IJVS 2017; 12(1); Serial No:26

IRANIAN JOURNAL OF VETERINARY SURGERY (IJVS)
WWW.IVSA.IR

Original Article

Comparison of Autogenic Costal Cartilage with Chitosan Scaffold in Canine Humeral Defect Healing

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Abstract

Objective- Current trends emphasize the acceleration of fracture healing on the ground that in doing so, the limitation of mobility and complications associated with recovery period are reduced. The present study aimed to compare autogenic costal cartilage with Chitosan scaffold in canine humeral defect healing.

Design- Experimental study

Animal-15 adult male mongrel dogs

Procedures- Dogs were randomized into three groups of five animals each. A window shaped defect was created in humerus on right hands. In the first group (controls), the defect was left untreated. In the second and third groups, chitosan and autogenic costal cartilage were placed into the defects, respectively. Radiographs of the defects were prepared at weeks 2, 4, 6 and 8 and finally the dogs were euthanized after 70 days. Histological sections were also obtained from the defect sites.

Result-The results indicated that the costal cartilage alone treated group was inferior to both chitosan treated and control groups, so cartilage did not seem to serve as a suitable alternative for grafting in canine bone defects.

Conclusion and Clinical Relevance- Taking into account the results and other recent reports, it could be concluded that chitosan scaffolds with greater capabilities can be used in canine bone defect healing, however, for ideal bone tissue regeneration, chitosan as a base has to be combined with other materials including those mentioned above. The present study showed that cartilage could not serve as a proper alternative for grafting.

Keywords– Autogenic Costal cartilage, Chitosan Scaffold, Bone Defect, Canine

Received: 15 April 2017; **Accepted:** 24 September 2017; **Online:** 08 October 2017

Introduction

Acceleration of fracture healing process is currently a major area of interest to practitioners because in doing so, the limitation of mobility and complications during recovery period are reduced to a large extent. Great deal of research is conducted investigating the materials which can help meet such a need.

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A wide range of bone defects due to traumas, tumors, osteomyelitis, loosened internal stabilizers or osteotomes for reformations, require surgical interventions, because repair without surgery is possible in cases of minor damages.[1]

Bone defect repair involves intermediate tissue regeneration such as fibrous connective tissue, cartilage and woven bones which occur before final bone restoration. Intermediate tissue plays a role in damaged site stabilization and provides a scaffold for the differentiation of new tissues.[1] Autograft is still the gold standard treatment in grafting, however, it suffers from some shortcomings such as complications in harvesting site and limited availability of bone for autografting.[2,3] Therefore, other alternatives to autograft are considered and investigated extensively

freeze dryer (Bench top, LSBC50, Germany) for a day

DOI: 10.22034/ivsa.2017.50822

including cartilage graft which can serve as an appropriate alternative.[2,4] The fetal physiologic process of endochondral ossification and cartilage formation during fracture healing as a part of bone regeneration, need for less oxygen compared to bone cells, which leads to longer endurance on the graft site and use of bigger segment of cartilage for grafting purpose, altogether make the cartilage a proper grafting material[2]. Given that the primary matrix of long and short bones during fetal development is of hyaline cartilage, we used costal cartilage in this study.[5]
Costal cartilage is translucent with the possibility to become hypertrophic and biopsied, leaving least damage on the site. During recent years, the use of such a cartilage or processed type in the culture medium has been investigated. In the present study, costal cartilage was placed in the defect created in the dog humerus. Polymer scaffold is another bone graft substitute made by joining together the small units or monomers. Finally, Chitosan scaffold is a natural polymer used in bone tissue engineering in the past years.[5] Chitosan is derived from deacetylated chitin, a constituent of exoskeleton in arthropods/crustaceans such as crabs or shrimps. It is among the biomaterials which can be used as a scaffold with varying pore/cavity size in bone tissue engineering. Biocompatibility and antibacterial property are the characteristics of this material and in contrast to most of synthetic polymers, Chitosan is hydrophilic and reportedly can serve as a suitable scaffold used in combination with other polymers.[6,7] in this study, the effects of chitosan scaffold alone on the humerus bone defect healing in dogs were evaluated. We used dogs on the ground that the results are comparable to those on humans, as indicated by Standard world Food and Drug Administration. Accordingly, some pharmaceutical companies seek to investigate the effectiveness of the drugs on dogs.[8] The current study, was aimed to evaluate the effects of costal cartilage and chitosan alone on the long bone defect healing in dogs and to compare the respective results.

