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Abstract of doctoral thesis

Cartilage regeneration using peroral supplementation and artificial scaffolds

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Content:

1. Abstrakt

Chrupavka je schopná regenerovat jen v omezeném rozsahu. Cílem této práce bylo zlepšit regeneraci hyalinní chrupavky pomocí perorální suplementace glykosaminoglykanů (GAG) a antioxidantů nebo pomocí umělých matric osázených autologními buňkami. Matrice osázené chondrocyty nebo mesenchymálními kmenovými buňkami (MSC) jsou schopné reparovat defekty chrupavky. Aby bylo možné komplexně charakterizovat biomechanické vlastnosti matric, vyvinuli jsme novou dynamickou metodu nárazem. Následně byla vyvinutá složená matrice z hyaluronanu, kolagenu typu I a fibrinu, u které byly viskoelastické vlastnosti a zátěžový diagram podobné nativní chrupavce. Tato kompozitní matrice byla následně použit na regeneraci osteochondrálního defektu a poškozené růstové ploténky.

Účinek perorální suplementace GAG a antioxidantů (vitamin E/selen) na regeneraci osteochondrálních defektů byl zkoumán u králíků. Po vytvoření definovaných osteochondrálních defektů v kolenním kloubu, dostávaly skupiny deseti zvířat směs GAG/vitamin E/selen (GAG skupina) nebo placebo (laktózu) po dobu 6 týdnů. Množství sulfatovaných GAG v reparovaném osteochondrálním defektu bylo signifikantně vyšší v GAG skupině. U obou skupin bylo zjištěno signifikantně vyšší množství GAG v chrupavce operovaného kolene než v neoperovaném koleně. V GAG skupině byla navíc naměřena vyšší viskozita synoviální tekutiny.

Regenerace osteochondrálních defektů pomocí složeného scaffoldu s autologními chondrocyty byla zkoumána u miniaturních prasat. Po šesti měsících byla složená matrice z hyaluronanu, kolagenu a fibrinu osázena chondrocyty schopna regenerovat defekt tvorbou hyalinní chrupavky a částečně fibrózní chrupavky, zatím co matrice bez buněk podporovala tvorbu fibrózní chrupavky.

Schopnost kultivovaných MSC reparovat poškozenou růstovou ploténku byla hodnocena u králíků. Matrice osazená autologními MSC, profylakticky implantována do iatrogenního defektu distální růstové ploténky femuru králíka zabránila tvorbě kostního můstku a vyústila do hojení růstové ploténky tvorbou hyalinní chrupavky.

2. Abstract

Cartilage can regenerate only in a limited extend. The aim of the study was to enhance hyaline cartilage regeneration using peroral supplementation of glycosaminoglycans (GAGs) and antioxidants, or using artificial scaffolds seeded with autologous cells. Scaffolds seeded with chondrocytes or mesenchymal stem cells (MSCs) have a potential for treatment of cartilage defects. For the complex characterization of biomechanical properties of scaffold, we developed the novel shock dynamic method. Subsequently, the composite hyaluronate-type I collagen-fibrin scaffold with the viscoelastic properties and dynamic loading response similar to the native cartilage was developed. The composite scaffold was then used for osteochondral regeneration and physeal cartilage regeneration.

The effect of peroral supplementation of GAGs and antioxidants (vitamin E/selenium) on the regeneration of osteochondral defects was investigated in rabbits. After introduction of defined osteochondral defects in the knee joint, groups of ten animals were given a GAG/vitamin E/selenium mixture or a placebo (milk sugar) for 6 weeks. The amount of sulfated GAGs in the osteochondral regenerates was significantly higher in the GAG group. In both groups, the GAG amount in the cartilage of the operated knee was significantly higher than in the non-involved knee. Moreover, the viscosity of the synovial fluid was significantly enhanced in the GAG group.

Osteochondral regeneration using autologous chondrocyte-seeded composite scaffold was tested in miniature pigs. After six months, chondrocyte-seeded composite hyaluronate-type I collagen-fibrin scaffold was able to regenerate the defect with the formation of the hyaline cartilage, and partially fibrocartilage while the scaffold alone supported the fibrocartilage formation.

The ability of cultured MSCs to repair damaged physis was evaluated in rabbits. Prophylactic implantation of autologous mesenchymal stem cell-seeded scaffold to iatrogenically damaged distal growth plate of rabbit femur prevented bone bridge formation and resulted in healing of the physeal defect with hyaline cartilage.

