

## Chondrocytic Potential of Allogenic Mesenchymal Stem Cells Transplanted without Immunosuppression to Regenerate Physeal Defect in Rabbits

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### Abstract

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Mesenchymal stem cells (MSCs) from bone marrow are multipotent cells capable of forming cartilage, bone, and other connective tissues. The objective of this study was to determine whether the use of allogenic mesenchymal stem cells could functionally heal a defect in the distal femoral physis in rabbits without the use of immunosuppressive therapy.

A iatrogenic defect was created in the lateral femoral condyle of thirty-two New Zealand white rabbits, 7 weeks old, weighing  $2.25 \pm 0.24$  kg. Each defect, 3.5 mm in width and 12 mm in length, in the right distal femoral physis was treated with allogenic mesenchymal stem cells in new composite hyaluronate/collagen type I/fibrin scaffold. The healing response was evaluated radiographically, by MRI (three weeks and four months after implantation) and also histologically, by Pearl's reaction and with immunofluorescence (four months after implantation). The results were compared with the data for the control defects (without stem cell implantation) in left distal femoral physes.

On average, right femurs with a damaged distal physis and transplanted MSCs grew more in length ( $0.55 \pm 0.21$  cm) compared with left femurs with a physeal defect without stem cell transplantation ( $0.46 \pm 0.23$  cm). Valgus deformity of right femurs with a physeal defect and transplanted MSCs was mild ( $0.2 \pm 0.1^\circ$ ). On the contrary, left femurs with a physeal defect without transplanted MSCs showed a significant valgus deformity ( $2.7 \pm 1.6^\circ$ ). For defects treated with allogenic mesenchymal stem cell implants, no adverse immune response and implant rejection were detected in this model. Histologically, no lymphocytic infiltration occurred. At four months after transplantation, hyaline cartilage had formed throughout the defects treated with allogenic MSCs. Labeled mesenchymal stem cells/differentiated chondrocytes were detected in the physeal defects based on magnetic resonance imaging and immunofluorescence.

The results of this study demonstrated that allogenic mesenchymal stem cells in a new composite hyaluronate/collagen type I/fibrin scaffold repaired iatrogenic defects in the distal femoral physes in rabbits without the use of immunosuppressive therapy. The use of allogenic mesenchymal stem cells for the repair of physeal defects may be an alternative to autologous MSCs transplantation. An allogenic approach would enable mesenchymal stem cells to be isolated from any donor, providing a readily available source of cells for cartilage tissue repair.

*Growth plate injury, bone bridge, limb deformity, physeal repair, rejection*

Trauma is a typical cause of defect in growth cartilage and the subsequent bone bridge formation. The standard surgical treatment method for bone growth defects due to closure of physeal growth cartilage consists of the resection of the bone bridge and corrective osteotomy (Bright 1984; Broughton et al. 1989; Klassen and Peterson 1982; Langenskiöld 1981; Macksoud and Bright 1989; Williamson and Staheli 1990).

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More recently, cultured chondrocytes (Foster et al. 1990; Gál et al. 2002; Lee et al. 1998; Lennox et al. 1983; Nečas et al. 2006) and mesenchymal stem cell (Ahn et al. 2004; Chen et al. 2003; Plánka et al. 2007) transplantations represent an attractive and promising approach to treat physal cartilage defects.

It is a well known fact that mesenchymal stem cells (MSCs) are multi-potent cells capable of differentiation not only into chondrocytes, but also into osteoblasts, adipocytes, tenocytes and myoblasts (Caplan et al. 1993; Jaiswal et al. 1997; Pittenger et al. 1999). After sampling of bone marrow blood by aspiration, MSCs can be isolated and, with regard to their proliferative capacity, cultivated and expanded to large numbers (Beresford 1989; Friedenstein et al. 1987; Lennon et al. 1996). Mesenchymal stem cells have a characteristic immunological phenotype and specific cell surface markers - SH- 2, 3, and 4. However, their surface lacks haematopoietic markers such as CD34 and CD45 (Bruder et al. 1997a; Haynesworth et al. 1992; Pittenger et al. 1999). It was also proven that MSCs remain functional not only after expansion by cultivation, but also after cryopreservation (Bruder et al. 1997b), which expands their potential for tissue therapy.

In our previous study, we showed that by delivering autologous mesenchymal stem cells on a suitable scaffold, we could enhance growth plate regeneration in a distal femoral physal defect and thereby prevent bone bridge formation, bone shortening and angular deformity in rabbits (Plánka et al. 2007). Thus, the results of the study demonstrated the potential of the use of autologous mesenchymal stem cells in physal cartilage repair. However, the transplantation of autologous MSCs in case of a physal injury to a patient in clinical practice would first require bone marrow blood sampling by aspiration from this patient, subsequent several weeks long cultivation (expansion) of mesenchymal stem cells and then implantation to the defective spot. A clinically applicable alternative, however, could consist of bone marrow blood aspiration from a donor, isolation, expansion and cryopreservation of mesenchymal stem cells with subsequent transplantation when necessary (in this case to the injured physal growth plate). This would constitute a possible "off-the-shelf" use of allogenic MSCs in therapy.

