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TGF- β_3 and TIMP-2 Co-transfection of Rabbit Bone Marrow Mesenchymal Stem Cells Compound Silk Fibroin / Chitosan Scaffolds Implanted in Vivo Rabbit Cartilage Defect Repair; Silk Fibrin/Chitosan Biological Scaffold*

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ABSTRACT Objective: TGF- β_3 widely present in bone tissue and cartilage tissue, can induce cultured mesenchymal stem cells into cartilage differentiation and growth. TIMP-2 can inhibits the degradation of the cartilage matrix caused by MMP and protect new cartilage. This test investigated the feasibility and effect differences of pure TGF- β_3 and TGF- β_3 , TIMP-2 co-transfection of rabbit bone marrow mesenchymal stem cells Filament fibroin (silk fibroin, SF) / chitosan (chitosan, CS) scaffolds implanted animals repair of rabbit articular cartilage defects *in vivo*. **Methods:** Twenty New Zealand white rabbits were divided into four groups, each five (scaffold group, untransfected group, reAAV-TGF- β_3 transfected group, reAAV-TGF- β_3 , reAAV-TIMP-2 co-transfection group). Under sterile conditions the third generation of the logarithmic growth phase of rabbit bone marrow mesenchymal stem cells (BMSCs) were took, with the purpose of carrying a recombinant adeno-associated virus gene transfection, the composite with transfected BMSCs and SF-CS scaffolds were implanted in rabbit articular cartilage defects: blank control group was implanted SF-CS scaffold, the negative control group was implanted untransfected BMSCs composite SF-CS scaffold, reAAV-TGF- β_3 turn transfection group implanted reAAV-TGF- β_3 transfected BMSCs composite SF-CS scaffold, reAAV-TGF- β_3 , TIMP-2 co-transfection group was implanted reAAV-TGF- β_3 , TIMP-2 transfected BMSCs joint compound SF-CS scaffold. After two months, the rabbits were killed, as well as visually assessed of cartilage defect repair situation by HE staining, and conduct characteristic of cartilage cells that stained with toluidine blue staining and type II collagen immunohistochemical staining. **Results:** After two months, cartilage defects in each experimental group were formed cartilage-like substance, and the co-transfection group induced cartilage were closer to hyaline cartilage, the difference of co-transfection group and pure TGF- β_3 transfected group score was statistically significant ($P < 0.01$). HE staining results suggest that co-transfected cartilage repair group's result was better than pure TGF- β_3 transfected group. **Conclusion:** In animal experiments, the pure TGF- β_3 transfected rabbit bone marrow mesenchymal stem cells can repair rabbit articular cartilage defects, the effect of TGF- β_3 and TIMP-2 co-transfection group repairing the defect is more effective, suggesting that TIMP-2 and TGF- β_3 have a synergistic effect on induced differentiation of stem cells into cartilage. TGF- β_3 , TIMP-2 co-transfection of rabbit bone marrow mesenchymal stem cells combined with SF-CS scaffold have a good effect on reconstruction rabbit articular cartilage injury.

Key words: Transforming growth factor; Matrix metalloproteinases inhibitors; Bone marrow mesenchymal stem cells; Cartilage defects

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Introduction

Bone marrow mesenchymal stem cell and the development of tissue engineering brings hope to cartilage repair after damage and defect^[1]. Studies have shown that although allogeneic BMSCs transplantation has immune response but weaker, eventually allogeneic BMSCs which be transplanted can repair cartilage defects^[2]. Adeno-associated viral vectors compared with other vectors like adenoviral has more stable physical and chemical properties^[3]. TGF- β is a family of peptides with multiple functions, including

