

# Nanotechnology and mesenchymal stem cells with chondrocytes in prevention of partial growth plate arrest in pigs

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**Introduction.** This study describes the results achieved using a combination of allogeneic mesenchymal stem cells (MSCs) with chondrocytes (CHC) and a new scaffold consisting of type-I collagen and chitosan nanofibers in the prevention of partial growth plate arrest after iatrogenic injury in pigs.

**Material and methods.** The miniature pig was selected as an experimental model to compare the results in the left femoral bones (MSCs and CHC in scaffold transplantation into the iatrogenic partial distal growth plate defect) and right femoral bones (scaffold alone transplantation). The experimental group consisted of 10 animals. Bone marrow from os ilium as the source of MSCs was used. A porous cylinder consisting of 0.5% by weight type-I collagen and 30% by weight chitosan, was the optimal choice. The length of the bone and angular deformity of distal femur after the healing period was measured and the quality and structure of the newly formed cartilage was histologically examined.

**Results.** Transplantation of the composite scaffold in combination with MSCs and chondrocytes led to the prevention of growth disorder and angular deformity in the distal epiphysis of the left femur. Compared to the right (control) femur, tissue similar to hyaline cartilage with signs of columnar organization typical of the growth plate occurred in most cases.

**Conclusions.** The promising results of this study reveal the new and effective means for the prevention of bone bridge formation after growth plate injury.

**Key words:** mesenchymal stem cells, growth plate defect, cartilage, bone bridge

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## INTRODUCTION

A growing number of experimental studies are examining ways of exploiting mesenchymal stem cells in the regeneration of injured hyaline cartilage. Earlier experiments raised the question whether it is possible to develop an ideal carrier that could be more easily implanted into a tissue defect and which would meet the requirements for mechanical support and temporary stem cell nutrition. Our study describes the results achieved using a combination of allogeneic mesenchymal stem cells (MSCs) with allogenic chondrocytes (CHC) and a new scaffold consisting of type-I collagen and chitosan nanofibers. The combination of MSCs and CHC drew on the assumption that the differentiation of MSCs would be affected, making it more accurate. Prior to the *in vivo* implantation, we used the knees of sixty pig cadavers to confirm the procedure of creating a defect in the growth plate and to ensure stable positioning of biocomposite in the defect. The development of a scaffold that would be easier to use was motivated not only by the prospect of more favourable

technical characteristics but also increased protection of the transplanted stem cells.

The new collagen carrier manifested significantly better mechanical properties already in the surgical phase of our experiment. The preceding laboratory tests showed the dynamic proliferation and reproduction of mesenchymal stem cells on the scaffold. A comparison of results was made between the experimental and control groups. In terms of preventing growth retardation in the injured extremity and reducing angular deformation of the affected femoral epiphysis, more favourable results had been anticipated.

## MATERIAL AND METHODS

Miniature pigs aged 9 months were selected as our experimental model and purchased, certified breeding from the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic in Libechev. The experimental group consisted of 10 animals (left legs – treated group, right legs – control group).

## Cell preparation

As the source of mesenchymal stem cells we used bone marrow from the os ilium<sup>1</sup>. After aseptically operative field preparation, bone marrow blood was aspirated by a bioptic needle (Iliac-Crest 15G/70-mm bioptic needle (Somatex)) from os ilium (*tuber coxae ala osis illii*) (Guo et al. 2004) into two 10 ml syringes with 5 ml of PBS (Phosphate Buffered Saline, Dulbecco) + 2% FBS (Fetal Bovine Serum, StemCell Technologies) and heparin 5 IU/ml. Under sterile conditions, the diluted bone marrow blood (about 20 ml) was deposited over 15 ml of FPP solution (Ficoll-Paque PLUS, StemCell Technologies).