Materials and Methods

Preparation of %2 Chitosan

Two grams of chitosan powder with medium molecular weight (Sigma, USA) was provided and dissolved in 0.5 M Acetic acid (Applichem, Germany) for deacetylation. To obtain a uniform solution, it was stirred for 5 hours at 50C on a magnetic stirrer (IKA, Basic 2RH, Germany) using magnet, stored in a fridge (Samsung, RL726, Korea) at 5 °C for 24 hours, then transferred to a freezer (Samsung, RL726, Korea) and stored at -18 °C for 24 hours, and finally stored in a long. To defreeze the product, it was re-stored for 24 hours at -18 °C in a freezer and finally transferred to a fridge and stored at 4 °C. Following these steps, a white porous spongy like product was obtained.

Surgical Operation

Fifteen adult male dogs weighing about 20 kg and aged 2-3 years were prepared for surgery. They were kept in special cages for 10 days to get used to new conditions. Feeding and conditions were the same for all the dogs which were fed meat and bone on daily basis. They had access to water at all times. Having approved their health status clinically and paraclinically, and subcutaneous administration of %1 Ivermectin, and anti-parasite medication(Razak Ivectin®1%) at a dose of 600 mg/kg and repeating in two weeks, the dogs were randomly assigned to three groups of five. To prepare them for surgery, they were anesthetized with a combination of ketamine (10mg/kg) and diazepam (5mg/kg) followed by inhalation anesthesia with halothane. The inner forearms were shaved and prepared for surgery after draping and scrubbing. To remove a window shaped piece of humorous, an incision was made in the craniomedial and the skin, connective tissue and muscle were removed so that humerus bone appeared where we cut a piece 2 cm long and 1 cm wide. Finally, a rectangular bone of that size from the inner surface of humerus was prepared. As for the control group, the window shaped gap was left empty. To harvest cartilage in the autogenic cartilage treated group, the skin on the distal end of 6^{th} and 7^{th} ribs was shaved simultaneously. After draping and scrubbing, an incision of required size was made on the respective cartilage for grafting and placed it in the defect. In the chitosan treated group, adequate amount of already prepared Chitosan sterilized by nano sterile apparatus was removed and placed in the gap/defect. Finally, after transplantation, muscles and subcutaneous tissues were sutured in two layers using vicryl no.1 thread and the skin by non-absorbable nylon no.1 thread. To prevent infection, penicillin at a dose of 20,000 U/kg IM was administered to the treated groups once a day for 3 days.

Radiological Evaluation

At weeks 2, 4, 6, and 8, post-operative interior-lateral x-rays of the operation site were prepared and modified Lane Sandhu scoring system was used for assessment and scoring. Accordingly, bone callus around, bone formation rate, and remodeling were scored 0-2, 0-4, and 0-2, respectively.[9]

Histopathological Evaluation

Dogs were euthanized 70 days post-surgery, after anesthesia with ketamine and diazepam followed by IV infusion of magnesium sulfate. Then, humerus bone and all surrounding soft tissues in all the dogs were separated. The bones were stored in %10 formalin and sent to the lab for histopathological study. The specimens were fixed in %10 formalin for 48 hours and decalcified in 15% Formic acid for 10 days. Dehydration, clearing, paraffin embedding were done by routine method. Then 5 µm thick sections were cut from the centers of each specimen and stained with Hematoxylin and Eosin for histologic examination. Finally, the sections were blindly evaluated and scored according to Heiple's scoring system (Table 1).[10]

Statistical Analysis

SPSS software was used for statistical analysis. The obtained results were analyzed by Kruscal-Wallis non-parametric ANOVA and in case of $p<0.05$, they were re-analyzed by Mann-Whitney test. The level of significance for the tests was set at $P<0.05$.

Results

Radiology

Evaluation of the radiographs revealed no significant difference between the three groups although bone formation in costal cartilage and Chitosan groups was respectively inferior and superior, compared to each other pair of groups (Table2) (Fig 1-3) .