TGF- β_1 , TGF- β_2 , TGF- β_3 and over twenty members of BMP subfamily^[4]. They are widely present in the cartilage tissue, bone tissue, activation of lymphocytes and platelets. Studies have shown^[5]: TGF- β_3 plays an important role in proliferation, differentiation, maturation and matrix metabolism of cartilage cells, and as a signal product plays a catalytic role in the cartilage repair. Chondrocyte survival of the extracellular matrix under the action of matrix metabolic enzymes may cause or aggravate the cartilage damage, matrix metalloproteinases play a major role. MMPs play function affected by its specific tissue inhibitor: Tissue inhibitor

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Metalloproteinase, in cartilage matrix of osteoarthritis by adding TIMP chondrocytes can significantly reduce the damage by inflammatory cytokines^[6]. TIMPs have been reported that there are four types, and TIMP-1 and TIMP-2 were more recent studied than others. Studies have shown^[7] adeno-associated viral as vectors, the TGF- β_3 and TIMP-2 two purposes gene transfection to integrate into the BMSCs genome of rabbit, it has autocrine TGF- β_3 and TIMP-2's ability to successfully let different BMSCs into chondrocytes phenotype through endogenous of induction in vitro. Therefore, this study aims to experimentally investigate the feasibility and effect differences of the pure TGF- β_3 and TGF- β_3 , TIMP-2 co-transfection of rabbit BMSCs composite SF-CS scaffold repair rabbit knee cartilage defect^[8].

1 Materials and Methods

Recombinant adeno-associated virus carried objective genes: reAAV-TGF- β_3 , reAAV-TIMP-2 made by the Affiliated Hospital of Qingdao University Medical Center Laboratory.

1.1 Adeno-associated virus carried objective genes transfected bone marrow stem cells

We executed one randomly from 21 white rabbits, peeled off the rabbit fur after local disinfection with 75% alcohol, remove lower limbs intactly (upper retention femoral head, the lower retention ankle), peeled off limb soft tissue release under sterile conditions, iodine volts soak 5 minutes before cutting ends of the bones, Rinseing bone marrow blood into a centrifuge tube with D-MEM low sugar medium, carefully aspirate the supernatant, adding fresh culture medium. Medium was changed every two days. After 7 days 0.25% trypsin digestion and passage. Each experimental group were used to carry recombinant adeno-associated virus gene transfection third generation BMSCs. Take the cell supernatants which the first 5 days after transfection, with enzyme-linked immunosorbent assay (ELISA) to TIMP-2 detection, the 3, 6, 9 days after transfection cell lysis, extraction of protein to ELISA test, testing the expression of TGF- β_3 genes, to verify the transfection success.

1.2 SF-CS biological scaffold materials

Solutions of silk fibroin and chitosan solution concentration of 1:1 according to the proportion of mixture, pour the solution into a mold, placed the mold in the refrigerator frozen, vacuum drying with freeze drying machine and then use crosslinker crosslinked, after vacuum drying again get the SF-CS stent materials, prepare it into a diameter of 3 mm, to 5 mm high cylinder, with sterile PBS repeatedly washing, soaking in the cell culture medium for 24 h. Digest and Centrifuge the successful transfected BMSCs, adjust the cell density of $1.2 \times 10^8/\text{ml}$, inoculation on the stents soaked in culture medium.

1.3 Animal Surgery

Take 20 female New Zealand white rabbits, weighing 2.5-3.0 Kg, divided into 4 groups, each group is 5. With 10% chloral hydrate anesthetized by intraperitoneal injection, skin preparation,

pending the corneal reflection disappeared, the knee incision under sterile conditions, in the same part of the medial femoral condyle with a hollow drill drill a diameter of about 3mm full-thickness cartilage defects.

reAAV-TGF- β_3 transfected group implanted MSCs be transfected by reAAV-TGF- β_3 and composited by SF-CS biological scaffold into the area of cartilage defect; reAAV-TGF- β_3 , reAAV-TIMP-2 co-transfection group was implanted reAAV-TGF- β_3 , reAAV-TIMP-2 co-transfection of MSCs composite SF-CS biological scaffold; untransfected group implanted MSCs composite SF-CS biological scaffold; stent group implantation SF-CS scaffold. Layer wound closure and the first 3 days after operation penicillin 500,000 U/d was subcutaneous injected to prevent infection.