To separate mononuclear cells from whole bone marrow, we used density gradient centrifugation at 400g for 30 min at room temperature using Ficoll-Paque TM PLUS (StemCell Technologies, Canada). Mononuclear cells in an opalescent layer between Ficoll and blood plasma were removed, washed in a culture medium (see below) and used for propagation under *in vitro* conditions. The average number of mononuclear cells from each isolation was  $25 \times 10^6$  cells. Cell number and viability were analyzed on Vi-CELL (Series×Cell Viability Analyzers) and about 95% of viable cells were detected. Cells were seeded in tissue culture flasks ( $1.3 \times 10^6/\text{cm}^2$ ) and cultured at 37 °C in 5% CO<sub>2</sub>. The culture medium was  $\alpha$  MEM medium (GIBCO, Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) and Gentamicin (50 mg/ml, Sigma-Aldrich). After 80% of confluence achievement the adherent MSCs were passaged using trypsin (0.5% trypsin-EDTA solution, Sigma-Aldrich) and reseeded at a density of 15,000 cells/cm<sup>2</sup>. After the 3<sup>rd</sup> passage, the MSCs were harvested and analyzed for expression of surface markers CD29, CD44, CD90, CD105 and CD45 by flow cytometry<sup>2</sup>.

The source of chondrocytes was a sample obtained from the non-weight-bearing articular surface of the distal femoral epiphysis in the same pig by bone marrow aspiration (see above) followed by their culturing in a nutrient medium. Cartilage was left immersed in collagenase overnight. The separated chondrocytes were centrifuged at 200 g for 5 min and placed in a culture flask with solution of Iscove's Modified Dulbecco's Medium and 15% Fetal Bovine Serum (FBS), 3 g/l NaHCO<sub>3</sub>, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 4 mM L-glutamine, 20  $\mu\text{g}/\text{ml}$  L-ascorbate-2-phosphate.

The cells were seeded in culture flasks (150 cm<sup>2</sup>) at a density of approximately  $1.3 \times 10^6$  cells/cm<sup>2</sup> and cultured at 37 °C in 5% CO<sub>2</sub>. The culture medium was  $\alpha$ -MEM (Gibco) supplemented with 10% FBS (Sigma-Aldrich) and Gentamicin (50 mg/ml, Sigma-Aldrich). After 24 hours, the non-adherent cells were removed and during the subsequent culturing (3 weeks) the medium was exchanged every 3 days. The first colonies of MSCs appeared after 4 to 5 days of culturing and 80% confluence was achieved after 10 days. The cells were passaged with a solution of 0.5% trypsin and EDTA (Sigma-Aldrich) for 5 min at 37 °C and replated at a density of 15,000 cells/cm<sup>2</sup>. For labeling with a fluorescent dye CM-DiI (Molecular Probes) at concentration 5  $\mu\text{g}/2.5$  ml PBS, cells were harvested on the day of transplantation, incu-

bated for 5 min at 37 °C and for 15 min at 4 °C. The cells were then thoroughly washed in PBS. To induce chondrogenic differentiation<sup>3,4</sup>, the labelled cells were placed in a medium composed of  $\alpha$ -MEM supplemented with 100 ng/ml recombinant human TGF- $\beta_1$  (R&D Systems), 100 nM dexamethasone (Medochemie), 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid 2-phosphate (Sigma-Aldrich) and 1% ITS (Insulin-Transferrin-Selenium-A Supplement, Gibco) for 30 min. Subsequently, the cells were centrifuged (700 g for 5 min) and cell pellets were prepared for deposition on the scaffold. After 5 days of culturing in a flask, the cells were passaged and to promote cell reproduction the same medium with 10% FBS was applied. On achieving confluence, the cells were passaged with trypsin and the second passage was used for seeding the cells. Each scaffold was seeded with  $10^6$  chondrocytes.

In both cases (CHC and MSCs) we used an allogeneic graft. However, we took particular care to ensure that both components came from the same animal.

The experimental procedure was in full agreement with the ethical norms for animal experiments and after agreement by the Ethical Commission (no. 46613/2003-1020).

