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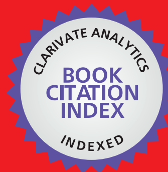

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Characteristics of antlerogenic stem cells and their potential application

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1. Introduction – growing antler as a model of tissue regeneration in mammals

Antlers are present in all males in 39 species of the deer family (*Cervidae*) and are a bony product, unlike horns which originate from epidermis in artiodactyls (*Bovidae*). Among reindeer antlers are also present in females, helping them to find fodder. The size and symmetry of the antlers in males are decisive for their hierarchy in a herd, manifest their actual condition and affect the selection of appropriate mating partners. Every year antlers of the deer family are shed and regrown over a short period of time. They develop as elongations of osseous pedicles present in the frontal bone of the skull. The pedicle arises from a specialised region in the frontal bone, the so-called antlerogenic periosteum (Price & Allen, 2004; Price et al., 2005a). Xenogeneic and allogeneic ectopic grafts of the antlerogenic periosteum confirm its antlerogenic potential. At the sites of the implantation a pedicle is formed and in some cases antlers develop. Both organs are built from a specific chondro-osseous tissue which is a mixture of cartilage and bone (Goss & Powel, 1985; Kierdorf & Kierdorf, 2000; Li et al., 2001). During growth of the pedicle, some of the cells of the antlerogenic periosteum migrate to the apical region of the pedicle, where they build its periosteum and participate in creating antler growth centres (Kierdorf et al., 2003; Li & Suttie, 2001). Histological studies of the early stages of the antler regeneration show that

these centres create undifferentiated and proliferating clusters of mesenchymal cells (Kierdorf et al., 2003; Li et al., 2005; Price et al., 2005a). The antlerogenic periosteum, the antlerogenic cells of the pedicle and antlers retain their features of embryonic cells for they contain large amounts of glycogen granules (Cegielski et al., 2006; Li & Suttie, 1998; Li & Suttie, 2001). Among the cells of the antlerogenic periosteum, pedicle and mesenchyme of the growing antlers are stem cells, which can differentiate into cartilage, bone and adipose tissue (Berg et al., 2007; Cegielski et al., 2006; Kierdorf et al., 2009; Rolf et. al, 2008).

During the reproduction period, antlers cease to grow and the skin covering them (velvet) is shed. This process coincides with the parallel growth of testosterone concentration, which converts into oestrogen in antler tissues (Bubenik et al., 2005; Muir et al., 1988). Oestrogen inhibits cell proliferation and increases cartilage mineralization via its receptors (Barrell et al., 1999). Until next spring the old antlers remain fixed to the osseous stem and next, they are cast as a result of bone resorption by osteoclasts on the antler-pedicle border. This process is parallel with the decrease in testosterone levels (Goss et al., 1992). The ossified antlers are not a dead structure, because one month before they are cast, inside of them, there are still blood vessels and bone remodelling processes by osteoclasts still take place (Rolf & Enderle, 1999). In red deer, as soon as the old set of antlers is cast, a new one is grown. The antler regenerating process begins before the old set is cast, which can be seen in the ring created around the apical region of the pedicle (Kierdorf et al., 1994). The antler growth is rapid: within three months the daily increments of 2 cm cause that the growing antlers represent one of the most rapidly growing organs in mammals. The most recent studies demonstrate that testosterone is responsible for antler growth in mature stags. IGF-1 is responsible mainly for body mass increase and its role in regulating antler growth is limited to young specimens (Bartos et al., 2009). Other factors affecting the antler growth are factors synthesised by its tissues: BMP2 and BMP4 (bone morphogenetic protein 2, 4) (Feng et al., 1995; Feng et al., 1997), TGFβ1 and TGFβ2 (transforming growth factor β1, β2) (Francis & Suttie, 1998), PTHrP (parathyroid hormone related peptide) (Faucheux et al., 2004), retinoic acid (Allen et al., 2002), FGF-2 (fibroblast growth factor 2) (Lai et al., 2007), VEGF (vascular endothelial growth factor) (Lai et al., 2007), EGF (epidermal growth factor) (Barling et al., 2005), pleiotropin (Clark et al., 2006), PEDF (pigment epithelium-derived factor) (Lord et al., 2007), NGF (nerve growth factor) (Li et al., 2007) as well as IGF-1 (insulin-like growth factor-1) (Gu et al., 2007). Altogether, nearly 800 proteins are present in a growing antler. Among them, there are antler-specific growth factors: MEKK1 (mitogen-activated protein kinase 1), SRP72 (signal recognition particle 72 kDa protein) as well as DRG1 (developmentally regulated GTP-binding protein 1) (Park et al., 2004). The apical region of the antler is the site in which growth and modified endochondral ossification processes take place, while membranous ossification occurs around the antler shaft (Price & Allen, 2004). The apical region of the antler consists of several layers: distal perichondrium and respectively – mesenchyme, transient layer, cartilage and bone (Cegielski et al., 2009; Li et al., 2002). Worth noticing is the mesenchyme, which consists of numerous undifferentiated and actively proliferating cells. They are characterised by low levels of type I collagen and alkaline phosphatase expression and no expression of type II collagen (Price et al., 1994; Price et al., 1996). *In vitro* studies show that IGF-1, IGF-2 and FGF-2 stimulate their proliferation, however BMP-2 and TGFβ-1 inhibit their proliferation (Price et al., 2005b). The mesenchymal cells possess a great potential to proliferate, which has been confirmed by presence of extracellular matrix protein – matrilin-2 – which is a marker

characteristic for differentiating cells (Korpos et al., 2005). Moreover, the mesenchymal cells synthesize many growth factors and other substances, which may affect the mesenchyme itself or adjacent layers of cells: PTHrP (Faucheux et al., 2004), retinoic acid (Allen et al., 2002), FGF-2 (Lai et al., 2007), EGF (Barling et al., 2005), pleiotropin (Clark et al., 2006) and PEDF (Lord et al., 2007). The mesenchyme participates in antler growth because constantly provides cells which differentiate into chondroblasts and next, into mature cartilage cells. The newly added cartilage undergoes mineralization and transforms into a bone (Szuwart et al., 1998). The transformation, as well as modelling of the cartilage, take place in the presence of osteoclasts (found in perivascular spaces of the cartilage) which derive from osteoclast progenitor cells influenced by locally secreted PTHrP (Faucheux et al., 2002). Apoptotic processes also play a vital role in antler growth and shaping processes, just as it happens during skeletal development, bone growth and remodelling (Colitti et al., 2005).

2. Histology of growing and mature antler

The terminal fragment of a growing antler (Fig. 1) consists of several layers. Microscopically, from the outside, we can distinguish:

A – hairy skin
B – perichondrium
C – mesenchyme and chondroprogenitor area
D – blood vessels and chondrocyte columns

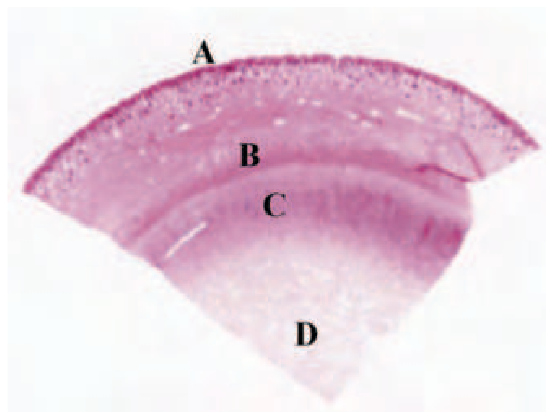


Fig. 1. Cross-section of terminal fragments of the growing antler showing following layers: (A) hairy skin, (B) perichondrium, (C) mesenchyme-chondroprogenitors, (D) central shaft formed of numerous blood vessels and chondrocyte columns. H+E staining, ×40

Antlers are covered by hairy skin, the so-called velvet, consisting of three basic layers: epidermis, dermis and subcutaneous tissue (Fig. 2a). Epidermis is keratinized stratified squamous epithelium built from five layers of cells: basal, squamous, granular, clear and horny (Fig. 2b). Dermis consists of proper connective tissue and has two layers: papillary and reticular. The wave-like organisation of the first layer constitutes the basis of good connection with epidermis (Fig. 2b). The second layer possesses numerous bundles of collagen fibres, hair roots and sebaceous glands (Fig. 2a). The subcutaneous layer has numerous blood vessels, collagen fibres and connective tissue cells. Below the skin region, there is a darker staining zone of tissue constituting the perichondrium (Fig. 2c), the outer

part of which is fibrous and the inner part contains cells. Below the perichondrium, there is a wide layer of undifferentiated cells constituting the mesenchyme of the antler. It surrounds the so-called central shaft built from numerous blood vessels stretching from base to apex of the growing antler. Among these vessels, there are chondrocytes organised in characteristic columns (Fig. 2d).

