A thin honeycomb-patterned film as an adhesion barrier in an animal model of glaucoma filtration surgery.

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biodegradable film for glaucoma filtration surgery in rabbits.
Methods. A 7μm-thick film made from poly(L-lactide-co-ε-caprolactone) was placed in the
subconjunctival space in one eye of rabbits, with or without full-thickness filtration surgery. The film
had a honeycomb-patterned surface that faced the subconjunctival Tenon tissue and the other side
was smooth. Filtration surgery was also performed in the fellow eye, which received either no
adjunctive treatment or 0.4 mg/ml mitomycin C (MMC; n=6 each). Intraocular pressure (IOP)

measurements and bleb evaluations using ultrasound biomicroscopy were performed periodically for 28 days after surgery followed by histological observation.

Results. Postoperative IOPs of the film-treated eyes were significantly lower than that of control eyes from day 10 to day 28 ($P<0.05$), but were not significantly different from those of MMC-treated eyes. The subconjunctival filtration space, detected by ultrasound biomicroscopy, disappeared in five control eyes, one MMC-treated eye, but none of the film-treated eyes. A bleb leak occurred postoperatively in two MMC-treated eyes. Histologically, in eyes without filtration surgery, fibrotic tissue with the film partly attached to it was noted on the honeycomb side, but was minimal on the sclera that faced the smooth side of the film. In eyes with filtration surgery, the honeycomb-patterned film lined the inner bleb wall with minimal inflammatory reaction.

Conclusions. The thin honeycomb-patterned film that attached to the inner bleb wall worked as an adhesion barrier in glaucoma filtration surgery in rabbits, which is worthy of further investigation.

A Thin Honeycomb-Patterned Film as an Adhesion Barrier in an Animal Model of Glaucoma Filtration Surgery

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Introduction

Success of filtration surgery depends mostly on suppression of postoperative scarring around the filtration site. Although anti-scarring agents, such as mitomycin C (MMC) and 5-fluorouracil (5-FU), help prevent postoperative scarring and improve glaucoma surgical outcomes, [1-7] their use has been associated with undesirable side effects, such as scleral ischemia, hypotony, corneal problems, endophthalmitis, filtering bleb dysesthesia and cataract. [8-13] Recently, to avoid such postoperative complications caused by MMC and 5-FU, various anti-scarring chemicals and mechanical adhesion barriers have been used in glaucoma filtration surgery in animal models. [14-31] Although favorable results were reported, no alternative methods to MMC or 5-FU treatment have been widely adapted until now.

A honeycomb-patterned film is a thin biodegradable film made from poly(L-lactide-co-ε-caprolactone).[32]   It has a honeycomb structure that allows tissue attachment on one side and a smooth structure that prevents adhesion on the other side. Therefore, the film can stick to the tissue surface on the honeycomb side, while preventing adhesion of other tissues to the smooth side. Recently, Narita et al [33] reported that this film prevented wound adhesion in a dog model of cardiac surgery. Therefore, this film, when attached to the subconjunctival Tenon tissue on the honeycomb side, may work as an adhesion barrier on the smooth side and prolong bleb survival after glaucoma filtration surgery. The purpose of this pilot study was to investigate the effectiveness and safety of a honeycomb-patterned film in an animal model of glaucoma filtration surgery.

Materials and Methods

Fourteen New Zealand White rabbits (weight range, 2.5 kg to 3.0kg) were used. All procedures involving animals were performed according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Experimental procedures were approved by the Committee on Animal Experimentation of Kanazawa University, Takara-machi campus, Japan.

Surgery was performed on both eyes of all rabbits and all surgeries were performed by the same surgeon (T.O). The bilateral surgery protocol was adapted because different rabbits had significantly different baseline IOPs and wound-healing reactions. Surgical procedures performed unilaterally as a preliminary experiment caused no serious postoperative complications (data not shown).

The Honeycomb-Patterned Film

The honeycomb-patterned film used in this study was about 7 μm thick, biodegradable and made from poly(L-lactide-co-ε-caprolactone) (Fig. 1). Briefly, poly(L-lactide-co-ε-caprolactone) was dissolved in chloroform at a concentration of 5 mg/ml at room temperature. A surfactant, dioleoylphosphatidylethanolamine (0.5% w/v), was added based on the weight of poly(L-lactide-co-ε-caprolactone). The honeycomb-patterned films were fabricated by simply casting under a current of highly humid air. [32]

The film has a unique surface structure, characterized by pores of a

hexagonal shape interconnecting horizontally along the film surface on one side. The opposite side of the film is quite smooth. [32] This honeycomb structure has a considerable influence on the adhesion, migration and proliferation of cells and the opposite smooth side prevents adhesion of the wound following surgical treatments. [32] Dissolution time of the film depends on the size and thickness of the film and the proportion of poly(lactic acid) to polycaprolactone. The film used in this study consisted of approximately 88% poly(lactic acid) and 12% polycaprolactone. It took approximately one year before a comparable film dissolved in dry tissue (unpublished data from Teijin Corporation).

