Modified acellular dermal matrix for chondrocyte implantation in repairing cartilage defects of rabbits

Jiang Jian[1], Sun Lei[1], Feng Hua[2], Chen Lei[1], Meng Shu-qin[2], Feng Zheng[1], Tao Jian-feng[1], Peter I. Lelkes[3]

Abstract

BACKGROUND: Acellular dermal matrix possesses good flexibility and simple trimming. The intracutaneous or subcutaneous injection of acellular dermal matrix powder has fibroblast migration and collagen deposition. It has been widely used in plastic and reconstructive surgery.
OBJECTIVE: To explore the feasibility of reconstructed acellular dermal matrix as a scaffold for chondrocyte implantation.
DESIGN, TIME AND SETTING: Comparative observation. The study was performed at the Peking University Medical Department and Beijing Jishuitan Hospital between August 2003 and February 2007.
MATERIALS: Neonatal calf dermis was provided by Beijing Yuanheng Shengma Biology Technology Research Institute. A total of 24 healthy adult SD rats, weighing 250 g, regardless of gender, and 36 New Zealand rabbits, aged 3 months, were selected.
METHODS: ①Calf full-thickness back skin was incubated with cell free buffer or ABS/AES for decellularization, followed by surface modification using growth factors. ②Three rectangle skin flaps at two sides of the spinal cord of rats were made, and implanted with acellular dermal matrix. The implants were harvested at 2, 6, and 12 weeks postoperatively. ③The rabbits were divided into experimental and control groups. The cartilage was obtained from the left articular facet to isolate chondrocytes. The chondrocytes were seeded on the acellular dermal matrix. The cartilage defect was made on the right hind limb of experimental rabbits, and implanted with acellular dermal matrix containing autologous chondrocytes. Biogel was dropped on the surface of carrier. In the control group, the cartilage defect was made on the right hind limb of rabbits and the wound was sutured. Two rabbits from control group and 5 from experimental group were selected respectively at 4, 12 and 24 weeks postoperatively.
MAIN OUTCOME MEASURES: Cross-linking effect comparison; repair effect of rabbit bone defects.
RESULTS: ①The acellular dermal matrix cross-linked by glutaraldehyde demonstrated an obvious inflammatory reaction with tissue bleeding and necrosis. Conversely, ADM treated with water-soluble cross-linking agent caused displayed good histocompatibility. ②The cartilage defects were repaired completely; the attached cells survived and proliferated and the acellular dermal matrix was degraded after 24 weeks of surgery.
CONCLUSION: The acellular dermal matrix decellularized with cell free buffer, digested with digestive buffer, cross-linked by water-soluble cross-linking agent, and further decorated with growth factor exhibited good histocompatibility, and was suitable for cell attachment and growth. The acellular dermal matrix scaffold almost degrades in the rabbits, with no rejection, and the bone defects were repaired after 24 weeks.

[1]Beijing Institute of Traumatology and Orthopedics, Beijing 100035, China; [2]Sport Medical Center, Beijing Jishuitan Hospital, Beijing 100035, China; [3]School of Biomedical Engineering, Drexel University, Philadelphia PA, USA

Jiang Jian, Beijing Institute of Traumatology and Orthopedics, Beijing 100035, China
lily8210@hotmail.com

Correspondence to: Sun Lei, Doctor, Associate professor Beijing Institute of Traumatology and Orthopedics, Beijing 100035, China
dr_sunlei@126.com

