FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 44, No. 3, 2006
pp. 201-206

Is the repair of articular cartilage lesion by costal chondrocyte transplantation donor age-dependent? An experimental study in rabbits

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Abstract: The repair of chondral injuries is a very important problem and a subject of many experimental and clinical studies. Different techniques to induce articular cartilage repair are under investigation. In the present study, we have investigated whether the repair of articular cartilage folowing costal chondrocyte transplantation is donor age-dependent. Transplantation of costal chondrocytes from 4- and 24-week old donors, with artificially induced femoral cartilage lesion, was performed on fourteen 20-week-old New Zealand White male rabbits. In the control group, the lesion was left without chondrocyte transplantation. The evaluation of the cartilage repair was performed after 12 weeks of transplantation. We analyzed the macroscopic and histological appearance of the newly formed tissue. Immunohistochemistry was also performed using monoclonal antibodies against rabbit collagen type II. The newly formed tissue had a hyaline-like appearance in most of the lesions after chondrocyte transplantation. Positive immunohistochemical reaction for collagen II was also observed in both groups with transplanted chondrocytes. Cartilage from adult donors required longer isolation time and induced slightly poorer repair. However, hyaline-like cartilage was observed in most specimens from this group, in contrast to the control group, where fibrous connective tissue filled the lesions. Rabbit costal chondrocytes seem to be a potentially useful material for inducing articular cartilage repair and, even more important, they can also be derived from adult, sexually mature animals.

Key words: Chondrocytes, costal - Transplantation - Cartilage - Repair

Introduction

The repair of chondral injuries is a very important issue and a subject of many experimental and clinical studies. Different techniques to induce cartilage repair are under investigation. Currently used methods include the transplantation of chondrogenic cells into the defects [1, 2, 3]. Transplanted chondrogenic cells, held in place by a covering periosteal flap, produce a hyaline-like repair tissue [4, 5, 6, 7].

The idea of introducing the costal chondrocytes into the articular defect in order to induce cartilage repair is under investigation for two main reasons. It could lower the number of interventions into the joint for harvesting chondrocytes while at the same time it allows to obtain sufficient amount of cartilage.

Several studies [15, 19, 23] have reported costal cartilage to be a promising source of cells for transplantation. In our previous experiment [unpublished data, 30, 33], transplantation of costal chondrocytes obtained from 4-week-old rabbits induced the repair of artificially created lesions in the articular cartilage. Good results could have been influenced by young rabbit (4-week-old) donor model. The present study was designed to determine age-related effects of donor cells on articular cartilage repair. Four-week-old rabbits were used as young donors and 24-weeks-old animals after sexual maturation as adult donors. One of the most important factors likely to influence the success or failure of repair is the age of the donor cells. Findings of other authors suggest that the age of undifferentiated multipotential cell [12, 24, 32], as well as human articular chondrocyte donors [21, 28], are a significant factor affecting cellular proliferation and phenotypic expression. In the present study, the influence of age of the costal chondrocyte donor on cell proliferation, incorporation into the lesion and extracellular matrix synthesis was examined. The aim of our study was to determine if adult costal cartilage may be also an useful source of chondrocytes for inducing repair of articular cartilage lesions.

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Materials and methods

Animals. New Zealand White male rabbits, 4-week old (weight, 0.6-0.8 kg), were used as young donors of costal chondrocytes, and 24-week old ones (weight, 3,5-4,2 kg) as adult donors. 20-week old rabbits (weight, 2.2-2.8 kg) were used as host for chondrocyte transplantation. The approval for this study was granted by Animal Ethics Committee of Białystok Medical University.

Surgical procedures were performed under general combined anesthesia. The rabbits were anesthetized with intramuscular injection of mixture of ketamine (Narkamon, 6-10 mg per kg b.w.) and xylazine (Xylavet, 2 mg per kg b.w.). After harvesting of costal cartilage and before the evaluation of repaired lesions, the animals were sacrificed by cervical dislocation. Transplantation of costal chondrocytes from young and adult rabbits was performed on fourteen 20-week-old New Zealand White male rabbits.