Subconjunctival Placement of the Honeycomb-Patterned Film without Filtration Surgery

Prior to experimental filtration surgery, we examined the effect of film that was placed in the subconjunctival space. Two rabbits were used. The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). An eyelid speculum was inserted and the eye was fixed with a corneal traction suture (8-0 vicryl; Ethicon, Piscataway, NJ). After a conjunctival incision 8 mm from the limbus, the sclera was exposed anteriorly to the limbus in both eyes. The honeycomb-patterned film, 6×6 mm^2, was sutured with 10-0 nylon on the sclera in one eye, with the honeycomb side facing up. Nothing was placed in the fellow eye (control). The conjunctival wound was closed with a running 10-0 nylon suture. Postoperative examination with slit-lamp biomicroscopy and indirect ophthalmoscopy was performed for 4 weeks. Subsequently, all rabbit eyes were

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subjected to histological evaluation.

Filtration Surgery Procedure

Twelve rabbits were randomly assigned to one of two groups (6 rabbits in each group). The filtration site of one eye received the honeycomb-patterned film, while that of the fellow eye was treated as follows: no adjunctive treatment (group 1) or MMC (group 2). In order to minimize the subjective bias regarding the surgical procedure, the operation was started in one eye and it was after creation of the scleral flap that the eye was randomly assigned the honeycomb-patterned film or not. Also, the dimension of the scleral flap and the sclerostomy was strictly checked by an assistant surgeon (T. H) during the surgery.

Rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). An eyelid speculum was inserted and the eye was fixed with a corneal traction suture (8-0 vicryl). The conjunctiva in the superior-nasal quadrant was incised 8 mm from the limbus and was carefully dissected anteriorly to the limbus. Then a 3×3 mm partial-thickness scleral flap was outlined. For the MMC-treated eyes (group 2), a surgical sponge measuring 5×5 mm was cut and soaked in 0.4 mg/ml MMC (Kyowa Hakko Kogyo Co, Tokyo, Japan). It was placed on the sclera, under the conjunctival flap, for 3 minutes. After MMC application, this area was irrigated thoroughly with balanced salt solution (BSS; Santen Pharmaceutical Co., Ltd., Osaka, Japan). A sclerostomy (1×2 mm) was performed under the scleral flap using a Kelly Descemet's membrane punch and the scleral flap was then

dissected. For the honeycomb-patterned film placement (all groups), a piece of film (6×6 mm) was placed on the sclerostomy site. The film, turned honeycomb side toward the subconjunctival Tenon tissue, was loosely secured by suturing on the sclera with 10-0 nylon at the two corners near the limbus and at the center of the two corners on the fornix side (Fig. 2). A peripheral iridectomy was not performed to avoid bleeding and the conjunctival incision was closed with a running 10-0 nylon suture. After surgery, a subconjunctival injection of steroid (1 mg of triamcinolone: Kenakolot-A; Bristol Pharmaceuticals KK, Tokyo, Japan) was performed away from the filtration site to decrease postoperative inflammation, and ofloxacin ointment (Santen, Pharmaceutical Co., Ltd., Osaka, Japan) was applied.

Preoperative and Postoperative Examinations

After topical anesthesia of 0.4% oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd., Osaka, Japan), IOP was measured using a Tonopen (Mentor, Norwell, MA) at baseline and 3, 7, 10, 14, 17, 21, 24 and 28 days after surgery. IOP measurements were conducted in a masked fashion. IOP was measured three times per eye and a mean reading was documented, except if the confidence interval was less than 95%. Under the general anesthesia described above, the conjunctiva, cornea, and anterior segment of each eye were recorded with a digital video (GVD-1000; SONY CO., Tokyo, Japan) under a surgical microscope following postoperative IOP measurements. Thereafter, ultrasound biomicroscopy (UBM; UD-6000, Tomey, Nagoya, Japan) was

performed to examine blebs. At the end of the examination, each eye received

levofloxacin eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan).

Animals were examined at specified intervals for 28 days after surgery

followed by histological evaluation.

Histological Evaluation

Animals were killed on day 28 by an overdose intravenous injection of

sodium pentobarbital under deep general anesthesia. Corneoscleral blocks of

the operation site (10×10 mm^2) with overlying conjunctiva were dissected from

all eyes. All blocks were fixed in 20% paraformaldehyde and embedded in

paraffin. Sections through the center of the operation site were stained with

hematoxylin and eosin (H&E) and observed by light microscopy. Histological

evaluations were conducted in a masked fashion regarding the surgical

procedures.

Statistical Evaluation

In eyes that received filtration surgery, comparison of IOP between

fellow eyes was performed using the paired t-test. Presence of filtration space

detected by UBM on day 28 was compared between film-treated and fellow eyes

by the χ^2 test. For all analyses, P-values less than 0.05 were considered

statistically significant.

