Expression of FGF2 and FGF2R on fibroblasts proliferation in wound healing after chitosan and pericardium application

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Abstract

In the case of surgery, it is necessary to apply a membrane barrier to provide an opportunity for the healing process for bone defects, it is because soft tissue cells migrate and develop faster than hard tissue cells. These conditions affect in the healing process of bone defects being disrupted because soft tissue that proliferates more quickly can fill the cavity of the bone defect. The membrane to be used must be biocompatible, able to regenerate bone, easy clinical application and should integrate with surrounding tissues. Thus, chitosan Is preferred as an alternative candidate for membrane barrier.

Objectives to examine the expression of fibroblasts growth factor-2 (FGF2) and fibroblasts growth factor-2 receptor (FGF2R) to determine the proliferation of fibroblasts after the installation of the chitosan barrier membrane.

Wistar rats were randomly into Six groups: the control group was sub into 3 smaller group based on days which is 7, 14 and 28 days same as test group; Wound was created on both side on soft tissue and hard tissue and Membrane was placed at the wound as GTR. The wistar rats were taken down on day 7, 14 and 28 days. The number of fibroblasts based on FGF2 and FGF2R expression were examined. Data was analysed using independent sample t-test ($p<0.05$).

The expression of FGF2 and FGF2R in the application of chitosan membrane application was greater than pericardium membrane on days 7, 14 and 28 days.

Chitosan membrane showed greater proliferation of fibroblasts in comparison to pericardium membrane.

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Introduction

Guided Tissue Regeneration (GTR) is a method of tissue regeneration. GTR can be either a bone graft or a barrier membrane. Barrier membranes can be used to temporarily separate soft and hard tissues.[1,2] In the case of surgery, it is necessary to apply a membrane barrier to provide an opportunity for the healing process for bone defects and to increase the wound healing process in soft tissues. The healing process in soft tissue is different from hard tissue, in soft tissue the cells migrate and develop faster than hard tissue cells.[3] This results in the healing process of bone defects being disrupted because soft tissue that proliferates more quickly can fill the cavity of the bone defect, therefore a membrane barrier application is needed with the aim of isolating hard tissue and soft tissue areas.

The criteria for the membrane barrier selection having biocompatibility, being able to regenerate bone, easy clinical application and being able to integrate with surrounding tissues.[4,5] Membrane barrier consists of several types, based on their degradation ability, they are divided into two, namely resorbable and non-resorbable. Based on the basic material, the membrane is divided into two, namely membranes from natural polymers and composite polymers. Natural polymer

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membranes can be derived from animal collagen, animal pericardium tissue, chitosan, gelatin and Silk Fibroin (SF). The membrane barrier circulating in Indonesia is dominated by membranes derived from animals, namely the pericardium membrane.[5,6]

Pericardium membrane using bovine pericardium tissue is a regenerative material that is widely used in Indonesia. This could be attributed to the majority of the Indonesian population is Muslim who does not use a membrane barrier derived from pigs. The pericardium membrane has drawbacks, namely the presence of chemicals contained as cross-linking agents in the membrane manufacturing process that can be toxic, as well as the risk of disease transmission between animals and humans.[7,8,9] Based on these limitations, it is necessary to develop alternative membrane barrier materials.

Utilization of shrimp produces shrimp shell by-products and chitosan is a deacetylation product from shrimp shell. Chitosan as a derivative compound has been shown to increase the release of inflammatory mediators such as PMNs and macrophages that play a role in the process of tissue regeneration.[10] Chitosan has been reported to be proven as a wound dressing for the regeneration of the soft tissue of the back in experimental rats.[11] The use of chitosan needs to be developed, especially in tissue engineering. Chitosan as a membrane barrier is still unclear.[11]

The healing process consists of three phases, namely the inflammatory phase, the proliferative phase and the remodeling phase. In the inflammatory phase, macrophages migrate to the wound area as a form of body defense. Growth factor produced by macrophages is an important component in the healing process and the formation of new tissue. One of the components of Growth factor that plays a role in the healing process is Fibroblast Growth Factor (FGF). In humans there are 10 types of FGF, FGF1 to FGF2 that play a role in the healing process. FGF2 is an important component in the healing process and is known as basic fibroblast growth factor and FGF2R, which is the receptor of FGF2 is a growth factor and signaling protein, which is encoded by the FGF2R gene.[12,13]

Therefore, it is necessary to examine the expression of FGF2 and FGF2R to determine the proliferation of fibroblasts after the installation of the chitosan barrier membrane.

