Original Article

Title

A novel method of tracheal anastomosis healing using a single submucosal injection of basic fibroblast growth factor: Initial report.

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Visual abstract

Key question

Does Basic fibroblast growth factor (bFGF) injection promote cartilage regeneration at the tracheal anastomosis?

Key findings

Single intratracheal injection of bFGF accelerated tracheal cartilage regeneration and strengthened the anastomosis.

Take-home message

Single injection of bFGF can be implemented with a simple procedure to reinforce anastomosis and has the potential to be widely used.

Abstract

Objectives

For the technical management of tracheal anastomosis, developing new and simple methods is required to relieve anastomotic tension. This study aimed to investigate whether basic fibroblast growth factor (bFGF) only once injected immediately before anastomosis promotes cartilage regeneration at the tracheal anastomosis and whether the regenerated cartilage has the effect of reinforcing the anastomosis in a rabbit model.

Methods

New Zealand white rabbits were anesthetized, and the cervical trachea was exposed through a cervical midline incision, followed by resection of the 10^{th} tracheal cartilage. The rabbits were categorized into two groups: the bFGF group (n=6) and the control group (n=6). In the former group, bFGF (25μg) was administered into the submucosal layer of the cartilage using a 27-gauge needle immediately before tracheal anastomosis. The animals were sacrificed 4 weeks later. Histological, mechanical, and biochemical evaluations were performed on this anastomosed trachea.

Results

At 4 weeks of age, the anastomoses were spindle-shaped and displayed maximum diameter at the injection site compared with those in the control group. Histological evaluation showed that cartilage tissue had regenerated between the 9^{th} and 11^{th} tracheal cartilage rings. Tensile test showed that the anastomoses displayed a significantly high strain/stress ratio ($p = 0.035$). The collagen type 2 and glycosaminoglycan levels were significantly increased, and the collagen type 1 level was significantly decreased ($p = 0.019$, $p = 0.013$, and $p = 0.045$, respectively).

Conclusions

A new wound-healing concept of airway anastomosis could be provided by the results that single injection of bFGF regenerated tracheal cartilage in rabbits and strengthened the anastomosis by bridging the regenerated and well-matured cartilage. Further investigation of this method will lead to potential clinical applications for reinforcement of tracheal anastomoses.

Keywords

Basic fibroblast growth factor; trachea; cartilage; type 2 collagen; glycosaminoglycan; tensile test

Abbreviations

bFGF: basic fibroblast growth factor

H&E: hematoxylin and eosin

Col I: collagen type I

Col II: collagen type II

GAG: glycosaminoglycan

ELISA: enzyme-linked immunosorbent assay

Introduction

The technical management of airway reconstruction is one of the most challenging problems associated with a wide range of tracheal resections in patients with malignant or benign diseases [1–3]. The important management issues include the strong tension at the anastomosis and anastomotic dislodgements. One solution is to increase the blood supply [4] by focusing on standard anastomotic wound healing. Omentopexy at anastomoses with impaired blood flow is a suitable approach [5]; however, this procedure is highly invasive because it involves additional abdominal manipulation. Another solution is tracheal grafting with esophageal interposition [6] or bioengineering [7]. A three-dimensional biocompatible engineered airway would be ideal as a grafting material, but technological advances are required for its fabrication. Therefore, it is desirable to develop a simple, safe, and reliable technique for airway anastomosis based on an entirely new viewpoint of airway wound healing.

In a rabbit airway reconstruction model, detailed in our previous study [8], we found that neocartilaginous tissue binds at the suture line between the maturing tissue-engineered cartilage plate and the native hyaline cartilage. *In vivo* maturing tissue-engineered

cartilage provides a suitable environment for neocartilage formation in the suture line between cartilages. The perichondrium of the native tracheal cartilage at the suture line produces chondrogenic stem cells to generate neocartilage. In the process of cartilage development, the paracrine phenomenon of macrophages induces anti-inflammatory activities at the junction site to join the neocartilage [9].

Basic fibroblast growth factor (bFGF), a chondrocyte growth factor, is a highly effective growth factor that acts on smooth muscle cells, endothelial cells, fibroblasts, and epithelial cells to induce chondrocyte proliferation [10–12]. Previous studies have shown that bFGF promotes the regeneration of tracheal cartilage, which suggests that artificial tracheal cartilage regeneration using bFGF can be employed to repair tracheal defects [13–16]. In our previous study, we reported that direct injection of bFGF into the submucosal space of the trachea can promote tracheal cartilage growth, with the proliferation of chondrogenic stem cells at the perichondrium and cartilage matrix produced by chondrocytes [17]. In other words, bFGF injection promotes the growth of maturing cartilage and induces the paracrine phenomenon.

In this study, we present a new concept for airway anastomosis that involves neocartilage formation. We reinforced the anastomosis with regenerated cartilage, which is more rigid than granulation tissue. As a preliminary study to establish this research framework, we focused principally on whether the regenerated cartilage, formed as a result of bFGF injection, could form a bridging structure at the anastomosis and whether the cartilage increased the strength of the anastomosis. Therefore, we performed this study in an experimental setting, which provided sufficient time from surgery to sacrifice, as well as lower tension and abundant blood flow at the anastomosis. We investigated the

morphological, histological, mechanical, and biological features of regenerative cartilage junctions between the native hyaline tracheal cartilages induced by bFGF injection immediately before anastomosis.

Materials and Methods

Ethics Statement

The protocol of this study was approved by the Animal Care and Use Committee of the University of Tokyo (protocol No. P-19-036), and all experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments of the University of Tokyo.

Preparation of bFGF solution

Trafermin (Fiblast® Spray, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan), a commercially available, recombinant human bFGF, has been authorized for use in patients by the Ministry of Health, Labour and Welfare of Japan. The solution was adjusted to a concentration of 0.25 μg/μL bFGF.

Surgical procedures

Twelve 10-week-old female New Zealand white rabbits weighing approximately 2 kg were anesthetized with propofol in a bolus of 10 mg/kg (Maruisihi Pharmaceutical Co. Ltd., Osaka, Japan) and isoflurane (Pfizer Japan, Tokyo, Japan). With the rabbit in the supine position, isoflurane was administered continuously for maintenance of anesthesia. The cervical trachea was exposed through a cervical midline incision, and the tenth tracheal cartilage was resected, whose resection generated small tension. An intubation tube (Portex Tracheal tube, 2.5 mm; Smiths medical Japan Ltd., Tokyo, Japan) was inserted

155 into the lower trachea for anesthesia management. The rabbits were categorized into two
156 groups: the bFGF group (n = 6) and the control group (n = 6). In the bFGF group, 12.5 μg
157 bFGF was administered into the submucosal layer of the cartilage with a 27-gauge needle
158 in the 2 o'clock and 8 o'clock positions of the cephalic tracheal stump. Next, 12.5 μg bFGF
159 was administered into the submucosal layer of the cartilage with a 27-gauge needle in the
160 4 o'clock and 10 o'clock positions of the caudal tracheal segment. The injection point was
161 set to deviate by 90 degrees at the cephalic and caudal sides of the anastomosis, as
162 shown in Figure 1b. Then, in the tracheal anastomosis between the 9^{th} and 11^{th} tracheal
163 cartilage, the membranous portion of the trachea was anastomosed with three stitches of
164 6-0 Nespiren (non-absorbable monofilament; Alfresa Pharma Corp., Osaka, Tokyo) in a
165 single ligation, and the cartilaginous portion of the trachea was anastomosed with five
166 stitches in the interrupted suture technique. After the procedure, we confirmed that there
167 was no air leakage from the anastomosis. Finally, the muscle and skin were closed
168 separately.
169
170 Morphological and histological examinations
171 Four weeks after the surgical procedure, all rabbits were sacrificed for morphological and
172 histological examinations. After tracheas were harvested, tissue specimens were obtained,
173 as shown in Figure 2. First, the membranous portion of the trachea was cut along its entire
174 length (Figure 2a and 2b). The rectangular tissue sample was hollowed out into a regular
175 circle with a diameter of 3 mm centered on the point of the bFGF injection (Figure 1b), and
176 the thickness and weight of the specimens were measured. The specimens were divided
177 into two groups for tensile test followed by histological examination and for enzyme-linked
178 immunosorbent assay (ELISA). The specimens for histological examination were
179 embedded in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo,

Japan) and frozen. Embedded tissues were subsequently sliced into 7-μm sections and stained with hematoxylin and eosin (H&E), toluidine blue, safranin O, or Masson trichrome.