3. Introduction

An articular cartilage absorbs forces, and enables smooth movement of the joint. Hyaline physeal cartilage is necessary for the growth of bones during the postnatal period.

Osteoatrhritis (OA), is characterized by retrogressive changes in both cells and matrix that result in the loss of both structure and function of the articular cartilage (Buckwalter and Mankin 1998b). NSAIDs are often used in the therapy of OA but they do not affect the cause of osteoarthritis. Various substances, such as chondroitin sulfate, D-glucosamine sulfate, and hyaluronic acid, have proved their chondroprotective effect in the treatment of OA. However, until now, it has not been possible to directly prove a causal link between orally applied agents and the structural changes within the hyaline cartilage or within cartilage regenerates (Brief et al. 2001). In spite of being supported by positive clinical results, these substances are discussed controversially and are not accepted as OA-modifying drugs (Felson and Anderson 2002).

Standard surgical techniques, such as debridement, penetration of subchondral bone, osteotomy, transplantation of autographs, and soft tissue grafts may decrease symptoms and improve joint function However, they are not able to restore the articular cartilage (Buckwalter and Mankin 1998b).

The novel treatment of chondral and osteochondral defects includes chondrocytes or mesenchymal stem cells embedded in biocompatible scaffold. The scaffolds allows chondrocytes to redifferentiate and to produce their own extracellular matrix. (Drury and Mooney 2003). Natural derived hydrogel scaffolds, such as fibrin, collagen, hyaluronic acid, fibrin provides the chondrocytes and MSCs similar environment like in native cartilage. However, they exhibit only poor mechanical properties that may lead to production of fibrocartilage (Buckwalter and Mankin 1998a).

From the mechanical point of view, the cartilage can be regarded as a so-called poroelastic material, involving viscous properties of interstitial fluid and elasticity of the matrix. Mechanical properties, viz. elasticity, strength and stiffness, are often used to characterize the physical nature of native cartilages. As the nature of cartilage tissue implies that the mechanical parameters are highly strain-rate-dependent, suitable mechanical characterization is rather complicated and it seems to be one of the principal limitations of broader application and implantation of tissue-engineered cartilage.

Limb long bone injuries are most often associated with damage of physis (e.g. distal physis of ulna or distal physis of femur) which is crucial for longitudinal growth of a bone (Maretta and Schrader 1983). Poor regeneration capacity of cells in damaged growth plates leads to formation of a bridge at the site of physeal injury. Fortunately, the main effort is concentrated on the treatment of already formed bone bridge and related deformities, and shortening of affected bone. However, a new approach using autologous chondrocytes in the prevention of the bony bridge formation has been reported by Gál et al. (2002).

4. Hypothesis and aims of the study

We suggest that peroral supplementation of natural mixture of GAG and antioxidants – vitamin E and selenium may improve cartilage regeneration. The aim of the study was to study the effect of GAGs and antioxidants on viscosity of synovial fluid, biochemical composition and structure of regenerated cartilage.

The biomechanical characteristics of the scaffold implanted into osteochondral defect strongly influence the effectiveness of the repair of the defect. The aim of the study was to develope the dynamic method for accurate characterization of biomechanical properties of the cartilage and artificial scaffolds. Moreover, our aim of the study was to develope an artificial scaffold with improved stiffness and viscoelastic properties that are similar to native cartilage and to study the ability of the chondrocyte-seeded scaffold to regenerate osteochondral defects in miniature pigs.

MSCs showed the potential to differentiate into chondrocytes. We suggested that the MSC-seeded scaffold implanted into physeal defects might improve the healing of physeal cartilage. The aim of the study was to prevent bone bridge formation and subsequent deformity formation in rabbits.

5. Materials and Methods

1. GAG supplementation

Anteromedial knee arthrotomy was performed and the patella was moved laterally. In 90° knee flexion, a 3.5 mm x 3.5 mm (width x depth) osteochondral defect was created in the trochlea femoris and the knee was cllosed and sutured in two layers.

Both groups of ten adult gray Chinchilla rabbits received the same food but those within the GAG study group were additionally fed 50 mg/kg of the GAG-mixture with 10 µg selenium and 10 mg vitamin E (KnorpelPlus®; Nutritect, Germany) in tablet form. The animals in the control group were given placebo tablets (milk sugar 50 mg/kg). Six weeks after the operation all animals were sacrificed. The amount and viscosity of the synovial fluid were measured.