The postoperative 20 rabbit into the cage free activities, feeding them in the same conditions, such as forage, temperature, and light. Execute them after 2 months. Remove distal femur, complete the femurs with 10% formaldehyde solution fixed organization for 24 hours. The cutting of the medial femoral condyle on rabbit ensure its thickness in more than 5 mm; 10% EDTA decalcified for a month, gradient alcohol dehydration, paraffin embedding and tissue biopsies. HE staining to observe the cartilage cells, and conduct characteristic of cartilage cells that stained with toluidine blue staining and type II collagen immunohistochemical staining.

1.4 Statistical Methods

The experimental data were measured $\bar{x} \pm s$, application SPSS 17.0 software for statistical analysis. Among each group a number of pairwise comparisons were used Bonferroni test, $P < 0.05$ was considered statistically significant.

2 Results

After transplantation, animal diet and activities were less than before, after five days of each group of animals can stand with limbs, primary healing of incision surgery after one week, animals diet resume normal, two weeks after the animals in each group limbs recovered. During the observation period, all animals without intra-articular infection and death.

General observation: scaffold group cartilage defect covered by a fibrous tissue, there is no cartilage-like tissue formation; untransfected group cartilage defects instead of a little cartilage-like tissue, cartilage repair without luster and the surface is irregular, not restore damage surface contour, texture is soft, with the surrounding cartilage boundary is obvious; reAAV-TGF- β_3 transfected group cartilage-like tissue formation in the cartilage defect, repair of the cartilage surface is regular but dull edge in contact with the surrounding cartilage more closely, partial restoration of the cartilage damage surface contour, texture relatively hard, quite boundaries with the surrounding cartilage (Figure 1); reAAV-TGF- β_3 , reAAV-TIMP-2 co-transfection group cartilage defect completely replaced by the class cartilage repair cartilage surface is smooth, transparent, edges tightly with the surrounding cartilage and nearly comp-

letely restored the contour of the damage surface, texture hard, on



Fig.1 In the reAAV-TGF-β3 transfected group, there are cartilage-like tissues formation in the defectt, obvious boundaries with the surrounding cartilage

obvious boundaries with the surrounding cartilage (Figure 2).



Fig.2 In the co-transfection group , there are cartilage-like tissues formation in the defectt, handly classed the boundaries with the surrounding cartilage

Table 1 Two months after the score were seen in each group(n=5)

Group	Score
Scaffold group	0.600± 0.548 ^①
Untransfected group	1.800± 0.447 ^②
ReAAV-TGF-β3 transfected group	3.800± 0.837 ^③
ReAAV-TGF-β3, reAAV-TIMP-2 co-transfection group	5.400± 0.548 ^④

Note: Levene test of homogeneity of variance (F = 1.202, P = 0.341), analysis of variance (F = 60.267, P = 0.000).

Mean by the Bonferroni method of multiple Comparison, α= 0.008, ① vs ② P = 0.041, ① vs ③ P = 0.001, ① vs

④ P = 0.000 ② vs ③ P = 0.001, ② vs ④ P = 0.000, ③ vs ④ P = 0.005.