In case of such allogenic transplantation it is therefore expected that the donors and recipients are fully immunologically unrelated. This gives rise to the issue of possible adverse immune reaction and possible rejection of allogenic mesenchymal stem cells by the recipient. The results of previous studies suggest that MSCs might be immune privileged cells (Bartholomew et al. 2002; DiNicola et al. 2002). It is believed that surface characteristics of mesenchymal stem cells make it possible for them to avoid rejection. It was proven that mesenchymal stem cells do not express mesenchymal stem cell Class-II molecules and co-stimulatory molecules (B7 and CD40), which are necessary for full activation of T cells responsible for transplant rejection (Devine et al. 2001; McIntosh and Bartholomew 2000). Previous *in vitro* immunological studies prove the fact that mesenchymal stem cells do not provoke immune reaction if combined with allogenic lymphocytic cells (Bartholomew et al. 2002; DiNicola et al. 2002).

This experimental study in the New-Zealand white rabbit focused on the testing of the ability (chondrogenic potential) of allogenic mesenchymal stem cells to elicit regeneration of iatrogenically damaged femoral distal growth plate without using immunosuppressive therapy. The goal was to confirm our working hypothesis that allogenic MSCs after *in vivo* transplantation into the injured physis do not provoke an adverse immune response, and to verify the ability of allogenic mesenchymal stem cells to stimulate and to participate in the healing of iatrogenically created physal defect by newly created hyaline cartilaginous tissue.

## Materials and Methods

The New Zealand white rabbit (from a facility approved for laboratory animal breeding) was chosen as an experimental animal model. Thirty-two healthy individuals, 16 males and 16 females, of the same age (7 weeks) and approximately same weight ( $2.25 \pm 0.24$  kg) were included in the study.

For the transplantation proper, we used allogenic mesenchymal stem cells (MSCs) prepared in the cytological laboratory of the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, Liběchov (IAPG AS CR). The cells came from unrelated rabbits used in another study. Twenty-one days before transplantation of MSCs to the recipient animal, a bone marrow blood sample was taken from the donor rabbits to culture the MSCs. The samples were taken under general anaesthesia. Induction was achieved by intramuscular administration of medetomidine at the dose of 200  $\mu\text{g}/\text{kg}$  (DOMITOR inj. a.u.v., Pfizer) and ketamine 10 mg/kg (NARKAMON 5% inj., Léčiva). Total intravenous anaesthesia (TIVA) was then maintained with propofol at the dose of 0.15 mg/kg/h (PROPOFOL ABBOTT inj., Abbott Lab). Hypodermic needle (20G/40 mm) was used to take bone marrow blood in the syringe (5 ml, Baxter) with 2 ml of PBS (Phosphate Buffered Saline, Dulbecco), 2% FBS (Fetal Bovine Serum, StemCell) and heparin at a dose of 5 IU/ml. Puncture of wings of both ilia of seven-week-old donor rabbits yielded approximately 2 ml of bone marrow blood.

### Isolation, culturing, labelling and differentiation of cells

Blood plasma was removed from collected bone marrow blood by centrifugation (400 g, 30 min). Afterwards, opalescent film of mononuclear cells was skimmed for further isolation and culturing of bone marrow mesenchymal stem cells (MSCs) under laboratory conditions at 37 °C and 5% CO<sub>2</sub>. Culture medium Dulbecco's Modified Eagles Medium (DMEM) (Gibco Laboratories, Life Technologies, Grand Island, NY) supplemented with 10% FBS and gentamycin (50mg/l, GENTAMYCIN, Sigma) was used. Cells were cultured on culture plates with the area of 0.75 cm<sup>2</sup>. After 24 hours, non-adherent cells were removed by rinsing the culture bottle with PBS solution. Culture medium was changed every three days. First colonies of MSCs occurred on days 4 to 5. After 10 days, 80% of the culture plates were covered with cells. MSCs were washed in 0.5% solution of Trypsin and EDTA (Sigma) and layered to achieve a concentration of 5,000 - 6,000 cells/cm<sup>2</sup>. The total number of isolated nuclear cells ranged from 15 to 30 million nuclear cells per each isolation (Plate XII, Fig. 1). Culturing of all samples of MSCs took 21 days.

Before they were transplanted to target tissue, the cultured bone marrow mesenchymal stem cells were labelled in two ways. First, they were labelled by a contrast consisting of paramagnetic iron oxide nanoparticles (Resovist, 0.5 mmol Fe/ml, Shering). Three days before transplantation, Resovist (at the concentration of 1 ml/ml) was added to the culture medium containing the cells. Second, the cells were labelled by lipophilic fluorescent stain CM-DiI (Chloromethylbenzamido derivate of DiI, CellTracker™ CM-DiI, Invitrogena) at the concentration of 5  $\mu\text{l}/2.5$  ml of PBS. This labeller can be detected histologically using the immunofluorescence method. CM-DiI stain was added to MSCs after they were centrifuged (700 g, 5 min); incubation took 5 minutes at 37 °C, and 15 minutes at 4 °C. Finally, MSCs were rinsed with PBS.