Histopathology

Histopathological study indicated little improvement in the bone defect healing in costal cartilage treated group. Despite the initiation of in situ bone marrow cavity formation indicating the conversion of cartilage to bone, cartilage tissue was present (Fig 4). In the control group, formation of cancellous bone in the fracture space and appearance of bone marrow cavities along with red bone marrow were evident (Fig 5). Also, in chitosan treated group, formation and remodeling of cancellous bone in the fracture space with enlarged bone marrow space and presence of red bone marrow were more evident than other groups (Fig 6). Statistical analysis showed a significant difference between control and costal cartilage groups in terms of cancellous bone criteria, cortical bone and union (P=0.01, P=0.01, and P=0.04, respectively). Accordingly, autogenic cartilage treated group was found inferior to the controls. The same findings were also true for Chitosan and costal cartilage groups (P=0.04, P=0.01, and P-0.04, respectively), i.e., the latter group was inferior to the former. Although chitosan group was relatively superior to costal cartilage counterpart, the difference was not statistically significant (Table 1).

Table 1. Heiple histopathological scoring system

Union	
No evidence of union	0
Fibrous union	1
Osteochondral union	2
Bone union	3
Complete organization of shaft	4
Cancellous bone	
No osseous cellular activity	0
Early apposition of new bone	1
Active apposition of new bone	2
Reorganizing cancellous bone	3
Complete reorganization of cancellous bone	4
Cortical bone	
Non	0
Early appearance	1
Formation under way	2
Mostly reorganized	3
Completely formed	4
Marrow	
None in resected area	0
Beginning to appear	1
Present in more than half of the defect	2
Complete colonization by red marrow	3
Mature fatty marrow	4
Total points possible per category	
Distal union	4
Cancellous bone	4
Cortex	4
Marrow	4
Maximum score	16

Discussion

Given that most bone formation occurs through endochondral ossification ,i.e., when cartilage is converted to bone, cartilage can be used in bone defect healing.[2] Therefore, in this study conducted on dogs, we harvested autogenic costal cartilage, placed it

in the bone defect of the dog's humerus and evaluated its effect on the defect healing. Parallel to that, we placed Chitosan scaffold in the defect created in another group of dogs and compared the efficacy of the two materials; cartilage and chitosan, in bone defect repair in an animal model.

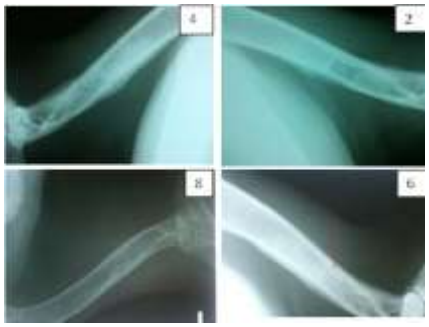

Figure1. Radiographs at weeks 2, 4, 6 and 8 in control group

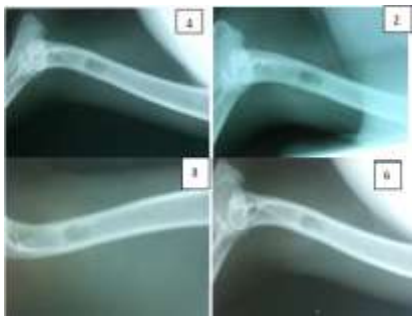

Figure 2. Radiographs at weeks 2, 4, 6 and 8 in autogenic costal cartilage treated group.

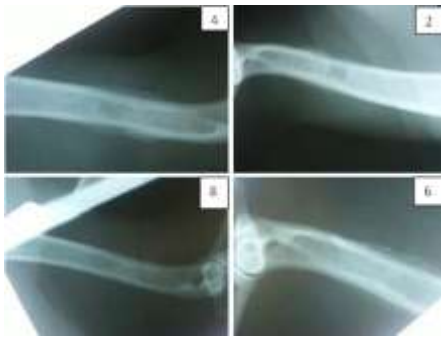

Figure 3. Radiographs at weeks 2, 4, 6 and 8 in autogenic chitosan treated group

Table 2: Results of radiograph evaluation in mean value (Max-Min)

Med(min-Mx)				
Weeks	**Control Group**	**Cartilage Group**	**Chitosan Group**	**Pa**
2	2(1-5)	3(1-3)	3(2-3)	0.48
4	4(2-5)	4(4-6)	4(2-5)	0.6
6	5(4-8)	5(4-7)	4(3-8)	0.92
8	8(5-8)	6(5-8)	7(3-8)	0.008*

[a]Kruscal-Wallis test was done and P value less than 0.05 was considered statically significant.