## Scaffold preparation

Based on *in vitro* evaluation performed at the Institute of Experimental Medicine of the Academy of Sciences of the Czech Republic in Prague, a collagen-based scaffold containing chitosan micro- and nanofibers was selected as the most suitable material for meeting specific requirements: the required biological features, stability in simulated body environment, biomechanical response and scaffold behaviour at the site of the planned implantation (*ex vivo* insertion of the scaffold into an iatrogenic defect in the articular surface and tests of the mechanical properties of the scaffold on prepared pig knee joints). The lyophilization process was optimized in order to obtain interconnected pores of approximately 150  $\mu\text{m}$ . As a result of successive testing of the suitable composition of a three-dimensional carrier of MSCs, a porous cylinder consisting of 0.5% by weight type-I collagen and 30% by weight chitosan (in proportion to collagen amount), cross-linked by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and catalysed by NHS (N-hydroxysuccinimide 98%), with chitosan-nanofibers-based structure, seemed to be the optimum choice. To facilitate manipulation during implantation, after lyophilization the cylinder-like substrates were placed such that they could be seeded with cells in a 96-well plate. Prior to implantation, the cylinders were seeded with  $2 \times 10^6$  allogeneic MSCs and  $1 \times 10^6$  allogeneic chondrocytes.

## Surgical procedure

Surgery was performed under general anesthesia. All animals were intramuscularly administered tiletamine-zolazepam (Zoletil 100, Virbac, France) at 2 mg/kg dosing, xylazine (Sedazine, Fort Dodge, USA) at 0.5 mg/kg dosing and ketamine (Ketaset, Fort Dodge, USA) at 2 mg/kg dosing. All substances were administered

together in a single syringe. After the start of sedation, i.e. 10 min after administration, an intravenous catheter was inserted in the ear vein and propofol (Propofol 1%, Fresenius, Austria) was administered at 0.5-1 mg/kg dosing (depending on effect). The course of anesthesia was consistent with previous experimental projects.

Following all the above mentioned procedures, each pig was subjected to radiological examination of the femur in craniocaudal and lateromedial projection. The animal was placed on the operating table in the right lateral position and after sufficient exposure to disinfectant solution, draping of the operative field followed including the placement of the protective sheet. Lateral arthrotomy was performed to reach the left distal end of the femur. The location of the growth zone was confirmed visually. A screw of 4.5 mm diameter (drill 4.5 mm, SYNTHES) was used to create a defect 12 mm in depth across the growth plate. After lavage of the defect in the joint, the combined graft consisting of 3 blocks of a collagen-chitosan scaffold, employing allogeneic mesenchymal stem cells in 2 and allogeneic chondrocytes in the middle block, was transferred into the drilled defect (Fig. 1) by press fit fixation. The right combination was obtained by layering 3 biocomposite components: the scaffold and MSCs constituted the first and deepest layer deposited in the defect, the identical scaffold seeded with chondrocytes created the middle section and a scaffold with MSCs formed the outer part. To ensure stability of the transplant, the opening was sutured using the edges of periosteum with monofilament fiber (PDS 0, ETHICON). The repositioning of femur in the knee joint was followed by successive suture of arthrotomy in the individual anatomical layers *lege artis*. Under supervision of an anaesthesiologist, the animal was positioned on the left hip and identical methodology was applied to perform lateral arthrotomy of the right knee joint. A scaffold without mesenchymal stem cells was deposited in the drilled defect. Suture of anatomical layers was performed in the same way as on the left side. Immediately after the surgery, the operative incision was treated with Novikov solution and the experimental animal was placed in a separate box to allow the effects of general anesthesia to subside. Analgesics were used during the follow up period. The observation period of the miniature pigs from implantation to *lege artis* euthanasia was 16 weeks. During that time we paid particular attention to the cleanliness of the incisions, injury prevention in individual animals and monitoring of possible postoperative complications. At the end of week 16 all animals were euthanized *lege artis* by intravenous administration of a mixture of embutramide, mebezonium iodide, tetracaine hydrochloride and dimethylformamide (T61, Intervet, the Netherlands) in a dose of 5 ml i.v. *pro toto*. Following euthanasia, both femurs were excised and placed in 10% solution of formalin for fixation before histological examination.