The cells were stimulated to differentiate towards chondrocytes as per the protocol (Miura 2002) for 30 minutes in  $\alpha$ -MEM medium with the supplement of 100 ng/ml of human recombinant TGF- $\beta_1$  (R&D Systems), 1% ITS (Insulin - Transferrin - Selenium, Gibco), 100 nM of dexamethasone (DEXAMETAZON, Medochemie) and 50 mg/ml of ascorbate-2-phosphate solution (ASCORBÁT-2-FOSFÁT, Sigma). After induction, the cells were centrifuged (700 g, 5 min) and prepared to be transferred to a scaffold.

### Preparation of the scaffold

The scaffold was prepared at 4 °C by blending 20.75  $\mu\text{l}$  of sodium hyaluronate (10 mg/ml, 1500 kDa (SODIUM HYALURONAT, Contipro)) with 31.1  $\mu\text{l}$  1 mg/ml of collagen type I solution (collagen type I was taken from calfskin kept in acid solvent) in 0.1-molar acetic acid (Kyselina octová 99.8%, Lachner s.r.o.). The scaffold was neutralized in 1-molar kalium hydroxide (potash) (HYDROXID DRASELNY, Penta). Afterwards, a pipette was used to add 36  $\mu\text{l}$  of MSCs suspension ( $2 \times 10^6$  of cells each time).

The medium containing 100  $\mu\text{g}/\text{ml}$  of ascorbate-2-phosphate, 200 mM of dexamethasone, 20% FBS and ITS was supplemented with 0.12 ml solution of human protein for Tissucol (Tissucol® Kit Baxter) in aprotinin (fibrinogen 70 - 110 mg/ml, aprotinin 3000 KIU/ml) and 0.12 ml of thrombin solution (4 IU/ml) in CaCl<sub>2</sub> (40  $\mu\text{mol}/\text{ml}$ , Tissucol® Kit, Baxter). Gel was formed on a microtitration plate (96 wells/300 ml/well diameter 0.628 cm) (TPT) at 37 °C. Afterwards, the culture medium was added, and the scaffold was placed in the incubator with humid atmosphere of 5% CO<sub>2</sub> at 37 °C.

A mixture of scaffold and MSCs prepared in this way was kept in the incubator under the above mentioned conditions until the cells were implanted to the recipient animal (after 1 - 6 hours).

### Surgical procedures

Surgeries were performed under general anaesthesia. Before induction to anaesthesia enrofloxacin (BAYTRIL 2.5% inj. ad us. vet., Bayer) at a dose of 5 mg/kg was administered intravenously. Induction was achieved by intramuscular administration of midazolam (1.00 mg/kg (DORMICUM inj., Roche)) + fentanyl (0.02 mg/kg (FENTANYL, Janssen) and medetomidine at the dose of 200  $\mu\text{g}/\text{kg}$  (DOMITOR inj. a.u.v., Pfizer). Total inhalation anaesthesia was then maintained by a mixture of oxygen, nitrous oxide (2 : 3) and isoflurane (FORANE, Abbott Laboratoires) using a non-re-breathing system (Bain). Heart rate, respiratory rate, invasive blood pressure, end-

tidal partial pressure of carbon dioxide and saturation of haemoglobin by oxygen was monitored (DATEX Cardiocap II). As this combination of drugs causes a strong respiratory depression, all animals were connected to a controlled ventilation device.

Rabbits were placed in dorsal recumbence and the surgical site was routinely prepared for aseptic procedure on both knees.

Lateral arthrotomy of the right stifle joint was performed by parapatellar incision. After visual localization of the growth plate, the battery-powered drill (Colibri system, SYNTHES, USA) was used to create a defect in the lateral part of the distal physis of the femur in order to cause damage exceeding 9% of the growth plate area (Gál et al. 2002; Janáry et al. 1998). Therefore, 3.5 mm drill bit (ACUFEX - MosaicPlasty Precision, Smith & Nephew, USA) was used to bore a canal 12 mm in depth from lateral surface of lateral condyle dorsolaterally above the insertion of *m. extensor digitorum longus*. The canal was drilled in dorsomedial direction in order to cause damage of the lateral part of the distal femoral physis including adjacent parts of the epiphysis and metaphysis. Before implantation of the scaffold with allogeneous MSCs, the canal was dilated using 3.5 mm dilator (ACUFEX - MosaicPlasty Precision, Smith & Nephew, USA). A mixture of the scaffold and MSCs was prepared in wells of a microtitration plate (TPT, from where the implant (in the form of a cylinder 3.5 mm thick and 10 mm long) was taken by the drill guide (ACUFEX - MosaicPlasty Precision, Smith & Nephew, USA) and carefully inserted using a delivery tamp (ACUFEX - MosaicPlasty Precision, Smith & Nephew, USA) into the defect drilled in the lateral femoral condyle. In order to fix the transplant in its position, the canal was closed (on the lateral surface of the lateral condyle of the femur) with a cylinder made from beta-tricalcium phosphate (ChronOS, SYNTHES) 3.5 mm thick and 2 mm long, that was cut out from a preformed ChronOS block using 3.5 mm tubular chisel (ACUFEX - MosaicPlasty Precision, Smith & Nephew, USA).