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INTRODUCTION

Articular cartilage is an important structure and function unit of synovial joint. The cartilage tissue lacks of vessels and undifferentiated cells to repair injury and defects, moreover, chondrocytes are embedded in dense collagen-proteoglycan matrix, which limits cell proliferation and migration. Therefore, cartilage repair by itself is difficult, and inappropriate treatment could result in abnormity of articular cartilage formation, even joint dysfunction[1-2]. Articular cartilage injury is common in sports injury, and is a difficulty in orthopedics field. Treatments of cartilage defects or biosubstitute of cartilage have been explored[3-4]. Cell transplantation alone has some problems, such as unfixed cells, limited repair area, slow healing, calcification, and unknown long-term effect. With developing tissue engineering technology, autologous or allogeneic cells have been cultured and enlarged in vitro and seeded onto three-dimensional scaffolds with good biocompatibility and biodegradation to form cell-material composite for implantation into body. Scaffold materials are important in repair of articular cartilage defects by tissue engineering. They should have properties as follows: ①good biocompatibility, with no toxic or adverse effects on cell and organism; ②beneficial to cell attachment and proliferation and expected growth on the material; ③biodegradable, with matched degradation speed with new tissue formation; ④three-dimensional structure, which benefits cell nutrient transport and vascularization[5]. The scaffold materials mainly include raw material and synthesis material. The raw materials for scaffold involve chitosan, glucosan, calcium alginate, collagen, fibrin, hyaluronic acid, and chondroitin sulfate. The raw materials is injectable, benefits cell embedding, and can be used in minimally invasive surgery, moreover, it has good biocompatibility and cellular affinity, but no toxicity. However, it has poor mechanical function, could not maintain spatial structure during *in vivo* hydrolysis; moreover, their quality is limited by place of production and sources, therefore, their production quality is poor[6]. Sponge collagen is thought to be appropriate chondrocyte carrier, but the poor processing performance, lack of flexibility and tensile strength limit its single application to serve as material of chondrocyte culture matrix[7]. Acellular dermal matrix (ADM) has good flexibility and simple trimming. It can be cut, folded, and twisted or made into powder for intracutaneous or subcutaneous injection,

which has fibroblast migration and collagen deposition. ADM has been widely used in various plastic and reconstructive surgeries[8].

To solve degradation speed, water permeability, bioactivity and tensile strength, we utilized and reconstructed collagen fiber of dermis as matrix to explore the feasibility of reconstructed ADM as a scaffold for chondrocyte growth.

MATERIALS AND METHODS

Design: Comparative observation.

Time and setting: The study was performed at the Peking University Medical Department and Beijing Jishuitan Hospital between August 2003 and February 2007.

Materials

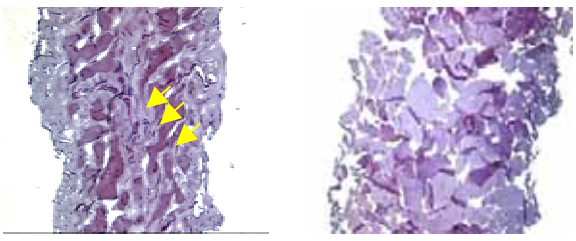
Neonatal calf dermis was provided by Beijing Yuanheng Shengma Biology Technology Research Institute. A total of 24 healthy adult SD rats, weighing 250 g, regardless of gender, were provided by Beijing Vitalriver Co., Ltd. (No. SCXK2006-0009), and 36 New Zealand rabbits, aged 3 months, were provided by Beijing Fuhao Experimental Animal Nursery (No. SCXK2005-0009). The experimental procedure was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[9]. Reagents and instruments are as follows.

Reagent and instrument	Source
DMEM, trypsin	INVIPROGEN
Fetal bovine serum (FBS)	HYCLONE
CO_2 incubator (14 D-78532 Tuttlingen)	BINDER
Scanning electron microscope (JSM-6380 LV)	JEOL, Japan

Methods

Acquisition of dermal matrix and various decellularization methods

The dermis of neonatal calf was cut into square pieces of 1 cm ×1 cm, and immersed into surface active agent (Figure 1a) and self-made acellular agent (Figure 1b) for decellularization.