Tensile tests

Circumferential tensile tests were conducted to compare the strength of the anastomoses between the bFGF group and the control group. The rabbit trachea and larynx were extracted and the whole specimen was used for the tensile test. The thicknesses of the rabbit trachea were measured using an outside micrometer. The thickness and width of each specimen were measured at four locations: the 4^{th}, 9^{th}, 11^{th}, and 16^{th} tracheal cartilage rings. Figure 5a shows the mechanical measurement setup for the stress and strain tensile tests. The specimen was pulled with a cylindrical rod without any bending moments so that pure tensile stress could be loaded. The data from the anastomosis were measured between the upper margin of the 9^{th} tracheal cartilage ring and the lower margin of the 11^{th} tracheal cartilage ring. As a standard, data from the long segment specimen were measured between the 6^{th} tracheal cartilage ring and the 15^{th} tracheal cartilage ring. The specimens were pulled at slow strain velocities. To ensure that the effect of strain velocities on the measurement results was small [18], the strain velocities during measurement were set between 0.0001/s and 0.0004/s at the long segment of the trachea. Based on our previous research results [19], we applied pull loads that were strong enough to trail the long segment up to approximately 1.2 times in length. The specimens were kept wet with physiological saline water spray during the tensile tests. For the results of the tensile tests, the slopes of the stress–strain curves in the anastomoses were expressed as a quotient divided by the slopes of the stress–strain curves in the long segment of the trachea to minimize the effect of the strain differences between individual tracheas.

205

206 ELISA for collagen and glycosaminoglycan

207 Specimens were homogenized in 800 μL of 0.05 M acetic acid and digested with 100 μL of
208 10 mg/mL pepsin solution (162-18721, Wako Pure Chemical Industries, Osaka, Japan) at
209 4°C for 48 h. After adding 100 μL of 1 mg/mL pancreatic elastase solution (058-05361,
210 Wako Pure Chemical Industries), the samples were incubated at 4°C for 24 h. Insoluble
211 material was removed by centrifugation at 10,000 rpm at room temperature for 5 min, and
212 the supernatant was collected for the determination of collagen type I (Col I), collagen type
213 II (Col II), and sulfated glycosaminoglycan (GAG). Quantitative measurement of Col I was
214 performed using a commercially available ELISA kit (Rabbit Type I Collagen Detection Kit,
215 Catalog #6016, Chondrex, Inc.). All samples were diluted 1:10, and the assay was
216 performed according to the manufacturer's protocol. Quantitative measurement of Col II
217 was performed using a commercially available ELISA kit (Type II Collagen Detection Kit,
218 Multi-species, Catalog #6018, Chondrex, Inc.). All samples were diluted 1:1000, and the
219 assay was performed according to the manufacturer's protocol. Quantitative measurement
220 of GAG was performed using a commercially available ELISA kit (Glycosaminogricans
221 Assay Kit, Catalog #6022, Chondrex, Inc.). All samples were diluted 1:20, and the assay
222 was performed according to the manufacturer's protocol. The concentrations of Col I, Col
223 II, and GAG were adjusted according to the total protein concentration in each sample
224 (i.e., they were expressed as a quotient divided by the concentration of the total protein
225 amount) to minimize the effect of the size differences between individual samples.

226

227 Statistical analysis

228 SPSS version 26 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 9.0
229 (GraphPad Prism Software Inc., San Diego, CA, USA) were used for statistical analyses

and to construct figures. The results were assessed using unpaired two-tailed Student's *t*-tests with Welch's correction. A p-value of <0.05 was considered statistically significant.

Results

All twelve rabbits survived without complications until sacrificed as planned; changes in breathing were not noted. Upon gross examination of the tracheas, differences in inflammatory signs between the bFGF group and the control group were not observed.

Macroscopically, the anastomoses in the bFGF group were spindle-shaped and had maximum diameter at the injection site (Figure 2a), whereas those in the control group showed a tapered shape (Figure 2b). The width of each cartilage ring was greater in the bFGF group than in the control group. Specimens were prepared with the same cross-sectional size, and the thicknesses and weights of the specimens were compared. Specimens in the bFGF group were thicker and heavier than those in the control group (Figure 2c and 2d, $p = 0.017$ and $p = 0.029$, respectively).

Histological examination of the cross-sections of the tracheas revealed that the cartilage tissue had regenerated between the 9^{th} and 11^{th} tracheal cartilage rings in the bFGF group (Figure 3a). The presence of cartilage was confirmed by H&E (Figure 4a), toluidine blue (Figure 4b), and safranin O staining (Figure 4c). Regenerated cartilage tissue was connected with the 9^{th} and 11^{th} tracheal cartilage rings, and it formed a cross-linked structure between the two tracheal cartilage rings. In the control group, conversely, the gap between the 9^{th} and 11^{th} tracheal cartilage rings was filled with soft granulation tissue and cartilage regeneration was not observed (Supplementary Figure S1a–c). Regarding collagen fibers surrounding the anastomoses, no significant differences were observed

between the two groups following Masson trichrome staining (Figure 4d and Supplementary Figure S1d).

Figure 5b shows the tensile test results of the two groups. Compared with the control group, the anastomoses in the bFGF group showed a significantly higher strain/stress ratio ($p = 0.035$), which indicates that the anastomoses in the bFGF group were more rigid than those in the control group.

Figure 6 shows the ELISA results. Figure 6a shows that the concentration of GAG in the bFGF group was significantly increased compared with that in the control group ($p = 0.013$). Figure 6b shows that the concentration of Col II in the bFGF group was also significantly increased compared with that in the control group ($p = 0.019$). Conversely, the concentration of Col I in the bFGF group was significantly lower than that in the control group ($p = 0.045$) (Figure 6c). All descriptive statistics is shown in supplementary table.

Discussion

In this study, we demonstrated that bFGF injected only once immediately before performing anastomosis promotes cartilage regeneration at the tracheal anastomosis and that the regenerated cartilage connects to the adjacent cartilage and bridges the gap to reinforce the tracheal anastomosis. To the best of our knowledge, this is the first report of an *in vivo* model demonstrating reinforcement of tracheal anastomoses using regenerated cartilage instead of wrapping materials.

To measure the degree of reinforcement, we believed that the tensile test was appropriate to directly evaluate tracheal anastomosis resistance. Previous reports have evaluated

tracheal strength using the compression test [14]; however, the compression test seems to only support evidence for resistance. We considered an approximately 1.2-fold extension sufficient because physiological extension of the trachea in the cephalocaudal direction is unlikely to occur unless long segment of trachea is resected and anastomosed. Moreover, based on our previous research with swine bronchi [19], strong traction on the bronchial wall results in the loss of tissue recovery, thereby leading to a loss of reliability of the tensile test data. Therefore, we concluded that approximately 1.2-fold traction would be an appropriate threshold. We considered whether the cartilage would be hard enough to reinforce the anastomosis as the stiffness of regenerated cartilage is significantly lower than that of native cartilage [20]. However, the collagenous tissue surrounding the cartilage was not histologically different between the two groups. The only difference between the two groups was the presence of regenerated cartilage. From these results, we concluded that the regenerated cartilage reinforced the anastomosis.