Overall, the results were in favor of chitosan, and chitosan group was found superior to cartilage and control counterparts. In 2006, Saifzadeh et al investigated the effect of elastic ear cartilage autograft on canine radial fracture healing, and reported improvement in defect healing after 8 weeks but no complete union in the gap. According to their results, the amount of callus formation in the defect was suggestive of probable complete union over time, though further studies are needed to confirm it.[11]

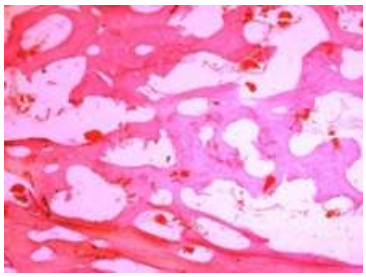

Figure 4. Formation of cancellous bone and bone marrow cavities in less than half area of defect in control group (×10, H&E).

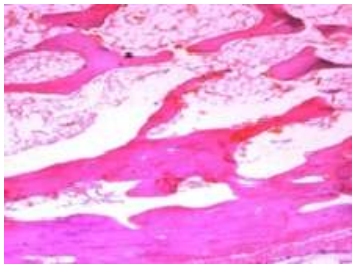

Figure 5. Remain of cartilage with formation of bone marrow cavities in them in the defect space in cartilage group (×10, H&E).

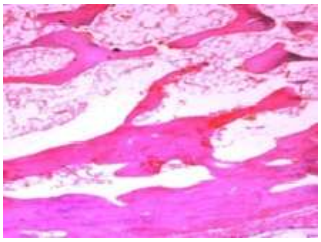

Figure 6. Formation of cortical and cancellous bone with formation of red bone marrow in more than half area off defect in chitosan group(×10, H&E).

In the present study, despite the 70 day treatment period, not much improvement was observed in the cartilage treated group although bone marrow cavities appeared. Montufar et al, 2004 placed murine cartilage tissue processed in a reactive environment, in the created defect and observed ossification. Nevertheless, whether the same results can be achieved in the long bones or not requires further investigations.[12] In another study by Pipenger et al, 2015 a proof-principle was demonstrated using nasal cartilage cells processed by collagen and growth factor. Accordingly, nasal chondrocytes have the capability to convert to bone cells and directly involved in bone formation with longer endurance for the regenerated bone, compared to reference bone cells (mesenchymal stromal cells derived from bone marrow) so that they can serve as an easily available source of cells for head and face bone reconstruction.[13] Bardsley et al, 2016 developed hypertonic cartilage grafts under lab condition by embedding rat nasal cartilage cells in poly-glycolic acid scaffold and processing by increased expression of collagen genes. As reported, they observed the conversion of the cartilage graft to bone once it was placed in the defect made in the skulls of the rats. To produce hypertonic cartilage cells, they initially investigated the conversion capability of sterna and nasal cartilages and followed the cell processing as stated above and once they found that nasal cartilage was superior, they used it in their study. However, they provided no explanation for the superiority of nasal cartilage to costal one.[2]

In the present study, we used costal cartilage as a transparent in repairing bone defects and as a mentioned above the results were not satisfactory enough, in agreement with Bardsley et al regarding the difference between nasal and costal cartilages. Xiaojing and colleagues in 2014, conducted an investigation into the use of Chitosan-collagen combination scaffold for the canine mandible bone defect healing and reported more new bone formation in the treatment group, compared to the controls. Although no significant difference was observed between bone-to-implant contact and new bone filled area, the new bone height at week 8, was significantly greater than that in the controls. They concluded that strengthened collagen-chitosan combination can serve as a good candidate for grafting in bone defects.[14] Parbaharan, (2016) investigated the structure of chitosan in combination with other materials such as calcium phosphate, synthetic polymers, bioglass, and hydroxyapatite and suggested that such chitosan based scaffolds could be used in bone tissue engineering and other body tissues as well. They also concluded that such scaffolds had adequate stability and mechanical strength to become more efficient in tissue regeneration.[15]