Chondrocyte isolation and culture. Using sterile procedures the ribs were dissected, cartilage specimens were minced and washed three times in 0.9% sodium chloride. Isolation of chondrocytes was done by using 0.25% collagenase (type I, Sigma), 0.05 DNase (Sigma) and 17.5 mM Nα-p-tosyl-l-lysine chlorometyl ketone (TLCK, Sigma) in an F-12 medium (Sigma) supplemented with 10% FCS as described previously [26]. The collagenase digest was passed through a 25-m pore size filter to isolate the cells. Primary cell cultures were established by incubation in Ham's F-12 (Sigma) medium supplemented with 10% fetal calf serum (FCS) and antibiotics (Penicillin, Streptomycin, Amphotericin; ICN Biomedicals) under 5% CO_2. Culture medium was changed every two days and the cultures were maintained until 100% confluency was achieved. Afterwards, the cells were prepared for transplantation: they were isolated from the dish by 0.25% trypsin (Sigma), washed three times in culture medium, collected by centrifugation (1500 rpm, 5 min), counted in a Burker device and placed in 50 µl of medium in a sterile syringe.

Chondrocyte transplantation. Surgical procedures were performed under general combined anesthesia as described above. After a medial parapatellar incision was made on the left knee, the patella was dislocated laterally and a full thickness articular cartilage lesion was created on the patellar groove of the femur with the use of a drill. The size of the lesion was 5×5 mm in width and 2 mm in depth. The same surgical approach was used for harvesting a free periosteal flap, of a size corresponding with the lesion, from the medial proximal tibia. The flap was sutured to the peripheral cartilage rim of the lesion by four 8-0 sutures. Three of the sutures were tied immediately while the fourth was left untied until the cultured chondrocytes were transplanted. The chondrocytes were transfered into the lesion with a sterile syringe. Finally, the knee wound was closed in separate layers. In two rabbits from every group the procedures were repeated on the right leg with lesion left without transplanted chondrocytes but with a periosteal flap as a control. The animals were allowed to move freely. The animal were divided into 3 groups. Group I - transplantation of the costal chondrocytes from 4-week old donors; Group II - transplantation of the costal chondrocytes from 24-week old donors; Group III - control with periosteum only. The lesions were examined 12 weeks after transplantation.

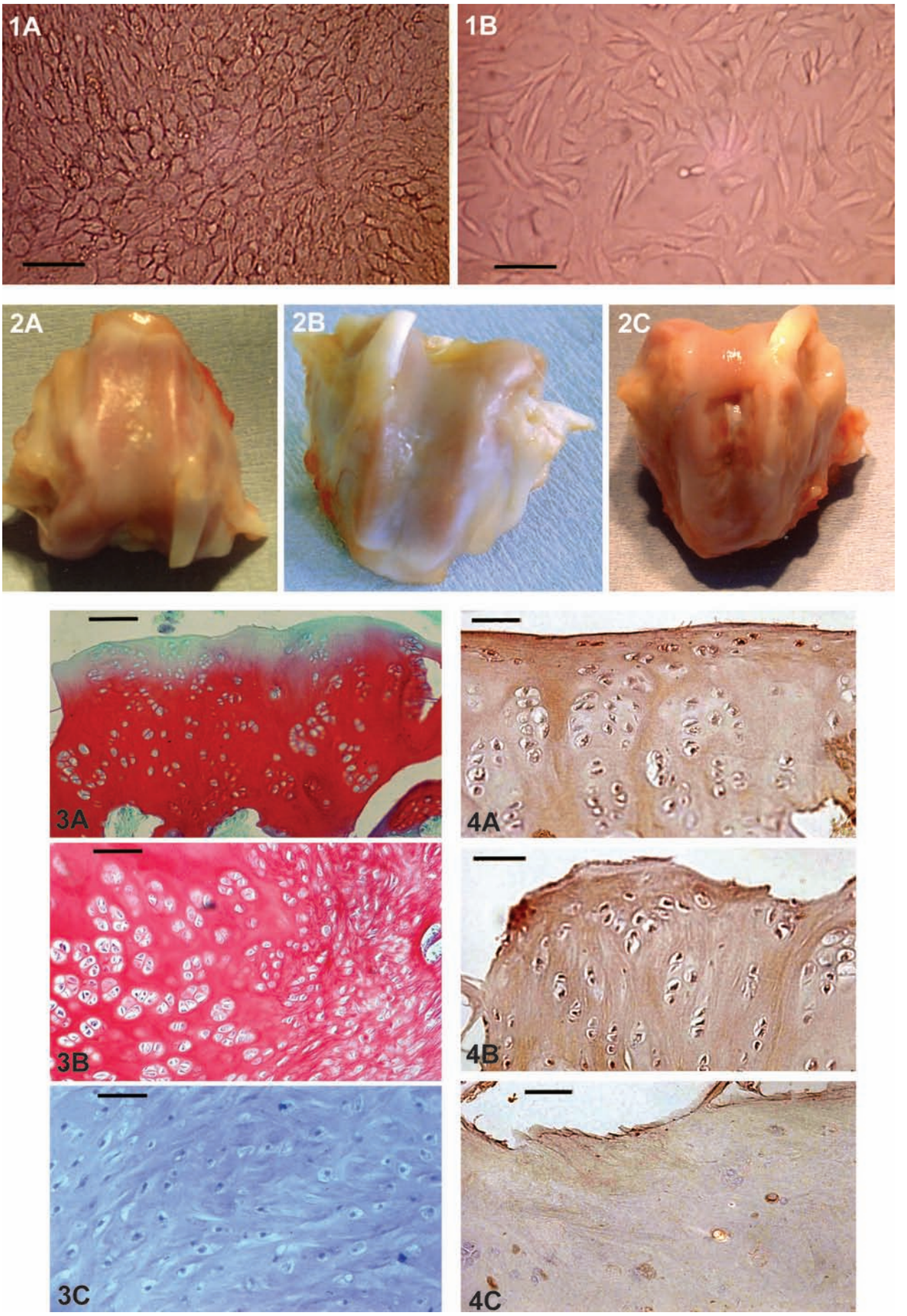
Table 1. Histological grading scale for the defects of cartilage [34].