The stifle joint was lavaged with Ringer lactate solution (Ringer Lactat I.V.Inf., Braun Medical AG). The joint capsule was closed with interrupted suture (polypropylene, Prolene 4/0, Ethicon). Subcutaneous layer was closed with continuous suture using 2/0 polyglactin 910 (Vicryl, Ethicon). The skin was closed with simple interrupted suture using 2/0 polyglactin 910 (Vicryl, Ethicon).

Afterwards, a defect in the lateral part of the distal epiphyseal plate of the left femur was created similarly. However, the canal drilled in the condyle was neither filled with an implant (i.e. the scaffold with allogeneous MSCs), nor with beta-tricalcium phosphate (ChronOS). This limb served as a control.

After the surgery, antagonization of all three anaesthetic components was performed using a combination of naloxon (0.03 mg/kg (INTRENON inj., Léčiva a.s.)) + flumazenil (0.1 mg/kg (ANEXATE, Hoffmann-La Roche Ltd.)) + atipamezol (1.0 mg/kg (ANTISEDAN inj. ad us. vet., Pfizer Animal Health)) that was administered intramuscularly. Analgesia in post-operative period was achieved by application of carprofen (RIMADYL inj. ad us. vet., Pfizer Animal Health) at a dose of 2 mg/kg/day for three days after the surgery.

Following recovery from surgery, the animals were allowed to walk freely and weight down as tolerated. During the whole study period, the animals were fed, handled and housed according to welfare principles (Sýkora et al. 1983). At the end of the experiment (4 months after the surgery), all animals were euthanized *lege artis*. First, they were put under general anaesthesia using intravenous thiopental at a dose of 20 mg/kg. Then they were given intravenous T 61 inj. ad us. vet. (Hoechst Roussel Vet.) at a dose of 1 ml *pro toto*.

Length and angular (valgus) deformity of the operated bone was measured from radiographs in a craniocaudal (CC) projection. The quality of graft incorporation was evaluated histologically and using Pearl's reaction. Detection of the transplanted allogeneic MSCs in the physeal defects was based on magnetic resonance imaging (MRI) and immunofluorescence. All procedures were carried out with the consent of the Ethical Committee (No. 46613/2003-1020).

#### Bone length discrepancy and femoral valgus deformity measurements

Each rabbit was subjected to radiological examination on the day of transplantation and immediately after euthanasia. Bone length discrepancy and valgus deformity were measured from radiographs. Measurement of the length of the right femur (with the physeal defect and transplanted MSCs) and the left femur (with the physeal defect without transplanted MSCs) was done from radiographs of the femur in a craniocaudal (CC) projection. The actual length of the femur and the angle of the valgus deformity of the distal femur were measured. The measurements were performed separately by three independent observers. The measured values were averaged to calculate the arithmetic mean.

#### Magnetic resonance imaging

*In vivo* detection of transplanted MSCs in the physeal defect was assessed by magnetic resonance imaging. Three weeks after the surgery and on the day of euthanasia, the rabbits were subjected to MRI examination - they were examined by the technique of T1 and T2 weighted images and by the sequence modified to highlight hyposignal of MSCs labelled with iron oxide (detection of paramagnetic iron oxide nanoparticles, Resovist). A three-week interval between the transplantation and the first MRI examination selected to eliminate possible formation of artifacts caused by post-operative haematoma (Anderson et al. 2004).

#### Histological findings

Healing of the defect was investigated histologically, using haematoxylin and eosin staining. Femurs were extracted and placed in a fixative 10% solution of buffered formalin for 48 hours. Femurs were decalcified in a solution of hydrochloric acid and ferric chloride (changed every 12 hours) for 5 to 8 days. Following complete

decalcification, the resected samples were sectioned in a way to reveal the longitudinal axis of the canal drilled. Parts of the growth plates with the lesions were excised together with at least 1 cm of the surrounding tissue. They were histologically processed and stained with haematoxylin and eosin. Sections 6 µm in depth were examined and photographed using an optic microscope Nikon Eclipse 1000. Incorporation of the transplant into the canal drilled through the growth plate was evaluated. The defects filled with MSCs in the right distal femoral physis were histologically compared with unfilled left femoral physeal defects.

On the basis of immunofluorescence detection of CM-DiI stain incorporated into the cell wall, these examinations should have proved, whether chondrocytes present in the defect come from the implanted colony of allogeneous MSCs or not.

#### Statistical evaluation

Means and standard deviations were calculated for the length and valgus deformity of the right femur (with the physeal defect and transplanted MSCs) and the left femur (with the physeal defect without transplanted MSCs) as well as for differences in length and angular deformities before MSCs transplantation and after euthanasia. The values were statistically analyzed using Wilcoxon matched-pairs test; STATISTICA (data analysis software system), version 7.1 (StatSoft, Inc. 2005).

## Results

In all thirty-two rabbits, we successfully cultured MSCs allotransplant in the required quality with the average count of cells  $29 \times 10^6$  ( $29 \pm 2 \times 10^6$ ). The allogeneic MSCs were successfully implanted into iatrogenic distal femoral physeal defects. There were no anaesthesia and post-operative complications in any of the animals. The wounds healed *per primam intentionem*, and no clinical adverse host response to allogeneous MSCs transplantation into iatrogenically damaged physes without immunosuppression could be detected.

