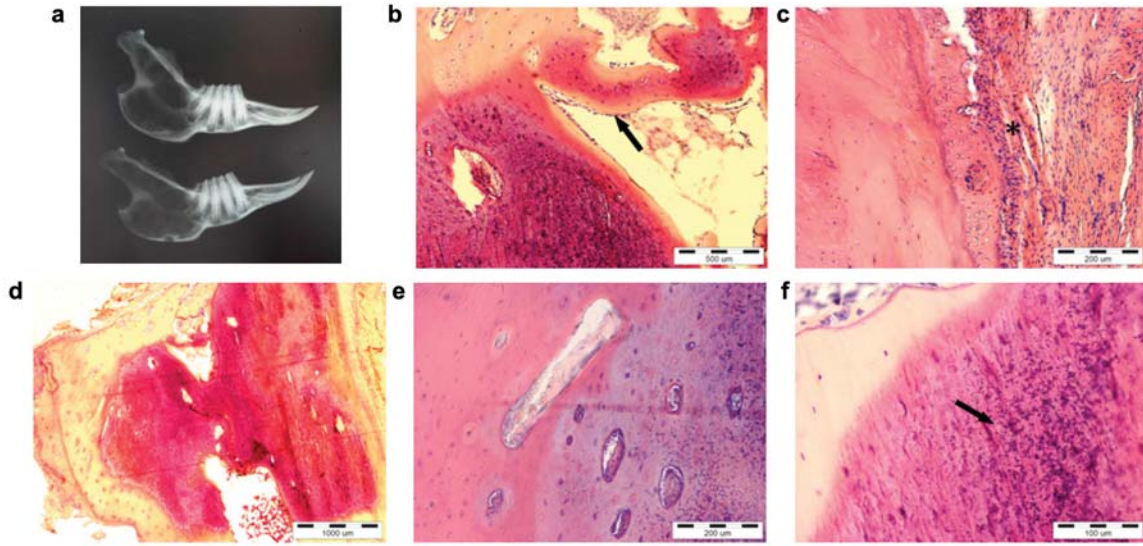


Figure 1. Regeneration of a lesion one month after implanting anterogenic cells. a: X-ray of left (top) and right (bottom, implant) mandible branch, showing a well-mineralized border between own bone and the lesion; b: forming of bone trabeculae with layers of active osteoblasts on their surface (↑); c: periosteum with numerous active osteogenic cells (*); d: center of regeneration area, showing mesenchyma in the form of membrane bounded by mineralized secondary bone tissue; e: vascularized areas of bone, with ongoing cell differentiation and matrix mineralization; f: dark, finely flocculent material marks areas of early crystallization and mineralization of vesicles (↑), in some areas intracellular substance became mineralized. b-f: H&E staining.