Category	Points
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly non-cartilage	3
Non-cartilage only	4
Matrix staining (metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic staining	3
Surface regularity	
Smooth (>3/4)	0
Moderate (>1/2-3/4)	1
Irregular (1/4-1/2)	2
Severely irregular	3
Thickness of cartilage	
>2/3	0
1/3-2/3	1
<1/3	2
Integration of donor with host adjacent cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Total maximum	14

Histological evaluation of cartilage repair. The rabbits were killed 12 weeks after operation. The entire knee was dissected and examined under the microscope. The surfaces of the grafts were inspected for color, integrity, contour and smoothness.

After macroscopic observation the distal part of the femur was excised and fixed in 10% buffered formalin for 6 days. Each specimen was decalcified with 2.5% formic acid buffered with citric acid and embedded in paraffin. Sections 5 µm thick were cut through the grafted area and stained with Safranin O, hematoxylin-eosin and Azan methods (azocarmine + aniline blue). The histological sections were examined for the quality of the repair tissue and were graded with use of histological scale described by Wakitani *et al.* [34] (Table 1).

Fig. 1. Rabbit costal chondrocytes after 14 days in culture. **A** - young donor group, **B** - adult donor group. Bar=50 µm. **Fig. 2.** Gross photographs of patellar groove of rabbit femurs, showing incorporation of the repair tissue into the surrounding cartilage with recognizable margins of the defect. **A** - young donors, **B** - adult donors, **C** - control group. **Fig. 3.** Micrographs of the reparative tissue stained with Safranin O. **A** - after transplantation of costal chondrocytes from young donors, showing numerous irregular groups of chondrocytes with abundance of extracellular matrix. Bar=100 µm. **B** - after transplantation of costal chondrocytes from adult donors. Note fibro-cartilaginous character of the repair tissue on the right side of the micrograph. Bar=50 µm. **C** - control animal, fibrous tissue. Bar=50 µm. **Fig. 4.** Micrographs of the reparative tissue immunostained for collagen II. **A -** after transplantation of costal chondrocytes from young donors; immunostaining is more intense in the superficial layers of the reperative tissue. **B** - After transplantation of costal chondrocytes from adult donors; immunostaining can be seen in some chondrocytes and collagen fibers. **C** - control animal, negative immunostaining. Bar=100 µm.


1A
1B
2A
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3A
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The scale is composed of five categories and assigns a score ranging from 0 to 14 points. The cell morphology was graded from 0 (for normal tissue, comparable with adjacent healthy cartilage) to 4 points (absence of cartilaginous tissue). Matrix-staining was graded from 0 (normal cartilage) to 3 points (no metachromatic staining). Surface regularity was graded from 0 (when more than three-quarters of the surface was smooth) to 3 points (when less than one-quarter was smooth). The thickness of the cartilage was graded from 0 (when the average thickness of the cartilage in lesion was more than two-thirds that of surrounding cartilage) to 2 points (when the average thickness was less than one-third that of the surrounding cartilage). Integration of the donor cartilage with the host adjacent cartilage was graded from 0 (no gap between the donor and the host cartilage) to 2 points (a complete lack of integration). Statistical analysis has not been performed due to the lack of linear or comparably equivalent grading system among the five categories.