Histological HE sections: stent group cartilage defect area consists of fibrous tissue replacement. There were no classes of chondrocyte generation in defect aera, toluidine blue staining in the surface of the defect area was no different chromatin; untransfected cartilage defect repair cartilage tissue mainly by category like cells, showing a small amount of fibrous tissue. The cells in defect were larger quantities, and there was less extracellular matrix in the aeea. Cartilage cells are more naive freshman class, cell disorder, new matrix layered obvious, repair cartilage and surrounding area has obvious boundaries, toluidine blue staining defect area in a small amount of abnormal chromatin surface and arranged irregularly; reAAV-TGF-β₃ transfected cartilage defect area constituted by the class of cartilage-like cells, visible lacuna structure, cell quantity, irregular arrangement, ECM less obvious stratification repair tissue matrix repair area and the surrounding tissue is still relatively clear boundaries, some sections showed that only one edge with the surrounding cartilage integration is good, toluidine blue staining on the surface defect repair area regular arrangement the different chromatin (Figure 3); reAAV-TGF-β₃, reAAV-TIMP-2 co-transfection group by a cartilage-like repair tissue cells, cartilage lacuna structure, cell quantity, arrangement tends to a certain direction, bracket materials have been completely degraded and absorbed, organizational base of a tide line appears, and complete a row, no lymphocytic infiltration and invasion of new blood vessels, new tissue close to full integration with the surrounding cartil-

age (Fig.4). Toluidine blue staining on the surface defect area is continuous with the adjacent cartilage neat arrangement of different chromatin. General observations in each group by Moran^[9] general observation index score, the results in Table 1. Histological observations in each group by Wakitani^[10] cartilage defects histological grading score, the results in Table 2.

3 Discussion

Articular cartilage lacks of blood vessels and innervation, so that the articular cartilage damage often cannot repair itself. So articular cartilage defects repairing in recent years has been a hot research topic in orthopedics^[11]. Clinically for the treatment of articular cartilage defects present methods are as follows (1) articular cartilage defect drilling techniques (2) autologous periosteum or perichondrium transplantation (3) osteochondral transplantation (4) autologous bone marrow cells or cartilage cell transplantation^[12]. Which autologous chondrocyte transplantation obtains a more satisfactory clinical outcome^[13], but also exposed some shortcomings, such as cartilage cells in vitro dedifferentiation may occur and periosteal hypertrophy^[13,14]. Experts and scholars at home and abroad in recent years have been committed to developing a new, more effective treatment of cartilage defects, namely phenotypic differentiation of cartilage tissue engineering research. There were Studies which in animal models and clinical application of research have obtained certain results, compared with traditional surgery, tissue engineering technology has a broader clinical prospects^[15].

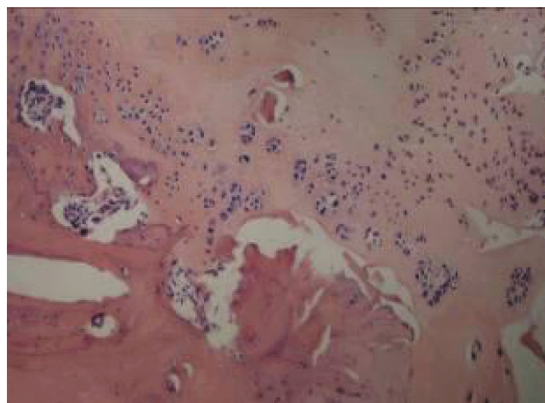


Fig.3 reAAV-TGF-β3 transfected cartilage group defect area is visible lacuna structure, cell quantity, irregular arrangement, ECM less obvious stratification repair tissue matrix repair area and the surrounding tissue is still obvious boundaries

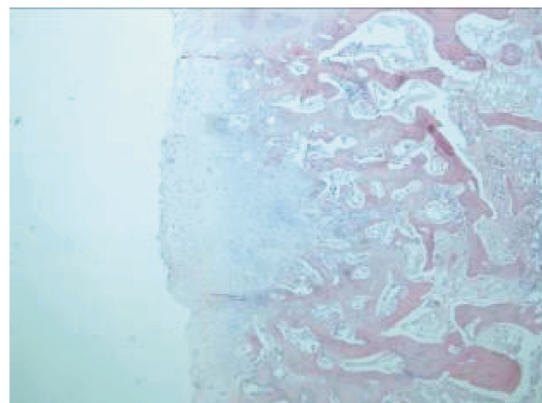


Fig.4 The co-transfection group cartilage repair tissue structure, cell quantity, arrangement tends to a certain direction, organizational base of a tide line appears, and complete a row, close to the new organization is fully integrated with the surrounding cartilage