<u>Results</u>

Subconjunctival Placement of the Honeycomb-patterned Film without Glaucoma Filtration Surgery

In honeycomb-patterned film-treated eyes, no significant inflammatory findings were observed in the follow-up period; that is, the anterior segment was silent and the conjunctiva and sclera were not remarkably injected as observed for control eyes. Histopathologically, several multinucleated macrophages were observed but foreign body granuloma formation was not detected. Lymphoid or neutrophil infiltration in the subconjunctival space was minimal in both the film-treated and control eyes (Fig. 3A, C). In the film-treated eyes, fibrotic tissue with the film partly attached to it was noted on the honeycomb side of the film, but was minimal on the scleral side, which faced the smooth side of the film (Fig. 3A, B). In control eyes, fibrotic tissue was observed on the sclera (Fig. 3C).

Intraoperative Administration of the Honeycomb-patterned Film Compared with No Treatment or MMC in Glaucoma Filtration Surgery

Postoperative IOP changes

Mean IOP was significantly lower than baseline until day 28 in honeycomb-patterned film-treated and MMC-treated eyes. In group 1, the IOP of honeycomb-patterned film-treated eyes was significantly lower than that of control eyes from day 10 to day 28 ($P<0.05$; Fig. 4A). Postoperative IOPs of the film-treated eyes were not significantly different from MMC-treated eyes (Fig. 4B).

Surgical Microscope Examinations

No eyes exhibited severe postoperative inflammation in the anterior

chamber. All corneas stayed clear and no blebitis or endophthalmitis was

observed during the postoperative period. The degree of conjunctival injection,

which was observed during days 3 to 14 and decreased thereafter, was similar

among control, honeycomb-patterned film-treated and MMC-treated eyes. Five

out of 6 MMC-treated blebs became totally and persistently avascular by days 7

to 14. Among the avascular blebs, two MMC-treated blebs had transconjunctival

aqueous leakage from days 21 to 24. Bleb leak and secondary hypotony

continued until day 28. All 12 eyes treated with the honeycomb-patterned film

had no bleb leak.

Bleb Appearance Examined by UBM

The subconjunctival filtration space after filtration surgery was monitored

by UBM. Disappearance of the filtration space occurred in five of 6 control eyes

(group 1), one of 6 MMC-treated eyes (group 2), but none of the 12 film-treated

eyes (groups 1 and 2). At day 14, 67% of control eyes had no filtration space.

The timing of the disappearance of the filtration space was almost coincident

with an IOP increase to baseline level. On day 28, the presence of the filtration

space in film-treated eyes was significantly more frequent than that of control

eyes ($P<0.05$), but not significantly different from that of MMC-treated eyes. In

film-treated eyes, a septum that suggested the presence of the film was imaged

by UBM (Fig. 5). Various blebs of large, flat and normal size were observed early

in the postoperative period in the film-treated eyes (Figs. 6A, B). However, all of

the flat-looking blebs became spontaneously elevated later (Fig. 6C).

Histopathological Features

The degree of subconjunctival fibrotic response in the surgical area was

similar among control and honeycomb-patterned film treated eyes, but was

markedly suppressed in MMC-treated eyes by H&E staining (Fig. 7). The

conjunctiva of the bleb area appeared normal in all control and

honeycomb-patterned film-treated eyes, but was disrupted in eyes with

avascular blebs in 5 of 6 MMC-treated eyes. Severe inflammation was absent in

all eyes and no signs of toxicity to the surrounding ocular tissues were observed

in honeycomb-patterned film treated eyes. The film lined the inner bleb wall with

minimal inflammatory reactions.

<u>Discussion</u>

In this study, the use of a honeycomb-patterned film appeared to

promote IOP reduction and bleb survival in glaucoma filtration surgery in rabbits.

In addition, no obvious intraoperative or postoperative complications were noted

using the film. Although MMC-treated eyes had a similar postoperative IOP

decrease to honeycomb-patterned film-treated eyes, avascular bleb formation

and transconjunctival aqueous leakage were found postoperatively in some

eyes. Histology showed a marked suppression of the postoperative fibrotic

response and disruption of the conjunctival epithelium in MMC-treated eyes as

reported previously. [34] In contrast, the film-treated eyes showed no abnormalities in the conjunctival epithelium.

Inhibition of wound adhesion in glaucoma filtration surgery to promote bleb survival can be classified as anti-scarring chemicals or mechanical adhesion barriers. Anti-scarring chemicals that have recently been tested in experimental glaucoma filtration surgery include inhibitors or antibodies against transforming growth factor, [14, 15] an antibody against connective tissue growth factor [15] an antibody against matrix metalloproteinase inhibitor, [16, 17] matrix metalloproteinase-3 , cyclosporine, [18] bleomycin, [19] suramin, [20, 21] Rho-associated protein kinase inhibitor, [22] p21$^{WAF-1/Cip-1}$, [23] fluorescence generated photoreaction products [24] and methylcellulose. [25] Amniotic membrane transplantation, which showed suppression of wound healing in experimental filtration surgery in agreement with its ability to down regulate TGFß signaling, [26] may also have a space-keeping effect as a mechanical barrier.