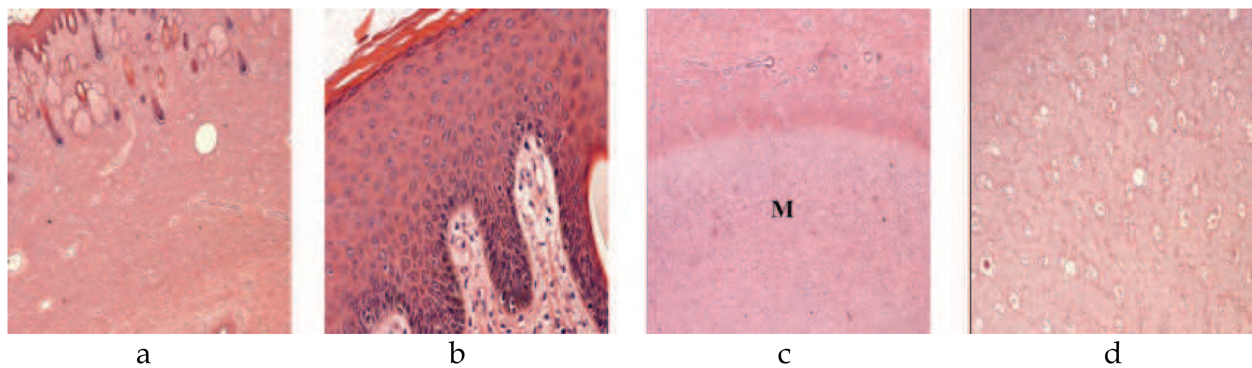


Fig. 2. a-d Histology of the apical fragment of the growing antler, longitudinal section. a – hairy skin (velvet) consisting of epidermis, dermis with hair and sebaceous glands, connective tissue, ×100; b – epidermis built from 5 layers of cells and papillary layer of dermis, ×300; c – perichondrium as a darker stained line below which, there is a wide layer of mesenchyme (M), ×100; d – numerous blood vessels and chondrocyte columns lying in the central shaft of the growing antler, ×200. All H+E staining

Immunohistochemical reactions allowed us to identify presence of cells expressing markers characteristic for stem cells (Bcrp1, c-kit) in the zone of intense proliferation – the mesenchyme (Fig. 3a). A large number of Ki-67- and PCNA-positive cells could be observed in the basal layer of epidermis, in skin glands, directly below secretory sections of skin glands, mesenchyme as well as within and in the vicinity of central blood vessels (Fig. 3b-d).

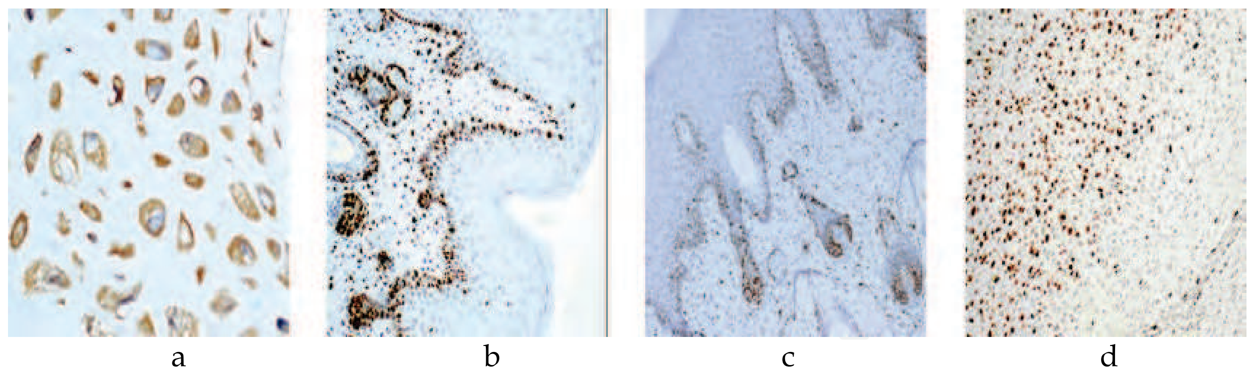
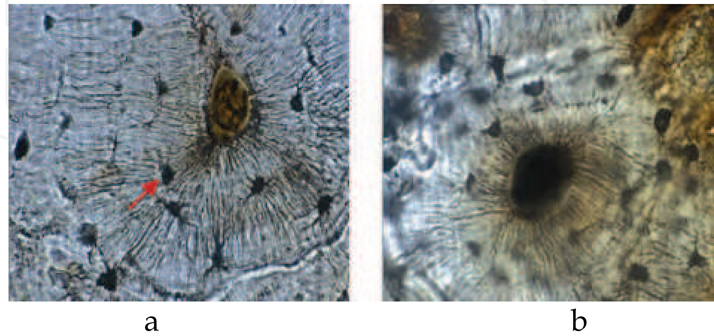


Fig. 3. a-d Immunohistochemical reactions for presence of stem cell markers and proliferating cell markers. a – stem cells (c-kit+) within mesenchyme of the growing antler, ×400; b – proliferating cells of the reproductive layer of epidermis and glands of the growing antler (Ki-67+), ×100; c – proliferating cells of the reproductive layer of epidermis as well as hair papillae of the antler (PCNA+), ×100; d – layer of undifferentiated cells constituting antler mesenchyme (Ki-67+), ×100

Unlike growing antlers, mature antlers consist of mineralized lamellar osseous tissue. The architectural unit is the osteon, which is built from the Haversian canal, around which two or three systemic plates are concentrically arranged (Figs. 4a, b). Both in and among these plates, there are osseous lacunae which possess osteocytes. All osseous lacunae within an osteon are connected by a network of bone canaliculi in which osteocyte processes are located.



a b

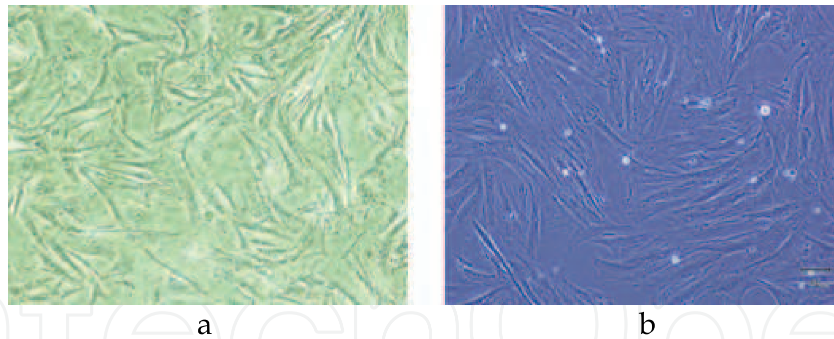
Figs. 4. a, b Cut of an ossified antler shows a typical organisation of lamellar osseous tissue. Around the blood vessel systemic plates, osteocytes (↑) and bone canaliculi can be seen. Stained with silver nitrate, ×400

3. Derivation of MIC-1 cell lineage

To start a culture of antlerogenic cells, we used fragments (100-900 µm) of mechanically disintegrated growing deer antler from which the hairy skin had been removed. By means of cell migration, proliferating cells were isolated and later placed in culture flasks. The culture medium used was DMEM (Dulbecco's Modified Eagle Medium) supplemented with L-glutamine (1 mM/ml), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were cultured under standard conditions of 5% CO_2 and temperature of 37°C. The culture was maintained over 4 months, with yield of 5×10^6/week per one 75 cm^2 culture flask. Obtained cells were frozen and stored in liquid nitrogen. For study purposes, they were again thawn.