Biochemical analysis. The total content of sulfated GAG was determined fluorometrically. The tissue was lyophilized and the dry weight was determined. The samples were then enzymatically split into 1 ml of a 0.5 % papain-buffer solution in a 65 °C bath and 1,9-dimethylmethylene blue was added to the aliquot. Finally, absorption was measured spectrophotometrically at 535 nm (Farndale *et al*. 1986). The GAG amount was determined by extrapolation from the standard curve with shark chondroitin sulfate (Sigma, Germany).

Histology. The defects with the surrounding cartilage were removed, denatured in formaldehyde, decalcified in EDTA, cut into 5 µm layers, stained by hematoxylin/eosin (HE) and histologically analyzed with regard to the type, orientation and number of cells as well as the character of the matrix.

Viscosity measurement. Due to the very small volume, the viscosity of the synovial fluid was measured using a fluorescence polarization of FITC-marked polylysine as the fluorescence probe using Perrin equation for viscosity determination as described by Amler *et al.* (1990).

2. Biomechanical Characterization of Cartilages

A pendulum-like apparatus setup permits tracking of material response to a single impact. Rapid increase of acting force should resemble physiological joint cartilage loading. Sample deformation is read simultaneously by a piezoelectric accelerometer (Bruel & Kjaer Type 4375) and Laser Doppler Vibrometer LDV (Polytec OFV-302). The signal acquired by both detectors is then collected by a preamplifier and computationally processed.

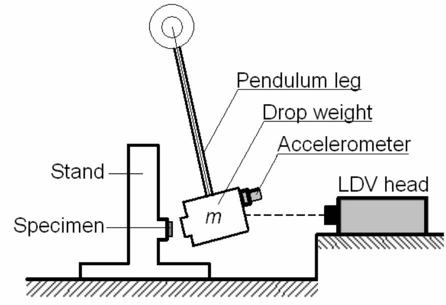

Fig. 1 Scheme of impact loading measurement setup.

Double time integration of the signal provided by the accelerometer (acceleration a) provides the value of actual sample deformation Δl:

$$\Delta l = \int_{t_1}^{t_2} (v(t) + v_0)\,dt$$

where $t1$ and $t2$ are the times characterizing initiation and termination of the impact process, v is the actual deformation velocity and v_0 is the constant determined by LDV, specifying the critical point in the impacting object movement (change of acceleration to deceleration), while the actual velocity during the impact is evaluated as

$$v(t) = \int_{t_1}^{t_2} a(t)dt$$

Strain ε is then determined as the ratio of actual sample deformation Δl and initial sample thickness l_0. Strain is used due to unequal thickness of examined samples. Acting forces as well as stresses can be evaluated simply by using Newton's force law

$$F = ma$$

where m is the mass of the impactor (m = 0.545 kg).

For static compressive material testing MTC 858.2 Mini Bionix testing machine was used. Artificial samples tested are examples of materials being developed or already used to replace the native cartilage tissue.

As the examined samples showed slightly uneven surfaces, prior loading of 1 N was considered. This normalization approach was applied in the data-processing phase, resembling real physical preloading used in standard mechanical testing.

3. Composite scaffold development and osteochondral regeneration

Chondrocyte isolation and cell culture. Small pieces of cartilage were taken from the left femoral trochlea of three miniature pigs under general anesthesia. After enzymic digestion, chondrocytes were collected and cultured for 14 days in Iscove´s Modified Dulbecco´s Medium supplemented with 15 % fetal bovine serum (FBS), 3 g/L $NaHCO_3$, 100 IU penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, 20 μg/mL L-ascorbate-2-phosphate sesquimagnesium salt, 50 ng/mL insulin-like growth factor, 10 ng/mL basic fibroblast growth factor, and 1 ng/mL transforming growth factor β2. During the subsequent 9 days the cells were cultured in the same medium but containing 10 % FBS.