Materials and methods

Research Samples

The study design used a posttest only control group design and the type of research was a laboratory experimental using experimental animals as research objects and was done at the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. The sample that would be used in this research is fourty two male Wistar rats. Criteria of the samples are male wistar weight around 250-300 grams and minimum of age is four months.

Research Methods

Subjects were divided into six groups, group 1 consist of 7 rats for the gamacha bonegraft insertion group, chitosan membrane on the right jaw at histopathological examination on the 7th day. Group 2 consist 7 rats for the gamacha bonegraft insertion group, pericardium membrane on the left jaw during histopathological examination on day. Group 3 consist of 7 rats for the gamacha bonegraft insertion group, chitosan membrane on the right jaw at histopathological examination on day 14. Group 4 consist of 7 rats for the gamacha bonegraft insertion group, pericardium membrane (BATAN) on the left jaw during histopathological examination on day 14. Group 5 consist of 7 rats for the gamacha bonegraft insertion group, chitosan membrane on the right jaw at histopathological examination on day 28. Group 6 consist of 7 rats for the gamacha bonegraft insertion group, pericardium membrane (BATAN) on the left jaw during histopathological examination on day 28.

The action was carried out by disinfecting the equipment to be used first using 70% alcohol, the treatment group was anesthetized intramuscularly with Ketamine (Ketalar®, Wamer Lambert, Ireland) (65 mg/kg body weight) and xylazine HCL (Rompun®, Bayer, Leverkusen, Germany) (7 mg/kg body weight) dissolved in sterile phosphate buffered saline (PBS). The maxillary gingival surfaces of rats were disinfected with sterile pellets previously dipped in a solution of povidone iodine (betadine). An incision was made at the apex of the maxillary gingival ridge between the second incisor and the first molar of the rat with a length of 6 mm, blunt

dissection using a blunt-tipped bent hemostat until the bone was visible. Next, the bone was burried with a diamond round bur measuring (0.084 mm in diameter) ± 2 mm deep, the space that was created was filled with gamacha® bonegraft which was then covered with chitosan membrane, on the right side a 0.2 mm thick chitosan membrane 3 mm in diameter was installed. above the bonegraft that has been given and on the other side a pericardial membrane is attached as a positive control. Then the wound was sutured with absorbable Vicryl 5.0 suture. The same treatment was carried out on the contralateral side except for the use of a membrane using a pericardial membrane as a control group. Suturing was performed on the wound area using Vicryl absorbable 5.0. Mice were returned to the cage after tagging and given the antibiotic Ampicillin (PT Meiji Indonesia, Vicillin®) a combination of Enrofloxacin (De Adelaar B.V., Interflox®) once a day for three days after treatment. Furthermore, they are reared by being given standard feed and drinking ad libittium. Mice from each group were subjected to necropsy and jaw decapitation according to the specified treatment time (7th day, 14th day and 28th day). Histopathological and immunohistochemical analyzes were performed on days 7, 14 and 28

Statistical analysis

The obtained data tested using Kolmogorov Smirnov sample normality. Then the Levene's Test homogeneity test was carried out to find out whether the sample groups had the same variation or not. Furthermore, the one-way ANOVA difference test was carried out to determine the mean difference between each group of variables with the significancy 0.05.

Ethical policy and institutional review board statement: Certificate ethic number 251/HRECC.FODM/V/2020.

Results

To determine the characteristics of fibroblast cells, a histopathological examination was performed using a microscope with a magnification of up to 1000x (shown by figure 1). Fibroblasts in bone are round in shape with a large nucleus.