Evaluation of antlerogeneic cells:

- Under an inverted phase contrast microscope. Cells growing in culture are spindle-shaped (fibroblast-like) (Fig. 5a) and their microscopic image is comparable to that of cultured human mesenchymal stem cells obtained from bone marrow (Ryan et al., 2005). The cells possess high proliferative potential (within 72 hours their number in culture doubles). Among cultured cells, there are small, round, undifferentiated, opalescent cells not attached to the surface and possessing no processes or any characteristic morphological features (Fig. 5b).



Figs. 5. a, b Primary culture of antlerogenic cells growing in a monolayer. a – differentiating and processes producing antlerogenic stem cells, ×200, b – numerous small, opalescent, non-adherent, undifferentiated cells, ×100. Inverted phase contrast microscopy

- Under light microscopy. Small, oval undifferentiated cells can be observed in fixed 24-h microcultures stained with H+E (Fig. 6).

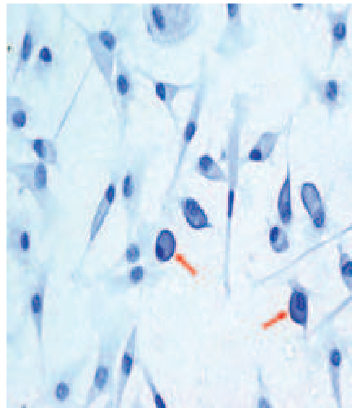


Fig. 6. Microculture of undifferentiated small, oval antlerogenic cells (↑), more mature cells possess processes, H+E staining, ×200

- Under scanning electron microscopy we observed cell morphology (Fig. 7).

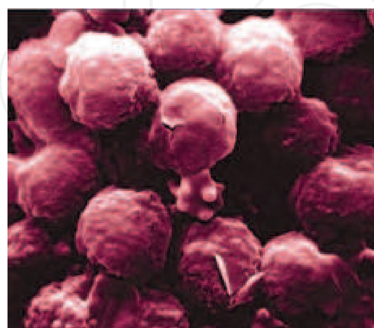


Fig. 7. *In vitro* culture of antlerogenic cells. Scanning electron microscopy, ×6600

- Immunohistochemical reactions for presence of stem cell markers: c-kit, Thy-1. Specific positive membranous reaction with anti-c-kit and anti-Thy1 antibodies was obtained

only in small, oval frequently dividing cells (Fig. 8a, b). No expression of the above mentioned antigens was shown in more differentiated cells with processes.

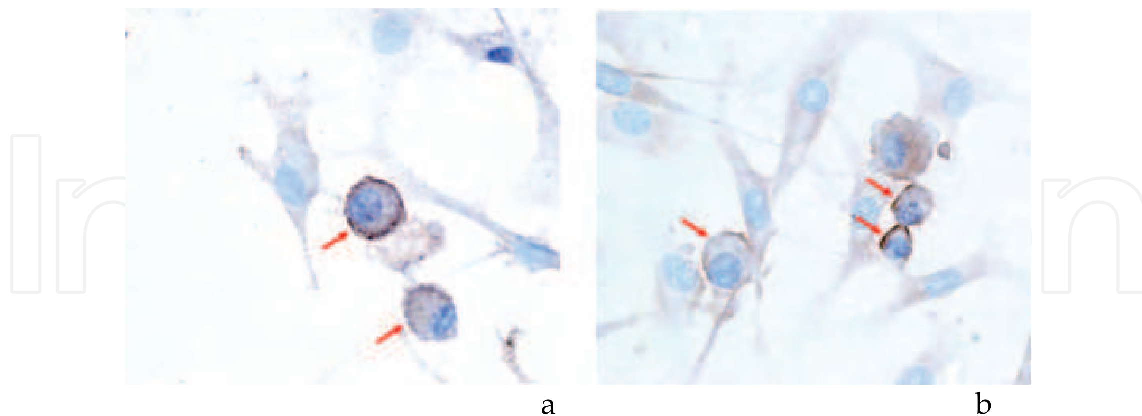


Fig. 8. a, b Microculture: a – immunocytochemical membranous reaction to c-kit (↑) protein presence in small, oval antlerogenic cells, ×400; b – immunocytochemical membranous reaction to Thy1 (↑) protein presence in small, oval antlerogenic cells, ×400

- Using PCR reaction we demonstrated expression of following genes: POU5F1, c-myc, MHC I as well as no expression of MHC II (Fig. 9a, b). From MIC-1 antlerogeneic cells we isolated the total RNA, conducted an reverse transcription and performed the PCR reaction with following primer pairs on the cDNA template (Table 1):

	Primer sequence	PCR product size	References
POU5F1 (OCT4)	5′-ATGACTTGTGTGGAGGGATGG-3′ 5′-GAACACCTTTCCAAAGAGAACC-3′	338 bp	Berg et al., 2007
c-myc	5′-GAGGGTCAAGTTGGACAGTGTCAG-3′ 3′-CTTGGACGGACAGGATGTATGCTG-5′	254 bp	Francis & Suttie, 1998
MHC I	5′-GGATGAAGCATCACTCAG-3′ 3′-CGCTGCTGCGCGCAGACC-5′	530 bp	Holmes et al., 2003
MHC II	5′-GTGTTACTTCACCAACGGGACG-3′ 3′-GTTGTGGTGGTTTAGAGCCTC-3′	207 bp	Swarbrick & Crawford, 1997

Table 1. Sequence of primers used in PCR reactions

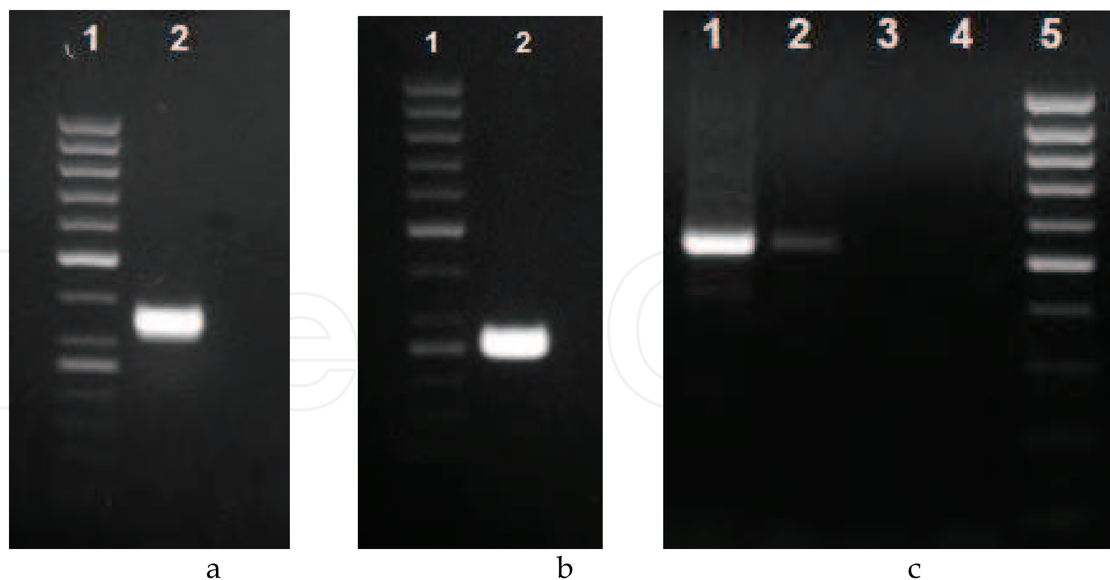


Fig. 9. a, b Expression of various genes in MIC-1 antlerogenic cells. a – lane 1: Molecular weight marker (50-1000 bp, Promega), lane 2 – POU5F1 (338 bp); b – lane 1: Molecular weight marker (50-1000 bp), lane 2: c-myc (254 bp); c – lane 1: MHCI (530 bp), lane 2: MHCI 1:10 cDNA (530 bp), lane 3: MHCII, lane 4: MHCII 1:10 cDNA, lane 5: Molecular weight marker (100-1000 bp, Promega)