Results of measurement of femur lengths showed that right femurs with a damaged distal growth zone and transplanted MSCs grew more in length ( $0.55 \pm 0.21$  cm) as compared with left femurs with the physeal defect without transplanted MSCs ( $0.46 \pm 0.23$  cm). Angular (valgus) deformity of right femurs with the physeal defect and transplanted MSCs was mild ( $0.2 \pm 0.1^\circ$ ). On the contrary, left femurs with the physeal defect without transplanted MSCs showed significant valgus deformity ( $2.7 \pm 1.6^\circ$ ). Differences in lengths and valgus deformity were statistically significant (1% level of significance,  $p = 0.001$ ).

Table 1 shows the results of examinations of the distal femoral physeal defects with MRI, the results of histological examination of samples, Pearl's reaction and the results of detection of transplanted allogeneic MSCs by immunofluorescence.

MRI examination proved the presence of paramagnetic nano-particles of iron oxide in the place of transplantation of marked allogeneic MSCs in the lateral section of the right femoral condyle in 85% of cases (in 27 out of 32 animals) (Plate XII, Fig. 2). Such marked MSCs were not proven by this method in five cases (rabbits AL12, AL13, AL16, AL25, and AL30).

Histological examination of sections of the distal portion of the femur coloured with haematoxylin-eosin proved the presence of chondrocytes in this physeal section after transplantation of allogeneic MSCs into places of experimentally created defects in the distal growth plate of the right femur. At eighteen weeks, hyaline cartilage had formed throughout the defects treated with allogeneic MSCs. Histologically, we found two forms of newly created cartilaginous tissue in the place of MSCs transplantation, namely enclaves of chondrocytes (Plate XII, Fig. 3a) or a massive filling of the physeal defect with hyaline cartilage (Plate XII, Fig. 3b). In five cases, no chondrocytes were found in the physeal defect treated with transplantation of allogeneic MSCs. These were the rabbits in which MRI proved no presence of paramagnetic nano-particles of iron oxide in the place of transplantation (rabbits AL12, AL13, AL16, AL25, and AL30). No adverse host response, in terms of lymphocytic infiltration on allogeneic MSCs transplantation without immunosuppression was histologically detected.

In 27 out of 32 experimental animals (Table 1) we proved the presence of transplanted mesenchymal stem cells, or cells differentiated from these implanted allogenic MSCs. In 26 cases we managed to prove the presence of these cells with MRI, histological examination, Pearl's reaction (Plate XIII, Fig. 4), and immunofluorescence simultaneously (Plate XIII, Fig. 5). In one case (rabbit AL20) we proved the presence of transplanted MSCs (or cells differentiated from them) with the aforementioned methods excluding immunofluorescence (Table 1).

Table 1. Results of MRI and histological examination (HE, Pearl's reaction, immunofluorescence examination) of dissections of right distal femoral physis in rabbits.

|      | MRI proof of MSCs with Resovist (after 3 weeks) | MRI proof of MSCs with Resovist (after 4 months) | Result of histological finding HE (after 4 months) | Proof of MSCs with Pearl's reaction (after 4 months) | Proof of MSCs with immunofluorescence (after 4 months) |
|------|---|--|--|--|--|
| AL01 | +   | +  | ++   | +  | +  |
| AL02 | +   | +  | ++   | +  | +  |
| AL03 | +   | +  | ++   | +  | +  |
| AL04 | +   | +  | ++   | +  | +  |
| AL05 | +   | +  | ++   | +  | +  |
| AL06 | +   | +  | ++   | +  | +  |
| AL07 | +   | +  | ++   | +  | +  |
| AL08 | +   | +  | ++   | +  | +  |
| AL09 | +   | +  | ++   | +  | +  |
| AL10 | +   | +  | ++   | +  | +  |
| AL11 | +   | +  | ++   | +  | +  |
| AL12 | +   | -  | -  | -  | -  |
| AL13 | +   | -  | -  | -  | -  |
| AL14 | +   | +  | ++   | +  | +  |
| AL15 | +   | +  | ++   | +  | +  |
| AL16 | +   | -  | -  | -  | -  |
| AL17 | +   | +  | ++   | +  | +  |
| AL18 | +   | +  | ++   | +  | +  |
| AL19 | +   | +  | ++   | +  | +  |
| AL20 | +   | +  | ++   | +  | -  |
| AL21 | +   | +  | ++   | +  | +  |
| AL22 | +   | +  | ++   | +  | +  |
| AL23 | +   | +  | ++   | +  | +  |
| AL24 | +   | +  | ++   | +  | +  |
| AL25 | +   | -  | -  | -  | -  |
| AL26 | +   | +  | ++   | +  | +  |
| AL27 | +   | +  | ++   | +  | +  |
| AL28 | +   | +  | ++   | +  | +  |
| AL29 | +   | +  | ++   | +  | +  |
| AL30 | +   | -  | -  | -  | -  |
| AL31 | +   | +  | ++   | +  | +  |
| AL32 | +   | +  | +  | +  | +  |

+) examination positive – MRI – finding of iron oxide labeled MSCs in the place of transplantation  
 - Pearl's reaction – presence of iron particles in the cytoplasm of chondrocytes  
 - Immunofluorescence – finding of fluorescence hue on the membranes of chondrocytes  
 - Histology – finding of chondrocyte enclaves in the physal defect

