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Original article

Tissue engineering strategy using mesenchymal stem cell-based chitosan scafolds in growth plate surgery: A preliminary study in rabbits

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ABSTRACT

Background: Growth plate injury in children could produce limb length discrepancy and angular deformity. Removal of damaged physis or bony bar and insertion of spacers produced variable results and for large defects in young children, the treatment is challenging. In this study, we used tissue-engineered mesenchymal stem cells (MSC-based chitosan scaffold) for restoration of the damaged physis. The usage of chitosan as a spacer was also investigated.
Materials and methods: An experimental model of growth arrest was created by removing lateral 50% of distal femoral physis of fourteen 4-week-olds albino rabbits. The left side growth plate defects were filled with MSC-based chitosan scaffold in 10 and scaffold alone in 4 rabbits. For all the rabbits, right-side defects were left alone as the control limb. After 3 months, femoral bones were harvested and gross inspection and radiology for measurement of angulations were done; histological study for evaluation of regeneration of physis was also done.
Results: The hemiphyseal resection procedures were successful and all of the operated limbs showed angular deformities. There was a trend toward less angular deformity in cases in which more concentration of MSCs with chitosan scaffold was used. In cases of transfer of MSCs with concentration of less than 1.5 millions, mixed results were observed and angular deformities were not reduced. Transfer of chitosan alone yielded poor results.
Conclusion: In this study, we have developed an in vitro construction of a transplantable tissue-engineered disk, using natural chitosan scaffold and MSCs. We investigated the efficacy of these disks for repairing the defect of growth plate cartilage at distal femoral physis. Our results showed that the beneficial effect of these cells on scaffold appeared in more concentration of cells.
Level of evidence: Level III. Low power comparative study.

1. Introduction

Growth plate injury in growing children could produce major problems, causing limb growth arrest leading to limb length discrepancy, angular or rotational mal-alignments [1]. The bony bars produced in the physis may be removed, and then replaced by various materials to reconstitute the continuity of the growth plate [2]. The outcome of such procedures is variable, depending on the patient's age, and also size and location of the bony bar [3]. The interposition materials used for such a purpose are quite variable and are usually acting only as passive spacers.

The idea of having active cells to re-build the damaged and destroyed growth plate cells is an intriguing concept. Polypotential stem cell regeneration and its transformation into various cell-lines is gaining popularity in different branches of medicine [4–6]. The use of mesenchymal stem cells for growth plate regeneration has been studied by Ahn et al. in 2004. The implantation of autologous MSC, stimulated by transforming growth factor-beta (TGF-β), into the physeal defect at the proximal end of the tibia in rabbits enhanced physeal cartilage repair [7]. In another study, Planka, in 2008, showed that transplantation of autogenous or allogenic MSC is equally effective in improving physeal cartilage repair in the damaged distal femoral physis of rabbit [8].

In order to transfer MSC to a bone defect, a carrier or scaffold is needed. Tissue engineering approaches use biocompatible and biodegradable scaffolds in combination with appropriate cells.

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Table 1
The summary of interventions in rabbits. The right limb growth plate defect was always the control side.

	Group	Spacer in left side	Duration	Left		Right	
				DMFA	HS	DMFA	HS
1	Excluded	0.2 M MSC and scaffold	2 weeks	77	13	80	12
2	Excluded	0.5 M MSC and scaffold	3 weeks	53	19	75	15
3	A	0.2 M MSC and scaffold	1 months	65	13	45	14
4	A	0.5 M MSC and scaffold	3 months	20	13	35	15
5	A	1.5 M MSC and scaffold	3 months	55	15	45	13
6	A	1.5 M MSC and scaffold	3 months	55	18	45	14
7	A	1.5 M MSC and scaffold	3 months	62	17	56	14
8	A	1.5 M MSC and scaffold	3 months	77	16	72	15
9	A	1.5 M MSC and scaffold	3 months	65	13	40	12
10	A	1.5 M MSC and scaffold	3 months	45	17	40	15
11	B	Scaffold	2 months	52	14	50	16
12	B	Scaffold	3 months	43	14	45	14
13	B	Scaffold	3 months	52	13	58	17
14	B	Scaffold	3 months	62	13	52	13

M: million; MSC: mesenchymal stem cells; DMFA: distal medial femoral angle; HS: histological score.