Regarding the cartilage components, Col II and GAG are the main components of cartilage matrixes, and the abundance of these proteins is related to the stiffness of the tracheal cartilage [21]. A previous study of engineered cartilage reported that, histologically, Col II but not Col I was present in the regenerated cartilage tissue [22]. Another study revealed that bFGF treatment of human bone marrow stem cells caused a reduction in the mRNA expression of Col I but an increase in the expression of Col II [23]. Our results agree with those of these previous studies. We also demonstrated that Col II and GAG concentrations were increased, whereas that of Col I was reduced in the bFGF group. The reduced production of Col I suggests that fibroblast proliferation was suppressed and the production of unnecessary granulation tissue at the anastomosis was inhibited.

Inappropriate granulation at the tracheal anastomosis may result in airway obstruction. From this viewpoint, bFGF injection might be preferable.

Previous reports have shown that the slow release of bFGF is beneficial for cartilage tissue engineering [16]. It is thought that the slow release of bFGF is important in continuous cartilage regeneration due to the short half-life of bFGF [24]. However, it has been recently reported that a single intratracheal injection of bFGF significantly promoted the growth of tracheal cartilage [25]. Moreover, 12 weeks after a single injection of bFGF, the effect on growth continued [26]. In the present study, we also used a single injection of bFGF, which proved effective. Based on these results, we speculated that an increase in bFGF concentration at some point was important for cartilage regeneration and that a sustained increase in concentration was unnecessary.

The specific role of bFGF in cartilage homeostasis remains unknown [27], but it is presumed that the perichondrium is involved in bFGF-induced cartilage regeneration. It has been previously reported that injected bFGF is absorbed from the submucosal lumens into the perichondrium of the tracheal cartilage [17]. Moreover, when the regenerated cartilage was covered with perichondrium that had grown from the perichondrium of the host cartilage, it showed continuity with the host cartilage stumps [16]. The perichondrium is known to have the potential to promote the production of chondrogenic matrix [28,29] and is considered a source of regenerated cartilage [30]. It is presumed that bFGF induces the enhanced growth of tracheal cartilage originating from perichondrium regeneration and chondrocyte proliferation in the trachea cartilage [17]. On the basis of the findings of previous reports, we speculate that the cartilage regeneration at the tracheal anastomosis

observed in this study may be attributed to the regeneration of the perichondrium and proliferation of chondrocytes induced by bFGF injection in the submucosa of the trachea.

Here, the question arises as to how the regeneration of the perichondrium and the proliferation of chondrocytes lead to cartilage regeneration. Considering our results, we speculated that there are two possibilities for the mechanism of cartilage regeneration in the bFGF group. 1) After the cartilage in the perichondrium is joined by fibrous tissue, cartilage stem cellmigrate to form cartilage based on the fibrous tissue, and this cartilage reinforces the joint. 2) Mesenchymal stem cells migrate from the perichondrium to the anastomosis, produce substrates for cartilage regeneration, and generate cartilage to bridge the anastomosis. We would like to discuss this issue, in terms of the differences from the control group and the effect of a single injection of bFGF. A scaffold is required for the chondrocytes to adhere to upon migration; however, a single dose of bFGF is unlikely to be effective for a long time due to its short half-life. Considering the period that elapses until the scaffold is formed, the second possibility is more likely. Further investigation is required to elucidate the mechanism of cartilage regeneration induced by single dose of bFGF.

Currently, we have no clinical experience of bFGF application to human bronchus, because Fibrast®, commercially available bFGF in Japan, is currently authorized as the medicine only for external application. However, we believe this method has several potential clinical implications. First, this method should be very useful in tracheal resection. This technique will allow us to skip laparotomy in cases that need to be covered with omentum. In addition, the additional procedure is very minimally invasive, as b-FGF is only injected into the bronchial stump before anastomosis. Second, we believe that this method

will be useful in bronchoplasty, such as sleeve resection. This method allows us to skip additional procedures, such as covering anastomoses with intercostal muscle or latissimus dorsi muscle. Moreover, b-FGF injection can be performed under thoracoscopically, therefore minimizing the invasiveness of surgery can be expected. Third, bronchoscopic injection can be performed, therefore this method may help repair bronchopleural fistulas only with bronchoscopic procedures.

This study had some limitations. First, the bFGF dosage was not adjusted in this study. High concentrations of bFGF cause acidification of the solution and necrosis of the injection site. Alternatively, large injection volumes cannot be stably injected into the submucosal tissue of the cartilage. Therefore, the dosage setting was unchanged in our study. Second, the timing and position of the bFGF injection was not adjusted. Because clinical application of this technique was considered, we believed it would be most feasible to perform the injection immediately before the anastomosis. We also believed that the selected injection site was the most appropriate for injecting bFGF as efficiently as possible into the tracheal cartilages and not into the tracheal membranous portion. Third, the trachea in this study was simply extended to 1.2 times its normal length. This force may not properly refer to the clinical reality of higher tensile forces after resection of longer trachea segments. Besides, the duration of this study was 4 weeks, which might be too long when reinforcement of the tracheal anastomosis is considered necessary. Although this study period was set according to previous papers, further investigation is required to evaluate the degree of cartilage regeneration and anastomotic strength over shorter time periods under higher tensile condition.

In conclusion, a new wound-healing concept of airway anastomosis could be provided by the results that single injection of bFGF regenerated tracheal cartilage in rabbits and strengthened the anastomosis by bridging the regenerated and well-matured cartilage. Further investigation of this technique will open up the possibility of clinical applications of bFGF injection for tracheal anastomotic reinforcement.

Author contributions statement

Conceptualization: FY, YY, MaKo, MaKa

Data curation FY, YY, MaKo, HK, TT

Formal Analysis: YY, MaKo, TT

Funding acquisition: FY, YY, MaKo

Investigation: FY, YY, MaKo

Methodology: FY, YY, MaKo, MaKa, TT

Project administration: YS, MaKo, MaKa

Resources: YS, MaKo, MaKa

Software: YY, MaKo, TT

Supervision: MaKa

Validation: YY, MaKo, TT

Visualization: FY, YY

Writing – original draft: FY, YY, MaKo

Writing – review & editing: TT, YS, MaKa

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Conflict of Interest statement

None declared.

Data Availability Statement

The data in this manuscript are neither held nor will be held in a public repository. All figures were originally drawn by authors (FY, YY).

Figure legends

Figure 1.

Operative procedures are shown. (a) The 10th tracheal cartilage ring is circumferentially resected, and the 9th and 11th tracheal cartilage rings are anastomosed. (b) The cephalic and caudal surgical stumps are shown. Red arrows show the basic fibroblast growth factor (bFGF) injection point of the trachea. The injection point is set to be deviated by 90° at the cephalic and caudal sides of the anastomosis.

Figure 2.

(a) Macroscopic findings of tracheal anastomosis in the bFGF injection group. Anastomoses in the bFGF injection group were spindle-shaped and displayed their maximum diameter at the injection site. (b) Macroscopic findings of tracheal anastomosis in the control group. (c) The tissue specimens in the bFGF injection group were significantly thicker than those in the control group ($p = 0.017$). (d) The tissue specimens in the bFGF injection group were significantly heavier than those in the control group ($p = 0.029$).

Figure 3.

(a) Microscopic findings of tracheal anastomosis in the bFGF injection group. (b) Microscopic findings of tracheal anastomosis in the control group.

Figure 4.

Frozen sections of tracheal cartilage were stained with hematoxylin and eosin (a), toluidine blue (b), safranin O (c), and Masson trichrome (d) under a high-power field. Arrows indicate regenerated cartilage, which bridged the 9^{th} and 11^{th} tracheal cartilages. The perichondrium was covered around the regenerated cartilage. Other collagenous fibers were absent around the cartilage.

Figure 5.