++) histological finding (HE) – defect filled with hyaline cartilage without columnar cell formation

-) microscopic examination found no chondrocytes in the defect

## Discussion

In our previous study focused on preventive transplantation of autologous MSCs into the defect in the femoral distal growth plate of the rabbit, hyaline cartilage was formed 4 months after implantation on the site of the original iatrogenic physeal defect (Plánka et al. 2007). Recently the focus of scientific interest has shifted from autologous MSCs implantations towards allogenic cell transplantations. Some authors suggest that there is no need for immunosuppressive therapy in cases of allogenic mesenchymal stem cell transplantation (Chiu et al. 2006; Batten et al. 2006; Plumas et al. 2005; Caparrelli et al. 2001). They describe the immunomodulation effect of MSCs, which itself leads to immunosuppression by inactivating CD4+ T lymphocytes (Batten et al. 2006), or induction of apoptosis of activated T lymphocytes (Plumas et al. 2005; Frank et al. 2004). The aforementioned immunomodulation ability of MSCs is taken a step further in our study - this characteristic was successfully used to cure systemic diseases (scleroderma, multiple sclerosis, rheumatoid arthritis, juvenile idiopathic arthritis and systemic lupus erythematosus). Implanted allogenic MSCs induced a certain type of immunosuppression used in the treatment of these autoimmune diseases (Lar et al. 2006). To our knowledge, this study is the first experimental work that describes a successful allogenic transplantation of mesenchymal stem cells without using immunosuppressive therapy in the repair of a damaged physis in the rabbit. Its results suggest that allogenic mesenchymal stem cells in the rabbit demonstrate a chondrogenic potential with the ability to elicit and participate in the repair of the iatrogenically damaged femoral distal growth plate with newly created hyaline cartilaginous tissue. The repair of cartilaginous tissue of the right femoral distal physis after transplantation of allogenic MSCs was clinically proven by different bone growth compared to the left femur (without transplanted MSCs). The right femur with a defect in the lateral section of the growth plate and transplanted MSCs was longer and less valgus deformed than the left femurs with the same physeal defect without MSCs transplantation. The average difference in the femoral length between operation and euthanasia (i.e. the scope of the bone growth) was, in case of the right femur after preventive transplantation MSCs  $0.55 \pm 0.21$  cm, whereas in case of the left femur it was only  $0.46 \pm 0.23$  cm. The average angular (valgus) deformity of the right femur with preventively transplanted MSCs into the iatrogenically created defect in the femoral distal physis was  $0.2 \pm 0.1^\circ$ , whereas in case of the left femur its valgus deformity was  $2.7 \pm 1.6^\circ$ . The difference in the measured values of both parameters was statistically significant at 1% significance level ( $p = 0.001$ ). The right femur, in contrast to the left femur, grew more in length. Its valgus deformity, compared to the left femur, was minimal and clinically non-significant.

The selection of an appropriate method of isolation, culturing, differentiation of cells and preparation of scaffold material for mesenchymal stem cell use in physeal cartilage repair is also a very important factor for successful MSCs implantation. In our study, the MSCs were separated from bone marrow blood taken from the ilium. Cultured MSCs were stimulated to differentiate towards chondrocytes as per protocol (Miura 2002) in  $\alpha$ -MEM medium with the supplement of human recombinant TGF- $\beta$ 1. The culture medium was supplemented also with 1% ITS, dexamethasone and ascorbate-2-phosphate which are commonly used ingredients of culture and differentiation medium (Li et al. 2005; Lisignoli et al. 2005; Indrawattana et al. 2004; Quintavalla et al. 2002). A new three-component scaffold (New Composite Hyaluronate/Collagen Type I/Fibrin Scaffold), whose characteristics are similar to the natural environment of cartilaginous extracellular matrix, was used. We also found a suitable technique of implant delivery to the site of the damaged distal physis of the femur using ACUFEX (MosaicPlasty Precision) instruments.

Under the conditions of this experiment, allogenic MSCs after *in vivo* transplantation into the injured physis without concomitant immunosuppressive therapy elicited no adverse

immune response. Clinically, no complications with defect healing in the physis or with the healing of the surgical wound were recorded after implantation of allogenic MSCs without immunosuppression. Histologically, no adverse local immune response in the form of lymphocytic infiltration in the place of MSCs implantation was recorded either. Moreover, 4 months after the implantation of allogenic MSCs a newly created hyaline cartilaginous tissue was histologically confirmed in the place of the iatrogenically created physal defect, which proves that the transplanted stem cells remained viable during the growth plate recovery and participated in the healing process of the physal defect.