The commonly used scaffolds include hydrogel [9], hyaloronate-collagen-fibrin [8], chitin [10], gelfoam [7], and agarose [11]. Natural cationic biopolymer chitosan is a partially deacetylated derivative of chitin. Chitosan is described as possessing biological and material properties suitable for biomedical and clinical applications [12,13]. It has a good mechanical stability and is biocompatible, promotes hyaluran synthesis and is also shown to enhance wound healing. Synergy of chitosan with hyaluronan develops enhanced performances in regenerating hyaline cartilage [14–16].

The aim of this study was to find the effect of allogenous cultured MSC on chitosan scaffold in regeneration and function of injured growth plate at distal femoral physis of rabbit. The effect of different concentration of MSC on restoration of physis was evaluated. The difference between chitosan alone and MSC-based chitosan scaffold on regeneration and function of the injured growth plate was also investigated.

2. Materials and methods

After obtaining the approval of the medical ethics committee, 14 albino male rabbits with an average age of 1–1.5 months were selected for this experimental study. Bone marrow was aspirated from an older (3 months of age) rabbit. Under ketamine and xylosine anesthesia, in sterile field, a large needle was inserted into the posterior iliac wing and 5 cm^3 bone marrow was aspirated into a heparinated syringe at Laboratory Animal Center, and sent for Stem Cell Culture laboratory of Transplant Research Center, Shiraz University of Medical Sciences during 2013.

The isolation and culturing of rabbit mesenchymal stem cells (MSCs) were done according to our previously reported method [17]. The cultured MSCs were counted and analyzed for viability by trypan blue staining analysis. The characterization of the rabbit MSCs was determined on a flowcytometr (FACS Calibur Becton, Dickinson, United States), and functional ability was assessed in response to specific culture conditions. Immunophenotyping of MSCs was done by expression of cell surface marker CD45, CD80 and CD90 (FITC conjugated from Dako, Denmark). After 3 weeks of incubation, the MSCs were ready for transplantation. The bone marrow-derived MSCs were transferred on 3–4 passage into 5×2 mm, disk-shaped chitosan scaffolds. Three different concentrations of MSC on chitosan scaffolds were prepared for comparing their effect on restoration of physis (Table 1, group A).

Chitosan-pourous scaffolds were prepared according to Suphasiriroj et al. [18] by dissolving the chitosan (ALDRICH code 961M0046 V) solution (2% w/v) in 1% (v/v) acetic acid. The appropriate amount of chitosan solution was transferred to polystyrene Petri dishes (2 cm^2). The resultant scaffolds (area 2 cm^2, thickness 3–3.5 mm) were sterilized and hydrated by immersing them in an ethanol series: absolute ethanol for 1 h, 70 and 50% ethanol for 30 min each. The solvent exchange of scaffolds was carried out by several changes of PBS (pH 7.4) and equilibration in PBS for removal of residual ethanol.

The recipient rabbits were then approached and under sterile operating room conditions and satisfactory general anesthesia, the distal lateral physis was exposed by lateral approach to the knee. The patella was dislocated medially and after visualization of the periphery of the physis, 50% of the physis with a small shell of proximal and distal cancellous bone was removed by a cylindrical trocar (Fig. 1). The defect produced as such in the left distal femur was filled with either MSC on chitosan scaffolds (10 rabbits group A) or chitosan scaffold alone (4 rabbit group B). The right physeal defects were left alone and used as control limbs. After suturing the wounds, topical antibiotic spray was applied over the wounds. No other form of antibiotics was used. The rabbits were free to move in their cages and received regular diet.