Immunohistochemistry. Immunohistochemical processing was performed using monoclonal antibodies against rabbit collagen type II (Novocastra). After removal of paraffin with xylol followed by absolute ethanol, sections were hydrated through a graded series of ethanol and placed in a Tris-HCL-buffered saline (0.05 M, pH 7.6). Incubation with a 0.1% trypsin solution (DIFCO) was performed for 30 min followed by endogenous peroxidase blocking with 3% hydrogen peroxide for 5 min. All sections were incubated with the antibody against collagen II (Novocastra) (1:15) at 25°C for 1 h in a darkroom. After washing three times with 0.05 M TRIS pH = 7.4 for 10 min the sections were incubated with a biotinylated secondary antibody (DAKO Kit LSAB 2) for 15 min. For identification of immunohistochemical reaction Streptavidin-peroxidase (DAKO Kit LSAB 2) and DAB chromogen were used according to manufacturer's protocol, followed by counterstaining with Meyer hematoxylin (DAKO). As a negative control, sections were incubated with a mixture of antibody and excess of minced healthy articular cartilage. After washing in distilled water and dehydration, the sections were mounted in Permount.

Results

Chondrocyte culture

Equal portions of costal cartilage from young and adult donors were prepared for chondrocyte isolation. Approximately two-fold lower number of cells was obtained from the adult animals. In every group, 2.0×10^6 cells per dish were seeded. Chondrocytes started to form the colonies from 2 to 5 days after seeding. After approximately 14 days, all cultures from young animals reached confluence. Cultures from adult donors reached confluence after approximately 22 days (Fig. 1). Most cells in culture showed fibroblast-like morphology.

Macroscopic observations

No signs of osteoarthrosis, such as osteophytes and erosion of cartilage were observed in the knees of rabbits receiving chondrocytes transplanted from young and adult donors. There was no significant difference in the appearance of the lesions in both groups. They were filled with a smooth, white, glossy tissue that resembled articular cartilage. The repair tissue was well incorporated into the surrounding cartilage. The lesions left without chondrocyte transplantation (control group) were filled with a red semitransparent tissue with discernible edges. Small fissures, fractures and loose attachment to the surrounding cartilage were present (Fig. 2).

Histological evaluation

The lesions containing transplanted costal chondrocytes from young and adult donors were filled with reparative tissue that resembled hyaline cartilage. It was firmly incorporated into the surrounding normal cartilage. Many chondroblasts with an abundance of extracellular matrix were observed. The reparative tissue showed intense metachromatic staining of the matrix. The cartilage occupied almost the full thickness of the original lesions and had comparable thickness in both groups. Some regions were filled with fibrous tissue. More fibrocartilage was present in the adult donor group. This was the main difference between the groups (Fig. 3 A, B). No trace of newly formed bone was found in any graft in both groups.

The lesions left without transplanted chondrocytes (control group) were filled mostly with numerous fibroblast-like cells surrounded by many collagen fibers with chaotic orientation. The reparative tissue filled the lesions in less than one-third of the thickness of the original cartilage. No metachromatic staining was present in any of the lesions and the reparative tissue resembled fibrous connective tissue (Fig. 3C).

The scores of histological grading of the repair tissue are shown in Table 2. The scores were slightly lower (better repair) in group receiving chondrocytes from young donors compared to group receiving chondrocytes from adult donors. The scores in the control group were inferior to both groups transplanted with chondrocytes. These results were in accordance with those of macroscopic and histological observations.

Immunohistochemistry

Staining for type II collagen was positive in all repair areas with hyaline morphology. Type II collagen immunostaining was observed in the extracellular matrix and in some chondroblasts. The intensity of the immunostaining differed throughout the newly formed tissue and was more intense in superficial layers of the lesions (Fig. 4A). In most specimens with mixed morphology, at least 50% of the matrix was collagen II-positive. In lesions with fibrocartilage repair less intense staining was showed. Type II collagen immunostaining in young and adult donor group (Fig. 4B) had similar appearance. No immunostaining was seen in the regions of the defect filled with fibrous tissue (Fig. 4C). No staining was present in the negative controls, either.

Discussion

Irrespective of the injury type, the intrinsic capacity of cartilage to repair chondral lesion is poor. Several different methods are either experimentally investigated or

already in clinical use. Biological articular resurfacing dates back to the early 1980s, but there is still an open debate as to which cells would be the best to induce the healing of articular cartilage. Experimental [1, 10, 11, 23, 29] and clinical [5] data concern the use of articular chondrocytes and mesenchymal stem cells [27, 32, 34] for inducing repair of articular cartilage. However, most of the repair strategies, fail to prevent future degeneration of the repair tissue and the surrounding host tissue.