As for mechanical barriers, Gelfilm, an absorbable film made from pig gelatin, was the first to be used experimentally and also clinically in 1955, [27] but was later found to cause severe inflammation when put in the anterior chamber. [28] More recently, Interceed, an oxidated regenerated cellulose, and Seprafilm, a polymer film made from sodium hyaluronate and carboxymethylcellulose, which are in clinical use for prevention of adhesions in gynecological and abdominal surgery, were tested for experimental glaucoma filtration surgery, but failed to show effectiveness. [29] Cross-linked hyaluronate hydrogel [30] and a small disk made of a biodegradable, porous collagen matrix were reported to promote postoperative IOP reduction compared to control eyes in experimental filtration

surgery. As a non-reabsorbable barrier, expanded polytetrafluoroethylene has been successfully used in trabeculectomy in human eyes. [31] Compared to other adhesion barriers, the distinguishing features of the honeycomb-patterned film are the unique surface structure on one side and its thinness. Our results suggest that the honeycomb-patterned surface caused different cellular reactions from the flat surface, which helped the film attach to the subconjunctival Tenon tissue. Furthermore, the 7 μm thickness of the film, which may not have a significant space-keeping effect in contrast to the much thicker materials used in other reports, was found to be sufficient for an adhesion barrier. Even though the material is reabsorbable, a thinner adhesion barrier may cause less traumatic effects to the overlying conjunctiva.

Although the honeycomb-patterned film (30 mm in diameter) was used without suturing in experimental cardiac surgery, [33] the film was sutured onto the sclera in this study so that it stayed precisely in the desired area because the size of the film was small (6x6 mm) and the subconjunctival space is highly mobile concomitantly with eye movements. The honeycomb-patterned film was attached to the subconjunctival Tenon tissue by allowing formation of fibrotic tissue on the honeycomb side when it was placed in the subconjunctival space without filtration surgery. After filtration surgery in a typical case, the film successfully attached to the Tenon tissue early in the postoperative period but in some cases, the film did not attach to it. This result suggests that over-filtration early in the postoperative period may prevent the film from attaching to the Tenon tissue. Although postoperative bleb size did not influence bleb survival, a more adhesive honeycomb design, which allows the film to attach to the Tenon

tissue more securely, may be desirable. In contrast, the smooth side of the film worked as a mechanical adhesion barrier between the subconjunctival Tenon tissue and the sclera, given that all of the flat-looking blebs observed early in the postoperative period in some film-treated eyes became spontaneously elevated afterwards.

Recently, UBM has been widely used to evaluate filtering blebs after glaucoma filtration surgery. [35] However, there have been no reports of bleb observation by UBM in animal models. In this study, UBM successfully showed the subconjunctival filtration space, even in a flat-looking bleb, and a septum, which suggested the presence of the honeycomb-patterned film. Therefore, UBM may be useful to evaluate blebs in animal models of glaucoma filtration surgery.

In conclusion, a thin, honeycomb-patterned film attached to the inner bleb wall worked as an adhesion barrier in glaucoma filtration surgery in rabbits. The effect on postoperative IOP reduction and bleb survival was comparable to that of MMC-treated eyes. This treatment may decrease the risk of serious postoperative complications of filtration surgery frequently found with MMC use, such as bleb leakage and bleb infection. Further studies are needed to optimize film design regarding the honeycomb size, dissolution and absorption time, and to determine the potential use of the honeycomb-patterned film as an adhesion barrier in glaucoma filtration surgery.

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Legends

Figure 1. A scanning electron microscope image of the surface structure of the honeycomb-patterned film. The pore size is approximately 5 μm in diameter. This characteristic structure enables it to attach to the tissue without glue. (Original magnification: × 5,000).

Figure 2. Schema of the surgical procedures used to place the honeycomb-patterned film. 10-0 nylon suture (black arrow); the honeycomb-patterned film (arrow head); filtration site (white arrow); the area of dissected scleral flap (dashed line); the conjunctival incision line (asterisks).

Figure 3. Histopathological features of the effects of the honeycomb-patterned film on the sclera. A: control eye; B: honeycomb-patterned film-treated eye; C: higher magnification of B. Several multinucleated macrophages were observed, but foreign body granuloma formation was not detected. Lymphoid or neutrophil infiltration in the subconjunctival space was minimal in both film-treated and control eyes. In the film-treated eye, fibrotic tissue (asterisks) was noted on the honeycomb side of the film (arrows), between the subconjunctival Tenon tissue and the film, but was minimal on the smooth side of the film, between the film and the sclera. At a higher magnification, the honeycomb structure of the film (arrows), which was partly attached to the fibrotic tissue, was observed. In a control eye, fibrotic tissue was observed on the sclera. T: subconjunctival Tenon tissue; S: sclera. Sections were stained with hematoxylin and eosin. (Original magnification: A × 200; B × 200; C × 1000).

Figure 4. IOP changes over time in group 1 (A) and group 2 (B).The mean IOP in honeycomb-patterned film-treated eyes in group 1 was significantly lower than

that of control eyes in group 1 from day 10 to day 28 (*P<0.05). There was no

significant difference in the mean IOP between honeycomb-patterned

film-treated and MMC-treated eyes in group 2.