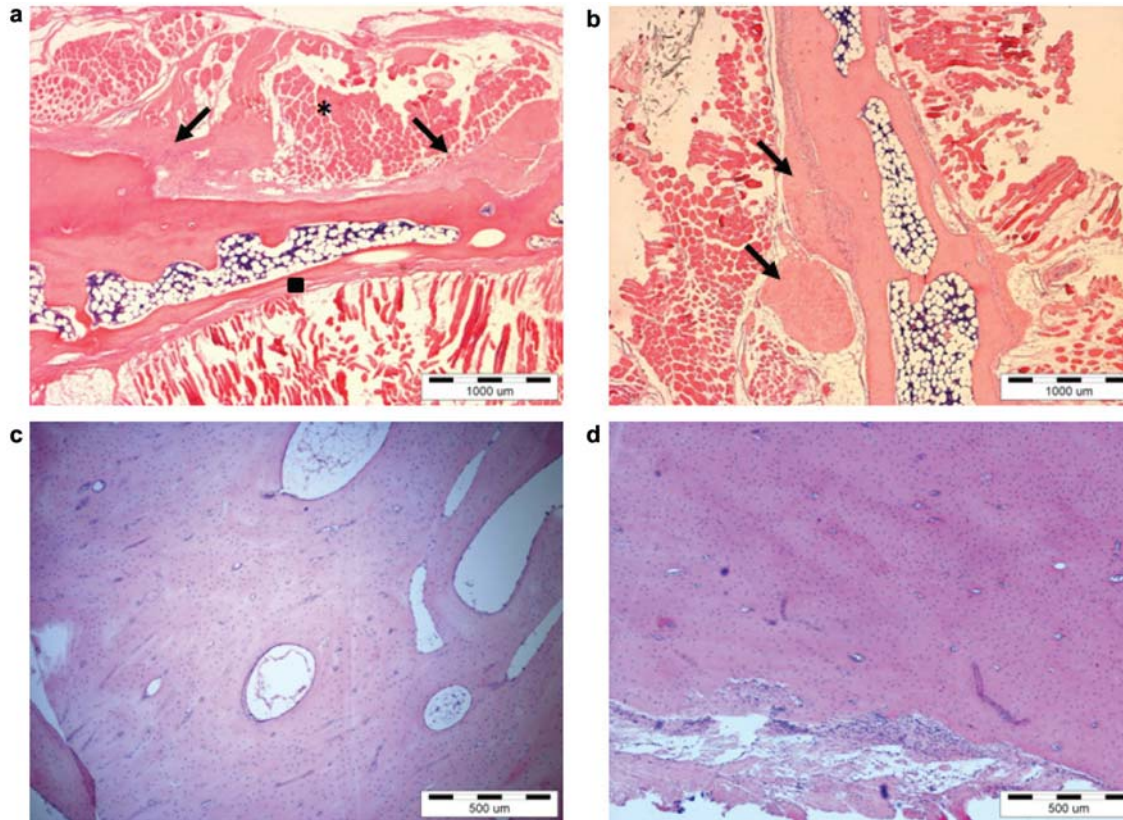


Figure 2. Regeneration of bone lesion two months after implanting anterogenic cells. a: High number of undifferentiated, proliferating cells (↑) and distinct two-layer periosteum (■) surrounded by muscle tissue (*); b: sporadically, between mandibular periosteum and the adjacent muscle layer, implanted cells form oval groups surrounded by a thin connective tissue sheath (↑); c: newly formed fibrous bone containing blood vessels and marrow cavity filled with red marrow; d: bone with thin layer of periosteum formed in control lesion. H&E staining in all sections.

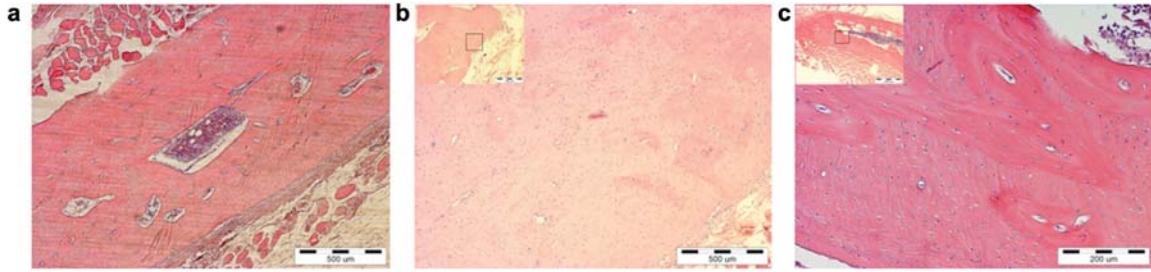


Figure 3. Regeneration of bone lesion after antlerogenic cell (AC) implantation: a, b: Six and twelve months after AC implantation respectively, showing restructuring of coarse fibrous bone into lamellar bone tissue; c: twenty-four months after implantation, showing mature lamellar bone, with visible osteons with two or three systemic lamellae around blood vessels. H&E staining in all sections.