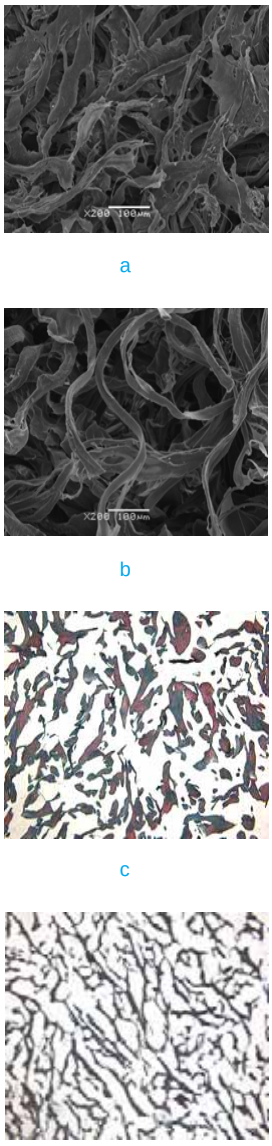
a: Surface active agent (SAA) b: Self-made acellular agent

Figure 1 Decellularization results by two methods (Arrow indicating the residual cells in the derma)

The paraffin sections were made to observe the dermis structure changes and remaining cell numbers. Results show that the residual cell number of dermal tissues following self-made acellular agent treatment was the least. Therefore, we selected this method. However, the fiber structure had no pores, which did not meet the requirements for carriers, so we modified this method.

Reconstruction of collagen fibers

The ADM was digested with trypsin (Figure 2a) and digestive solution at certain concentration (Figure 2b), and the fibers following treatment with trypsin (Figure 2c) and digestive solution (Figure 2b) were observed by scanning electron microscopy after Masson staining, and the pore was measured by LeicaQwin software. Staining showed that the ADM was composed of type I and III collagen fibers. The trypsinized fiber displayed rough surface, while the digested fiber had improved fiber surface structure, with the even pores (maximum porosity 530 μm) and three-dimensional structure. Therefore, digestive solution was the best method to reconstruct collagen fibers.



a

b

c

d

a, c: Trypsin; b, d : Digestive solution at certain concentration

Figure 2 Reconstruction of collagen fibers after digestion and its porosity measurement

ADM biocompatibility following various cross-linking methods

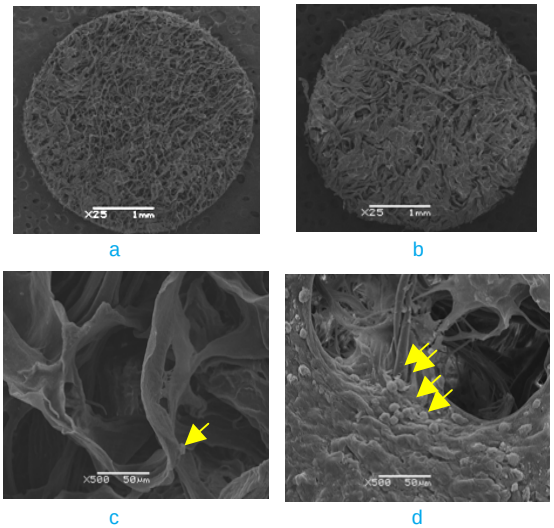
The dermis treated with self-made acellular agent and digestive solution was washed, freeze dried, and respectively cross-linked with glutaraldehyde and water-soluble cross-linking agent, then immersed in 10 mL PBS solution for 7 days.

The rats were anesthetized with 100 mg/kg ketamine. Three 0.5 cm×1.0 cm rectangle skin flaps, with 1.0 cm between two adjacent flaps, were made at two side of the spinal column, pedicled at the center of back. The cross-linked ADM (0.5 cm×1.0 cm) was subcutaneously implanted into the spinal column, and the wound was sutured. The implants were harvested 2, 6, 12 weeks postoperatively, sectioned with paraffin, and stained with HE to observe the compatibility with surrounding tissues. The results show that cross-linked ADM by water-soluble cross-linking agent had good biocompatibility.

Influence of ADM surface modification on cell growth

The cross-linked ADM by water-soluble cross-linking agent was prepared in 3.5 mm round sliced, freeze dried (Figure 3a), modified by growth factors (Figure 3b), and compared with non-modified ADM.