Scaffold preparation. The scaffolds were prepared in the wells of a 24-well plate at about 4 °C by mixing 33 μL of sodium hyaluronate (10 mg/mL, 1500 kDa) with 49.4 μL of 1 mg/ml type I collagen in 0.1 M acetic acid (Collagen type I from calf skin, acid soluble) and neutralized with 1 M KOH. 66 μl of cell suspension (4.3×10^6 chondrocytes) or 66 μl of media, respectively, was then added. Into this medium containing five times higher concentrations of growth factors compared to culture medium and 20 % FBS, we added 0.16 ml of Tissucol solution in aprotinin (fibrinogen 70-110 mg/ml, aprotinin 3000 kallidinogenase-inactivator units/mL), and 0.16 ml of thrombin solution (4 IU/mL) in $CaCl_2$ (40 μmol/mL, Tissucol® Kit, Baxter). Two identical scaffolds were prepared for each animal and implanted into osteochondral defects of trochlea femoris of minipig.
Cell viability. On day 7 and 14 after seeding, the scaffolds were incubated with 2′,7′′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (1mM) in an incubator with a humidified atmosphere, 5 % CO2 at 37 °C for 30 min, washed with PBS and subsequently stained with 5 μg/mL propidium iodide. The scaffold were scanned using confocal microscope Leica SP2 AOBS (excitation at 488 nm and 539 nm and emission at 505-535 and 638-699 nm for BCECF and propidium iodide, respectively).
MRI examination. MRI examination was performed on a whole-body MR system Siemens Vision 1.5 T (Erlangen, Germany) using a standard CP head coil. Subjects were measured in a supine position with the knee in the middle of the head coil. MR images were obtained in sagittal orientation using T1-weighted spin echo (TR/TE = 500/12 ms) and FLASH (TR/TE = 999/11 ms) sequence, FOV = 280 mm, slice thickness 3 mm. The same T1-weighted sequence was repeatedly applied after contrast agent (Gd-DTPA) application.
Histological examination and immunohistochemistry. HE statining was performed as mentioned above. Staining with Alcian blue at pH 2.5 and the PAS reaction was done to verify acidic and neutral GAG synthesis. Immunohistochemical staining was done to identify type II collagen using a monoclonal antibody against type II collagen, clone II-II6B3. Subsequently, the biotinylated secondary antibody and

streptavidin-peroxidase (both Immunotech) were used. Reactions were visualized using the DAB Chromogen Kit (Immunotech). The slides were counterstained with hematoxylin.
Histological and histochemical scoring system. The repair of osteochondral defects in all groups was compared by the scoring system for histological and histochemical results with maximum 24 points, modified from van Susante *et al.* (1999). The blinded analysis was performed by two authors.
Histomorphometric analysis. Histomorphometric analysis of photomicrographs of the histological preparations was performed using several randomly selected areas of each defect (Microscope IX 51, Olympus). The amount of hyaline cartilage, fibrocartilage, and fibrous and/or fibrovascular tissue was measured as their percentage of the total area of defect according to Breinan 2001. The blinded analysis was performed by two authors using software Ellipse (ViDiTo, Kosice, Slovakia).
Statistical analysis. Quantitative data of histomorphometric analysis were presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using One Way Analysis of Variance (ANOVA) and Student-Newman-Keuls Method. The level of significance was set at 0.05.

4. Physeal defect regeneration

Stem cells isolation and culture. Bone marrow blood was taken under general anesthesia from wings of both illiums in each rabbit to culture MSCs. Isolated MSCs were cultured in Dulbecco's Modified Eagle´s Medium supplemented with 10% FBS and gentamycin (50mg/l).
Physeal defect preparation and scaffold implantation. The battery-powered drill was used to create a defect in lateral part of distal physis of femur in order to cause damage exceeding 9% of the growth plate area. The canal was drilled in dorsomedial direction in order to cause damage of lateral part of distal femoral physis including adjacent parts of epiphysis and metaphysis. The defect was filled with

the MSC-seeded scaffold and fixed with beta-tricalcium phosphate (ChronOS, SYNTHES).

Bone length discrepancy and femoral valgus deformity measurements. Each rabbit was subjected to radiological examination on the day of transplantation and right after euthanasia. Valgus deformity was measured from radiographs. Measurement of the length of right femur (with physeal defect and transplanted MSCs) and left femur (with physeal defect without transplanted MSCs) was done from radiographs of femur in craniocaudal (CC) projection.

6. Results

1. Peroral supplementation of GAGs and antioxidants

Histological analysis showed a higher organisation level of the cartilage-like repair tissue in the healed osteochondral defect in the GAG group in comparison with the placebo group.

The amount of the sulfated GAG in the osteochondral defects of the GAG group was significantly higher than in the control group. (Table 1) In both groups the total GAG content in the cartilage of the operated knee joint was also significantly higher ($p < 0.05$) than that in the non-operated knee joint.

