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# A survey on osteogenic effect of collagen-membrane derived from *Rutilus kutum* swim bladder in rat calvaria

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## ABSTRACT

**Background:** The collagen membrane which obtained from bovine pericardium and human skin in Guided Bone Regeneration (GBR) is costly and may even cause transmission of diseases. Replacing conventional collagen membranes with a more easily accessible and cheaper ones will have economic benefits. The aim was to determine the osteogenic effect of collagen-membrane derived from *Rutilus kutum* swim bladder on rat calvaria.

**Materials and Methods:** The study was experimental. Thirty-six male albino rats of the Wistar strain were included in the study. The 5 mm surgical defects were created on calvarias and filled with allograft bone material and covered by *R. kutum* swim bladder (Group I), bovine derived pericardial membrane (Group II) and without membrane cover (Group III). The specimen were euthanized after 3, 5 and 8 weeks. The surrounding connective tissue was evaluated in term of osseous formation. Kruskal–Wallis, Univariant analysis of variance, and *post hoc* tests were used for statistical analysis. The $P < 0.05$ was considered statistically significant.

**Results:** A significant differences between groups in terms of osseous formation ($P = 0.001$) was noted. The difference of osseous formation was significantly higher in 5 and 8 weeks than 3 weeks after operation in all groups ($P = 0.03$ and $P = 0.006$, respectively). The osseous formation in Group I and II were significantly higher than Group III ($P = 0.023$ and $P = 0.001$).

**Conclusion:** The *R. kutum* swim bladder had osteogenic effect on rat calvaria. *R. kutum* swim bladder can be a new source in natural derived collagen membrane in GBR.

**Key Words:** Bone formation, bone regeneration, guided tissue, osteogenesis, regeneration

## INTRODUCTION

Regeneration of damaged tissues and maintain the function are major goals of Guided Bone Regeneration (GBR) and Guided Tissue Regeneration (GTR). Membranes in a GTR system work as a barrier and prevent the invasion of nonosteogenic cells into the defect and create a route to guide the bone regeneration. Absorbable collagen membranes have the best biocompatibility with soft and hard tissues, exert the chemotactic effects and activate the gingival fibroblasts. Applicable properties such as higher tendency of osteoblasts in link to collagen, provide collagen as a good membrane for GTR.[1,2] Hemostasis, low immunogenicity, semi permeability, and augmentation of tissue thickness are other advantages of collagen membranes.[3,4]

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The main sources of nonsynthetic resorbable collagen membranes are tendon, dermis, and pericardium of bovine and pig. Despite the good bio-compatibility, disease transmission, and rapid degradation are disadvantages of commercial available, nonsynthetic derived collagen membranes.[5,6] Besides, derived collagen membrane from organs such as bovine heart and human skin is difficult and expensive. A natural, cost-effective substitute has economic and therapeutic importance.

The fish swim bladder is a gas-filled organ covers by tunica extra and tunica intra. The internal layer composes of vascular connective tissue. The outer layer is a febrile, nonmuscular connective tissue. The connective tissue of swim bladder structured from collagen Type II. *Rutilus Kutum* (*Caspian kutum*) is a member of the *Cyprinidae* family. Simple availability, cheaper price of fish compared to cow and easier accessibility to collagen of swim bladder are important factors to consider the fish as an alternative source to drive collagen membrane. The aim was to survey on osteogenic effect of collagen-membrane derived from *R. kutum* swim bladder in rat calvaria.

## MATERIALS AND METHODS

The study was experimental. The study was approved by the ethical committee of Shahed University for animal management and surgery under registration number IR.Shahed. REC.1394.145.

### Animals

Thirty-six albino rats of the Wistar strain (age: 8 weeks, weight: 200 g) were entered the study and divided into three groups (12 rats in each group).

### The fish swim bladder preparation

The fish swim bladder was removed in sterile condition. All pieces of febrile fragments of swim bladder were sterile with 25 KGy of Gamma ray. The swim bladder was cut into pieces with 1 cm width, 1 cm length and 0.5 mm thickness. The febrile fragments were immersed and kept in ethyl alcohol 70% and 0.2 ml gentamicin for 1 week.[7] After 1 week, the pieces were placed in distilled water for 1 h and were incubated in 37°C for 2 h. The pieces were kept in sterile container and placed in refrigerator at 7°C.