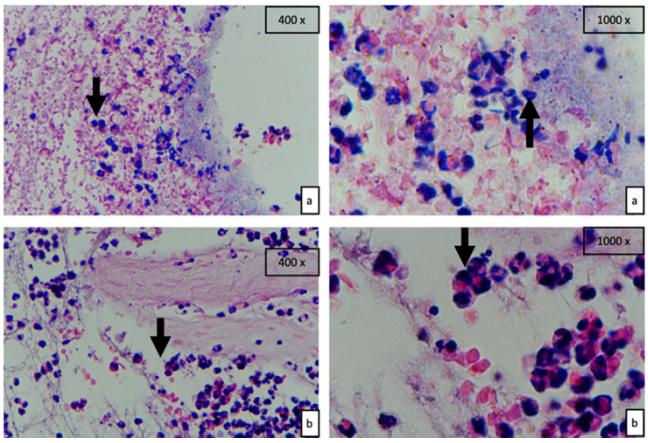

Figure 1. HPA overview of fibroblast cells in Wistar rat bone tissue preparations with 400x and 1000x magnification (Black arrows indicate fibroblast cells). a. pericardium b. chitosan.

Immunohistochemical examination of FGF2 expression

The effect of chitosan and pericardium membrane administration on FGF2 expression was evaluated by immunohistochemical examination to determine the distribution of fibroblast cells expressing FGF2 with calculations at 1000x magnification. The black arrow in the figure 2 until figure 4 indicates FGF2 expression.

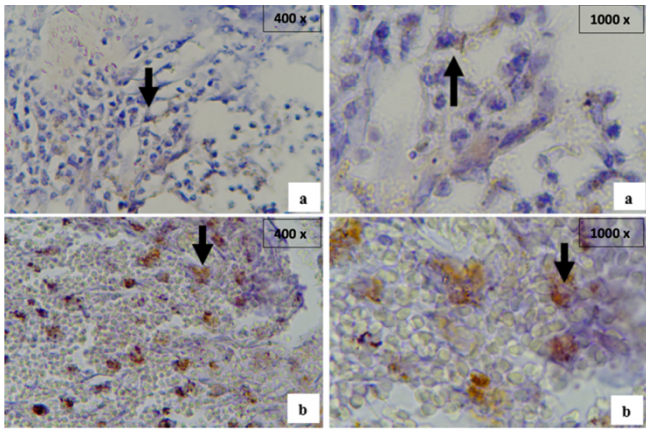

Figure 2. Fibroblast through FGF2 on day seven. A. pericardium, b. chitosan.

Immunohistochemical examination of FGF2R expression

The effect of chitosan and pericardium membrane administration on FGF2r expression was evaluated by immunohistochemical examination to determine the distribution of fibroblast cells expressing FGF2r with calculations at 1000x magnification. The black arrow in the figure 5 until figure 7 indicates the expression of FGF2r.

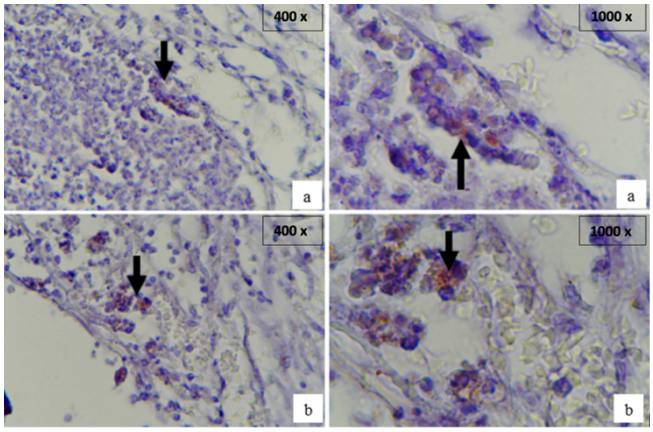

Figure 3. Fibroblast through FGF2 on day fourteen. A. pericardium, b. chitosan.

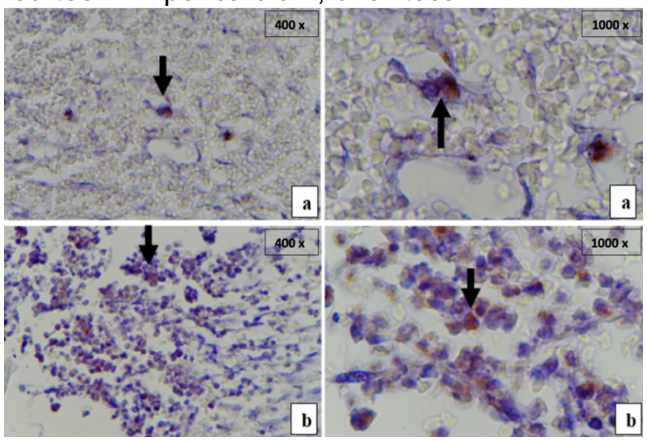

Figure 4. Fibroblast through FGF2 on day twenty-eight. A. pericardium, b. chitosan.