- Under electron microscopy. Active cells possess a large nucleus with loose chromatin and nucleoli. The small amount of cytoplasm surrounding the nucleus contained vast amounts of rough endoplasmic reticulum, mitochondria and vacuoles. The presence of glycogen granules makes these cells similar to embryonic cells. The cell surface was covered by numerous microvilli (Fig. 10).

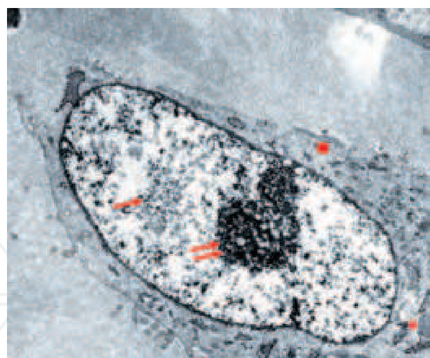
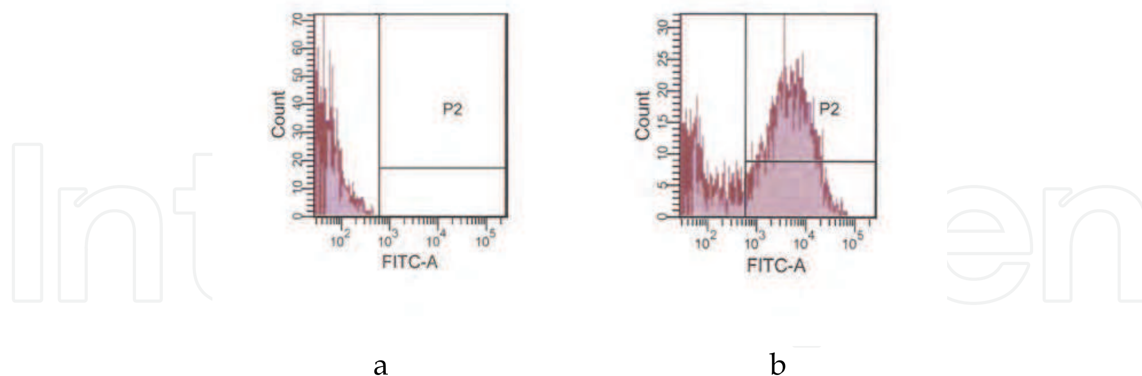


Fig. 10. Undifferentiated antler cell from an *in vitro* culture. Cell nucleus contains loose, transcriptively active chromatin (↑) and a nucleolus (↑↑). The cytoplasm contains rough endoplasmic reticulum (■) and mitochondria (*). Cell membrane forms numerous short processes. EM ×15000

- Marking of antlerogenic cells using a retroviral vector pMINV EGFP. Analysis conducted in a flow cytometer demonstrated 30% of EGFP+ cells (Fig. 11) Transduction of antlerogenic stem cells with an EGFP gene marker enabled us to follow the fates of cells engrafted into auricular cartilage defects.



a b

Fig. 11. Evaluation of EGFP expression in antlerogenic cells transducted with a pMINV EGFP by means of flow cytometry. a – no expression of EGFP in non-transduced cells; b – EGFP protein fluorescence level in transduced cells

4. Xenogeneic engraftment of antlerogenic cells and tissue regeneration

Autologous cartilage and bone tissue are commonly used materials for transplants in facial reconstructive surgery. The limited amount of tissue possible to obtain in children, surgical procedures conducted during its collection and, additionally – in case of bone graft – the risk of no graft adhesion with the host's tissue and its resorption, emphasise the imperfection of this method (Rotter et al., 2005). Despite common use of allogeneic cartilage and bone in regeneration of defects, the limiting factor may constitute their availability and possibility of losing them (Komender et al., 2001; Więcko et al., 1980). Chondrocytes amplified *in vitro* and used in tissue engineering may undergo differentiation (Barbero et al., 2003). Studies over the application of *in vitro* amplified osteoblasts show on the other hand, that differentiated cells do not provide proper environment for autogeneic cell regeneration and they require being administered together with growth factors (Sakata et al., 2006; Schliephake et al., 2001).

Another problem we addressed was the aseptic inflammation of the digital flexor muscles and tendons and interosseus muscle inflammation in horses. It affects about 10% of horses from the overall number of orthopaedic patients and constitutes, nowadays, a serious problem for doctors and horse owners. It causes long-term exclusion of horses from their previous activities. The most recent therapies rely on intratendonous injections under ultrasonographic control of: growth factors (platelet-rich plasma, IGF-1), bone marrow and autologous stem cells (Aspenberg, 2007). The last of the mentioned methods is exceptionally difficult to perform. It is necessary to collect the bone marrow, which constitutes an additional procedure. Time required for cell amplification is 3 to 4 weeks, which makes it difficult to administer the cells into the tendon at an optimal moment for after this time, the haematoma formed at the site of injury already undergoes organisation (Richardson et al., 2007).

In recent years, new possibilities for application of various stem cell types in regeneration and reconstruction of all types of tissues, including the connective tissue, appeared (Phinney & Prockop, 2007). Among others, a technology of obtaining chondrocytes from human stem cells and technology of obtaining mesenchymal cell precursors able to trigger cartilage

growth and regeneration were devised (Barberi et al., 2005; Vats et al., 2006). More and more often, also mesenchymal stem cells (MSC) are used in combination with natural or synthetic carriers, as well as BMP proteins, for bone reconstruction (Jafarian et al., 2008; Seto et al., 2001). The MSC cells can be obtained, among others, from bone marrow, adipose tissue, periosteum and dental pulp. Their advantages are their great proliferating potential, multipotentiality, as well as little immunogenicity and lack of tendencies to neoplasia (Shanti et al., 2007). Bearing in mind the common difficulties in obtaining the proper material for engraftment and searching for alternatives for human or autologous stem cells, we made an attempt to use a xenogeneic graft of cells obtained from a growing antler of the red deer (*Cervus elaphus*) to reconstruct of auricular cartilage and mandibular bone in rabbits, as well as tendons in horses (Cegielski et al., 2008a; Cegielski & Kalisiak, 2008). A stable MIC-1 antlerogenic cell line (name of the line introduced in patents) – mesenchymal stem cells – was derived from a growing antler of the red deer (Cegielski et al., 2006). The cells cultured by us are characterised by rapid growth and are practically "immortal," require uncomplicated standard procedures, which are generally accepted and used in cell culture laboratories. They can easily be transported, frozen and stored in liquid nitrogen and, if necessary, used for studies. The use of specific antibodies in immunocytochemical reactions allowed us to mark and localise these stem cells both *in vivo* in a growing antler, as well as *in vitro* in cell cultures (Cegielski et al., 2006).

4.1 Cartilage tissue

The aim of this study was application of a xenogeneic graft of stem cells obtained from a growing red deer antler (*Cervus elaphus*) to regenerate defects of ear auricular cartilage in rabbits.