Table 2 Two months after the histological score in each group specimens(n=5)

Group	Score
Scaffold group	13.800± 0.447 ^①
Untransfected group	10.000± 0.707 ^②
ReAAV-TGF-β3 transfected group	6.400± 0.894 ^③
ReAAV-TGF-β3, reAAV-TIMP-2 co-transfection group	3.800± 1.483 ^④

Note: Levene test of homogeneity of variance (F = 1.578, P = 0.234), analysis of variance (F = 102.414, P = 0.000).

Mean by the Bonferroni method of multiple Comparison, α= 0.008, ① vs ② P = 0.001, ① vs ③ P = 0.000, ① vs ④ P

= 0.000, ② vs ③ P = 0.001, ② vs ④ P = 0.000, ③ vs ④ P = 0.005

The importance of TGF superfamily has been found in the phenotype differentiation of stem cell. Zheng Dong, et al Studies have found that TGF-3 has the strongest effect in promoting mesenchymal stem cells which come from transforming growth factor-β family into chondrocytes phenotype differentiation, proliferation and inflammatory mediators affect its action [16]. It has been successfully induced a kind of substance similar to the hyaline cartilage on the basis of the existing theories, but found that induced articular cartilage can not withstand the inflammatory cytokines, hypoxia, high pressure and other harsh environments [17]. Under physiological conditions, the cartilage cells metabolism in a dynamic balance. Participate in a variety of macromolecules and maintain this balance, including: some damage factors like MMPs, IL-1β, TGF-α and some protective factors such as: TIMP, α2M and so on. Their expression and activity will directly affect the homeostasis of articular cartilage metabolism of particular importance is the dynamic balance between MMPs and TIMP.

Some studies have shown [18] when the activity of protection factor TIMP decreased and the activity of damage factors MMPs increased, the changes will lead to metabolic disorders of collagen, proteoglycans and others in ECM of articular cartilage, and this process of osteoarthritis disease is very common. When the seed cells through biological scaffolds getting into cartilage defect, even simply acute injury caused by cut the articular cavity will produce large amounts of inflammatory cytokines, such as collagenase 3, t-

hese inflammatory factors inevitably affect stem cells to chondrocytes table type differentiation, as well as to damage the cells has differentiated into cartilage phenotype [19]. Has been proved that carry TGF-β₃, TIMP-2 stem cells can induced endogenous cartilage phenotypic differentiation in vitro [20]. Therefore, this experiment envisaged put TIMP-2 gene via AAV viral vectors, together with TGF-β₃ gene sequences integrated into the chromosome of BMS-Cs, and composite SF-CS biological scaffolds implant into rabbit articular cartilage defects. We expect stem cells can be endogenous induced phenotypic differentiation into chondrocytes, while they can express TIMP-2 themselves, to protect themselves and reduce the damage of collagenase. To enhance the ability to resist damage of tissue engineered cartilage.

The co-transfection group cartilage defects has white class cartilage formed and integration with the surrounding cartilage is good, cartilage surface is smooth and neat. HE staining of articular cartilage defects see a large number of pit cells, toluidine blue staining of cartilage lacunae see blue-stained cells, II collagen immunohistochemical staining further confirmed newborn cells into chondrocytes. reAAV-TGF-β₃ transfected cartilage defects with cartilage-like material formed dull class, only one side edge of the integration with the surrounding cartilage is good, cartilage surface irregular, HE staining showed a large number of pit cells, stained with toluidine blue see lacuna inner cell blue dye, II collagen immunohistochemical staining further confirmed newborn cells into chondro-

ocytes. Statistical analysis showed that: reAAV-TGF- β_3 transfect-ed rabbit cartilage defect repair is superior to non-transfected group, and the difference was statistically significant ($P < 0.05$); co-transfected cartilage repair effect is better than reAAV-TGF- β_3 transfect-ed group, and the difference was statistically significant ($P < 0.05$).