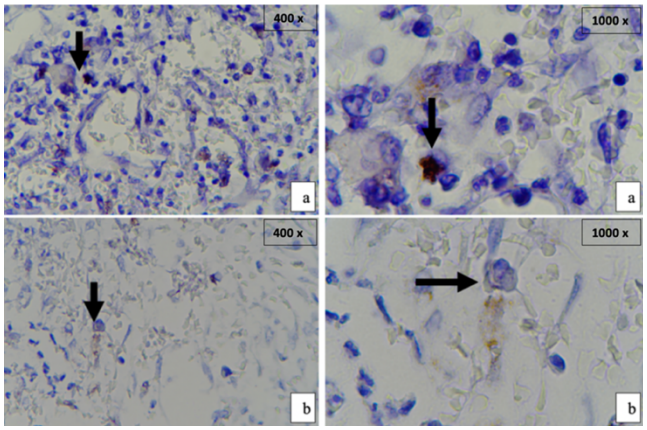

Figure 5. Fibroblast through FGF2r on day seven. A. pericardium, b. chitosan.

Observation of the number of fibroblast cells expressed by FGF2 and FGF2R shown by figure 11 and figure 12.

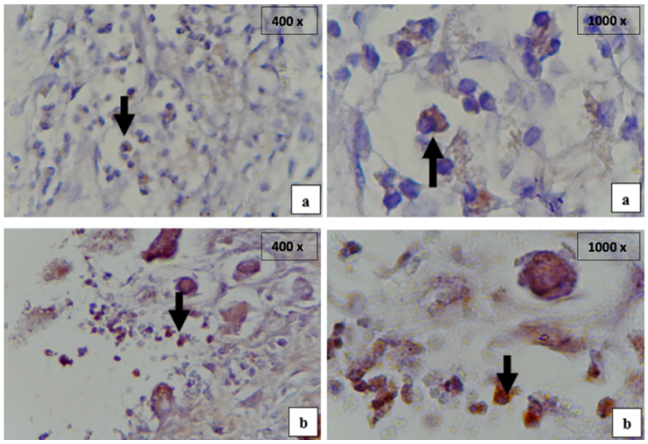

Figure 6. Fibroblast through FGF2r on day fourteen. A. pericardium, b. chitosan.

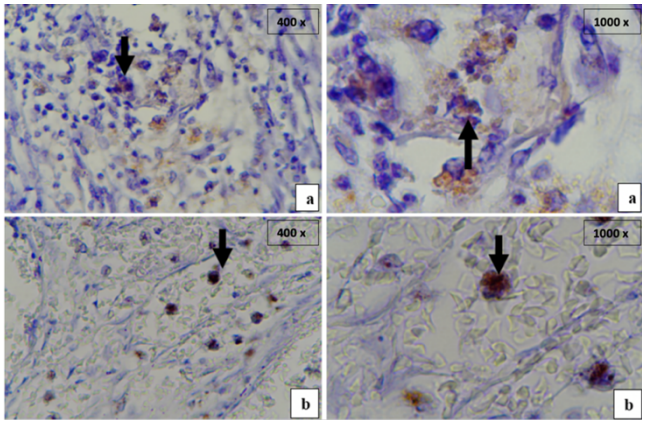

Figure 7. Fibroblast through FGF2r on day twenty-eight. A. pericardium, b. chitosan.

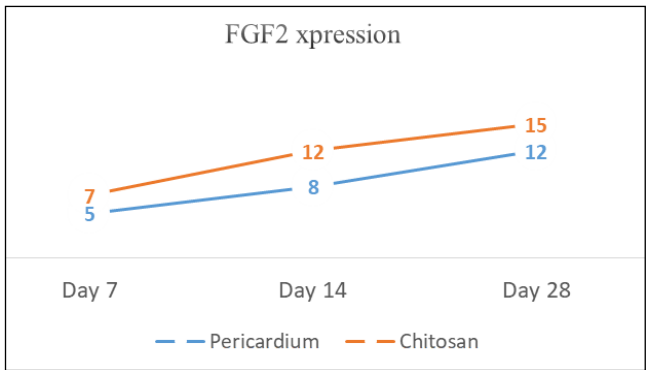

Figure 11. Mean chitosan and pericardium by FGF2 expression.