(a) Experimental setting of the tensile test. The harvested trachea was positioned on the measuring table of the scope, and a tensile test was performed for mechanical measurement of the stress and strain of the tracheal anastomosis. (b) The result of the tensile test is shown. The slopes of the stress–strain curves (Y-axis) in the anastomosis were expressed as a quotient divided by the slopes of the stress–strain curves in the long segment of the trachea. The anastomoses of the bFGF injection group were significantly stiffer than those of the control group ($p = 0.035$).

Figure 6.

Results of ELISA for glycosaminoglycan, type 1 collagen, and type 2 collagen. The concentrations were adjusted using the concentration of the total amount of proteins. (a) A comparison of glycosaminoglycan levels in the tracheal anastomosis is shown. Glycosaminoglycan levels in the bFGF injection group were significantly higher than those

in the control group ($p = 0.013$). (b) A comparison of type 2 collagen levels in the tracheal anastomosis is shown. Type 2 collagen levels in the bFGF injection group were significantly higher than those in the control group ($p = 0.019$). (c) A comparison of type 1 collagen levels in the tracheal anastomosis is shown. Type 1 collagen levels in the bFGF injection group were similar to those in the control group ($p = 0.045$).

Supplementary Figure legends

Figure S1.

Frozen sections of tracheal cartilage were stained with hematoxylin and eosin (a), toluidine blue (b), safranin O (c), and Masson trichrome (d) under a high-power field. Cartilage regeneration was not observed between the 9^{th} and 11^{th} tracheal cartilages in the control group.

Central Image.

The rabbits were categorized into two groups: the bFGF group (n=6) and the control group (n=6). In the former group, single intratracheal injection of bFGF was administered into the submucosal layer of the cartilage using a 27-gauge needle immediately before tracheal anastomosis. Tracheal cartilage regeneration was accelerated in the bFGF group.

References

1 Donahue DM, Grillo HC, Wain JC, Wright CD, Mathisen DJ. Reoperative tracheal resection and reconstruction for unsuccessful repair of postintubation stenosis. J Thorac Cardiovasc Surg 1997;114:934–938; discussion 938-9.

2 Wright CD, Grillo HC, Wain JC, Wong DR, Donahue DM, Gaissert HA, et al. Anastomotic complications after tracheal resection: prognostic factors and management. J Thorac Cardiovasc Surg 2004;128:731–739.

3 Bibas BJ, Terra RM, Oliveira Junior AL, Tamagno MFL, Minamoto H, Cardoso PFG, et al. Predictors for postoperative complications after tracheal resection. Ann Thorac Surg 2014;98:277–282.

4 Ishihara T, Nemoto E, Kikuchi K, Kato R, Kobayashi K. Does pleural bronchial wrapping improve wound healing in right sleeve lobectomy? J Thorac Cardiovasc Surg 1985;89:665–672.

5 Mayer E, Cardoso PFG, Puskas JD, De Campos K, Oka T, Dardick I, et al. The effect of basic fibroblast growth factor and omentopexy on revascularization and epithelial regeneration of heterotopic rat tracheal isografts. J Thorac Cardiovasc Surg 1992;104:180–188.

6 Kato R, Onuki AS, Watanabe M, Hashizume T, Kawamura M, Kikuchi K, et al. Tracheal reconstruction by esophageal interposition: An experimental study. Ann Thorac Surg 1990;49:951–954.

7 Martinod E, Chouahnia K, Radu DM, Joudiou P, Uzunhan Y, Bensidhoum M, et al. Feasibility of Bioengineered Tracheal and Bronchial Reconstruction Using Stented Aortic Matrices. JAMA 2018;319:2212.

8 Komura M, Komura H, Otani Y, Kanamori Y, Iwanaka T, Hoshi K, et al. The junction between hyaline cartilage and engineered cartilage in rabbits. Laryngoscope 2013;123:1547–1551.

9 Fujihara Y, Abe T, Hoshi K. Controlling the Phenotype of Macrophages Promotes Maturation of Tissue-Engineered Cartilage. Tissue Eng Part A 2020;26:1005–1013.

10 Przybylski M. A review of the current research on the role of bFGF and VEGF in angiogenesis. J Wound Care 2009;18:516–519.

11 Nakajima H, Sakakibara Y, Tambara K, Iwakura A, Doi K, Marui A, et al. Therapeutic angiogenesis by the controlled release of basic fibroblast growth factor for ischemic limb and heart injury: toward safety and minimal invasiveness. J Artif Organs 2004;7:58–61.

12 Quatela VC, Sherris DA, Rosier RN. The Human Auricular Chondrocyte: Responses to Growth Factors. Arch Otolaryngol - Head Neck Surg 1993;119:32–37.

13 Tatekawa Y, Kawazoe N, Chen G, Shirasaki Y, Komuro H, Kaneko M. Tracheal defect repair using a PLGA-collagen hybrid scaffold reinforced by a copolymer stent with bFGF-impregnated gelatin hydrogel. Pediatr Surg Int 2010;26:575–580.

14 Komura M, Komura H, Tanaka Y, Kanamori Y, Sugiyama M, Nakahara S, et al. Human tracheal chondrocytes as a cell source for augmenting stenotic tracheal segments: the first feasibility study in an in vivo culture system. Pediatr Surg Int 2008;24:1117–1121.

15 Igai H, Chang SS, Gotoh M, Yamamoto Y, Misaki N, Okamoto T, et al. Regeneration of Canine Tracheal Cartilage by Slow Release of Basic Fibroblast Growth Factor from Gelatin Sponge. ASAIO J 2006;52:86–91.

16 Igai H, Yamamoto Y, Chang SS, Yamamoto M, Tabata Y, Yokomise H. Tracheal cartilage regeneration by slow release of basic fibroblast growth factor from a gelatin sponge. J Thorac Cardiovasc Surg 2007;134:170–175.

17 Komura M, Komura H, Konishi K, Ishimaru T, Hoshi K, Takato T, et al. Promotion of tracheal cartilage growth by intra-tracheal injection of basic fibroblast growth factor (b-FGF). J Pediatr Surg 2014;49:296–300.

18 Li JT, Armstrong CG, Mow VC. The effect of strain rate on mechanical properties of articular cartilage in tension. ASME Biomech. Symp., 1983:117–120.

19 Yamauchi Y, Saito Y, Yasuda A, Shirai S, Kondo H, Uehara H, et al. Construction of a computational mechanical model of bronchi for practical simulation of the optimal positive intrathoracic pressure conditions during general thoracic surgery. Biomed Mater Eng 2021;Preprint:1–12.

20 Han J, Cuomo R, Zhao Y, Pan B, Yang Q. The Morphology and Bending Behavior of Regenerated Costal Cartilage with Kawanabe-Nagata Method in Rabbits – the Short Term Result of an Experimental Study. J Investig Surg 2020:1–5.

21 Bastiaansen-Jenniskens YMM, Koevoet W, de Bart ACWCW, van der Linden JCC, Zuurmond AMM, Weinans H, et al. Contribution of collagen network features to functional properties of engineered cartilage. Osteoarthr Cartil 2008;16:359–366.

22 Komura M, Komura H, Otani Y, Suzuki K, Satake R, Kodaka T, et al. Tracheoplasty with cartilage-engineered esophagus environments. J Pediatr Surg 2015;50:1093–1098.

23 Park D-S, Park J-C, Lee J-S, Kim T-W, Kim K-J, Jung B-J, et al. Effect of FGF-2 on collagen tissue regeneration by human vertebral bone marrow stem cells. Stem Cells Dev 2015;24:228–243.

24 Edelman ER, Nugent MA, Karnovsky MJ. Perivascular and intravenous administration of basic fibroblast growth factor: vascular and solid organ deposition. Proc Natl Acad Sci 1993;90:1513–1517.

25 Komura M, Komura H, Komuro H, Ikebukuro K, Hikita A, Hoshi K, et al. Tracheal cartilage growth by intratracheal injection of basic fibroblast growth factor. J Pediatr Surg 2017;52:235–238.