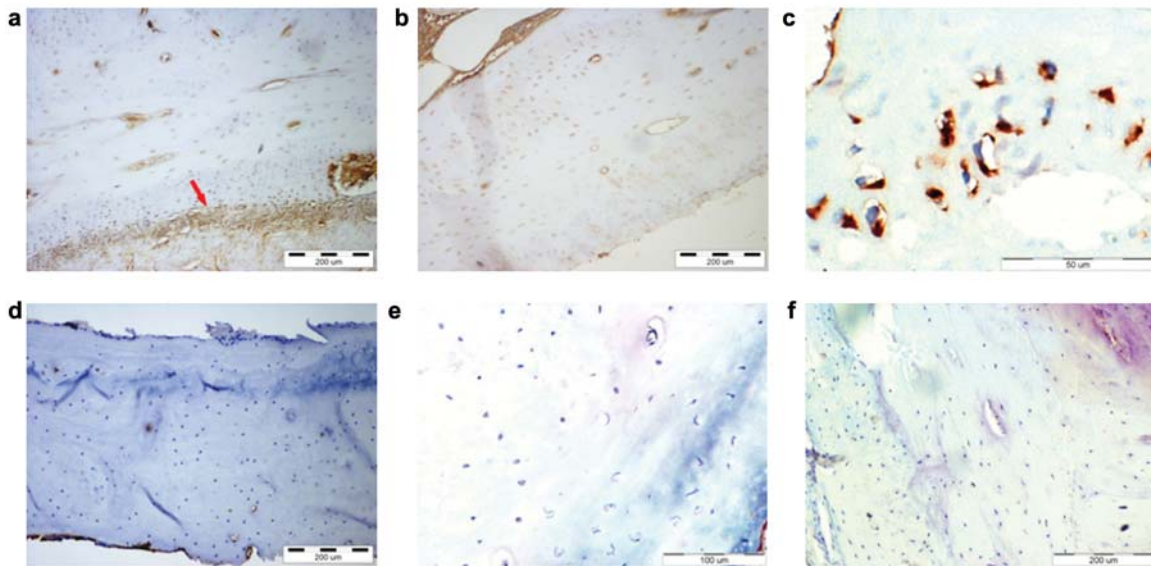


Figure 4. Immunohistochemical reactions performed one month after the surgery: a: BMP-4 expression in the osteoblasts on the side of periosteum (↑) and bone at the antlerogenic cell (AC) implantation site; b: BMP-4 expression in the osteoblasts of bone in the control defect; c: positive osteopontin reaction in osteoblasts on the side of periosteum at the AC implantation site; d: no expression of osteopontin in the control defect; e: no reaction of T-lymphocytes (CD3 antigen) at AC implantation site; f: no macrophage reaction (CD68 antigen) at the AC implantation site.

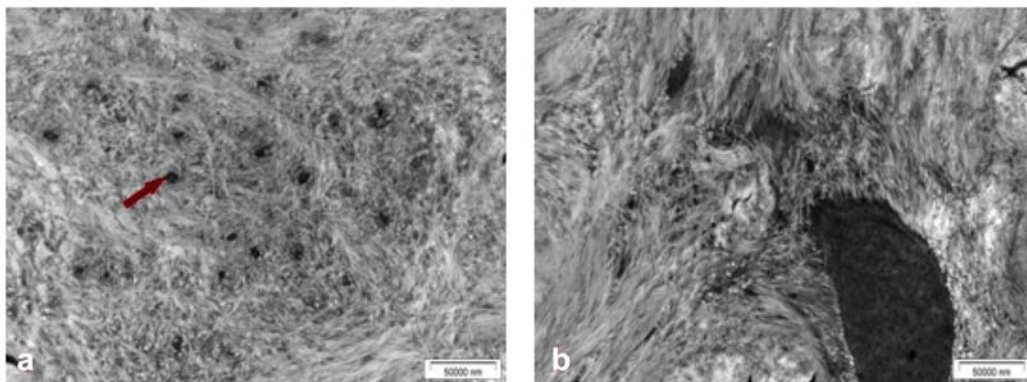


Figure 5. Ultrastructure of bone one month after antlerogenic cell implantation. a: Zone of newly formed osseomucoid and early mineralization and crystallization of matrix vesicles (↑), showing numerous bundles of collagen fibers; b: newly formed osteocyte with visible cytoplasmic processes, surrounded by a dense network of collagen fibers.

bone marrow implanted into bone lesions resulted in incomplete regeneration. Differentiated cells do not create the proper environment for regeneration and require appropriate growth factors (33, 34). Autologous bone marrow cells or combination of MSCs with BMP-2 protein allow for better regeneration, resulting in more structured bone formation (4, 5). Unfortunately, the regenerative capabilities of bone diminish with age as a result of a decreasing number of osteoprogenitor cells or MSCs of the bone marrow (35). Taking into account all these limitations, we have made an attempt to use antlerogenic MSCs (ACs) to reconstruct lesion in the rabbits' mandibular bones.