Figure 5. Typical bleb appearance of honeycomb-patterned film-treated eyes on

postoperative day 3 (A, C) and day 28 (B, D). A, B: photographs taken with a

surgical microscope. A mildly elevated diffuse bleb remained throughout the

experimental period. Conjunctival injection noted on postoperative day 3

gradually disappeared. C, D: images of ultrasound biomicroscopy. A large

filtration space (asterisks) with a septum, which suggested the presence of the

honeycomb-patterned film (dashed lines), was observed. The film attached to

the subconjunctival Tenon tissue anteriorly.

Figure 6. Various bleb appearances of honeycomb-patterned film-treated eyes

at early postoperative periods. A large bleb (A) and a flat-looking bleb (B) on

postoperative day 7. The honeycomb-patterned film did not attach to the

subconjunctival Tenon tissue in the large bleb. The flat-looking bleb became

spontaneously elevated afterwards (day 28: C) and the honeycomb-patterned

film attached to the subconjunctival Tenon tissue. Arrows point to septa, which

suggest the presence of the honeycomb-patterned film.

Figure 7. Histopathological features of the surgical area in eyes with blebs and

IOP reduction. A: control eye, B: honeycomb-patterned film-treated eye, C:

MMC-treated eye with no bleb leak, D: MMC-treated eye with bleb leak. The

cornea is toward the right side in each photo. Asterisks indicate the

subconjunctival filtration space. Arrows point to the conjunctival epithelium. The

degree of subconjunctival fibrotic response was similar among control and

honeycomb-patterned film-treated eyes, but was markedly suppressed in MMC-treated eyes. The conjunctival epithelium of the bleb area appeared normal in control (A) and honeycomb-patterned film-treated eyes (B), but was attenuated in the MMC-treated eye with no bleb leak (C). In addition, a conjunctival epithelial defect was observed in the MMC-treated eye with bleb leak (D). Honeycomb-patterned film lining the inner bleb wall was observed (black arrow in B). Sections are stained with hematoxylin and eosin. (Original magnification: A × 100; B × 100; C × 100; D× 50).

* Response to Reviewers

Okuda et al. A honeycomb-patterned film that prevents wound adhesion in filtration surgery
- 1 -

April 21, 2008

Editor-in-Chief,
George A. Cioffi, M.D.
Devers Eye Institute,
1040 NW 22nd Ave., Suite 200,
Portland, OR 97210
jcioffi@deverseye.org

Re: JOG-D-08-00051 (A thin honeycomb-patterned film as an adhesion barrier in an animal model of glaucoma filtration surgery)

Dear Dr. Cioffi:

Thank you very much for your letter of April 4th, 2008, with regard to our manuscript (JOG-D-08-00051) together with the comments from the Editorial Board Member and reviewer. We are sending herein the revised manuscripts and figures. Our corrections according to the reviewers' statements are as follows. We believe the manuscript has been improved satisfactorily.
Thank you very much in advance for your kind consideration.

Sincerely yours,

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Response to comments

To the reviewer 1:

1. I would like to see the MMC concentration included in the abstract and an expanded legend for figure 4 which I found confusing.

Reply: We added the concentration of MMC (0.4 mg/ml) to the abstract of the revised manuscript (page 2, line 10). The legend for figure 4 was changed to avoid confusion (page 21, lines 2 – 3). We also added "group 1" and "group 2" to figure 4A and 4B, respectively, in the revised manuscript.

To the reviewer 2:

1. Figure 2 – a white arrowhead unaccounted for in the figure legend.

Reply: A white arrowhead was not necessary for figure 2, and it was deleted in the revised manuscript. The white arrow remains and depicts the filtration site, which is stated in the legend.

2. Figure 3 – It appears Figure 3 may be out of order.

Reply: We are very sorry for this careless mistake. We corrected the legend of figure 3 in the revised manuscript (page 20, lines 11 – 12 and line 22).

3. Figure 7 –

   More labels are needed to orient the viewer in A.

Reply: We added more labels (asterisks and arrows) to figure 7 in the revised manuscript.

B appears to show a ruptured lens capsule or Descemet's・・

Reply: As pointed out by the reviewer, it was actually a ruptured lens capsule. It was not an intraoperative or postoperative complication, but was ruptured when the corneoscleral block was dissected from the eye for histological evaluation. Because we do not think it is important to show the lens capsule in the figure, we took another photo omitting the lens capsule and made a new figure.

C does not appear to show the bleb wall – due to leaking one assumes? An intact MMC bleb may serve as a better comparison to 7B.

Reply: Figure 7C shows a conjunctival epithelial defect in the eye with a bleb leak. In response to the reviewer's suggestion, we made a new figure with lower magnification for better orientation. We would still like to show the leaking bleb in the revised manuscript because it is one of the most serious complications of MMC use. We have included it as figure 7D. However, we also added a histological section of an intact MMC bleb (figure 7C) to the revised manuscript as the reviewer suggested.