#### Radiological measurements

Part of our experiment was a comparison of two radiographs. One was taken prior to experimental injury to the



Fig. 1. Implantation of scaffold with MSCs into the defect.

growth zone of the distal end of the femur. The other was taken 16 weeks after the injury. The measurements were performed in craniocaudal projection and a comparison of values obtained from the left (experimental) and right (control) femur was made. Length was measured from the top of the femur head to the most distal point of the femoral condyles. The deformity angle of distal epiphysis of femur was measured as an angle between the longitudinal axis of bone and the tangent intersecting the tops of both condyles.

#### Histological examination

All preparates of the distal end of femur placed in fixation solution were transported to the Institute of Pathology and Anatomy of the Faculty Hospital, Brno. Immediately after rinsing off the fixation solution, the bones were immersed in decalcifying Löwy solution which was regularly changed every 2 days. The actual process of decalcification took 10 to 12 days and its completion was followed by preparation of a block of bone tissue containing experimental or control defect in the growth plate. A microtome was used to cut the block into sections 0.2  $\mu\text{m}$  thick traversing the specific area and three specimens were prepared: for hematoxylin and eosin staining, fluorescence microscopy and immunohistochemical detection of type-II collagen. In microscopic examination of the specimen stained with hematoxylin and eosin, the coherence of the newly-formed tissue with the surrounding structure of the original growth plate cartilage and the maturity of cartilage in the defect were evaluated. The properties of the newly-created physis were assessed on a scale of 0 to 3 points and modified O'Driscoll score was applied with regard to the renewal of columnar cell formation<sup>5-8</sup>. Immunohistochemical reaction detected type-II collagen fibers and fibre content in the intracellular matrix of the newly-created tissue in the defect of the growth plate. After removal of paraffin from the tissue sections, they were inactivated by endogenous peroxidase (3%  $\text{H}_2\text{O}_2$  in methanol, 10 min), digested in proteinase K (10 min) and incubated with primary antibody (Novocastra NCL-COLL-IIp, polyclonal, rabbit).

**Table 1.** Histological evaluation.

Final result	Quality of new cartilage		Col II
	O'Driscoll score	Columnar organization of CHC	
0	0-7	no	negativity
1	8-15	no	low positivity
2	15 and above	no	medium positivity
3	15 and above	yes	high positivity

**Table 2.** Measurements of growth and angle of valgisation in distal epiphysis of femur.

Animal	Longitudinal growth of left femur (cm)	Longitudinal growth of right femur (cm)	Valgisation extent in distal epiphysis of left femur (°)	Valgisation extent in distal epiphysis of right femur (°)
A1	0.5	0.4	1	3
A2	0.8	0.7	2	5
A3	0.9	0.7	1	6
A4	0.7	0.3	2	5
A5	0.6	0.5	2	4
A6	0.5	0.6	1	5
A7	0.7	0.4	2	3
A8	0.8	0.6	1	5
A9	0.9	0.8	2	4
A10	0.7	0.5	0	5

This was followed by incubation using a detection system (EnVision+/HRP Rabbit, Dako) for 45 min at room temperature. Visualization by means of diaminobenzidine (DAB) was the next step (with a thermostat at 37 °C for 5 min). Nuclei were stained with hematoxylin and the specimens were then dehydrated with ethanol, highlighted with xylene and mounted on glass slides using mounting medium (Entelan TM). The positivity was again graded according to the number of detected fibers (Table 1). The numerical values from individual examinations were used in a statistical comparison of the treated (left femurs) and control group (right femurs).