Type II collagenase (2 g/L)-digested rabbit chondrocytes were diluted to cell suspension at a concentration of 3×10^8/L, seeded on modified or non-modified ADM, and incubated in DMEM culture solution containing 10% calf serum for 2, 4, and 8 days. Cell growth and attachment were observed by scanning electron microscopy. Few cells attached on non-modified ADM at 4 days, and a small number of cells were attached on the ADM at 8 days (Figure 3c). The collagen pore of modified ADM did not significantly change, which benefited cell attachment and growth; a large number of attached cells were observed on the collagen fibers (Figure 3d) with microvillus and secretion.



a b c d

a,c: ADM untreated with growth factor; b, d: ADM treated with growth factor

Figure 3 Effect of surface modification of acellular dermal matrix (ADM) with growth factors on cell growth (Arrow indicating chondrocytes)

Repair of rabbit cartilage defects

Growth factor-modified ADM was sterilized and made into 3.5 mm round slices. The rabbits were divided into experimental and control groups. The primary cartilage of patellofemoral articular facet of the left hind limbs was obtained under sterile condition and digested with 0.2% type II collagenase at 37 ℃ for 4 hours to isolate chondrocytes. The cells were seeded on compound ADM (3×10^7/L), and incubated in DMEM culture solution containing 10% calf serum for 24 hours. A cartilage defect, 3.5 mm diameter, 2 mm depth, was drilled at the patellofemoral articular facet of the right hind limbs of the same rabbit in the experimental group on the next day, and implanted with cultured cell-ADM composite.

The biogel was dropped on the carrier and the wound was sutured layer by layer. A cartilage defect, 3.5 mm diameter, 2 mm depth, was drilled at the patellofemoral articular facet of the right hind limbs of the same rabbit in control group on the next day, and sutured layer by layer. Two rabbits from control group and 5 from experimental group were respectively sampled at 4, 12, and 24 weeks postoperatively, observed by microscopy, sectioned by paraffin, followed by HE staining to compare the defect repair effects.

Main outcome measures

Cross-linking effects; rabbit cartilage defect repair.

Design, enforcement and evaluation

The study was designed and performed by Jiang Jian and Chen Lei, and evaluated by Meng Shu-qin and Feng Zheng.

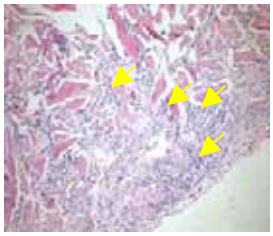
RESULTS

Comparison of cross-linking effect

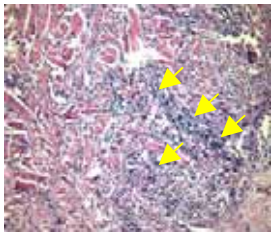
After soaking in PBS for 7 days, the volume of ADM cross-linked by glutaraldehyde and water-soluble cross-linking agent did not remarkably change.

HE sections showed that the collagen scaffold exhibited mesh-shaped arrangement, with no collagen swelling. However, their biocompatibility was significantly different. Glutaraldehyde cross-linked ADM implantation induced continuous inflammatory reaction, strong rejection and poor histocompatibility, and fiber encapsulation was observed at 2 weeks; inflammatory cell infiltration such as neutrophil, lymphocyte, and mononuclear phagocyte, was seen in ADM pores, and no bleeding or necrosis appeared in ADM (Figure 4a); at 12 weeks, fiber encapsulation surrounding the ADM, inflammatory cell infiltration, necrosis and small vessels growth in ADM were observed (Figure 4b). However, water-soluble cross-linking agent cross-linked ADM displayed good histocompatibility, with no bleeding or necrosis.