For the experiment, we chose California white rabbits – 8-month-old females weighing each about 4 kg. The Spongostan® fragments matched the size of the defect and constituted scaffolding for antlerogenic cells. The cells in the amount of 2×10^6 cell/ml, rinsed in MEM medium without serum, were placed on Spongostan® through delicate centrifugation (Fig. 12).

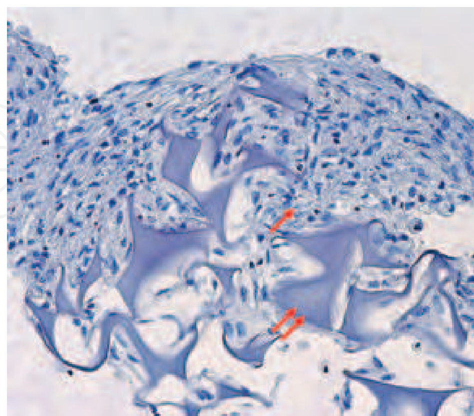


Fig. 12. Cultured antlerogenic cells (↑) placed on Spongostan® (↑↑) and prepared for engraftment. H+E staining, ×200

Surgical procedures included performing a defect half-way up the outer, dorsal part of the right ear auricle. At surgical site a centrally peduncled skin/perichondrium flap measuring 1.5 cm × 1.5 cm was prepared. The exposed cartilage fragment measuring 1 cm × 1 cm was excised and removed. The animals had the cartilage defects implanted with antlerogenic cells suspended on Spongostan®, antlerogenic cells transduced with a retroviral pMINV EGFP vector or Spongostan® alone saturated solely with physiological saline solution (control). The implants were covered with skin/perichondrium flaps. Using clinical observations as guidelines, after a period of 4 and 9 weeks from the surgery, we obtained specimens from the implantation sites, including the implant as well as endogenous cartilage. The collected material was then assessed under optical microscopy (H+E, toluidine blue), electron microscopy and by immunohistochemical examinations (reactions against antigens: Thy1, CXCR4, EGFP, CD3, CD20, CD68).

The implanted cells participated in cartilage reconstruction as a new cartilaginous scaffolding had been created from fibrous cartilage in which sporadic intracartilaginous ossification was observed. Peripherally, in the sites of recovery, there was a visible border between the old and the newly formed cartilage (Fig. 13). Numerous dividing chondrocytes and forming isogeneic groups were present (Fig. 14). After four weeks, the growth in cartilage thickness decreased, however, after nine weeks, the cartilage defect was completely and even excessively reconstructed. Vascularised, mixed chondro-osseous tissue was formed, just like the one participating in formation of deer antlers (Price et al., 2005a). The cartilage reconstruction process (calcification and ossification centres) resembled the remodelling processes taking place after engraftment of auto- and allogeneic cartilage (Lattyak et al., 2003; Więcko et al., 1980). As in application of autogeneic chondrocytes suspended on a polyethylene carrier, after the implantation of antlerogenic cells, we observed no connective-tissue capsule (Wellisz, 1993). In rabbits, at the sites where Spongostan® saturated solely with physiological saline solution was engrafted, we observed no cartilage regeneration, but only a scar formation process.

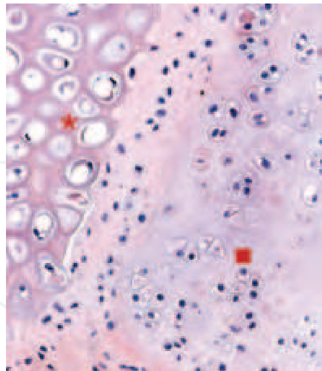


Fig. 13

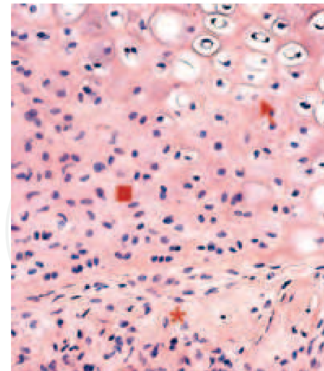
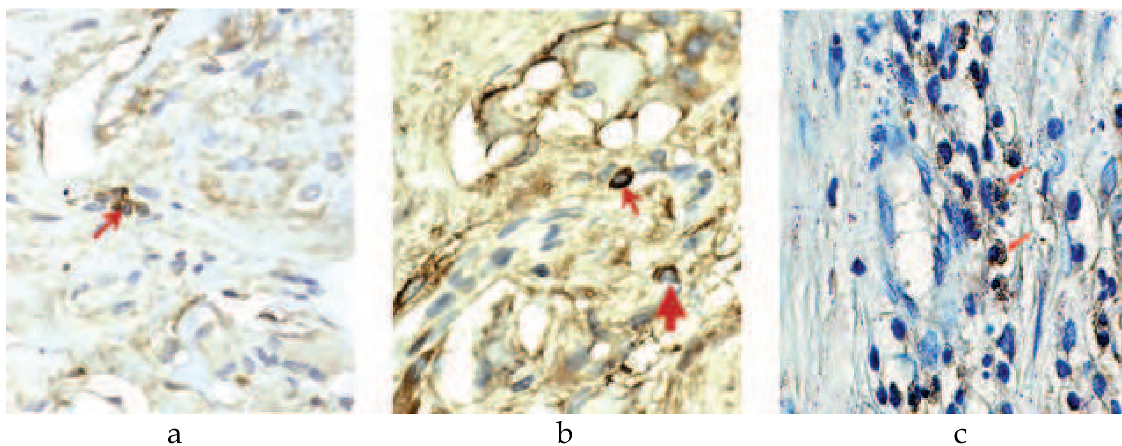


Fig.14

Fig. 13. Engraftment of antlerogenic cells within rabbit's ear auricle. Visible border between the old (*) and the newly formed (■) cartilage, H+E staining, ×200

Fig. 14. Engraft, antlerogenic cells taking part in regeneration of rabbit's cartilage defect. Visible numerous chondroblasts (*), dividing chondrocytes (■) as well as mature cartilage cells (↑), H+E staining, ×200

Conducted immunocytochemical reactions allowed us to localize, at the implantation site, cells showing expression of proteins characteristic of stem cells: Thy1 and CXCR4. The marked cells were located in the vicinity and within blood vessels as well as in the area of proliferating, undifferentiated cells at the implantation/autologous ear auricle cartilage border (Fig. 15a, b). To confirm the participation of antlerogenic stem cells in cartilage regeneration, we engrafted into defect areas cells transduced with a vector containing EGFP marker gene. Stem cells transduction with a retroviral vector containing EGFP marker gene constitutes one of the most commonly used methods to follow the fate of engrafted cells (Szyda et al., 2006). The immunohistochemical reaction conducted four weeks from the engraftment, showed the presence of EGFP-positive cells within the area of cartilage reconstruction (Fig. 15c). The reaction was specific only for small, undifferentiated cells, which proves that antlerogenic stem cells were not destroyed by the cells of the host. Moreover, they did not require any environment (tissue niches) to retain their undifferentiated state and stimulated the rabbit's cartilage to regenerate.