	Defect [%]	**Cartilage - operated knee [%]**	**Cartilage - non-operated knee [%]**
GAG group	8.8 ± 1.1 *	9.6 ± 1.7 +	5.9 ± 1.7
Control	6.0 ± 1.6	6.8 ± 1.5 +	3.6 ± 1.6

Table 1. The amount of sulfated GAG as share of the total weight of the regenerated tissue in the osteochondral lesions (mean ± SEM, * p<0.001, +p<0.05]

The amount of synovial fluid was increased in the placebo group while the viscosity of the synovial fluid was significantly enhanced in the GAG group (90 ± 2.9 vs. 69.4 ± 4.9 poise, $p < 0.001$). (Fig. 1)

2. Development of composite scaffold for cartilage regeneration

We developed the shock dynamic method for biomechanical characterization of cartilage. With regard to the distinct poroelastic and viscoelastic material properties of cartilages, blunt impact response evaluation seems to be the most promising method to obtain comprehensive mechanical data. Simple laboratory realization, repeatability and sufficient accuracy are the main features of this approach.

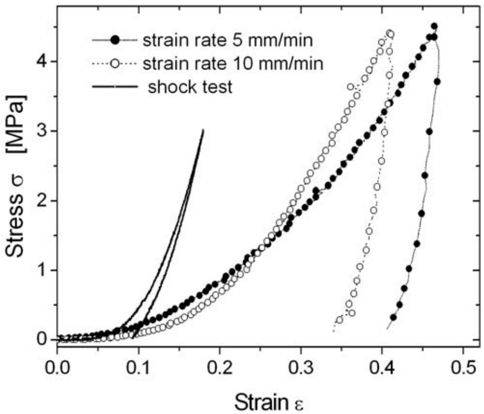

Fig. 2 Comparison of single pig joint cartilage sample stress-strain curves for static (strain rate 5 mm/min and 10 mm/min) and impact (shock test) loading.

The reaction of poroelastic material to static, dynamic and impact loading differs substantially. An example of cartilage biomechanical characteristic comparison obtained by both the proposed impact dynamic method and static loading test using strain rates of 5 mm min^{-1} and 10 mm min^{-1} is shown in Fig. 2.

The nonlinear shape of each loading curve results predominantly from large strains of unconfined compression, as the interstitial fluid flow within the tissue is sufficient for its escape from the material structure at low loading rate.

Healthy joint cartilage response consists of nearly linear part characterizing elastic matrix, followed by steep non-linear part caused mainly by viscous fluid. In damaged cartilage, the diagram changes usually occur in favor of linearity due to damaged porous structure.

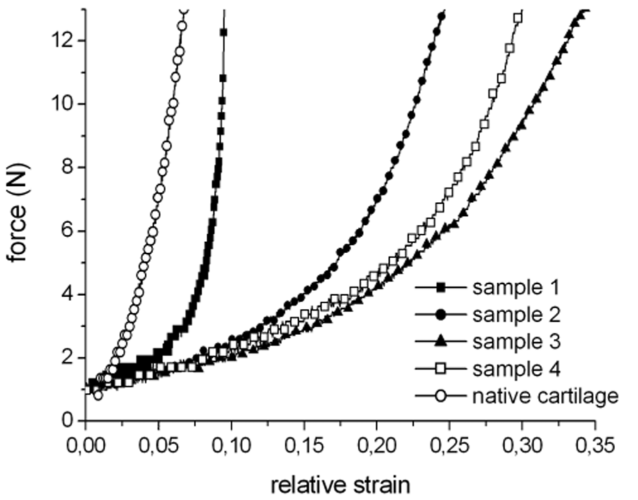

Fig.3 Loading diagrams of different composite scaffolds in comparison to native rabbit cartilage. Four different composite scaffolds (six pieces of each) were prepared, using 3 × 10^4 cells per scaffold. Hyaluronan/collagen volume ratio was 12.5/18.8 (sample 1), 16.3/13.6 (sample 2), 8.8/24.2 (sample 3). The sample 4 was prepared from Tissucol and Thrombin solutions without hyaluronan and collagen. Fibrin scaffold was recently used in clinical application. (Handl et al. 2006)

Four different chondrocyte-seeded hyaluronan-type I collagen-fibrin composite scaffolds displayed excellent viability of chondrocytes (84 % to 97 %) after a 7- and 14-day cultivation. The stiffness of the tested composite matrix (scaffold 1) was significantly higher than that of the fibrin gel (scaffold 4), but also better than that of scaffold 2, or 3. (Fig. 3)

3. Regeneration of osteochondral defects in miniature pigs

The composite scaffold 1 with the best biomechanical properties was subsequently implanted in osteochondral defects in miniature pigs.