Figure 1
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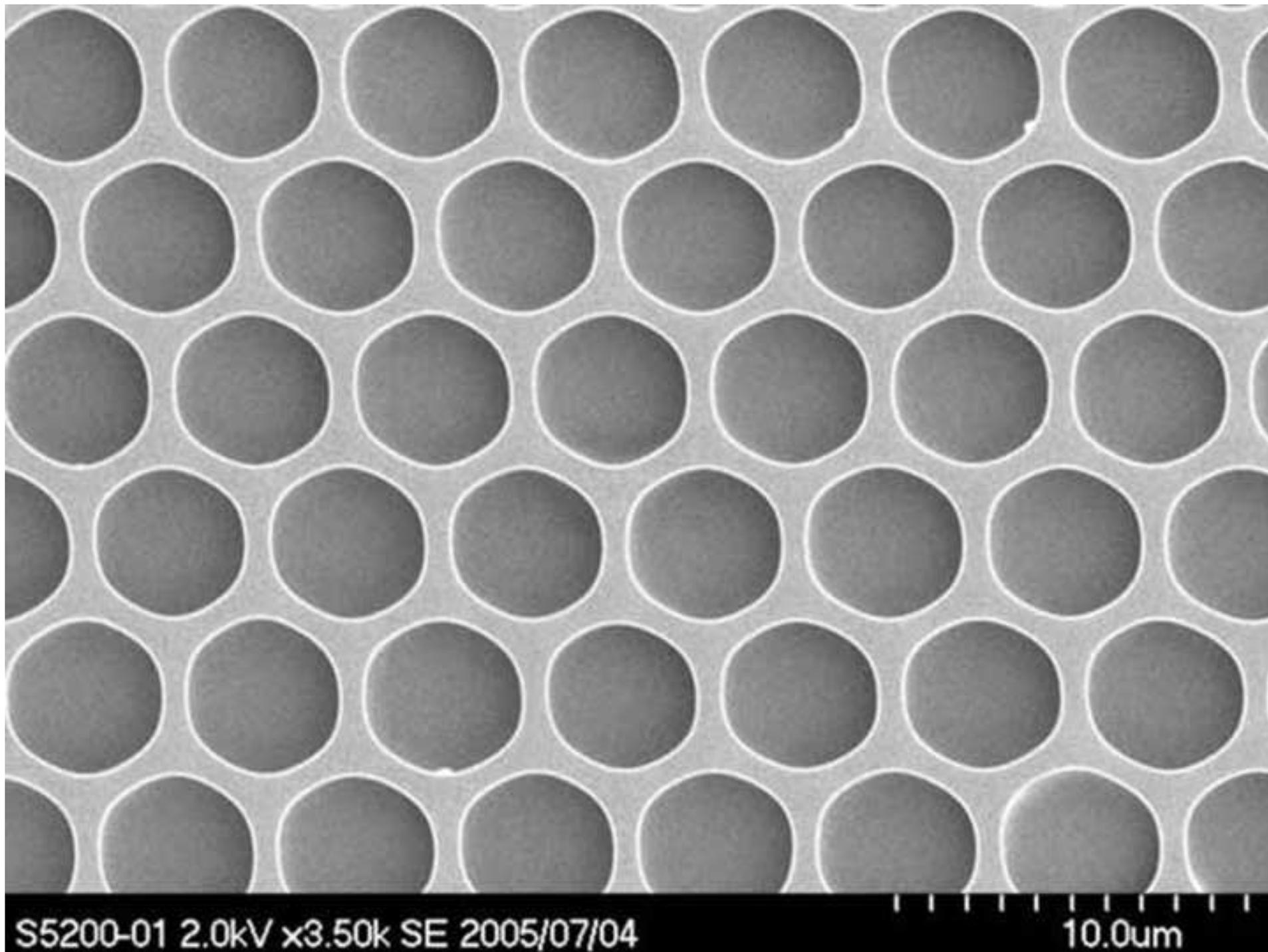
Figure 2