26 Komura M, Komura H, Komuro H, Konishi K, Obana K, Ikebukuro K, et al. Long-term follow-up of tracheal cartilage growth promotion by intratracheal injection of basic fibroblast growth factor. J Pediatr Surg 2018;53:2394–2398.

27 Ellman MB, Yan D, Ahmadinia K, Chen D, An HS, Im HJ. Fibroblast growth factor control of cartilage homeostasis. J Cell Biochem 2013;114:735–742.

28 Ohlsén L. Cartilage formation from free perichondrial grafts: an experimental study in rabbits. Br J Plast Surg 1976;29:262–267.

29 Zang M, Zhang Q, Davis G, Huang G, Jaffari M, Ríos CN, et al. Perichondrium directed cartilage formation in silk fibroin and chitosan blend scaffolds for tracheal transplantation. Acta Biomater 2011;7:3422–3431.

30 Özbek S, Kahveci R, Kahveci Z, Filiz G, Özcan M, Srmal Ş. Effect of Lyophilized Heterologous Collagen on New Cartilage Formation From Perichondrial Flaps in Rabbits: An Experimental Study. Ann Plast Surg 2003;50:528–534.

Figure 1

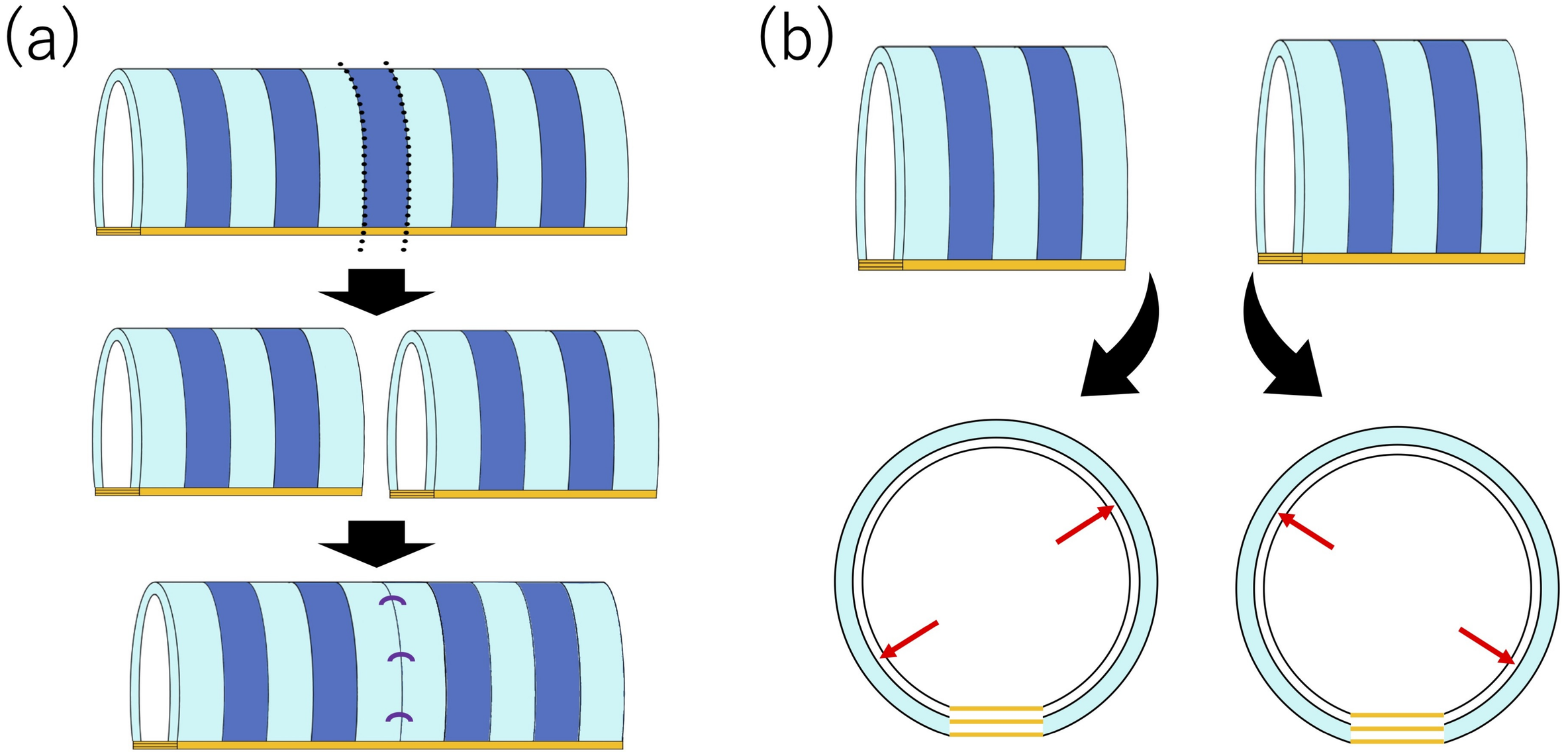
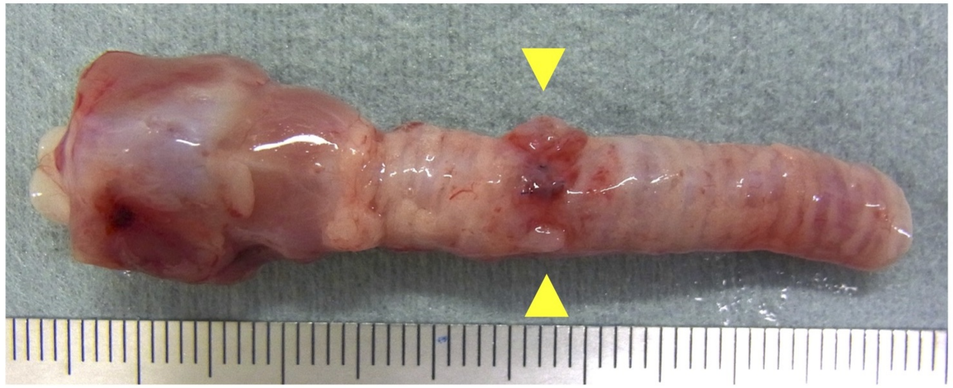


Figure 2

(a)



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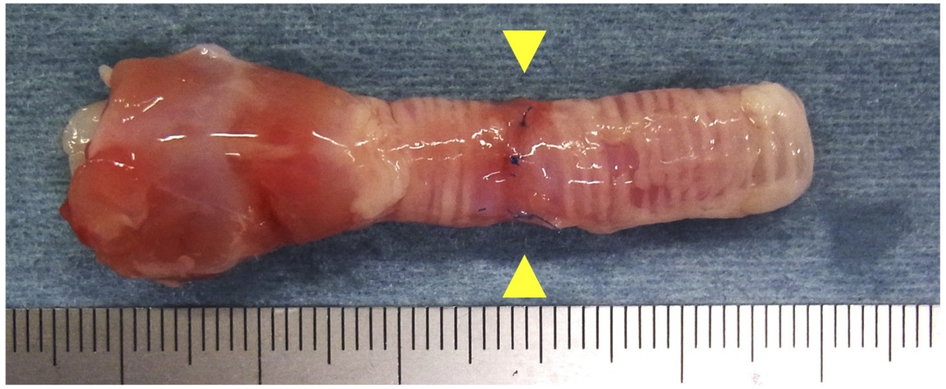