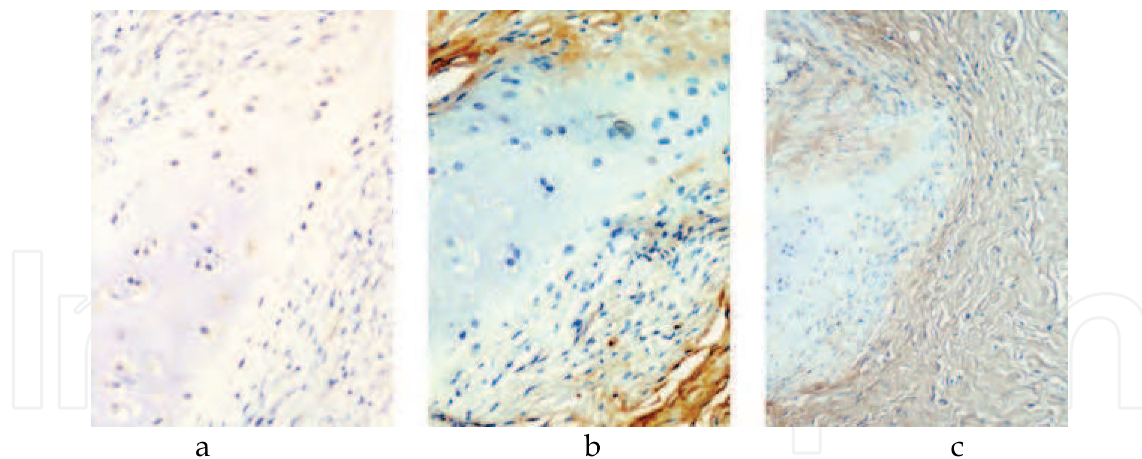


Figs. 15. a-c Immunohistochemical reactions demonstrating presence of antlerogenic stem cells within the implant. a – immunohistochemical localization of CXCR4+ cells (↑), ×200; b – immunohistochemical localization of Thy1+ cells (↑), ×200; c – immunohistochemical localization of EGFP+ transduced antlerogenic cells (↑), ×400

Participation of antlerogenic stem cells in reconstruction of the damaged tissue may be complex. The reconstruction always occurred from the periphery of the implant outwards and it is possible that stem cells could participate in regeneration of the damaged tissue not by differentiation into cells required for its reconstruction, but by local production of trophic factors modelling the microenvironment at the site of damage as well as inducing survival and proliferation of host cells. To these factors may belong, among others, proteins regulating hematopoesis, angiogenesis, wound healing and immune response (Barbash et al., 2003; Phinney & Prockop, 2007). Nakamura et al. observed regeneration of damaged cardiomyocytes in immunosuppressed mice after implantation of swine MSC. The authors suggest that regeneration is the effect of influence of the trophic MSC-released substances on native tissues (Nakamura et al., 2007). The *in vitro* studies demonstrated that MSC cells secrete cytokines which influence survival and secretory functions of other cells (Le Visage et al., 2004; Parekkadan et al., 2007). While an antler regenerates, certain growth factors are expressed and these are: EGF, FGF-2, VEGF and BMP (Barling et al., 2005; Feng et al., 1995; Lai et al., 2007). In our opinion, engrafted antlerogenic cells may participate in formation of

proper environment for growth of host's cells together with the above mentioned factors. In natural conditions, cartilage is a non-vascularised tissue and it is chondrocytes which probably produce the angiogenesis inhibiting factor (Moses et al., 1990). After implantation of antlerogenic cells into a cartilage defect, we observed numerous small blood vessels on the border of regeneration. This may show that angiogenesis is taking place, creating good conditions for the graft to be accepted. It is worth mentioning that abundant vascularisation of the antler constitutes the most striking difference between its morphology and morphology of other cartilages (Lai et al., 2007). Rich blood supply is necessary here so as to meet high metabolic requirements imposed by rapid cartilage growth. The fact that antlers constitute a valuable model for angiogenesis studies has recently been suggested by Clark and Lai, who demonstrated the presence of VEGF and its receptor VEGFR as well as pleiotropin in tissues of a growing antler (Clark et al., 2006; Lai et al., 2007).

The experiment we conducted did not use immunosupression, and the engrafted xenogenous antlerogenic cells were in direct contact to the perichondrium and remaining cartilage of the host's auricle. Despite this fact, clinically, we observed every time proper wound healing *per primam* and visible increase in cartilage thickness. In an experiment conducted by Ulusal et al. (Ulusal et al., 2005) first, appearing already in the period of 4 to 6 days after the end of immunosupression, symptoms of complete auriculae's allogeneic implant rejection in rats were: edema, localized epidermal desquamation, and formation of erythema. A typical reaction of the host to introduction of an allogeneic implant are massive lymphocyte concentration and the presence of plasma cells (Brown et al., 1980; Romaniuk et al., 1995). On no stage of the conducted experiment, we could observe inflammatory reactions, only in the fourth week we observed minor localized clusters of lymphocytes at the site of cell engraftment. Additionally, immunohistochemical reactions characteristic for T lymphocytes (CD3), B lymphocytes (CD20) and macrophages (CD68) showed none of the mentioned cells at the implantation site, confirming that the host's immune response was minimal (Fig. 16a-c). Because antlerogenic cells are poorly differentiated, they cause minor induction of immune response and good reception of xenogeneic graft. One explanation of the phenomenon responsible for no rejection of the allogeneic grafts with stem cells is the lack of major histocompatibility complex class II antigens (MHC II) and inhibition of T and B lymphocytes activity (Chamberlain et al., 2007; Ryan et al., 2005). Only 6 weeks after Spongostan® with cells or Spongostan® alone were engrafted, we could observe foreign body giant cells (FBGCs) on the surface of the remaining carrier fragments (Fig. 17). In parallel studies, we observed that the FBGCs are produced in the process of macrophage fusion in response to the introduction of a foreign body to an organism (Cegielski et al., 2008b). The carrier we used was completely degraded and resorbed as a result of activity of giant cells. It is probable that Spongostan® had no influence on the survival rate of cells it carried.



Figs. 16. a-c Negative immunohistochemical reactions for presence within the implant of: a – T lymphocytes (CD3+), ×200; b – B lymphocytes (CD20+), ×200; c – macrophages (CD68+), ×200

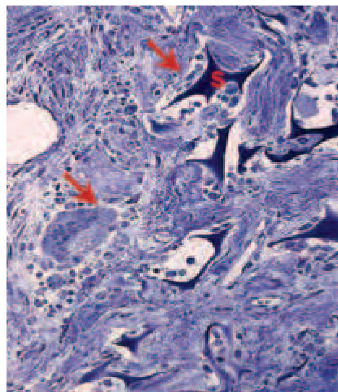


Fig. 17. Spongostan® (S) degradation by multinuclear foreign body giant cells (FBGC) (↑). These cells are visible in the vicinity of the degraded Spongostan®, which takes a typical star-like shape. Toluidine Blue staining, ×200

Electron microscopy analysis of the material collected from implantation sites showed a great number of collagen fibres and relatively numerous chondroblasts and fibroblasts. Active cells contained large amounts of rough endoplasmic reticulum with frequently distended cisterns, vacuoles as well as nuclei with large amounts of loosely packed chromatin (Fig. 18a). Some of the cells underwent apoptosis and membrane-bound cytoplasm fragments separated from them participated in production of extracellular substance (Fig. 18b). Apoptosis plays a vital role during skeletal development as well as bone growth and remodelling. Active apoptotic processes are also responsible for regulation of antler regeneration process, among others – by limiting excessive cell proliferation which may lead to initiation of neoplastic processes (Colitti et al., 2005).

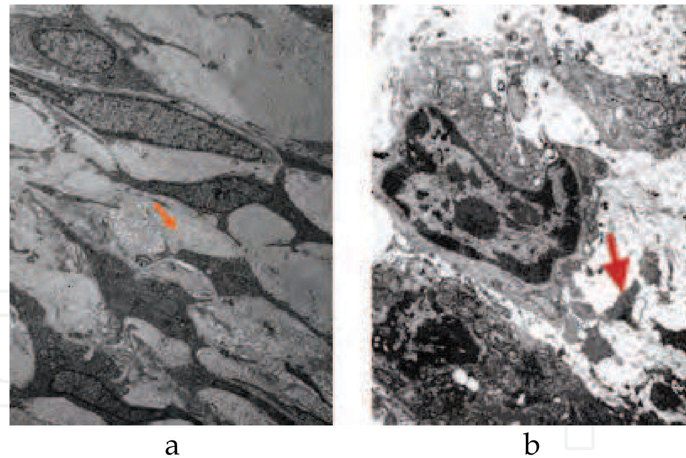


Fig. 18. a, b 9th week after antlerogenic cells implantation: a – numerous collagen fibres (↑), EM ×15000; b – cell containing a nucleus with condensed marginal chromatin, membrane-bound cytoplasm fragments (↑) by separating from the cell become part of extracellular substance, in which numerous collagen fibres are visible, EM ×15000

4.2 Osseous tissue

The aim of the study was the use of a xenogenous stem cell graft from a growing antler of the red deer to regenerate mandibular bone defects in rabbits.