#### Statistical evaluation

To analyse the data, we used both paired and unpaired tests. The non-parametric Wilcoxon matched-pairs test was used to evaluate whether there was any difference between the right limb without treatment and the left treated proximity in related parameters (longitudinal growth during experiment, difference in valgisation angle). To compare the proximities in categorical parameters (quality of cartilage healing, PAS, IHC, fluorescence, Pearls), the parameters were first binarized and the data were analysed using the McNemar matched-pairs test and the Fisher's exact test. The data were analysed using the IBM SPSS Statistics 19 for Windows (Release 19.0.0, IBM Corporation 2010).

## RESULTS

The source of stem cells was bone marrow harvested from an unrelated animal, with successful aspiration of 10 ml in the case of most animals. Each transplant bore 2 x 10<sup>6</sup> MSCs. Immunophenotyping indicated more than 95% of the cells were positive for CD29, CD44 and CD90 and



**Fig. 2.** Defect healing with newly-created hyaline cartilage with a sign of columnar formation, arrows pointing at the edge of defect, PAS staining, x40 magnification (animal A2 - left femur).

**Table 3.** Quality of healed cartilage and type-II collagen detection in the growth plate defect.

Animal	Quality of new cartilage		Immunohistochemistry – Col II	
	Left femur	Right femur	Left femur	Right femur
A1	0	0	2	1
A2	3	0	2	1
A3	0	0	1	1
A4	1	0	2	1
A5	3	0	3	0
A6	3	0	2	1
A7	3	0	2	1
A8	2	0	3	1
A9	3	0	2	1
A10	2	0	2	1

they could be considered homogeneous. The expression of CD105 and CD147 was low. We obtained sufficient chondrocytes by taking the non-weight-bearing articular surface of distal femoral epiphysis.

The measurements of femur lengths showed that the left femur where combined scaffold with MSCs and CHC had been implanted in a physal defect grew more in length ( $0.71 \pm 0.11$  cm) than the right femur with a physal defect without transplanted MSCs and CHC ( $0.55$  cm  $\pm$   $0.2$  cm) ( $P=0.011$ ). Angular (valgus) deformity in the left femur with MSCs and CHC transplanted in a physal defect was  $1.4^\circ \pm 0.7^\circ$ . On the contrary, the right femur with a physal defect without transplanted MSCs showed significantly greater valgus deformity  $4.5^\circ \pm 1.0^\circ$  ( $P=0.005$ ) (Table 2).

Histological examination revealed that the defect in the left femur was filled with cartilage-like tissue in seven cases. In a half of all cases in treated group, direct signs indicating hyaline cartilage were found (Fig. 2) and the remaining cases showed at least signs indicating fibrocartilage. The average positivity evaluation was  $2.0 \pm 1.0$ . In the defect of the right femur, a bone bridge was found in all cases ( $P=0.008$ ). The newly-formed tissue underwent immunohistochemical testing using antibodies to detect type-II collagen, with positive results ( $2.1 \pm 0.36$ ) in nine cases in treated group. Examination of the newly-created tissue in the defect of the right femur (control group) showed very low positivity for type-II collagen detection ( $0.9 \pm 0.1$ ;  $P=0.002$  – Table 3). Fluorescent stain was detected in only 6 cases and only in trace amounts. Nevertheless, we are able to say that the chondrocytes were differentiated from the transplanted MSCs which had been previously stained with CM-DiI since in the control group no fluorochrome was detected ( $P=0.031$ ).

We found manipulation with the scaffold easy, without events of travelling from the defect or significantly changing its shape. Transplantation of this composite scaffold in combination with MSCs and chondrocytes led to the prevention of growth defect and angular deformity in the distal epiphysis of the femur. Compared to the control group (only a scaffold implanted), tissue similar to hyaline cartilage with signs of columnar organization typical of

the growth plate occurred in most cases and favourably confirmed the presence of chondrocytes in differentiation *in vivo with effect on stem cell differentiation.*