Fiber encapsulation was observed at 2 weeks; inflammatory cell infiltration was seen surrounding the ADM, but no bleeding or necrosis appeared in ADM (Figure 4c); at 12 weeks, fiber encapsulation surrounding the ADM and small vessels growth in ADM were observed with no inflammatory cell infiltration, bleeding or necrosis (Figure 4d).



a



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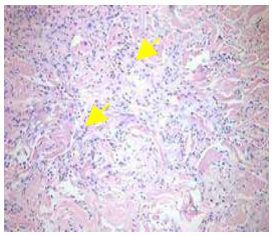

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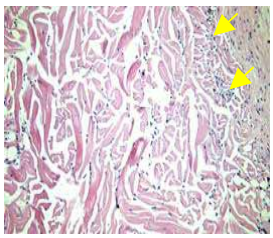


d

a,b: glutaraldehyde; c,d: water-soluble cross-linking agent

Figure 4 Biocompatibility of acellular dermal matrix after cross-linking by different reagents (Arrows indicating white blood cells inflammatory reaction)

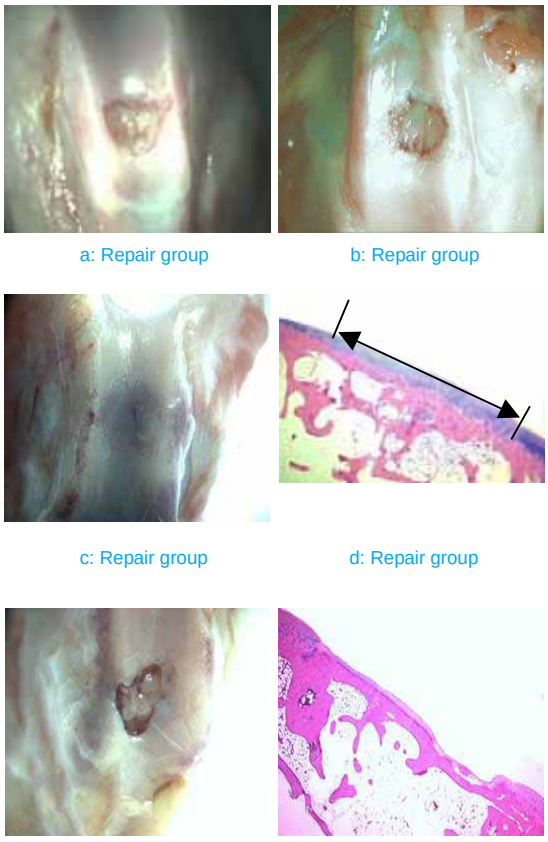
Repair of rabbit cartilage defects

The cartilage defects of experimental group were almost repaired 12 weeks after ADM implantation, and completely repaired at 24 weeks. Moreover, ADM was completely degraded *in vivo*.

Microscopic observation of experimental group showed that at 4 weeks, the implants and autologous tissue were well compatible, with no fiber encapsulation, adherence, diapyesis, hyperemia or hyperplasia (Figure 5a); at 12 weeks, the implants closely attached to autologous tissues, and a layer of translucent cartilage-like substance was observed on the surface of implants (Figure 5b); at 24 weeks, the implants closely attached to autologous tissues, with fused boundary, smooth surface, and similar color to normal cartilage (Figure 5c). HE staining showed that the implants were completely degraded at 24 weeks, tightly attached to the autologous tissues, with fused boundary but no inflammatory reactions; in addition, chondrocytes were seen in the implantation region, accompanied by a large amount of cartilage matrix among the chondrocytes; immature chondrocyte formed in the binding region of cancellous bone and the implants (Figure 5d).

In control group, the wound exhibited dark black, with a large amount of hyperemia, severe necrosis, soft tissue hyperplasia, and fiber

Encapsulation at 4 weeks; the wound was concave and enlarged, and the surrounding tissues necrosed at 12 weeks; at 24 weeks, no wound healing was observed, and subcartilaginous tissues were exposed (Figure 5e). HE staining showed wound concave and cartilage defects with no new formed cartilage tissues at the defects, and the defects expanded to the cancellous bone covered by a layer of fiber tissues at 24 weeks (Figure 5f).