After 3 months, the rabbits were euthanized and femoral bones were harvested for gross inspection, radiologic evaluation and histological analysis of the growth plates. Antero-posterior x-rays were obtained on each harvested bare femur. The distal medial femoral angles were measured on gross specimens and also on radiographs of the femurs in the case and control groups (Fig. 2 and Table 1). The findings were documented, analyzed and compared with normal distal femoral angles.

The femurs were then sent to a pathologist to study the distal femoral physis for regeneration of growth cartilage cells in group A, group B and in the control right femurs. The histological scoring was done according to O'Driscoll et al. [19]. In this scoring system, 9 parameters are considered for the quality of regenerated cartilage including cellular morphology, integrity and thickness of regenerated cartilage tissue and bonding to adjacent native cartilage. The maximal histological score is 24 [19]. According to this scoring system which includes different criteria for cartilage repair, higher score means more mature tissue grade and better response [19]. Statistical analysis was done using SPSS software, version 15. Wilcoxon signed rank test and Mann-Withney test were used for data analysis.

3. Results

The cell-analysis showed viability between 98% and 100% in rabbit marrow-derived samples. The MSCs were characterized by immunophenotyping, and differentiation potential into osteoblast and adipocytes (Fig. 3A, B). The rabbit MSCs were cultured on

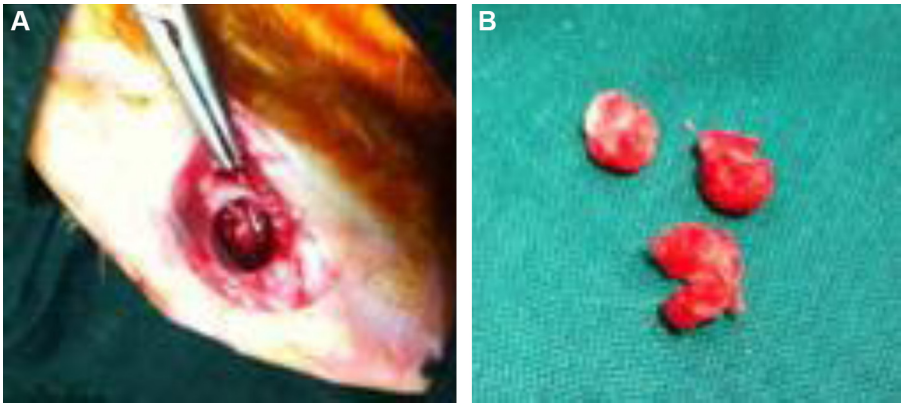

Fig. 1. Surgical procedure and removing the physis. A. The clamp tip is pointing to the physeal defect and white line in the middle of the hollow is physeal cartilage. B. Three pieces of harvested physeal cartilages and surrounding bone.

transplantable tissue-engineered chitosan scaffolds (Fig. 4A, B) in the form of disks with a diameter of 5 mm and thickness of 2 mm.

Fourteen rabbits were enrolled in the study. Two rabbits from group A died 2 and 3 weeks after operation. The cause of death was unrelated to surgery. Therefore, 14 left distal femurs and 14 right control distal femurs were evaluated for inducing angular deformity by removing 50% of the lateral growth plate (Fig. 1), and also correcting or decreasing the angular deformity when scaffold filled with MSC were utilized.

The hemiphyseal resection procedure was successful in producing angular deformity. In no limb, implantation of MSC and/or scaffold could produce a totally normal growing cartilage to prevent the angular deformity (Table 1).

The gross and radiographic assessment of the distal femora was done in all the 28 specimens. Fig. 3 shows the histological scoring done on all specimens. The two rabbits in group A which died 2–3 weeks after the surgery were excluded from further analysis.

In group A (Table 1), the distal metaphyseal angle (DMFA) varied from 20° to 77° (mean: 55.50) for the left side and 35° to 72° (mean: 47.25)for the right sides (*P*-value: 0.09). The histologic scoring varied from 13 to 18 (mean: 15.25) for the left side, and from 12 to 15 (mean: 14.00) for the right sides (*P*-value: 0.12).