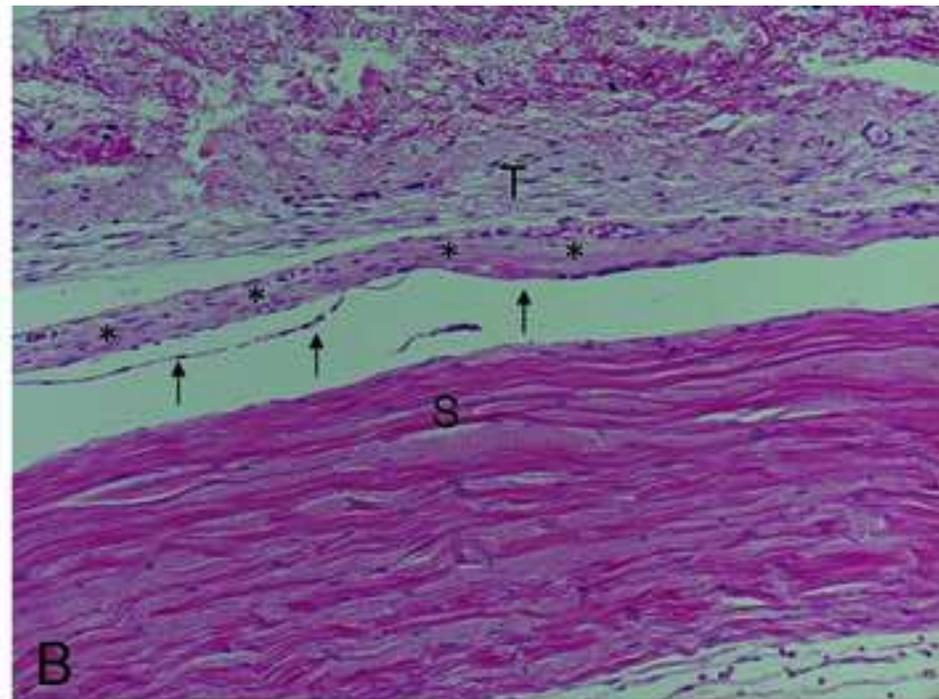**Click here to download high resolution image**
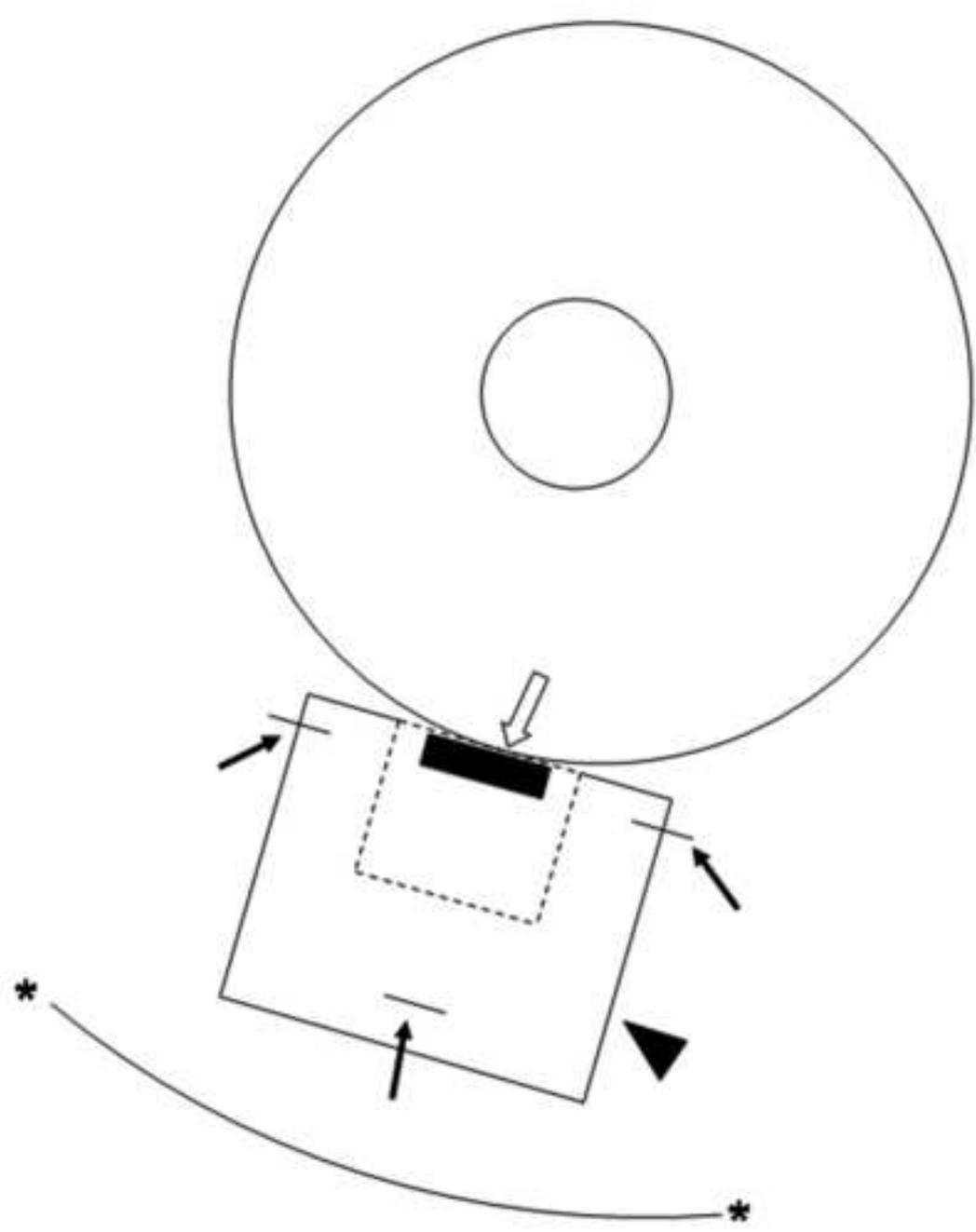
Figure 3