One month after the implantation of MIC-1 cells, on microscopic evaluation ongoing regenerative processes were clearly visible. Ossification sites and gradually forming periosteum were observable on the rim of the lesion. The regeneration center was filled with vascularized, membranous mesenchymal tissue, where differentiation of osteogenic cells occurred. Previously, we observed angiogenesis in our study related to auricle reconstruction in rabbits (20). Angiogenesis is an important part of proper formation of bone tissue and in this study may be caused by VEGF secreted by ACs. The process of formation and mineralization of the new bone started at the rim of the lesion and continued inwards. Empty space was gradually filled with bone trabeculae, with osteoblasts on their surface. Presence of BMP-4- and osteopontin-positive cells in the periosteum and newly formed bone confirmed the process of bone formation in the area of MIC-1 implantation. Similarly, Marukawa *et al.* (36) observed expression of BMP-2 in rabbit's mandible that underwent distraction osteogenesis. The electron microscopy also confirmed early phase osteogenesis, with numerous osteocytes surrounded by dense network of collagen fibers and ongoing matrix mineralization. There was no immune response in animals after AC implantation. After 1 and 2 months, a lack of CD3, CD20 and CD68 expression in the area of regeneration was observed. In available research on xenogenic implants, MSCs are well tolerated and non-immunogenic (37, 38). *In vitro*, MSCs modulate the function of B lymphocytes and suppress proliferation of T lymphocytes, which may induce tolerance to xenogenic implants (39, 40). MSCs take part in tissue regeneration due to their ability to differentiate into the tissue's cells or by producing trophic factors modulating the microenvironment and inducing survival and proliferation of host cells (41, 42). These are, for example, proteins regulating hematopoiesis, angiogenesis, wound healing processes and immunological response (43). During the regeneration of deer antler, various growth factors are expressed, such as EGF, FGF-2, VEGF and PTHrP (16-18). We believe that implanted ACs may also participate in the creation of suitable microenvironment for cellular growth in host tissues with participation of all these factors.

After two months, at the implantation site, we observed a reconstructed, two-layer periosteum and thick fibrous bone with irregular lamellar pattern, containing blood vessels and marrow cavities filled with red bone marrow. In our experiment, the histological picture of the regenerated bone resembles bone tissue created after implanting autologous MSCs from bone marrow into lesions in mandibles of dogs and apes. After 6 and 16 weeks, trabecular bone with marrow cavity was present in implantation sites (4, 5). Interestingly, between the new periosteum and overlying muscles, we observed oval aggregations of undifferentiated, proliferating cells surrounded by a thin capsule of connective tissue. Isolation of certain areas may be caused by using xenogenous cells or scaffold. A living organism may react to a xenograft by chronic inflammation or may try to isolate the given material by surrounding it with a fibrous capsule of various thickness usually 0.1-10 μm . The thinner the capsule, the greater the biocompatibility between the implant and host tissues (44). In our experiment, this phenomenon was observed sporadically and probably did not influence the regeneration process.

After six months, in the radiological evaluation, we observed a fusion between the sites of the lesions and surrounding bone. Microscopic assessment confirmed favorable remodeling of the thick fibrous bone into more organized lamellar bone. Further long-term observations, 12 and 24 months after implantation, showed the presence of mature lamellar bone covered by thin, uniform periosteum. Similarly, in another long-term xenogenic model, human MSCs were introduced into skull and mandible lesions, where they participated in the regeneration processes without any inflammatory response. Bone lesions were considerably rebuilt as early as 6 weeks, and by 96 weeks only a small increase of the bone mass and progressive resorption of the carrier with parallel formation of new blood vessels were observed (45).

In our experiments, ACs taken from *Cervus elaphus* and implanted into lesions in rabbit mandibles were definitely not rejected. ACs participated in the process of mandible reconstruction, probably together with host tissues. Participation of ACs in reconstruction of destroyed tissues, the excellent integration of newly formed bone with the host's own bone, low immunogenicity, quick proliferation *in vitro* and low production costs are important advantages of ACs. In the near future, ACs may become a real alternative to autologous MSCs.

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