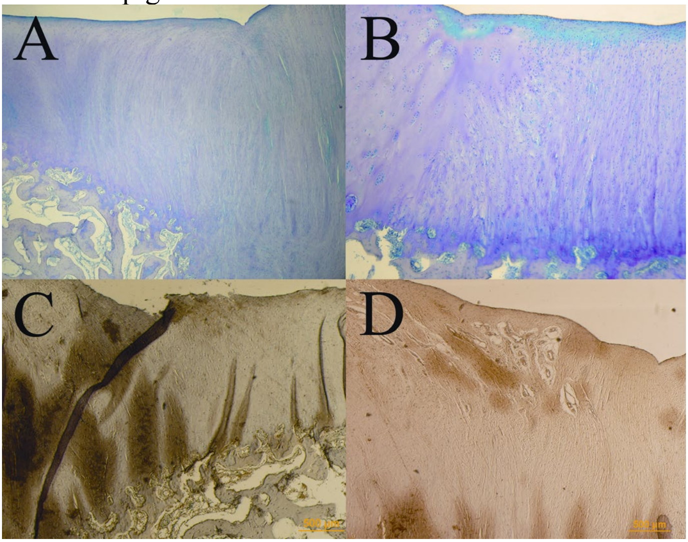

Fig.4 Histology of osteochondral defects A of the chondrocyte-seeded scaffold group (A, C) and non-seeded group (C, D) after 24 weeks using Alcian blue staining and PAS reaction at pH 2.5 (A, B), and immunohistochemical staining using monoclonal antibody against type II collagen (C, D). Magnification × 100 (B), × 40 (A, C, D).

In the A defects of chondrocyte-seeded scaffold group, hyaline cartilage and fibrocartilage was formed, containing type II collagen, acidic and neutral glycosaminoglycans while the non-seeded scaffold group was predominantly filled with fibrocartilage. (Fig. 4) Defects in the control group were predominantly filled with fibrous tissue.

Histomorphometric analysis of photomicrographs revealed a significantly higher amount of hyaline cartilage in the cell-seeded scaffold group in A defects than in other groups. Both scaffold groups in A defects showed significantly less fibrous tissue than cell-seeded defects B and the control group.

4. Regeneration of physeal defects in rabbits

Prophylactic transplantation of autogenous mesenchymal stem cells to iatrogenically damaged distal growth plate of rabbit femur prevented bone bridge formation and resulted in healing of the physeal defect with hyaline cartilage. (Fig. 5) Immunofluorescence examination showed that the chondrocytes newly formed in growth zone are the result of differentiation of implanted MSCs.

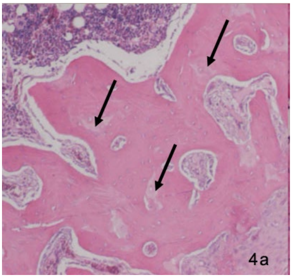

Fig.5 Histological examination of the distal femoral physis in rabbits after autogenous MSCs transplantation (HE stain, magnification × 40), arrows points to isolated cartilages islets in the defect of growth cartilage.

Fig. 6 Valgus deformity of the distal section of the left (L) femur (without transplantation of MSCs into the growth zone defect) and the right (P) femur in rabbit. Measured from X-ray photographs of femurs in CC projection after euthanasia (4 months after transplantation of MSCs).

Femur growth in traumatized physis was maintained even after transplantation of autogenous MSCs. (Fig. 6) As compared with the opposite femur (with physeal defect but without transplanted MSCs), the bone showed no significant shortening or valgus deformity (p=0.018).

7. Discussion

Changes of both structure and chemical composition of glycosaminoglycans observed in OA result in reduced biomechanical properties of the cartilage. In animal studies, substances like chondroitin sulfate or glycosamine sulfate not only cause an increase in the growth rate and production of proteoglycans, but also specifically interrupt catabolic processes such as the collagenolytic activity of chondrocytes or support an antiinflammatory function in the joints (Piperno et al. 2000). In clinical studies, the peroral supplementation of GAG could improve clinical symptoms and radiological parameters in patients with cartilage damage or OA. However, no link between peroral supplementation of these drugs and structural changes in cartilage was reported. In our study, the mixture of GAGs and antioxidants demonstrated their positive effect on GAG synthesis, organization of the tissue and viscosity of synovial fluid.