When we compared the limbs with 1.5 million MSC at the left side with the corresponding right side control limbs (rabbits: 5–10), the statistical differences between the two limbs' angular deformity and histological scores were significant. For these rabbits, mean DMFA for the left side was 59.38° and for the right side, it was 49.67°, (*P*-value: 0.027). Comparing histological scores, the mean for the left side was 16 and that for the right side was 13.8, (*P*-value: 0.027).

There was a trend towards less angular deformity in cases in which more concentration of MSC was used (left sides of cases: 5 to 10 in Table 1). The histological scores were also better in those cases, and the cartilage cells showed more columnar differentiation and more thickness and the morphology of the regenerated cartilage tissue was similar to normal physeal cartilage.

Mesenchymal cell concentration less than 1.5 millions produced mixed results and could not affect angular deformities. The histological scores in these cases were no better than the right-side control limbs (cases 3 and 4, Table 1).

Chitosan scaffold, when used alone (group B, Table 1), could not stop the appearance of angular deformity. The fibrous tissue and bone were formed in the growth plate defects filled with scaffold alone as rapidly as the contralateral control side, and both the histological scores and angular deformities were similar in the left and right femurs.

4. Discussion

The growing children could be the victims of major musculoskeletal deformity resulting from physical injury. A variety of causes, including trauma, burn, frostbite, infection, and tumor might cause physeal defects. When such an injury happens, the chance of spontaneous repair is very slim, except for very small damages [20]. Angular deformity and/or limb length inequality are the ill effects of this injury. The extent of deformity depends on anatomical site of physis, the size and the place of damage in the physis and the growth remaining potential in the physis. Small and central locations produce less deformity than large and peripheral lesions [3].

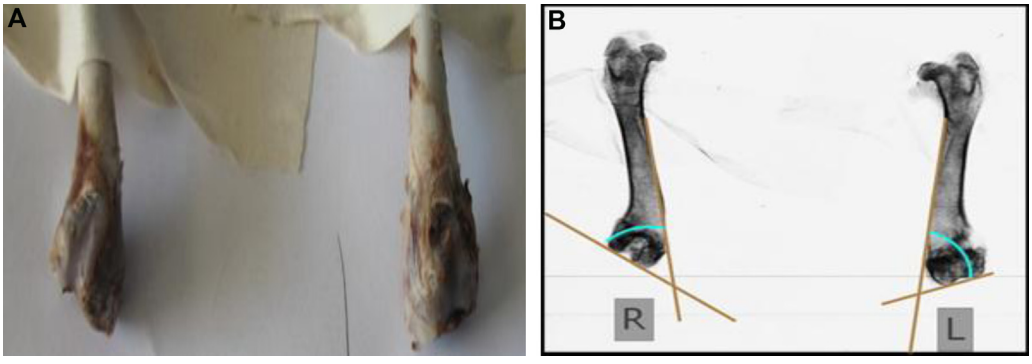

Fig. 2. Angular deformity on gross inspection and radiology. A. Gross appearance of the distal femur. B. Radiographic measurement of the same rabbit limbs. Note the lower valgus angulation on the left with MSC on scaffold compared to the control right femur.