The mandibular bone defect measuring 0.5 cm was performed with a steel surgical drill boring the bone along with the periosteum and reaching the marrow cavity. In the experimental group, inside the bore-hole we placed a fragment of Spongostan® of a matching size saturated with the suspension of antlerogenic cells in the amount of 2×10^6 cell/ml. In the control group, rabbits were implanted with Spongostan® saturated with physiological saline solution. After a period of 1, 2 and 6 months after engraftment, we collected the mandibles for examination.

Preliminary results show, that in the control group autoregeneration of lesion occurred (Fig. 19). In the experimental group antlerogenic cells participated in regeneration of mandibular bone (Fig. 20).

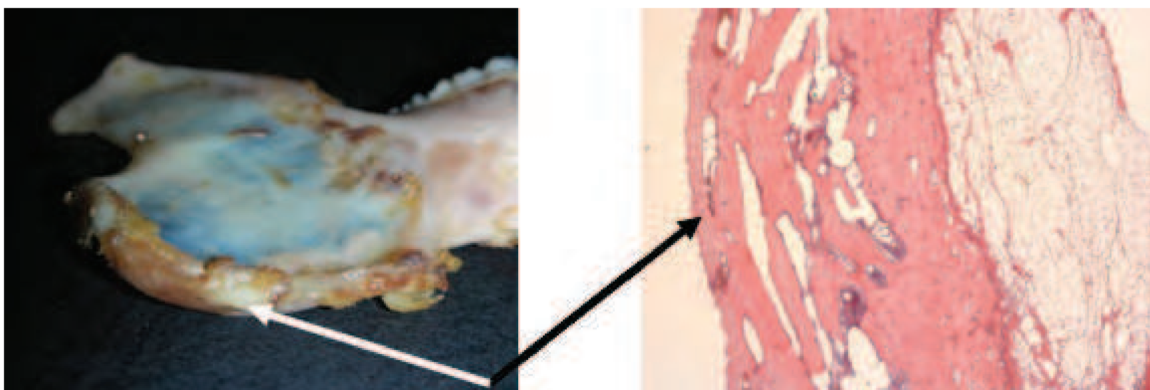


Fig. 19. Control bore-hole, bone autoregeneration after one month, H+E staining, ×40

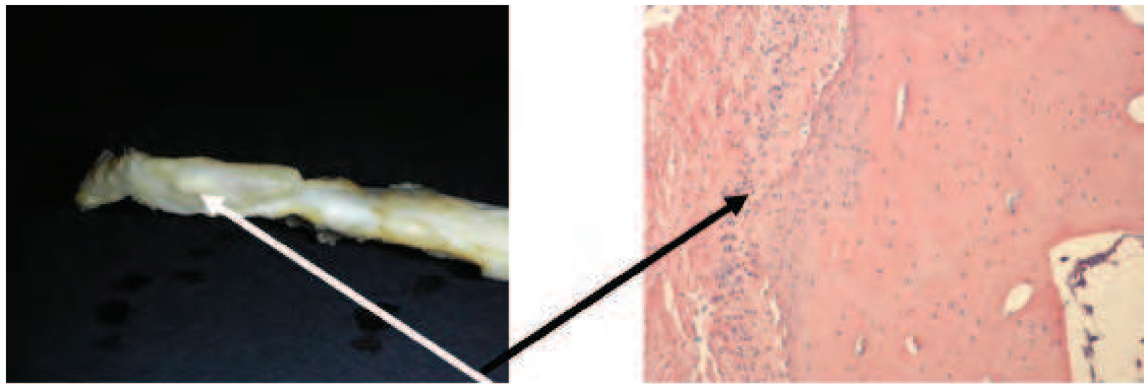


Fig. 20. Bone regeneration with antlerogenic cells at implantation site after one month, H+E staining, ×100

However, between the new periosteum and the muscle layer, we observed oval aggregates of undifferentiated proliferating cells with large amounts of collagen fibres surrounded by a thin connective-tissue capsule (Fig. 21). Isolation of certain areas may be a sign of application of the xenogeneic cells or a carrier. A living organism can react to an xenograft with chronic inflammation, indicating the lack of biocompatibility or can try to isolate the given material by surrounding it with a fibrous capsule of various thickness (usually 0.1-10 μm). The thinner the connective-tissue capsule, the bigger the biocompatibility of the carrier and host cells (Kos et al., 2003). In our experiment, this phenomenon was sporadically observed and probably it did not affect the regenerative processes. On the periphery of the defect, there were well visible ossification areas as well as gradually reconstructed periosteum. In the centre of the regeneration site, there was well vascularized mesenchymal tissue present in a form of a membrane. Angiogenesis constitutes a necessary condition for proper bone formation process. Formation of blood vessels can be a result of the activity of angiogenic factors (VEGF) secreted by antlerogenic cells, just like it takes place in a growing antler (Clark et al., 2006; Lai et al., 2007). The process of formation and mineralisation of the bone occurs from the periphery towards the centre of the regeneration area (Fig. 22).

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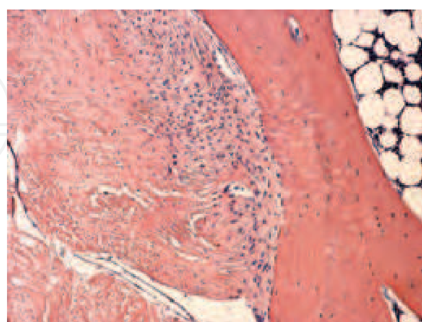

Fig. 21. Two months after implantation, along the mandibular arm we observe the engrafted cells together with a large number of collagen fibres forming oval aggregates surrounded by a thin connective-tissue capsule, H+E staining, ×100

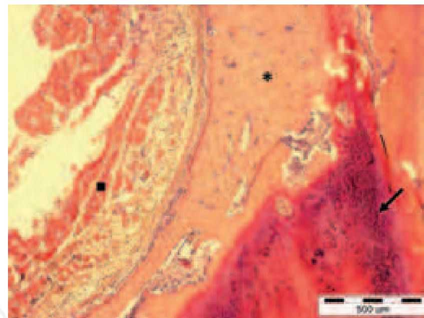

Fig. 22. The area of regeneration of a 1-month bore-hole after implantation of antlerogenic cells. The muscle layer (■) and a visible osseous tissue lying underneath (*). Dark finely flocculating material is the site of early vesicle crystallization and mineralization (↑), H+E staining

After two months, at the implantation site, we observed a reconstructed, two-layered periosteum and a fibrous bone possessing an irregular system of bone plates, containing blood vessels and marrow cavities filled with red marrow. The histological image of the regenerated osseous tissue in our experiment resembles the bone formed after engraftment of autologous MSC of the bone marrow into defects of mandibular bone in dog and monkey. In these experiments, the material collected from implantation sites after respectively 6 and 16 weeks contained trabecular bone with marrow cavity (Jafarian et al., 2008; Seto et al., 2001). 6 months after implantation, we observed the progressing remodelling of the fibrous bone into a more organised lamellar osseous tissue.
The regeneration method in our study proceeded similarly to another long-term xenogeneic model. Human MSC inserted into cranial and mandibular bone defects in mice participated in regeneration of the damaged tissue without inflammatory reaction. MSC cells modulate the function of B lymphocytes and inhibit T lymphocytes proliferation, which induces tolerance for example to allogeneic grafts (Ryan et al., 2005). Both, histological and immunohistochemical evaluations, were performed after 1, 2 and 6 months, and confirmed participation of xenogeneic 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 bony tissue, that underwent mineralization process and was later remodelled into trabecular bone. Antlerogenic cells participated in regeneration of mandibular bone most probably together with tolerating them host cells. In our opinion, the engrafted cells could have participated in forming of an environment favourable to host's cell growth just like it happened during cartilage tissue regeneration. The results of the experiment with rabbits allow us to believe that the antlerogenic cells could be used in reconstruction of bony tissues in other species as well.