Table 1 showed statistical test of FGF2 on day seven, fourteen and twenty-eight show p of 0.01, 0.009, 0.04 ($p < 0.05$), which means there are significant differences in the whole group of FGF2. FGF2R on day seven, fourteen and twenty-eight, obtained p of 0.002, 0.003 and 0.002 ($p < 0.05$).

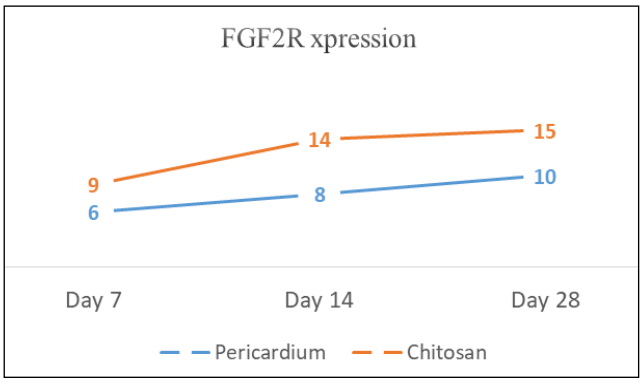

Figure 12. Mean pericardium and chitosan by FGF2R expression.

	Group	Mean ± SD	P value
Day seven	FGF2 expression in pericardium[a]	5.83 ± 1,169	0.01
	FGF2 expression ion chitosan[b]	7.67 ± 0,816	
	FGFR2 expression in pericardium[c]	6 ± 1,414	0.002
	FGFR2 expression in chitosan[d]	9.67 ± 1,633	
Day fourteen	FGF2 expression in pericardium[a]	8.33 ±1,966	0.009
	FGF2 expression ion chitosan[b]	12.17 ± 2,137	
	FGFR2 expression in pericardium[c]	8 ± 1,414	0.003
	FGFR2 expression in chitosan[d]	12.33 ± 2,160	
Day twenty-eight	FGF2 expression in pericardium[a]	12.67 ± 1,862	0.041
	FGF2 expression ion chitosan[b]	15.33 ± 2,066	
	FGFR2 expression in pericardium[c]	10 ± 1,789	0.002
	FGFR2 expression in chitosan[d]	14.50 ± 1,871	

Table 1. Mean, standard deviation (SD), and p value of FGF2-FGFR2 expression of the pericardium and chitosan. The different superscript letters are statistically different (ANOVA, $P < 0.05$).

Discussion

The pericardium used as a comparison in this study has been used as a regenerative material for a long time, but there are some disadvantages of using pericardium that can be overcome using chitosan such as too fast degradation, the commonly used cross-link technique will prolong degradation times of up to six months,[14] alter membrane properties and animal disease transmission.[9]

In the process of wound healing and remodeling, there are several important phases including hemostasis, inflammation, proliferation and remodeling, the peak of cell proliferation begins 2 or 3 days after trauma and continues for 3 to 4 weeks. This event is characterized by the presence of fibroblasts in the wound area and encounters an inflammatory process. Fibroblasts peak between days seven and fourteen.[15] This study will compare the number of fibroblast cells through the expression of FGF2 and FGF2R between administration of chitosan membrane and pericardium membrane. Examination of the number of fibroblasts themselves would be carried out on days seven, fourteen and twenty-eight after postoperative membrane installation in the maxillary alveolar bone of rats. Each group will be treated equally and totaling 7 samples per group.

Clinically, wound healing in chitosan group rats did not show signs of inflammation in all day groups. As shown by Caetano's study, in comparing the progress of wound healing, it was found that on day 2 there was no significant difference between the group using the chitosan membrane and the control group (saline). Differences began to appear on day seven. On day fourteen both groups had completely healed, although the chitosan group showed better quality wound tissue.[16] The epidermis partially closed the wound on day four and completely on day fourteen.[17] Research on dressings using chitosan as an ingredient showed that wound healing began between days seven and fourteen, when the control group had not closed the wound.[18] In in vivo analysis, the asymmetric membrane helped complete wound closure in 9 days, whereas in the control group this was achieved in 11 days.[19] Other studies that added other components such as silver nanoparticles[17] or nano zinc oxide (nZnO)[18] also showed a significant degree of wound healing.