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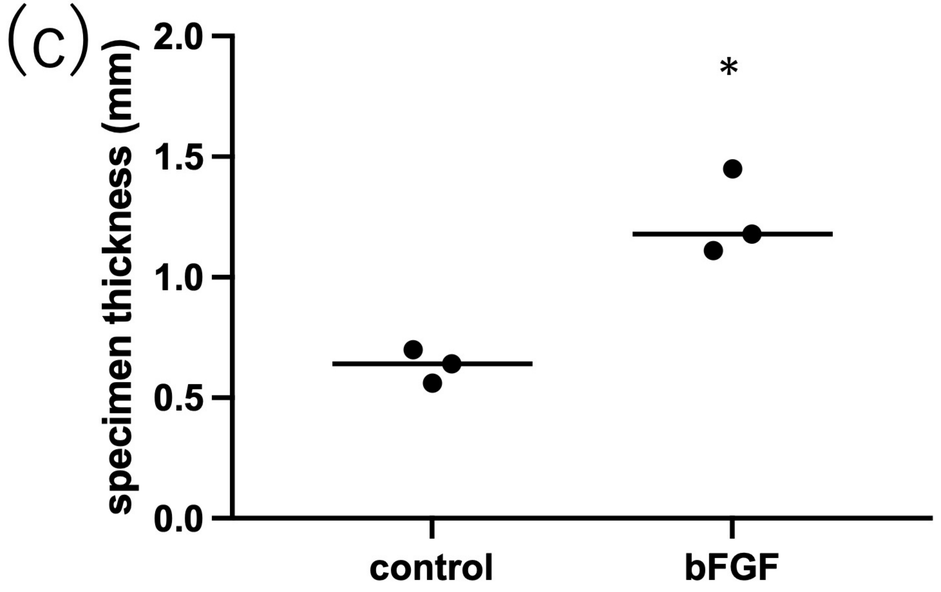

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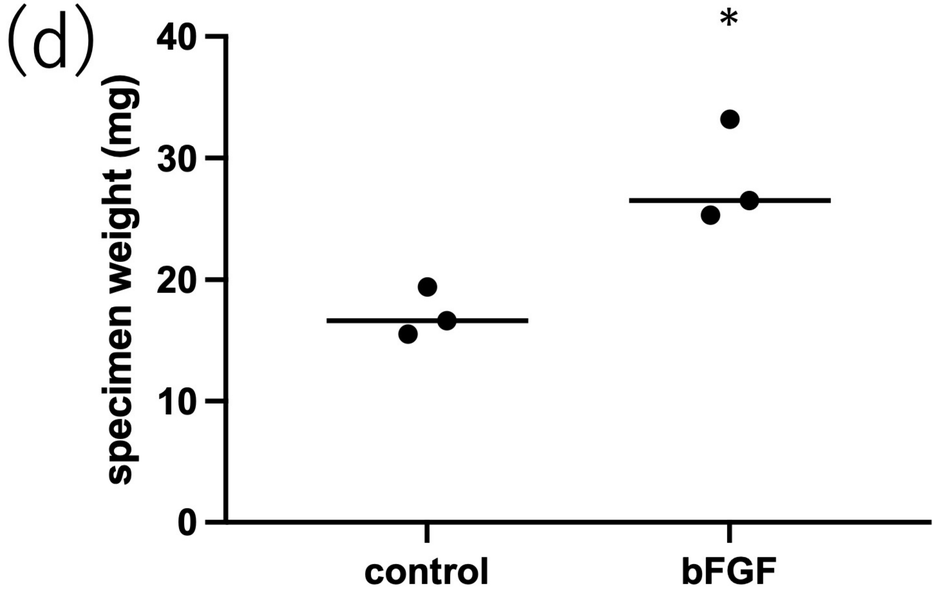
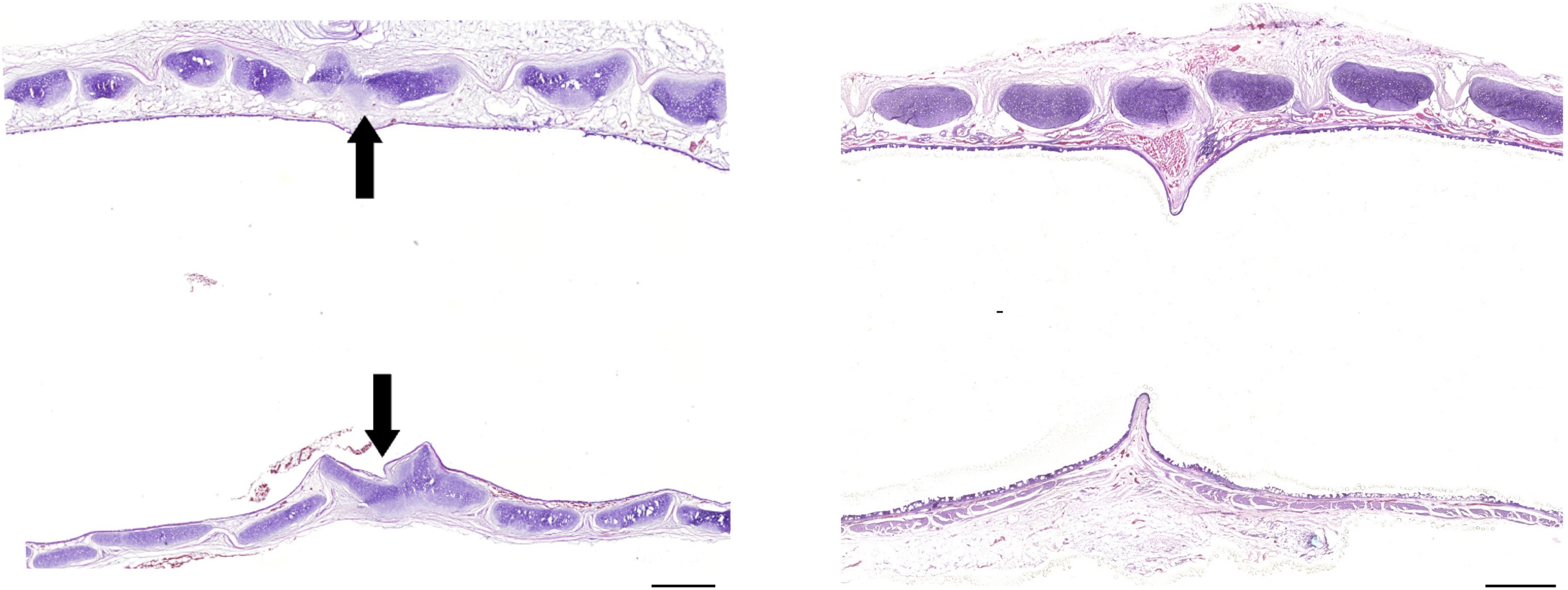


* $p<0.05$

Figure 3

(a)

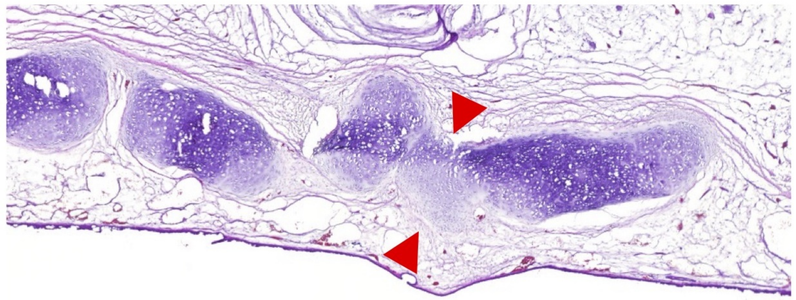
(b)



Bar: 1000μm

Figure 4

(a)



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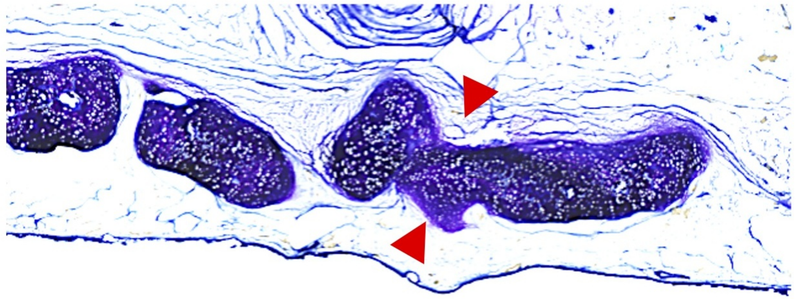