4.3 Tendinous tissue

The aim of the study was the use of a xenogeneic graft of stem cells from a growing antler of red deer to regenerate tendons in horses. Antlerogenic cells synthesize great amounts of collagen (Fig. 23), which is used in a dynamic remodelling of an antler (Price et al., 1996). Tendons and ligaments are built from thick bundles of collagen fibres, among which lie tendinous cells – tenocytes. They are surrounded by loose connective tissue, forming the internal peritendineum. Outside of the tendon, there is the external peritendineum, which participates in formation of a synovial tendon sheath. Guided intratendinous injection of

antlerogenic cells with great regenerative potential could rapidly start reconstruction processes.
Considering the results of preliminary studies, we knew that administration of antlerogenic cells to horses does not induce the immune response or lead to development of local inflammatory reaction. Despite the fact that horses are animals greatly susceptible to allergic responses, the test we performed on three horses, which was based on subcutaneous injection of 1.5 ml antlerogenic cells suspension into points of the middle 1/3 of horse's neck (injection sites were shaved and sterilized with 70% alcohol) after three-week-long observation was successful. There was no inflammatory reaction at the site where the cells were injected or hypersensitivity of anaphylactic type. Next, we performed intratendinous injections of antlerogenic cells in two horses suffering from tendon inflammation: in the former peripheral inflammation of superficial digital flexor tendon and muscle occurred, in the latter – interosseous muscle inflammation in the middle of the metacarpus. During six-week-long observation after administration of the cells, we noticed no reaction, despite slight painfulness at the sites of the injection present during 2 first weeks. Follow-up ultrasound examinations conducted at two-week intervals showed intense remodelling of the damaged structures (Fig. 24a-c). In order to assess objectively the effectiveness of this new method, further eighteen-month-long observation of patients lasts and clinical examinations on a bigger number of horses are necessary. However, the preliminary results are promising and encourage further studies.

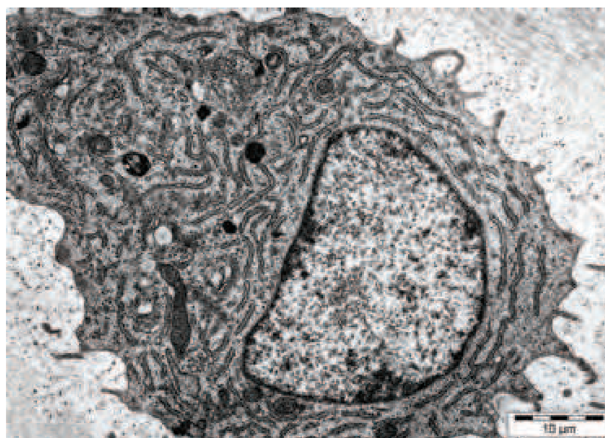

Fig. 23. Growing antler cell (chondroblast) containing numerous canaliculi of the endoplasmic reticulum, mitochondria and collagen fibres

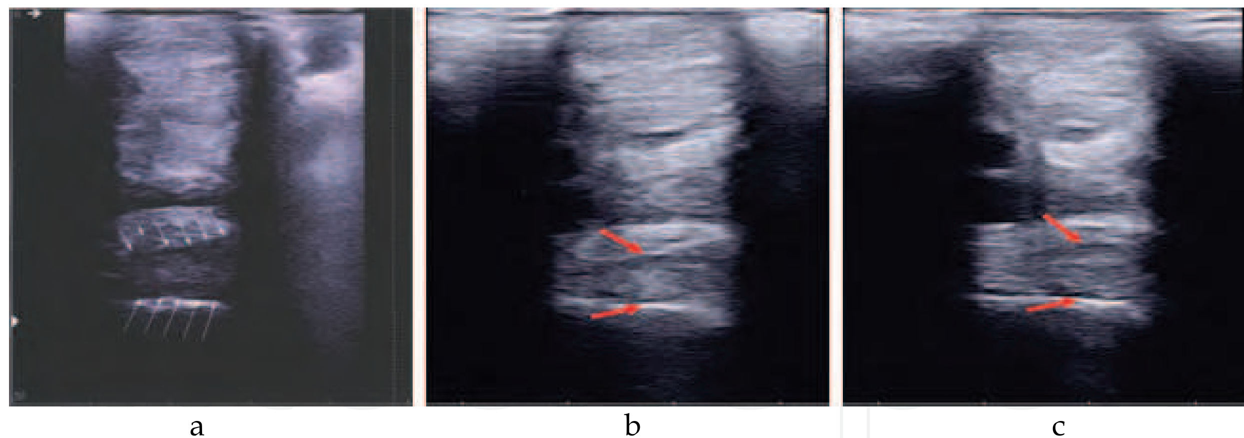

Fig. 24. a – ultrasound image of digital flexor muscle tendon and interosseous muscle in a horse. Arrows indicate the injury of the interosseous muscle; b – ultrasound image of the same horse 4 weeks after the injection; c – ultrasound image of the same horse 8 weeks after the injection. Visible reconstructing processes around the site of antlerogenic cells injection

5. Perspectives of antlerogenic cells application in medicine

More and more often stem cells are used in treatment of injured tissues and organs. In reconstructive surgery of the skull and face, autologous stem cells are successfully used to regenerate bones: mandibular and cranial bones (Lendeckel et al., 2004; Shayesteh et al., 2008; Warnke et al., 2004). A visible improvement has also been gained in the case of using them in treatments of osteogenesis imperfecta and knee joint cartilage defect in human (Horwitz et al., 2002; Kuroda et al., 2007). An alternative for human cells could be properly modified xenogeneic cells. The participation of antlerogenic cell in reconstruction of damaged tissues, poor immunogenicity, good integration of the reconstructed tissues with host's own tissue, rapid *in vitro* amplification as well as low production cost of the engraft constitute its advantages. From the results of the experiments concerning the participation of antlerogenic stem cells in regeneration of cartilaginous and osseous tissue defects in rabbits we know that after the completed reconstruction process, majority of the administered cells undergoes apoptosis and the remaining cells are eliminated by neutrophils. A two-year-long period of observation of experimental animals and lack of any undesirable responses allows us to conclude that there is great probability in using these cells in widely understood regenerative medicine. Our concept is confirmed by studies conducted by other authors, who prove that specificity of the stem cells differentiation process depends on the site of their engraftment. For example, swine mesenchymal stem cells implanted into a heart of a rat do not differentiate into chondroblasts or osteoblasts (as results from their purpose), but stimulate regeneration of injured cardiomyocytes (Nakamura et al., 2007). Full application of stem cells in regenerative medicine will require, however, learning about the differentiation and behaviour of these cells both *in vitro* and *in vivo*, as well as selecting proper carrier for them.

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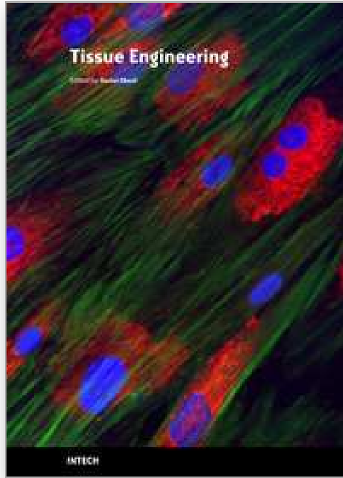

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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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