The scaffolds already used in clinical practice have been prepared mainly from natural chemicals, such as collagen, hyaluronic acid, fibrin, alginate. The effectiveness of the matrix-based therapy depends on the biocompatibility of scaffold, as well as its biomechanical properties, which are crucial for transmitting the applied stress to cells and tissue. The disadvantages of fibrin include its inapropriate mechanical properties, such as low viscoelasticity and stiffness, rather quick shrinkage and degradation of the gel. The viscoelastic behavior of our composite fibrin scaffold was positively influenced by high-molar-mass hyaluronic acid, and the stiffness was increased by the content of collagen.

The viscoelastic properties of the cartilage cannot be characterized by a simple value of Young´s modulus or Poisson´s ratio. Highly nonlinear and strain-rate-dependent properties of cartilage may be characterized by stress-strain or force-strain diagram where the linear part corresponds to elastic properties of the sample and the nonlinear part corresponds to viscous properties of the sample. Compared to many different experiments that have been performed to assess the mechanical properties of native hyaline cartilages *in vitro*, blunt impact response evaluation seems to be the most promising method to obtain comprehensive mechanical data.

It has been reported that pure fibrin sealant Tisseel® implanted into osteochondral defects in rabbits did not allow any cell migration into the gel after eight days. After four months, only incomplete irregular fibrous or fibrocartilaginous tissue formation were observed. Composite scaffolds prepared from two or more biopolymers are supposed to utilize the benefits of both components. Biocompatible, biodegradable polymers, such as collagen, alginate, fibrin or hyaluronic acid, support cell attachment, proliferation or control cell differentiation. Hyaluronic acid as a natural component of the cartilage plays the most important role in chondrocyte differentiation (Park, S. H. *et al.* 2005). In the *in vivo* study, our composite scaffold, composed of hyaluronate, type I collagen and fibrin, led to hyaline and fibrocartilaginous cartilage formation in the autologous chondrocyte-seeded group. We suppose that the superior biomechanical properties of our scaffold were essential to resist the dynamic loading during movement.

If the bony bridge takes 7 to 9 % of the physis area, a shortening and/or deformity of the bone is observed (Gál *et al.* 2002). We used a distal femoral physis defect as a model, because the distal femoral physis is critical for longitudinal growth of the femur. In addition, the injuries of the distal femur were more frequent than in the proximal femur. Clinical therapy of physeal injuries has been targeted as therapy of bone shortening and deformity. More recently, autologous chondrocytes-seeded graft was able to prevent bone bridge formation. In our study, we used TGF-β1, dexamethasone and ascorbate-2-phosphate to stimulate chondrogenic differentiation of autologous MSCs. After four weeks, chondrocyte islets, or hyaline cartilage were visible in the defect. Moreover, significantly increased bone growth with no valgus deformation was observed compared to the untreated leg. This approach may avoid complicated and protracted surgical therapy of the consequences of physeal closure.

8. Conclusions

Peroral supplementation of a mixture of both GAGs and radical scavengers (vitamin E and selenium) resulted in higher amount of sulfated GAGs in the repaired tissue of the osteochodral defect compared to the contralateral joint. This was accompanied by the increased viscosity of the synovial fluid in the treated joint. The combination of GAGs and radical scavengers can be used as supporting therapy in the treatment of osteochondral defects (Handl *et al.* 2007).

We developed a shock dynamic method for biomechanical analysis of the cartilage and scaffolds. This method is suitable for characterization of highly nonlinear and strain-rate dependent properties of cartilage. A novel composite hyaluronate-type I collagen-fibrin scaffold was developed. The scaffold exhibited improved biomechanical properties in comparison to fibrin glue. The viscoelastic properties and dynamic loading response of the composite scaffold were similar to the native cartilage (Varga *et al.* 2007).

Autologous chondrocyte-seeded composite hyaluronate-type I collagen-fibrin scaffold implanted into osteochondral defects in miniature pigs was able to regenerate the defect with a formation of the hyaline cartilage, and partially fibrocartilage while the scaffold alone supported the fibrocartilage formation (Filová *et al.* 2007).