In 2016, Nivedhitala et al examined the structure of chitosan- gelatin combination scaffold and suggested that the pores within such scaffolds play a critical role in the connection, formation and infiltration of cells. According to these researchers, scaffolds made from polymers have good mechanical properties but not adequate bone conduction for bone regeneration, so ceramic particles such as calcium phosphate, hydroxyapatite, and bioglass are introduced into them to promote the conduction.[16,17] In the present study, although chitosan scaffold alone with no combination of any other material was used, the chitosan treated group was found superior to the control and cartilage groups, indicating the properties of chitosan and presence of higher porosity, compared to cartilage. Perhaps the crushing of cartilage prior to insertion into the defect and longer period of study yields better results on the use of cartilage.

Taking into account the results and other recent reports, it could be concluded that chitosan scaffolds with greater capabilities could be used in canine bone defect healing, however, for ideal bone tissue regeneration, chitosan as a base has to be combined with other materials including those mentioned above. As for cartilage use in canine bone defect healing, the present study results showed that cartilage could not serve as a proper alternative for grafting and to improve its efficiency, it is suggested that cartilage be processed beforehand and made ready for grafting.

Acknowledgements

Authors would like to say thanks for Shahrekord University financial support

Conflicts of interest

None.

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نشریه جراحی دامپزشکی ایران
سال۲۰۱۷ ، جلد ۱۲ (شماره ۱)، شماره پیاپی ۲۶

چکیده

بررسی مقایسه‌ای اثر غضروف دنده‌ای اتوژنیک با داربست کیتوزان، در ترمیم نقیصه استخوانی، در استخوان بازوی سگ

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هدف- امروزه سرعت بخشیدن به روند التیام شکستگی‌ها بسیار مورد توجه می‌باشد. زیرا با تسریع روند التیام شکستگی، محدودیت‌های حرکتی و مشکلات دوران بهبودی برای بیمار کمتر می‌شود. در این مطالعه، ما به مقایسه اثر غضروف دنده‌ای اتوژنیک با داربست کیتوزان در ترمیم نقیصه استخوانی در استخوان بازوی سگ پرداختیم.

طرح- مطالعه تجربی آینده نگر

حیوانات-۱۵ قلاده سگ نر بالغ

روش کار- سگ‌ها به ۳ گروه پنج تایی تقسیم شدند، و نقیصه استخوانی پنجره‌ای شکل در استخوان بازوی راست آنها ایجاد گردید. در گروه اول به عنوان گروه کنترل، نقیصه خالی گذاشته شد. در گروه دوم کیتوزان و در گروه سوم غضروف دنده‌ای اتوژنیک قرار داده‌شد. در هفته‌های ۲، ۴، ۶ و ۸ رادیوگراف از محل نقیصه‌ها تهیه گردید. نهایتا پس از۷۰ روز سگ‌ها آسان‌کشی شدند و مقاطع هیستوپاتولوژی از محل نقیصه‌ها تهیه گردید.

نتایج- براساس نتایج مشخص شد که استفاده از غضروف دنده‌ای به تنهایی ضعیف‌تر از گروه درمانی با کیتوزان و کنترل عمل کرده‌است بنابر این استفاده از غضروف دنده‌ای به تنهایی نمی‌تواند به عنوان گزینه خوبی برای پیوند در نقیصه استخوانی سگ عمل کند.

نتیجه گیری و کاربرد بالینی- با توجه به نتایج این مطالعه و مطالعات اخیر کیتوزان می‌تواند به عنوان یک داربست با قابلیت‌های بالا در نقیصه‌های استخوانی سگ عمل کند ولی به تنهایی نمی تواند شرایط ایده آل را برای بازسازی بافت استخوان فراهم سازد. لذا باید به عنوان پایه در ترکیب با سایر مواد استفاده شود. در خصوص غضروف نیز مطالعه ما نشان داد که استفاده از غضروف به تنهایی ، نمی‌تواند به عنوان گزینه خوبی برای پیوند در نقیصه استخوانی سگ عمل کند.

کلمات کلیدی- غضروف دنده‌ای اتوژنیک، داربست کیتوزان، نقیصه استخوانی، سگ