a: Repair group b: Repair group

c: Repair group d: Repair group

e: Control group f: Control group

Arrow indicating the cartilage defects after repairing

Figure 5 Repair of rabbit cartilage defects

DISCUSSION

Type II collagen is predominantly distributed in articular cartilage. As type I collagen is easy to obtain and beneficial to chondrocyte regeneration, it is frequently used as cell culture scaffold. However, type I collagen lacks of mechanical strength, and rapidly degrades *in vivo*[10-11]. Dermis mainly comprises collagen. The transplantation of allogeneic dermis as carrier of

autologous epidermis[12] shows that the rejection of allogeneic skin transplantation results from epidermic cell, endothelial cell and fibroblast[13-14], therefore, the antigenicity of allogeneic dermis could be reduced by eliminating cell components in dermis. In early period, repetitive freeze thawing and trypsinization have been used to prepare ADM, but these methods could no completely remove the cell components of dermis, leading to intensive host rejection following ADM transplantation, and the preparation process is excessively complex. With technique improving, ADM has been prepared by DispascII-Triton, hypertonic saline-SDS, hypertonic saline-enzyme digestion, and NaOH corrosion. Although these methods effectively cell components of dermis under certain condition, they greatly damage the basal membrane. It is important to minimize cell components and sustain the matrix during ADM preparation.

Bovine type I collagen has been extensively used to make tissue-engineered scaffold due to its abundant source and success clinical application[15]. Therefore, we selected bovine skin to prepare ADM. Through histology examination, self-made acellular agent effectively eliminated the cell components in the dermis, and the decellularized ADM only had some non-cell components, such as extracellular matrix protein and collagen, with no obvious immunogenicity[16]. However, the ADM treated by self-made acellular agent exhibited mild collagen fiber swelling. Ideal tissue-engineered cartilage scaffold or cell carrier should have three-dimensional structure. The matrix material has pores and high porosity, with maximal porosity of 80%-90%; moreover, the large internal surface area benefits cell attachment and ingrowth, as well as nutrient penetration and metabolite discharging[17-18]. Therefore, we further modified the ADM.

In the present study, the collagen fibers of ADM were digested by various methods. Results show that the collage fibers digested by digestive solution at certain concentration exhibited smooth surface and even pores (average porosity 500 μm), which are beneficial to cell attachment and ingrowth, nutrient penetration and metabolite discharging. However, the pores are difficult to restore in liquid.

Some researchers believe that after 4-6 weeks ADM transplantation, when ADM degradation could reach dynamic balance with matrix amount secreted by fibroblasts, ADM degradation is terminated, and ADM is regarded as autologous tissue[19]. To maintain good appearance in long-term culture and *in vivo* environment, we cross-linked the ADM using different methods. Physical methods do not utilize exogenous material, but the degree of cross-linking is low. A variety of chemical cross-linking agents have been used to improve the degree of cross-linking, mechanical performance and biocompatibility[20]. In the present study, the influence of various cross-linking agents on ADM biocompatibility shows that water-soluble cross-linking agent was better, and the concentration, cross-linking duration and washing methods were confirmed. Cross-linking could maintain stereochemical structure and plasticity of carriers and delay degradation of collagen carrier. As ideal scaffold material of bioengineering, certain surface activity is necessary, which helps cell attachment, and provides good microenvironment for cell growth, proliferation and matrix secretion[17-18]. Cartilage capsule is mainly composed of chondroitin sulfate, and some noncollagen proteins, which bind together to form giant molecule chondromucoprotein[21]. Considering the requirement of carrier pores, we simulated the components of cartilage matrix under physiological state, and modified the ADM with growth factors. As the dermis is not transparent, cell growth could not be observed by inverted microscopy[22], so we used scanning electron microscopy. Results of the present study demonstrate that carrier modified by growth factors displayed cell attachment, and benefits long-term growth of cells.

Articular cartilage formation and maintenance depend on chondrocytes. Chondrocytes derive from mesenchymal cells, and can increase matrix volume during skeleton morphogenesis and development. In mature tissues, chondrocytes account for 10% of total cell volume. The chondrocytes have active metabolism, and react to various environment stimulation to promote cartilage matrix formation and cell proliferation, stimulating new cartilage formation[23]. To detect the repair effect of modified carrier on cartilage defects, the ADM with autologous chondrocytes was implanted into the cartilage defects. Results show good biocompatibility and repair efficacy at 24 weeks.