In addition to the stent group and untransfected group general observation score undifferentiated, the rest of the score differences between experimental groups were statistically significant ($P < 0.05$), reAAV-TGF- β_3 , reAAV-TIMP-2 co-transfection group whether cartilage repair degree from the naked eye, or from histological experimental group were significantly better than the other, and the differences were statistically significant ($P < 0.05$). Tip TGF- β_3 in animals can induced stem cell differentiation into cartilage phenotype, and TIMP-2 on inducing differentiation of stem cells in cartilage phenotype have a synergistic effect. This study also has some defect as follows: firstly, rabbits feeding time is shorter, can only verify the cartilage phenotype transformation, but cells in the experimental period is not completely converted into hyaline cartilage cells; second, if the currently available near hyaline cartilage can withstand the articular harsh environment needs to be verified.

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TGF- β_3 与 TIMP-2 联合转染兔骨髓间充质干细胞复合丝素蛋白 / 壳聚糖生物支架修复兔软骨缺损 *

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摘要 目的: TGF- β_3 广泛存在于骨组织、软骨组织中, 能诱导体外培养的间充质干细胞向软骨分化、生长。TIMP-2 能抑制 MMP 对软骨基质的降解, 保护新生软骨组织。本实验探讨单纯 TGF- β_3 和 TGF- β_3 , TIMP-2 联合转染兔骨髓间充质干细胞复合丝素蛋白壳聚糖 / (silk fibrin/chitosan, SF/CS) 生物支架植入动物体内修复兔膝关节软骨缺损的可行性及效果差异。方法: 将新西兰大白兔 20 只分为 4 组, 每组 5 只 (支架组、未转染组、reAAV-TGF- β_3 转染组、reAAV-TGF- β_3 , reAAV-TIMP-2 联合转染组)。在无菌条件下取兔第三代对数生长期骨髓间充质干细胞 (bone marrow mesenchymal stem cells, BMSCs), 用携带目的基因的重组腺相关病毒进行转染, 将转染成功的 BMSCs 与 SF-CS 生物支架复合, 分别植入兔膝关节软骨缺损处: 未转染组植入 SF-CS 生物支架, 未转染组植入未转染的 BMSCs 复合 SF-CS 生物支架, reAAV-TGF- β_3 转染组植入 reAAV-TGF- β_3 转染的 BMSCs 复合 SF-CS 生物支架, reAAV-TGF- β_3 , TIMP-2 联合转染组植入 reAAV-TGF- β_3 , TIMP-2 联合转染的 BMSCs 复合 SF-CS 生物支架。两月后处死家兔, 肉眼观察以及 HE 染色评定缺损软骨修复情况。并进行软骨细胞特征性染色即甲苯胺蓝染色及 II 型胶原免疫组化染色鉴定。结果: 两个月后除支架组外各实验组兔膝关节软骨缺损处均有软骨样物质形成, 且联合转染组诱导的新生成成分更接近缺损处周围正常软骨。联合转染组与 reAAV-TGF- β_3 转染组; 联合转染组与未转染组; reAAV-TGF- β_3 转染组与未转染组的评分差异均具有统计学意义 ($P < 0.05$)。HE 染色结果提示联合转染组软骨修复效果较单纯 TGF- β_3 转染组更好。结论: 单纯 TGF- β_3 转染兔骨髓间充质干细胞对兔膝关节软骨缺损有修复作用, TGF- β_3 与 TIMP-2 联合转染组修复缺损效果更明显, 提示 TIMP-2 与 TGF- β_3 具有协同效应。

关键词: 转化生长因子; 基质金属蛋白酶抑制剂; 骨髓间充质干细胞; 软骨缺损

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