### Membranes examined

The bovine pericardium (Cenomembrane. Kish Co, Iran) 1–1 cm with 6–9 mm thickness and bone powder with a mixture of 1–1 ratio of mineralized and demineralized allograft (DBM and MBA Powder – 125-840 um, cenobone FDBA, 500–1000 μ, cortico cancellous powder-kish Co, IRAN) was used.

Rats were divided into three groups; there was 12 mice in each group:

- Group I: Defects were filled with cenobone and covered with fish swim bladder
- Group II: Defects were filled with cenobone and covered with cenomembrane (First control group)
- Group III: Defects were filled with cenobone (Second control group).

### Surgical procedure

The rates were anesthetized by intraperitoneal injection of 2 mg/kg ketamine10% (Alfasan Co, Holland) and 2 mg/kg xylazine 2% (Alfasan Co, Holland) in a ratio of 1–3. Parietal area was shaved and disinfected with Povidone Iodine (Daru Darman Co, Iran). The area was anesthetized by injecting 4 cc of 2% lidocaine. A longitudinal 2 cm incision was made in midsagittal plane of cranium with scalpel No. 15. The periosteal layer was separated completely. Skin and periosteum full thickness flap was elevated and 5 mm bicortical critical sized defect was created by 5 mm Trephine bur drill (Salvin Co, Canada) under cold normal saline irrigation.[8,9] The membranes were randomly allocated and rats were coded. The wound was closed using horizontal mattress 4.0 resorbable sutures (Supa Co, Iran) [Figure 1]. In each group, coded rats were randomly categorized to euthanize in 3, 5, and 8 weeks after operation.

Rates were kept warm until conciseness. The animals were received three injected of Keflin (Daroupakhsh co, Iran) every 8 h for 1st day. Rates were euthanized using ketamine10% (Darupakhsh Co, Iran) and 2 mg/kg xylazine 2% (Alfasan Co, Holland) 3, 5, and 8 weeks' postoperation. The bony defects were created with 5 mm diameter trephine drill (DiaTessin Co, Switzerland). All rates were kept in standard situation and were fed with laboratory food pellets and water during the study based on ARRIVE guidelines.[10]

### Histomorphometry

Obtained samples were immediately fixed in formalin 10% and then decalcified with nitric acid 70%. Four mm thickness slides were stained with hematoxilin-eosin stain. All histopathologic samples were coded by a laboratory technician who was