As cross-linked carrier has high mechanical strength and certain elasticity, it could be fixed by suture. In this study, some ADM collagen carriers shed during defect repair. Therefore, in subsequent experiments, large cartilage defect repair experiments should be conducted and the carrier and normal cartilage tissue should be sutured under arthroscope to observe the repair effect. Moreover, the chondrocyte amplification technique should be explored, and the cell-carrier composite could be cultured and implanted to rabbit cartilage defects to further observe chondrocyte attachment, growth, and repair efficacy of tissue-engineered cartilage and to determine the capacity of chondrocytes to synthesize and secrete extracellular matrix in the scaffold.

In conclusion, modified, cross-linked collage scaffold could maintain good structure during long-term culture, with good biocompatibility. Results of the present study show that ADM collagen scaffold completely degraded in the cartilage defects, with no rejection, and attached cells survived for a long period of time and proliferated to repair the cartilage defects. Decellularized, modified, and cross-linked ADM could serve as cartilage carrier.

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改建脱细胞真皮基质作为软骨细胞移植载体修复兔软骨缺损○

江　健[1]，孙　磊[1]，冯　华[2]，陈　磊[1]，孟淑琴[2]，冯　正[1]，陶剑锋[1]，Peter I. Lelkes○[3] ([1]北京市创伤骨科研究所，北京市　100035；[2]北京积水潭医院运动医学中心，北京市　100035；[3] School of Biomedical Engineering, Drexel University Philadelphia PA，USA)

江　健，女，1982 年生，北京市人，汉族，2007 年北京大学医学部毕业，主要从事软骨组织工程载体的开发研究。
通讯作者：孙　磊，博士，副教授，北京市创伤骨科研究所，北京市　100035

摘要
背景：脱细胞真皮(Acellular Dermal Matrix, ADM)具有良好的柔韧性、易于修剪，制成微粉状皮内或皮下注射后，可见到成纤维细胞的移入及胶原的沉积，目前已被广泛应用于整形修复临床实践。
目的：验证脱细胞真皮经改建后作为软骨细胞移植载体的可行性。
设计、时间及地点：对比观察，于 2003-08/2007-02 在北京大学医学部和北京积水潭医院完成。
材料：新生小牛的真皮层由北京元亨圣马生物研究所提供；体质量 250 g 的 SD 健康成年大白鼠 24 只，雌雄不限。3 月龄的新西兰大白兔 36 只。
方法：①取新生小牛背部真皮组织，进行脱细胞处理，分别用戊二醛和水溶性交联剂进行交联；再用生长因子对其进行纤维表面修饰。②取 SD 大白鼠，于大鼠脊柱两侧各设计 3 个长方形皮瓣，分别植入两组交联后的脱细胞真皮基质。术后 2，6，12 周取出植入物。③取新西兰大白兔，分为实验组和对照组，从其左侧关节面取原代软骨，分离软骨细胞并接种于复合后的ADM上进行培养。将实验组兔右侧后肢造成软骨缺损，植入含有自体软骨细胞的 ADM，再将生物胶滴加于载体表面；对照组兔右侧后肢造成软骨缺损后分层缝合伤口。分别于第 4，12，24 周取材，对照组每次取 2 只，实验组每次取 5 只。
主要观察指标：①交联效果的比较。②兔软骨缺损修复结果。
结果：①经戊二醛交联后的 ADM 植入大鼠皮下后有强烈的炎症反应，并有组织出血坏死；而经水溶性交联剂交联的 ADM 的组织相容性较好。②在植入接种有自体软骨细胞的 ADM24 周后大白兔股关节的软骨缺损修复完好，附和的细胞能够存活且有增殖，ADM 本身基本降解。
结论：经脱细胞及纤维改建和交联及生长因子修饰的 ADM 孔隙均匀，组织相容性好，适于细胞黏附及长期生长。ADM 胶原支架在兔体内可基本降解，未见排异反应，移植 24 周后见骨缺损修复。

关键词：脱细胞真皮；组织工程；软骨细胞；软骨修复
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