In 85% of cases (27 out of 32 animals) we proved using MRI, histology, Pearl's reaction and immunofluorescence, the presence of transplanted mesenchymal stem cells, or cells differentiated from these implanted allogenic MSCs. In one instance, when chondrocytes were possible to detect with standard histological colouring, they were not detected by immunofluorescence (Table 1). Proof of the presence of paramagnetic nano-particles of iron oxide by MRI (3 weeks and 4 months after implantation of MSCs) and immunofluorescence proof of cells stained with DiI (4 months after implantation of MSCs) in the place of transplantation of labelled allogenic MSCs in the lateral section of the right femoral condyle testifies that the implanted stem cells underwent chondrogenic differentiation and started to generate cartilaginous tissue in the damaged physis. Fluorescence-labelled cells were detected in a smaller than original amount 4 months after transplantation. There are several possible explanations to this. One version suggests that the immunofluorescence-labelled MSCs were impossible to detect after such a long period because of dilution of the fluorescent cytoplasmic label during mitosis and the subsequent differentiation of mesenchymal stem cells. It is also possible that the MSCs differentiated into mature chondrocytes (chondroblasts) and then succumbed to apoptosis.

The findings of this study - that the allogenic MSCs can be successfully transplanted into a physal defects without immunosuppression - support the hypothesis that the mesenchymal stem cells may be immune-privileged cells (Bartholomew et al. 2002). It was also proven that the mesenchymal stem cells do not express mesenchymal stem cell Class-II molecules or co-stimulatory molecules (B7 and CD40) (Hock et al. 2001). Besides, they may also secrete certain factors that suppress the immune response (Krebsbach et al. 1997). The allogenic transplantation of MSCs into a partial defect of the growth zone that was carried out in the study proves the capacity of differentiation and survival of these cells in the target tissue without transplant rejection or the occurrence of graft-versus-host disease, even without immunosuppressive therapy. In spite of the very favourable results, we must continue verifying the method in other animal species before clinical application. And, last but not least, we must carry out long-term monitoring of animals with implanted MSCs from the standpoint of possible adverse complications of stem cells.

It also appears to be desirable to test the application of xenologous MSCs with similar indication on suitable models. Immunotolerance of mesenchymal stem cells was also described in xenotransplantations. In 2000, Liechty et al. carried out a successful transplantation of human MSCs into a sheep foetus *in utero*. It was an innovative idea - to expose an underdeveloped immunity system of a sheep foetus to the effects of a xenotransplant. Other studies describe xenotransplantation of human cells into the bones of laboratory rats (Fatkhudinov et al. 2005) or transplantation of mouse MSCs into the tissues of mature, fully immunocompetent rats (Saito et al. 2002). No transplant rejection was described here either. The aforementioned results may constitute a guide to the scenario when it will be possible to produce, *in vitro* multiply and cryopreserved allogenic or xenologous mesenchymal stem cells for immediate clinical use without any immunosuppression.

In conclusion, our results demonstrated that hyaline cartilage formation occurs in



iatrogenic distal femoral physeal defects treated with allogenic mesenchymal stem cells. Newly formed cartilage in the growth plate area became well integrated with the host physis and functional. The continued growth of the right femur from its distal physis was apparent. This study is the first report, to our knowledge, of the use of allogenic mesenchymal stem cells for repair of experimentally damaged physis in rabbits.

### **Chondrogenní potenciál allogenních mezenchymových kmenových buněk transplantovaných bez imunoprese k regeneraci fyzárních defektů u králíků**

Mezenchymové kmenové buňky (MSCs) kostní dřene jsou multipotentní buňky schopné diferencovat v kost, chrupavku a další pojivové tkáně. Cílem této studie bylo zjistit, zda allogenní mezenchymové kmenové buňky mohou bez imunopresní terapie funkčně zhojit defekt v distální růstové ploténce femuru králíka.

U 32 novozélandských bílých králíků, stáří 7 týdnů a tělesné hmotnosti  $2,25 \pm 0,24$  kg byl v laterálním kondylu femuru iatrogeně vytvořen defekt. Do tohoto defektu, o šířce 3,5 mm a hloubce 12 mm, vyvrtného distální fýzy pravého femuru byly implantovány allogenní mezenchymové kmenové buňky v novém kompozitním nosiči (hyaluronát/kolagen typu I/fibrin). Hojení defektu ve fýze bylo hodnoceno rentgenologicky, magnetickou rezonancí (3 týdny a 4 měsíce po implantaci), histologicky, Pearlsovou reakcí a imunofluorescenčně (4 měsíce po implantaci). Výsledky byly porovnány s údaji zjištěnými vyšetřením kontrolních defektů (bez implantace kmenových buněk) v distální fýze levého femuru.

Pravé femury s poškozenou distální fýzou a transplantovanými MSCs rostly do délky více ( $0,55 \pm 0,21$  cm), než levé femury s defektem v distální fýze bez transplantace kmenových buněk ( $0,46 \pm 0,23$  cm). Valgózní deformita pravých femurů s defektem fýzy a transplantovanými MSCs byla nepatrná ( $0,2 \pm 0,1^\circ$ ). Naproti tomu levé femury s defektem distální fýzy bez transplantace MSCs vykazovaly signifikantní valgózní deformitu ( $2,7 \pm 1,6^\circ$ ). V tomto modelu defektů distální fýzy femuru ošetřených transplantací allogenních mezenchymových kmenových buněk nebyla klinicky pozorována žádná nežádoucí imunitní reakce ani rejekce implantátu. Stejně tak histologicky nebyla zjištěna žádná lokální nežádoucí reakce v podobě lymfocytární infiltrace. Čtyři měsíce po transplantaci allogenních MSCs vytvořena hyalinní chrupavka. Značené mezenchymové kmenové buňky/diferencované chondrocyty byly po transplantaci detekovány ve fyzárních defektech magnetickou rezonancí a imunofluorescenčně.