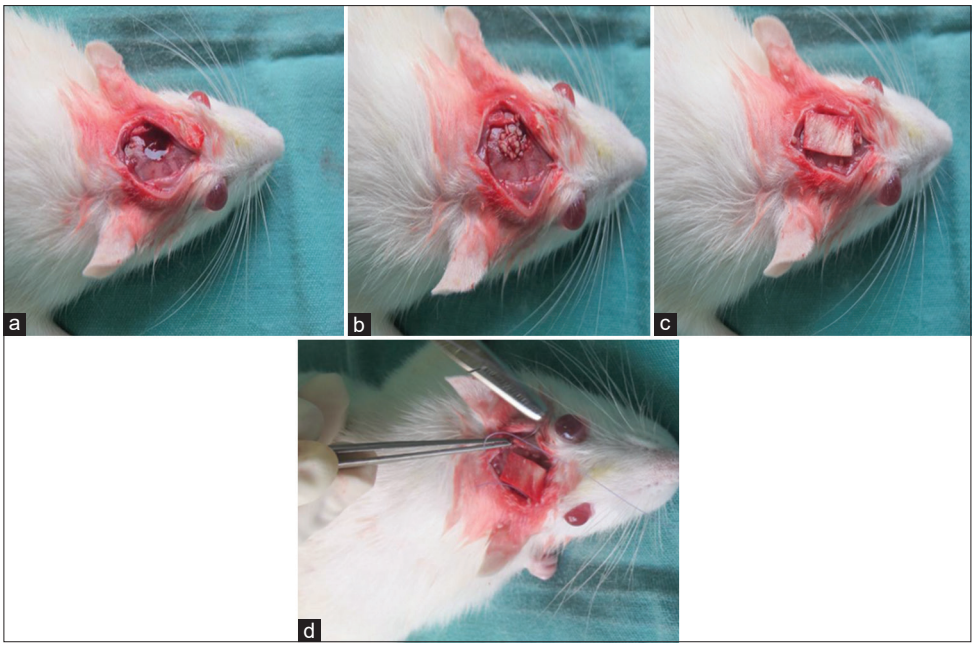


**Figure 1:** (a) Five mm defect created on calvaria of rat. (b) The bony defect was filled with bone powder. (c) Defect covered with fish swim bladder membrane (Group III). (d) The wound was closed using horizontal mattress sutures.

unaware of groups. The amount of new osseous formation was assessed quantitatively. A light microscope (Euromex Bioblue.lab Range; Holland) equipped with a digital camera (CMEX5 Camera; Holland) was used. The samples were studied under ×400 magnification in blind performance by one calibrated person under supervision of an experienced pathologist who were unaware of sample classifications. Newly woven/osteoid formed particles were assessed as (area of newly formed bone/area of the original defect) ×100.[11]

The amount of new formed osseous particles [Figure 2] were calculated in 3, 5 and 8 weeks' postoperation. Granulation formation, necrosis, fibrosis, and inflammation were descriptively evaluated. Inflammation scored as -: No inflammatory infiltration, + : Few chronic and acute inflammatory cells, ++ : Scattered inflammation, +++ : Focal inflammation. Fibrosis formation described as+ : <1/3 of slide surface, ++ : <2/3 of slide surface, +++ : More than 2/3 of slide surface. Necrosis scored as+ : <1/3 of slide surface, ++ : <2/3 of slide surface, +++ : More than 2/3 of slide surface. Granulation tissue scoring was evaluated as 0: Without granulation tissue, 1: Granulation tissue formation. Foreign body reaction was evaluated by the presence of multinucleated giant cells under the term of granulation tissue.[12,13]

### Statistical analysis

The Chi-square and Kruskal–Wallis tests were used to compare the osseous formation. The osseous formation in different weeks was assessed with Univariant analysis of variance. The statistical analysis was completed using SPSS (Version 22; IBM Company, Chicago, IL, USA). The $P<0.05$ was considered statistically significant.

## RESULTS

### Three weeks' postoperation

In Group I, focal, dense inflammatory cells infiltration was noted in histologic sections. Fibrotic tissue, necrosis, and granulation formation were not observed. The bone defect was packed with irregular-shaped of immature bone.

In Group II, the grade of inflammation was + in two cases and negative in two. Necrosis, granulation tissue and fibrosis were absent.

In Group III, the grade of inflammatory infiltration was – in three cases and + in one. Fibrotic tissue was noted in 2 cases and formation of granulation tissue in 1.

### Five weeks' postoperation

In Group I, inflammatory infiltration had chronic nature with score +. In one case, inflammatory infiltration was ++. Necrosis, granulation tissue, and fibrosis were absent.

In Group II, inflammatory infiltration was negative in three cases and + in one case. Necrosis, granulation tissue, and fibrosis were not noted.