## DISCUSSION

Failure of bone growth after growth plate injury used to require invasive orthopaedic surgery with lengthening and external fixation<sup>9</sup>. Current experiments like the present study are in contrast, seeking to primarily prevent deformities and growth defects. The first studies dealt with implantation of cartilaginous or periosteal tissue in defects in the growth plate. In Wirth's study<sup>10</sup> sheep tibia were used. A comparison of both materials favoured cartilaginous tissue over periost which led to quick growth of bone formations. The transplantation of chondrocyte grafts goes back to treatment of osteochondral lesions, particularly the use of mosaic arthroplasty. The method formerly referred to as promising, gradually became routine<sup>11-13</sup>. Foster et al. was probably the first to describe the method of transplanting chondrocytes in a defect in the growth plate in 1990. Authors of the experimental study on sheep, confirmed the method of filling the defect in the growth plate with autologous chondrocytes on a collagen scaffold. They described continuous proliferation of the chondrocyte culture, preservation of the cartilage-like structure and renewed architecture of the growth zone. The studies that followed tested different carriers of autologous chondrocytes - agarose<sup>14</sup>, atelocollagen gel<sup>15</sup>. The study carried out at our institution in 2002 used the miniature pig as a model and provided very good results<sup>16</sup>.

The study applied mesenchymal stem cells obtained from bone marrow (BMMC, BMMSCs), which has become the most frequent source of MSCs (ref.<sup>17,18</sup>). MSCs from periosteum or fat tissue<sup>18</sup> tend to be less common.

One of the main problems in all similar methods is the transfer of cells into tissue *in vivo*. Moreover, in experimental animals it is not possible to achieve temporary immobilisation which is presumed in human traumatology. As a result, the potential of experiments shifts to the development and testing of new scaffolds. One of the

goals of our study was to test a new scaffold which would be more resistant to mechanical load of bone and would provide increased support for the transferred cells. The tested product draws on the long-term research of our colleagues at the biotechnology laboratory of the Faculty of Chemistry, Brno University of Technology. The initial experiments employed a gel scaffold based on tissue adhesive<sup>17,19-21</sup>, and this scaffold form was unable to meet the requirements for resistance, let alone easy and simple surgical procedure. Subsequent studies used a three-dimensional scaffold with self-supporting construction of collagen fibers<sup>22,23</sup>. Thanks to easy manipulation and favourable results achieved in the previous studies<sup>17,24,25</sup>, it was unnecessary to change the strategy fundamentally in terms of scaffold choice. However, further testing of cell viability was performed and scaffold architecture was reinforced to achieve higher rigidity and easier manipulation. In addition, the new scaffold construction made possible hydrodynamic seeding with MSCs and CHC which is a more effective method and one that provides more protection to transferred cells<sup>26</sup>. Another innovation was layering, with the periphery seeded with MSCs and the central part with chondrocytes.

Among the most frequently used differentiation factors in experiments *in vitro* and *in vivo* are TGF- $\beta$ 1 (ref.<sup>18,27,28</sup>) and TGF- $\beta$ 3 (ref.<sup>29</sup>). A member of the large family of TGF factors is also cartilage-derived morphogenetic protein 1 (CDMP1) which was successfully transferred to pig BMMSCs by gene transfer and induced their stimulation to chondrogenesis. The latest studies are searching for other factors that could be of use and one of these is the successfully tested chondroitin sulfate C (ref.<sup>30</sup>). The differentiation procedure we followed in our study<sup>3</sup> proved useful in our previous experiments and it was further tested in other studies<sup>4</sup>.

A first questions arising from the results, is possible applications in human medicine. It is only a matter of time before legalisation of the employed biocomposite will take place and made available in the treatment of pediatric injuries. Testing autologous transplantation seems the first and most reasonable step, followed by allogeneic transplantation. Despite this procedural obstacle, the first tests with human MSCs are already underway, with a current study in China describing promising regeneration of hyaline cartilage in mice and rabbits using human MSCs in combination with TGF- $\beta$ 3 (ref.<sup>31</sup>).

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