Výsledky této studie dokazují, že allogenní mezenchymové kmenové buňky v novém kompozitním nosiči (hyaluronát/kolagen typu I/fibrin) zhojily iatrogení defekty v distální fýze femuru králíků bez použití imunopresní terapie. Použití allogenních mezenchymových kmenových buněk může tedy být v léčbě fyzárních defektů alternativou autologní transplantace MSCs. Tato allogenní transplantace by mohla v praxi znamenat izolaci MSCs od nepříbuzného dárce, tedy snadno dostupný zdroj buněk potřebných pro hojení chrupavčité tkáně.

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Fig. 1. Expansion of a colony of allogenic MSCs after 5 days (1a), after 10 days (1b), after 15 days (1c) and after 21 days (1d) of cultivation.



Fig. 2. The arrow shows an artifact created from paramagnetic iron nano-particles with Resovist contrast agent incorporated into transplanted MSCs.



Fig. 3. Histological examination of femoral distal physis of a rabbit after transplantation of allogenic MSCs (H E)

3a - cartilage enclaves in the femoral distal physis defect (arrows), magnification  $\times 400$

3b - newly created hyaline cartilage in the place of physal defect (arrow), magnification  $\times 20$

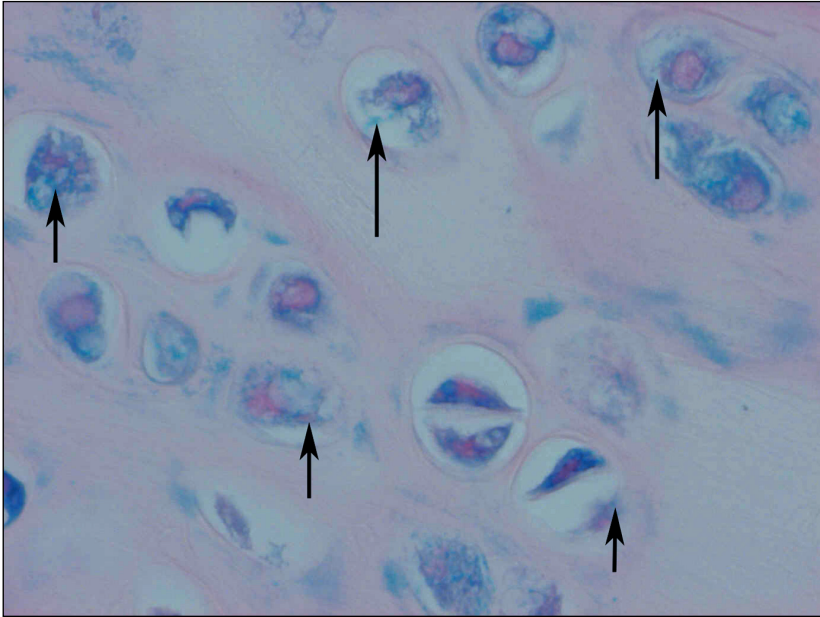


Fig. 4. Resovist granule (arrows) in the chondrocytes cytoplasm (rabbit AL11) differentiated from MSCs - Pearls reaction ( $\times 400$ )

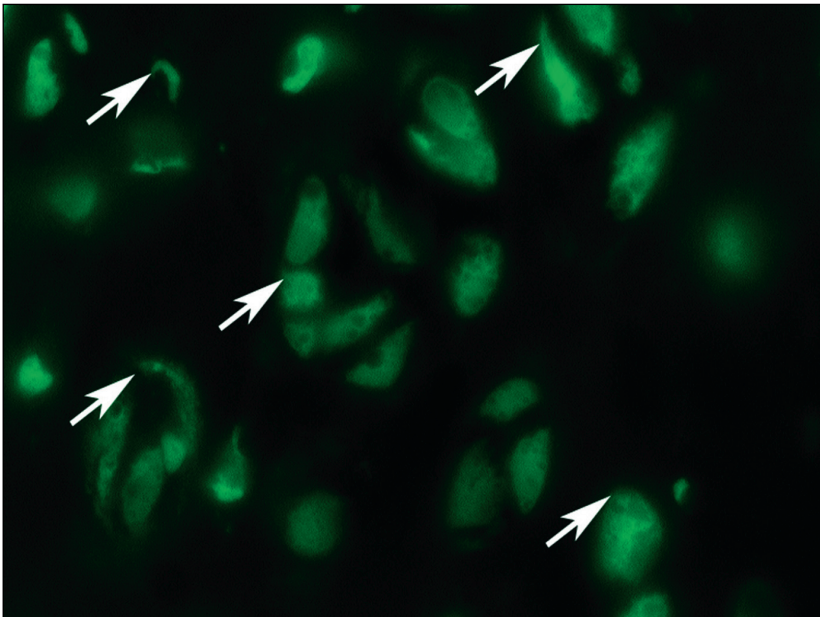


Fig. 5. Immunofluorescence hue DiI on chondrocytes membranes (arrows) differentiated from implanted allogenic MSCs (rabbit AL24) ( $\times 400$ )