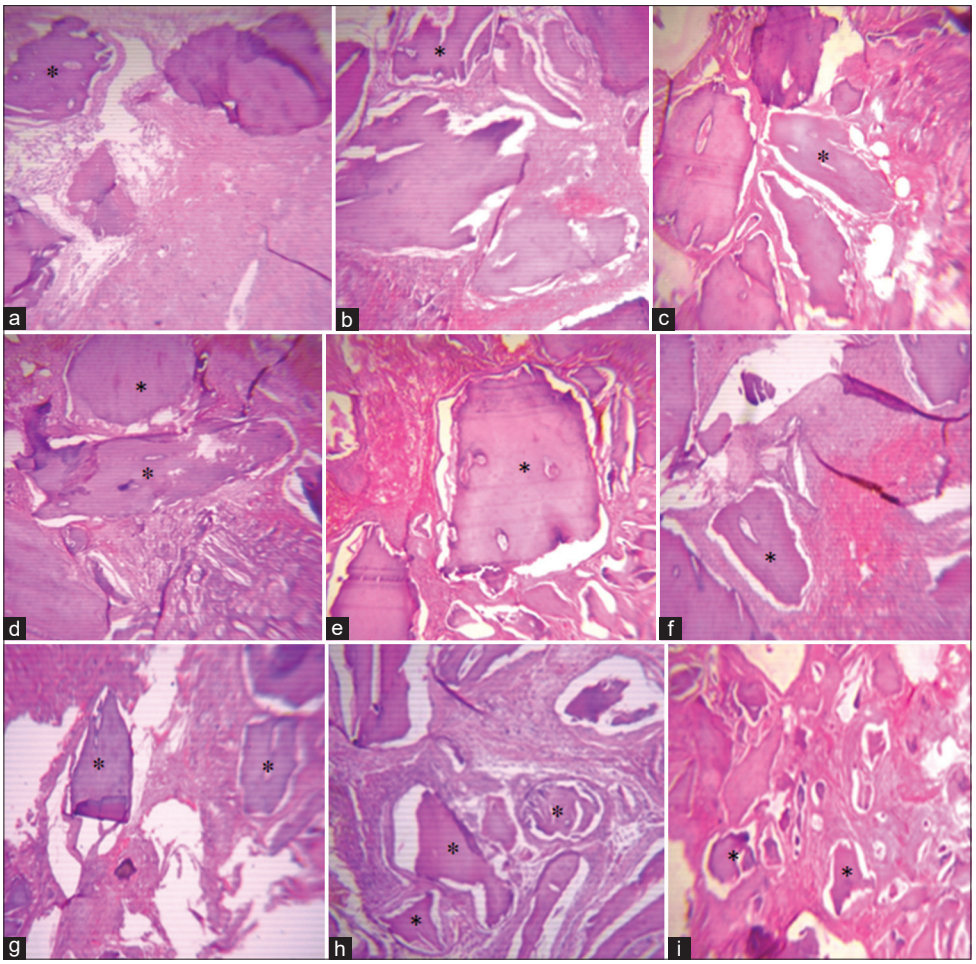


**Figure 2:** The histologic features of woven bone formation in 3, 5, and 8 weeks' postoperation. The newly formed bone marked. (a): Group I in 3 weeks, (b) Group I in 5 weeks, (c) Group I in 8 weeks. (d) Group II in 3 weeks, (e) Group II in 5 weeks, (f) Group II in 8 weeks. (g) Group III in 3 weeks, (h) Group III in 5 weeks, (i) Group III in 8 weeks (Hematoxillin Eosin staining, ×100 magnification).

In Group III, necrosis and granulation tissue were absent. The inflammatory infiltration was negative in three cases and + in one case. Fibrosis formation was noted in two cases.

### Eight weeks' postoperation

In Group I, No inflammatory infiltration was noted in 3 cases. The inflammatory infiltration was + in one case. Necrosis, granulation tissue, and fibrosis were not noted.

In Group II, the formation of necrosis, granulation tissue and fibrosis were negative. The inflammatory infiltration was negative in two cases and + in two other cases.

In Group III, slight inflammation was still present in one specimen. Inflammatory infiltration was negative in other cases. Fibrosis formation was noted in two cases. Necrosis and granulation tissue were not noted.

In 3, 5, and 8 weeks' postoperation bone defect was filled with numerous small to large pieces of newly formed particles of woven bone in all groups. Figure 3 shows the percentage of osteogenesis in three groups in different weeks.

The Univariant analysis of variance revealed a significant differences between groups in terms of osseous formation ($P = 0.001$).The *post hoc* test indicated that the difference of osseous formation was significantly higher in 5 and 8 weeks than 3 weeks after operation in all groups ($P = 0.03$ and $P = 0.006$, respectively). In all times, the osseous formation in Group I and II was not significantly different ($P = 0.3$). The osseous formation in Group I and II were significantly higher than Group III ($P = 0.023$ and $P = 0.001$).