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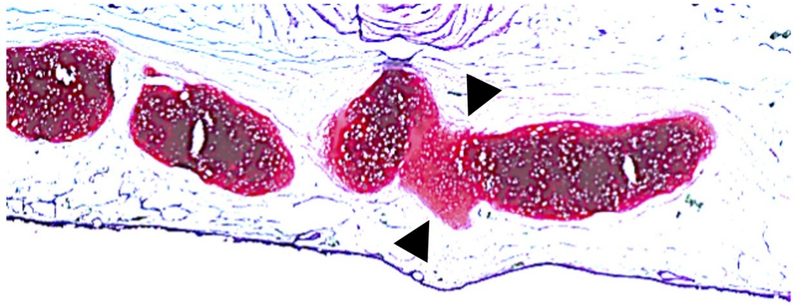

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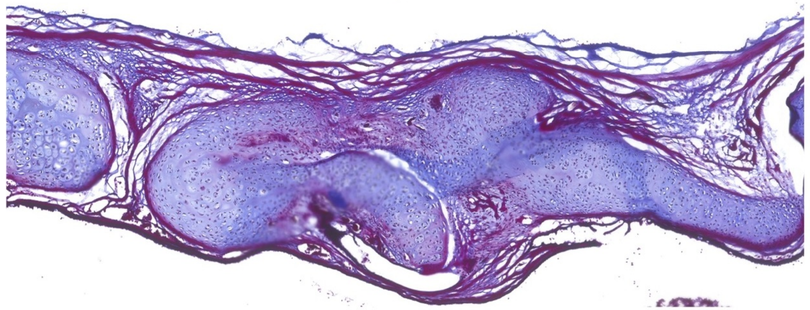
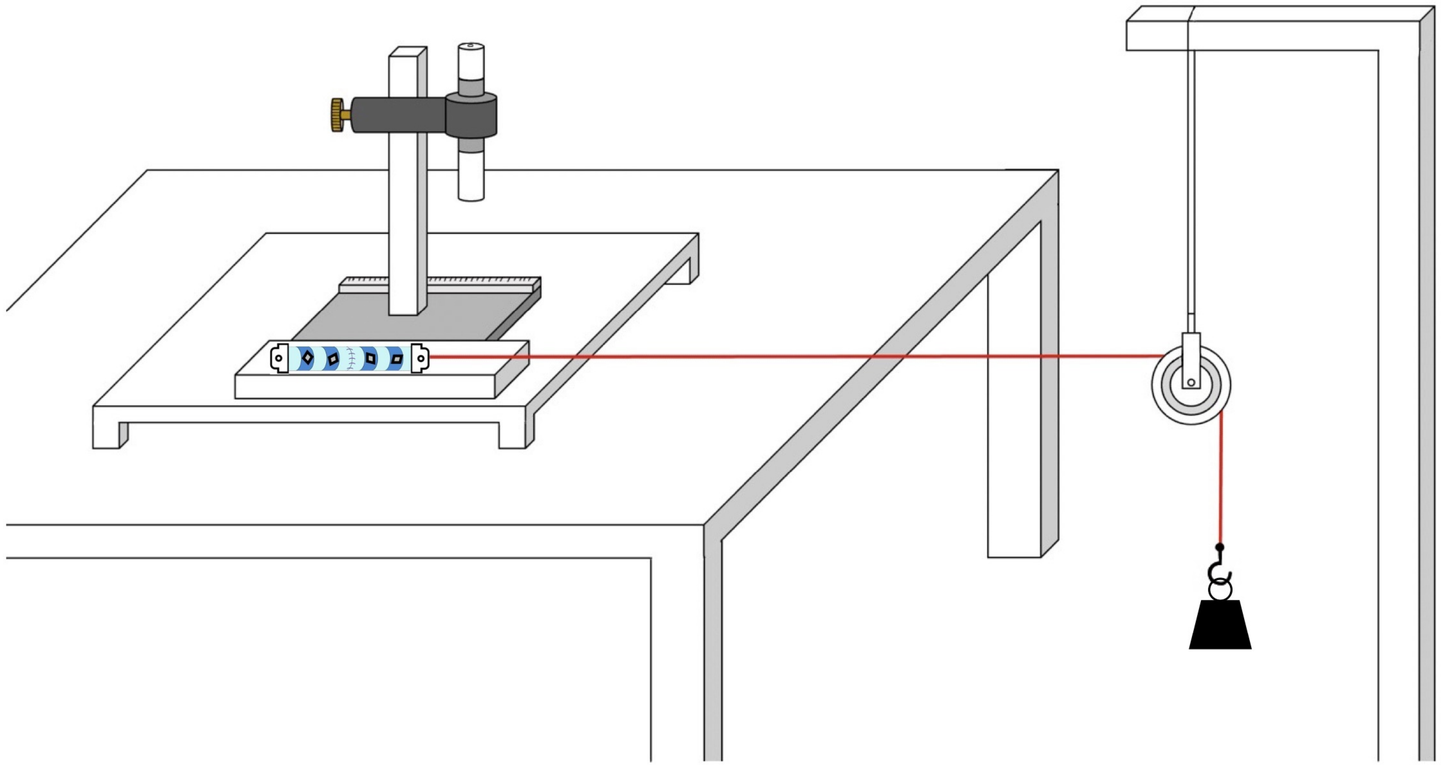


Bar: 1000μm

Figure 5

(a)