The amount of FGF2 expression on day seven was 7 and jumped high to day fourteen to 12 and 15 on day twenty-eight, the pattern of a significant increase on day fourteen also occurred in the FGF2r group. This is in line with a study comparing the effect of chitosan membrane on mandibular defects of rats showing the number of fibroblasts began to increase on days three and seven and on day fourteen was the peak of fibroblast numbers.[20]

In this study, in general, the number of fibroblasts expressed with FGF2 and FGF2R in chitosan was higher than in the pericardium group on the initial day (day seven). This statement is in line with previous studies which stated that fibroplasia was already high on days 7-14 and 21 with quite high differentiation[16] while pericardium-based membranes showed that fibroblasts had begun to be detected 10 days after placement around the membrane.[21] Another study conducted in 2014 showed the number of fibroblasts on the day seven after the application of the chitosan-based membrane was higher than in the control group's wounds until the day fourteen.[16]

This study showed that the number of fibroblasts continued to increase but began to decrease in number on day twenty-eight in both the FGF2 and FGF2R expression groups. This is also as shown in a previous study which showed that fibroblasts began to decrease at day twenty one but were still higher than the control group (not given a membrane).[16,20] The same trend also occurred in the pericardium group with FGF2R expression, a drastic increase in the number of fibroblasts from day seven and peaked on day fourteen but began to decrease on day twenty eight, this event is also the same as the study conducted by Al Falahi which showed the proliferation of fibroblasts with pericardium membranes. On day seven is high, day fourteen is high, day twenty-one is low, day twenty-eight is low.[22] What is interesting in this study, the opposite occurred in the pericardium FGF2 expression on day twenty eight, which peaked and experienced a significant increase compared to day fourteen with an increase of 5 points.

Fibroblasts play an important role in wound healing and tissue repair and the process of new bone formation, and fibroblasts in this study were used as indicators expressed by FGF2 and FG2R. Overall, chitosan has a higher number of fibroblasts than the pericardium and is indicated to help further bone formation. This is in line with research which showed that the formation of new bone in the group using the chitosan membrane was significantly higher than in the control group at four weeks[5,23], whereas with the new pericardium it appears after twelve weeks new points of new bone formation, after 24 weeks, the defect is completely covered with new bone.[24]

FGF2 plays an important role in bone regeneration and the amount of FGF2 alone determines the rate of new bone formation.[25] Previous studies have shown that FGF2 is a potent mitogen of Mesenchymal stem cells (MSC), also incubation of MSCs with FGF2 maintains the differentiation potential of MSCs.[26] Recent research by Chang showed that fibroblasts can be directly converted into osteoblasts.[27] Recent studies have shown that chitosan-based hydrogels increase graft size and cell retention, helping MSCs to differentiate.[28] The addition of FGF2 in the pericardium membrane showed an increase in the number of MSCs over time, on day three the number of MSCs was 1.5 times more than the control group. On day seven, the ratio of the number of MSCs in the pericardium FGF2 group increased to 2:1 compared to the control group.[29]

Furthermore, FGF2 has been shown to stimulate Human bone marrow stem cells (HBMSC) derived from MSC proliferation.[30] A recent study revealed that if the chitosan membrane was given additional aspirin, an additional 50 mg showed more beneficial properties to promote the development of bone marrow stem cells (BMSCs). The same study showed the addition of aspirin to the manufacture of chitosan membranes which have shown biocompatibility and osteogenic potential.[31] In another study, BMSCs were similar in the first 5 days but faster in the acellular porcine pericardium group than in the collagen group (control). On day seven, neat proliferation was more visible in the treatment group (acellular porcine pericardium), while more thin cells were seen in the BG (bioglide) group.[32]

Conclusions

Based on the results of experimental laboratory research that has been carried out, it can be concluded the number of fibroblast cells through the expression of FGF2 on the administration of chitosan membrane was more than the pericardium membrane and the number of fibroblast cells through the expression of FGF2r on the administration of chitosan membrane was more than the pericardium membrane.

Declaration of Interest

The authors report no conflict of interest.

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