The Kruskal–Wallis test revealed that the inflammatory infiltration was significantly higher in Group I than in II and III in 3 weeks'

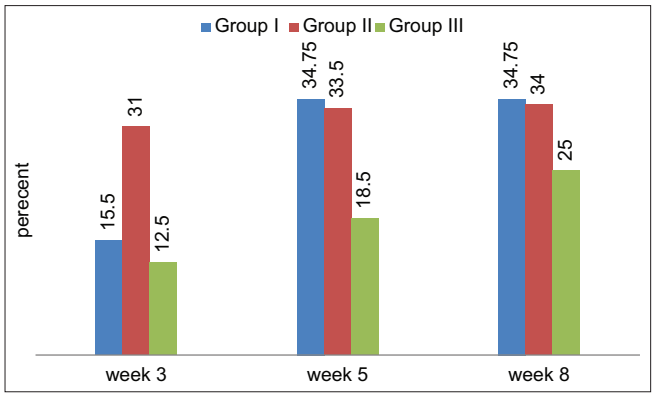


**Figure 3:** Percent of osteogenesis of three groups in different weeks.

postoperation ($P$ = 0.01). After 5 weeks in Group I, the inflammatory infiltration was significantly higher than II and III groups ($P$ = 0.04). The inflammatory infiltration was not significant between three groups after 8 weeks ($P$ = 1).

## DISCUSSION

The results show that osseous formation of *R. kutum* fish swim bladder was not significantly different from bovine derived pericardial membrane. The finding showed that the *R. kutum* fish swim bladder membrane could be a potential substitute for bovine derived pericardial collagen membrane.

Collagen membranes have desirable chemical and physical proprieties in GTR.[14,15] Collagen stimulates clot stability and enhances regeneration. Chemotaxis and bioresorption are other appropriate properties of collagen membranes.[16] Gielkens *et al*. reported that there was no significant difference in the use of Poly (DL-lactide-epsilon-caprolactone), collagen and expanded polytetrafluoroethylene membranes in cover the grafts.[17]

Bovine-derived collagen membranes has an appropriate biocompatibility and do not provoke antibody response.[16] In spite of this, difficult technology due to using massive animals and cost-effective outcomes are concerning issues in natural derived membrane production from animals. The present idea was to determine the osteogenic effect of collagen-membrane derived from *R. kutum* fish swim bladder as an available substitute for bovine.

The inflammation had no significant difference between the *R. kutum* swim bladder derived collagen membrane, bovine derived pericardial membrane and control groups in 8 weeks postoperation. The inflammatory cells infiltration was significantly decreased from 5 to 8 weeks postoperation in *R. kutum* fish swim bladder derived collagen membrane group. It was compatible with degree of inflammation in bovine derived pericardial membrane and control group. The finding suggests proper biocompatibility of *R. kutum* fish swim bladder derived collagen membrane. This is in agreement to Unsal *et al*. who concluded an examination on collagen membrane derived from different structure including bovine collagen, fascia temporalis and dura mater.[18] The finding, consistent with the present study, suggests that the other collagen sources may be a viable alternative to existing collagen membranes.

The osseous formation in three groups was increased over time. The rate of newly formed particles of bone in the 3rd, 5th and 8th weeks after surgery were significantly higher in groups with *R. kutum* fish-derived collagen membrane and bovine-derived pericardial membrane in compare to specimens without membrane. The finding is consistent with previous studies that showed the use of collagen membranes had significant effect on bone regeneration.[19]

The study demonstrates that the *R. kutum* fish swim bladder had an osteogenic effect. Based on results, it can be a natural alternative in production of nonsynthetic membranes. The study did not face any particular limitations, but easy access to the collagen tissue was the strength point for research. The study was an experimental evaluation, clinical studies along with three dimensional histologic measurements of newly made bony particles suggested for further studies.

## CONCLUSION

*R. kutum* fish swim bladder had osteogenic effect in bone defects. The *R. kutum* fish swim bladder can be introduced as a collagen membrane in GBR. This finding is also of economic importance because it is possible to use the swim bladder of dead fishes in a recycling process.

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### Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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