Autologous MSCs-seeded scaffold implanted into iatrogenically damaged distal growth plate of rabbit femur prevented bone bridge formation and resulted in healing of the physeal defect with both a hyaline cartilage and a fibrocartilage. This was not accompanied by any significant valgus deformity and shortening of the bone (Plánka *et al.* 2007).

9. References:

AMLER E, JASINSKA R, DRAHOTA Z, ZBOROWSKI J: Membrane lateral pressure as a modulator of glycerol-3-

phosphate dehydrogenase activity. FEBS Lett 271:165-168, 1990

BREINAN HA, MINAS T, HSU HP, *et al.*: Autologous chondrocyte implantation in a canine model: change in composition of reparative tissue with time. *J Orthop Res* **19**: 482–492, 2001.

BRIEF AA, MAURER SG, DI CESARE PE: Use of glucosamine and chondroitin sulfate in the management of osteoarthritis. J Am Acad Orthop Surg 9: 71-78, 2001.

BUCKWALTER JA, MANKIN HJ: Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect* **47**: 477-486, 1998a.

BUCKWALTER JA, MANKIN HJ: Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr Course Lect* **47**: 487-504, 1998b.

DRURY JL, MOONEY DJ: Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24**: 4337-4351, 2003.

FELSON DT, ANDERSON JJ: Hyaluronate sodium injections for osteoarthritis: hope, hype, and hard truths. Arch Intern Med 162: 245-247, 2002.

GÁL P, NEČAS A, ADLER J, *et al.*: Transplantation of the autogenous chondrocyte graft to physeal defects: an experimental study in pigs. Acta Vet Brno 71: 327-332, 2002

HANDL M, TRC T, HANUS M, *et al.:* Therapy of severe chondral defects of the patella by autologous chondrocyte implantation. Acta Chir Orthop Traumatol Cech 73:373-379, 2006

MARETTA SM, SCHRADER SC 1983: Physeal injuries in the dog: A review of 135 cases. J Am Vet Med Assoc 182: 708-710

PARK SH, PARK SR, CHUNG SI, *et al.*: Tissue-engineered cartilage using fibrin/hyaluronan composite gel and its in vivo implantation. *Artif Organs* **29**: 838-845, 2005.

PIPERNO M, REBOUL P, HELLIO LE GRAVERAND MP, *et al.*: Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 8: 207-212, 2000.

10. Author´s publications

Publications *in extenso*, which are the basis of the PhD Thesis

a) with impact factor

M. Handl, E. Amler, K. Bräun, J. Holzheu, T. Trč, A. B. Imhoff, A. Lytvynets, E. Filová, H. Kolářová, A. Kotyk, V. Martínek: Positive Effect of Oral Supplementation with Glycosaminoglycans and Antioxidants on the Regeneration of Osteochondral Defects in the Knee Joint. Physiol. Res. 56: 243-249, 2007, IF = 2.093(06)

E. Filová, M. Rampichová, M. Handl, A. Lytvynets, R. Halouzka, D. Usvald, J. Hlucilová, R. Procházka, M. Dezortová, E. Rolencová, E. Košťáková, T. Trč, E. Šťastný, L. Koláčná, M. Hájek, J. Motlík, E. Amler: Composite Hyaluronate-type I Collagen-Fibrin Scaffold in the Therapy of Osteochondral Defects in Miniature Pigs. Physiol. Res. 2007; 56 (Suppl. 1): S5-S16, IF = 2.093(06)

F. Varga, M. Držík, M. Handl, J. Chlpík, P. Kos, E. Filová, M. Rampichová, T. Trč, E. Amler: Biomechanical Characterization of Cartilages by a Novel Approach of Blunt Impact Testing. Physiol. Res. 2007; 56 (Suppl. 1): S61-S68, IF = 2.093(06)

Plánka L., A. Nečas, P. Gál, H. Kecová, E. Filová, L. Křen, P. Krupa: Prevention of Bone Bridge Formation using Transplantation of the Autogenous Mesenchymal Stem Cells to Physeal Defects: An Experimental Study in Rabbits. Acta Vet Brno 2007, 76: 253-63, IF = 0.491(06)

b) without impact factor

Publications *in extenso* not related to PhD thesis

a) with impact factor

M. Handl, E. Filová, M. Kubala, Z. Lánský, L. Koláčná, J. Vorlíček, T. Trč, M. Pach, E. Amler: Fluorescent Advanced Glycation End Products in the Detection of Factual Stages of Cartilage Degeneration. Physiol. Res. 2007; 56: 235-242, IF = 2.093(06)

b) without impact factor