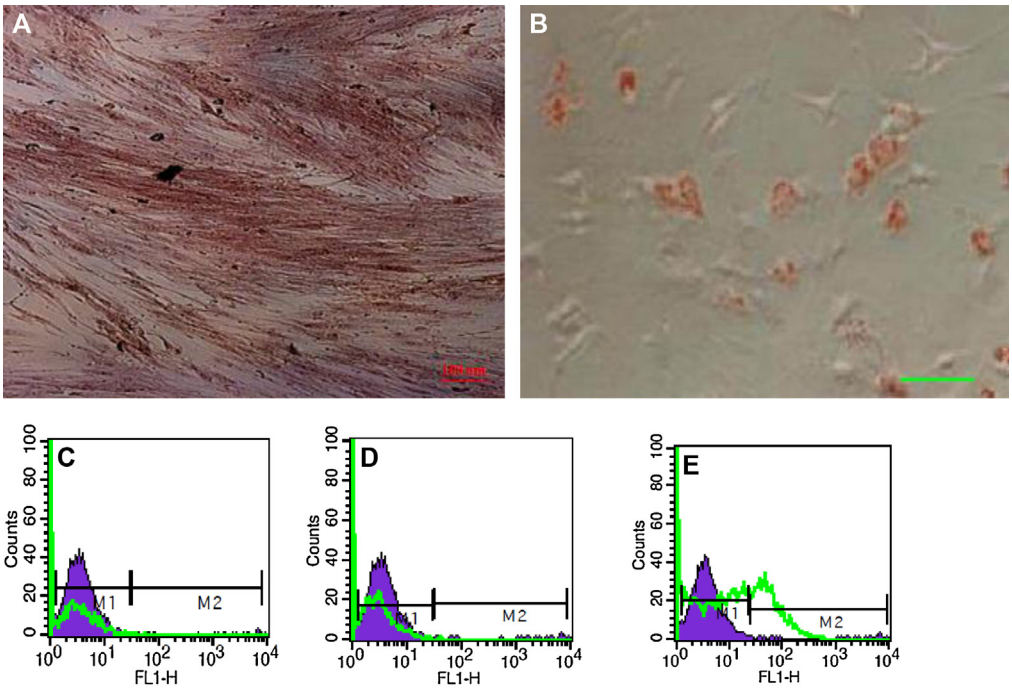

Fig. 3. Characterization of isolated human MSCs. Osteogenic and adipogenic differentiation of rabbit bone marrow mesenchymal stem cells in the 4th passage. A. Osteogenic differentiation was positive for alizarin red staining. B. The adipose droplet in differentiated cells after staining with oil red. Scale bar for: A: 100 um; B: 50 um. Flow cytometric analysis at passage numbers 3 demonstrated that the cells were negative for surface expression of CD45 and CD80, respectively (C and D), but they were positive for surface expression of CD90 (E).

The management possibilities for these defects include:

- "wait and see" – for very small defects or in patients near skeletal maturity;
- use inert materials as spacers in place of the bony or fibrous bar that is formed at the site of physeal damage when it is less than 25–30% of physis;
- totally obliterating the physis and dealing with the length inequality in situations when the defect is large [3].

Since there is no easy, quick, well-established solution, with high success rate for the bony bars formed in physeal damage, the searches for better remedies continue.

The mesenchymal polypotential stem cells are potentially effective elements. The ability of MSC to repair the physeal cartilage defects have been studied in the past in few articles: Planka et al. produced physeal injury in the distal lateral femoral physis to the extent of 9% of the physis and filled the defects with 2 million MSC on a special composite scaffold of hyaloronate-fibirn-collagen in each sample. They could induce almost 4° of angular deformity correction with this technique [8]. In another report, Chen et al. produced large (50%) defects at the proximal tibial physis and used cultured MSC derived from periosteum and agarose to fill the defect. While the concentration of implanted MSC is not clear, near total correction of physeal defect and normalization of growth was reported [11].

The transferrable MSC can be autogenous or allogenous. Infusion of allogenic MSC in patients with muccopolysaccharidosis and leukodystrophy with nerve and bone tissue problems resulted in some improvement in the nerve function and bone density. The infused allogneic MSCs were found to be alive in the bone marrow and be functional [21]. It seems that MSC has the ability to escape allogenic immunogenicity and can survive in the host tissue [22].

Ahn et al. used autogenous MSC for physeal cartilage repair in the proximal tibia of rabbit. They used gelfoam and gelatin as scaffolds, and investigated the effect of stimulation of MSC by TGF-β. They stimulated the MSC towards cartilage cell formation and, therefore, the injected cells were actually cartilage cell