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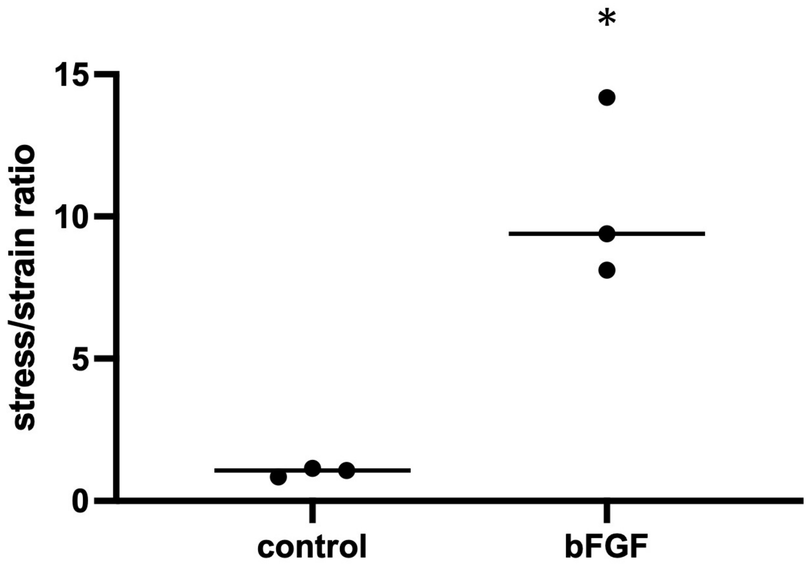
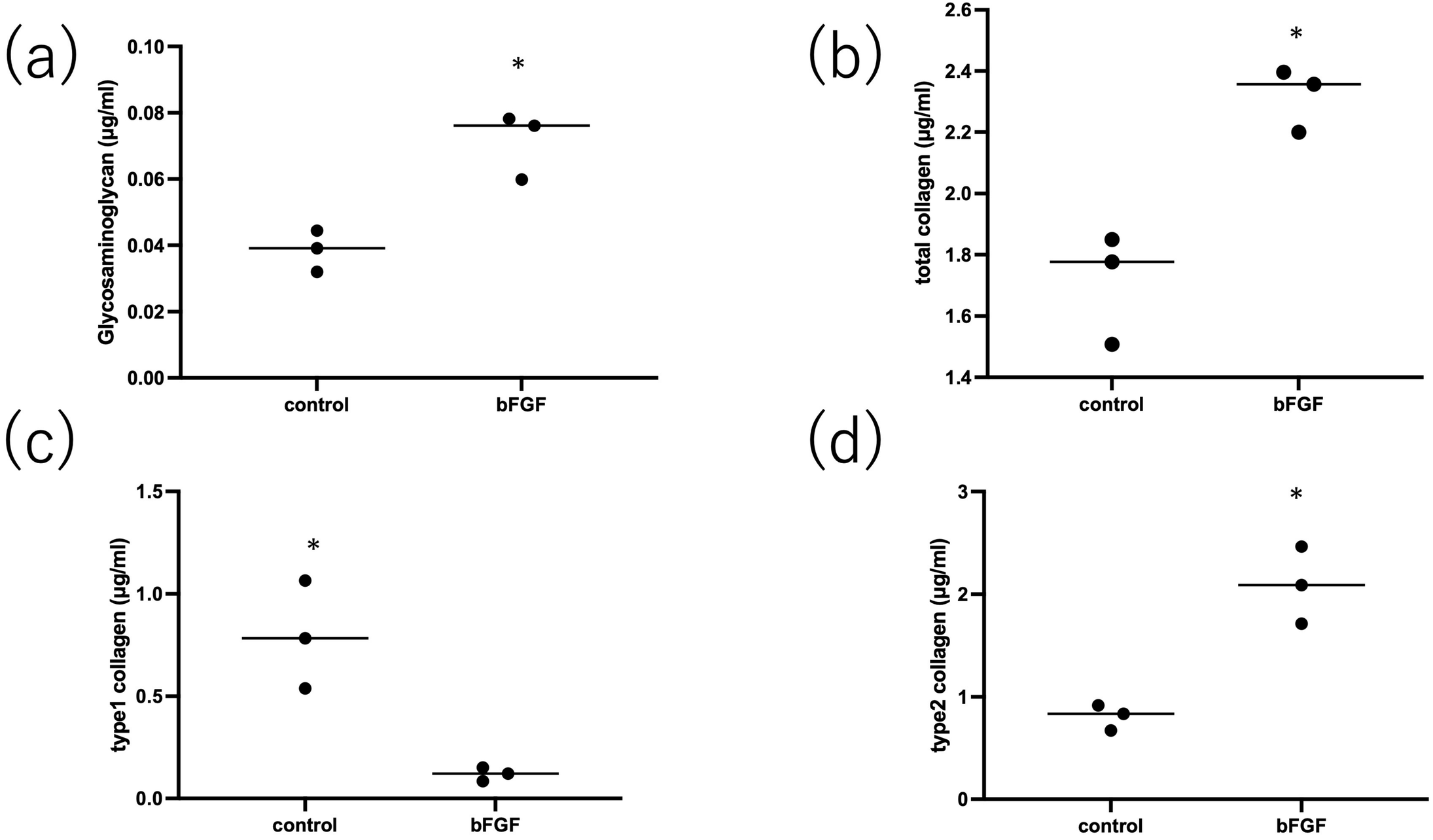


* $p<0.05$

Figure 6

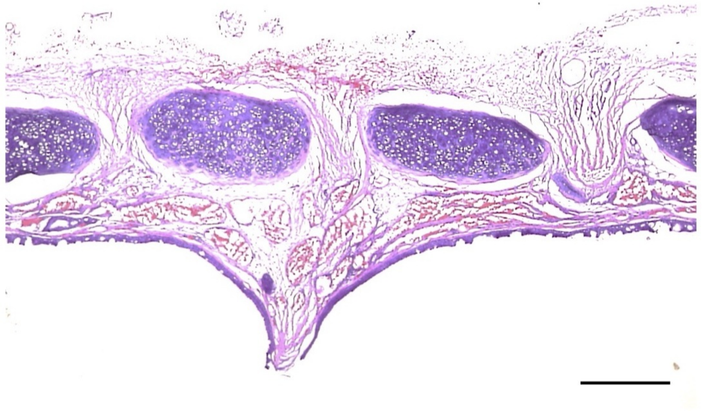


* $p<0.05$

** $p<0.01$

Supplementary Figure S1

(a)



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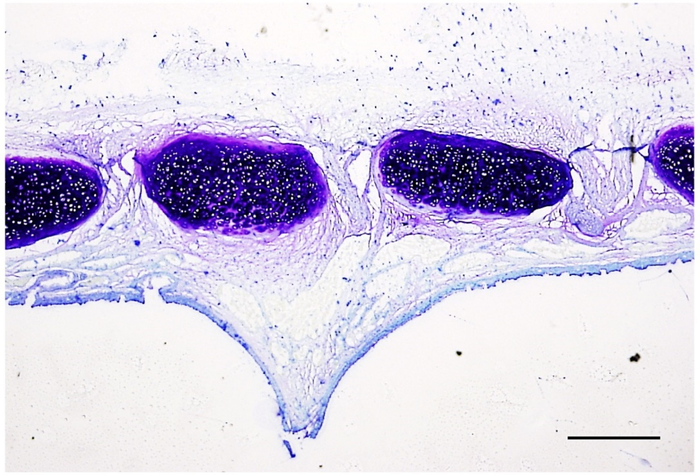

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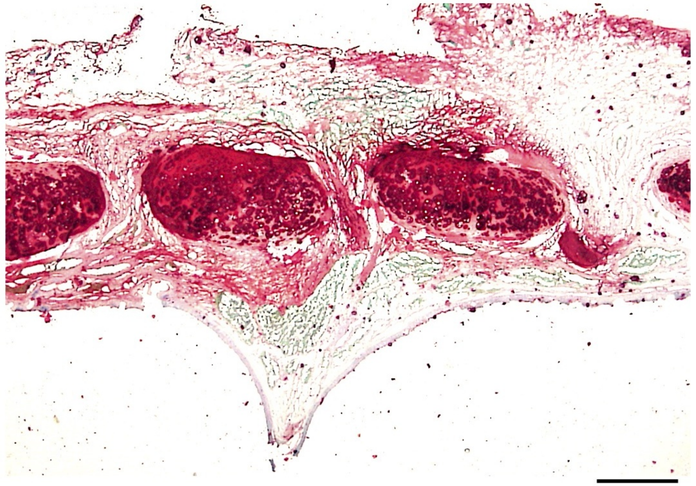

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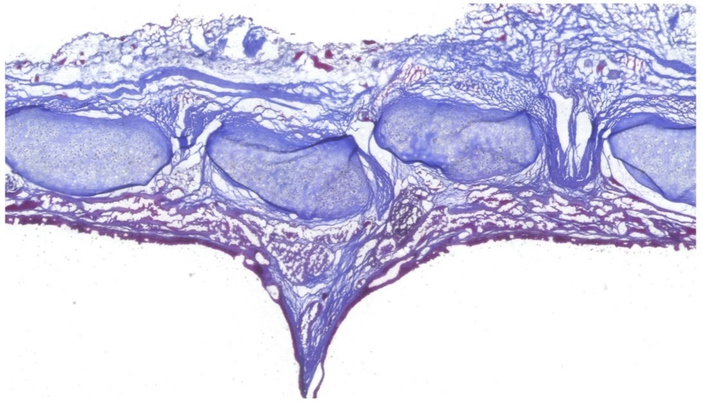

Bar: 1000μm

Supplementary table: descriptive statistics

	control			bFGF		
specimen weight (mg)	17.170	±	2.011	28.330	±	4.257
specimen thickness (mm)	0.633	±	0.070	1.247	±	0.180
stress/strain ratio	1.018	±	0.156	10.560	±	3.201
GAG (μg/ml)	0.039	±	0.006	0.071	±	0.010
Col2 (μg/ml)	0.808	±	0.124	2.089	±	0.375
Col1 (μg/ml)	0.796	±	0.263	0.119	±	0.033