For most cartilage regeneration studies, the large quantities of chondrocytes are needed. This requires either large portion of cartilage collected from the donor or many passages of the cultured cells. To avoid these problems, we have focused on costal cartilage as a source for chondrocytes for transplantation. The harvesting of costal cartilage does not cause additional damage to the articular surface and lowers the number of interventions into the joint while at the same time it allows to obtain sufficient amounts of cartilage.

Very few studies were devoted to the use of costal chondrocytes for inducing repair of articular cartilage defects. Moskalewski and Bator [25] used dog costal chondrocytes for transplantation into soft tissue. Kitaoka *et al.* [15] and Lohman *et al.* [19] employed costal chondrocytes for articular regeneration, Mori *et al.* [23] used costal cartilage implants fused with bone for articular cartilage restoration, and Mierisch *et al.* [22] has assessed the fate of costal chondrocytes transplanted into articular defects.

In our previous studies [30, 33], we used cultured rabbit costal chondrocytes for inducing articular cartilage repair with promising results. However, we have used 4-week old rabbits as donors of chondrocytes for transplantation. Chondrocytes derived from very young animals may have higher proliferation rate and chondrogenic potential than those from adult donors. Taking into consideration the influence of donor age on the quality of articular cartilage repair by costal chondrocytes transplantation may give better insight into this method of repair.

In our research we obtained a large number of quickly dividing cells. In the present experiments, labeling of collagen type I in cultures was not performed. In our pilot study, however, no trace of collagen type I was found in cultures of costal chondrocytes from donors of various age. Brittberg *et al.* [6] has proved that fibroblast-like character of dedifferentiated chondrocytes may be reversed by placing them in three-dimensional suspension.

Cartilage from adult donors required longer isolation time, and the number of chondrocytes collected in this group was lower than in young donors. However, all cultures reached confluence at a comparable time (14 to 21 days). According to various authors [9, 31] the duration of chondrocyte culturing is critical for valuable repair. More fibrous tissue is present at the repair site when chondrocytes have been cultured for a longer period of time.

Table 2. The mean scores of the histological grading of the repair tissue

Category	Young donors group (n=7)	Adult donors group (n=7)	Control group (n=4)
Cell morphology	1.28	1.42	3.25
Matrix staining	1.14	1.42	2.75
Surface regularity	1.42	1.28	2.00
Cartilage thickness	0.85	1.00	1.25
Integration with host cartilage	0.85	1.14	1.75
Total	5.54	6.62	11.00

Macroscopic observations of reparative tissue in lesions from both groups showed good incorporation into the surrounding cartilage, which may be of greater importance than the quality of the tissue itself [8]. Good incorporation into the defects protects the surrounding healthy cartilage from further deterioration [16].

In the present study, articular cartilage lesions transplanted with chondrocytes from young and adult donors showed histological appearance and extracellular matrix similar to that of hyaline cartilage. Although we have noticed slight differences between the two groups with chondrocyte transplantation (Table 2), the control group showed markedly inferior results in all categories. Collagen type II is the most ubiquitous type of collagen in normal articular cartilage. In the current study, distribution of collagen type II immunostaining was very similar in groups with chondrocytes transplanted from both young and adult donors.

Several authors have noticed that partial regeneration of chondral defects could be induced by cells either migrating from the surrounding tissue or from subchondral bone marrow [3, 13, 14 18]. However those cells were not able to induce healing comparable with that caused by transplanted chondrocytes. Our control group confirmed this.

Another problem to be considered is the endochodral ossification process of costal cartilage. Ossification of costal chondrocytes-derived tissue could be even more distinct with the use of more mature chondrocytes for transplantation. In the research of Ksiazek [17] and Malejczyk *et al.* [20], endochondral ossification was present as soon as fourteen days after intramuscular implantation of costal chondrocytes. However in our experiment no trace of newly formed bone was observed in either group.

Several studies [12, 21, 24, 28] have reported age-related decline in chondrogenic activity of articular chondrocytes and mesenchymal stem cells. Our data points to the potential of costal chondrocytes derived from young and also adult rabbits, as a good source for transplanta-

tion, despite some small differences between those two groups.

The rabbit model employed in this study is a useful tool for further investigations of the pathological mechanism of articular cartilage defects and possibilities for the repair irrespective of age of the cell donor.

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Received: December 30, 2005
Accepted after revision: March 1, 2006