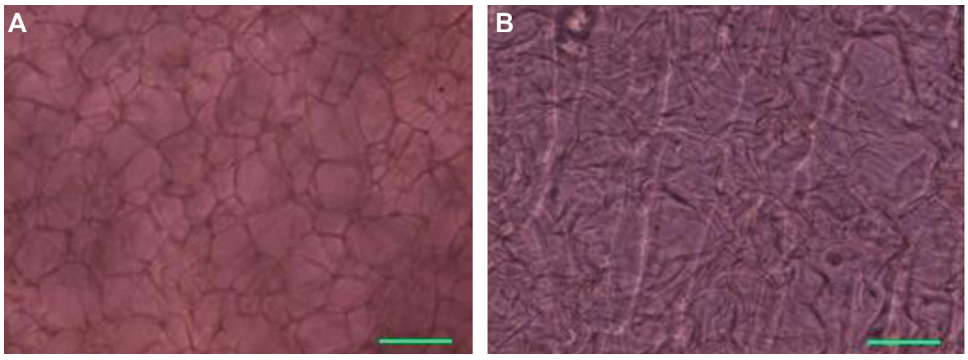

Fig. 4. In vitro construction of transplantable tissue-engineered chitosan scaffold. A. Porous chitosan scaffold without cells. B. Porous chitosan scaffold with rabbit cultured MSCs. Scale bar for A and B: 50 um.

transplantation. The implantation of cartilage cells resulted in less angular deformity, and even produced better results when the implanted cells received stimulation by TGF-β [7]. Planka et al. compared allogenic and autogenous implantation of MSC for physeal cartilage repair and reported that there was no difference between allogenic and autogneous cells in repairing the physeal cartilage defect [8].

The stem cells have to be carried to the intended sites via the scaffolds. The scaffold itself can act as a spacer and help the reconstitution of the damaged physis from the remaining physeal cartilage cells. Chitosan scaffold has been found to be biodegradable and biocompatible with excellent structural stability [10]. It has been used for MSC transfer in the past [12,13]. In order to differentiate between the positive spacer effects of chitosan versus the regeneration potential of MSC, we used chitosan alone in some and chitin carrying allogenic stem cells in some other animals and found that chitin was not a good spacer, as the angulation of the distal femurs in chitin group was similar to the contralateral control side. This raises the concerns about chitin as an inert spacer. The possible inflammatory reaction against chitin might be the reason for failure of chitin as a spacer. Another reason for this finding could be the size of the defect, as 50% physeal defect is too large to be managed by a simple spacer.

We studied the use of allogenic MSC on chitosan scaffold for repairing large (50%) defects of growth plate cartilage at the distal femoral physis. The beneficial effect of these cells was seen only in 1.5 million-cell concentrations, and in lower concentration of cells, the effect was not different from using scaffold alone. Our findings are similar to those of Planka et al., except that they used smaller defect and higher concentration of cells in a larger number of rabbits. They considered only few degrees of angular correction as significant achievement. In our study, we had a mean of 10° of angular difference between the two limbs, and the size of the defect was much larger (50% of the physis). Large defects of 50% have been used in the past in the rabbit's proximal tibia, and restoration of physis by implantation of MSC in agarose has been successful [11].

The main limitation of this study was the small number of animals studied and the fact that a higher concentration of MSC was not used in all of the animals. In addition, the fate of the implanted cells in imposing angular correction cannot be definitely determined as has been suggested by Planka et al. by MRI detection of risovist in the implanted cells [8]. The histologic evaluation of the implanted physis was, however, a unique feature of our study. We are pursuing the same idea with larger number of animals and with higher concentrations of MSC.

The results of our work indicate that large quantities of physeal defects can be partially reconstructed by transplantable tissue-engineered disks using natural chitosan scaffold and allogenic MSCs. Although still "theoretical" but further confirmation of this idea by other studies, using different scaffolds and stem cell concentrations can have potential clinical applications for treatment of congenital, tumoral or traumatic growth deficiencies in the future.

In conclusion, implantation of our tissue-engineered allogenic MSC-based scaffolds can promote the repair process of large physeal cartilage damage at the distal femoral physis in rabbits, and larger cell concentration is more effective. Optimal MSC concentration and effect of the size of damage need further investigation with larger number of animals.

Disclosure of interest

The authors declare that they have no conflict of interest concerning this article.

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