Figure 3
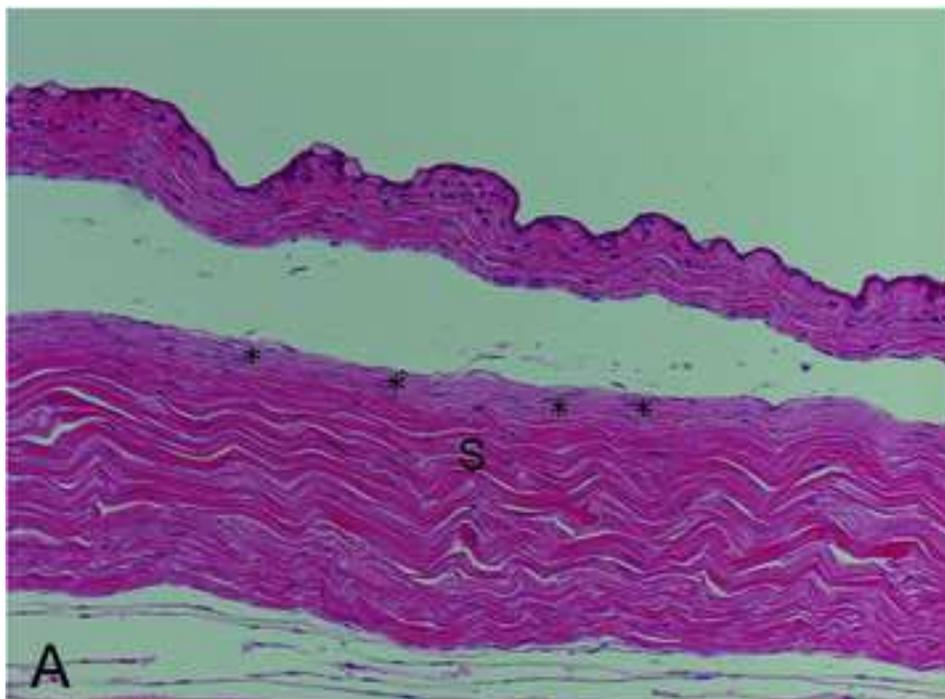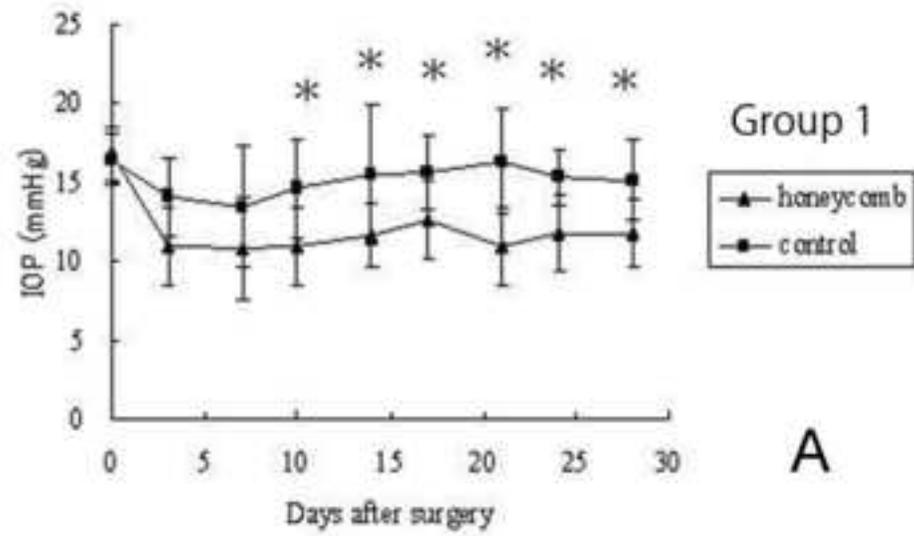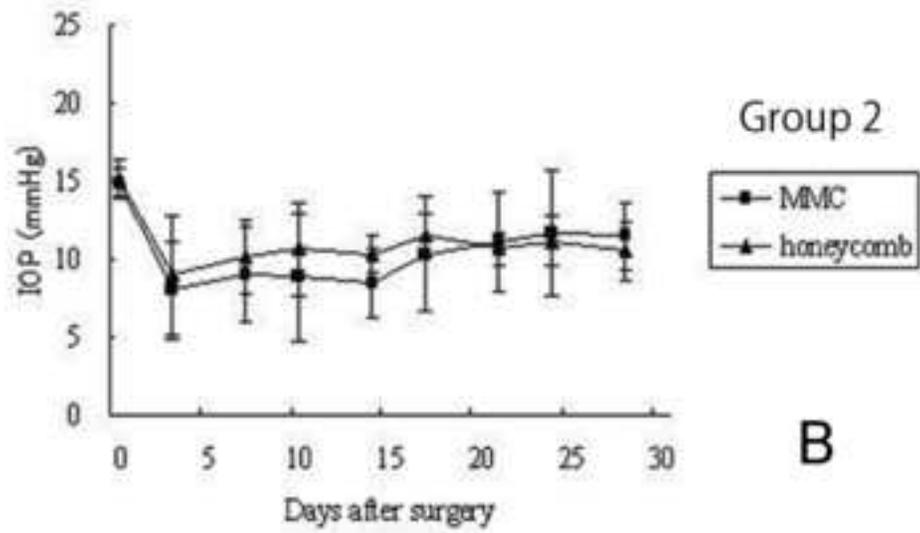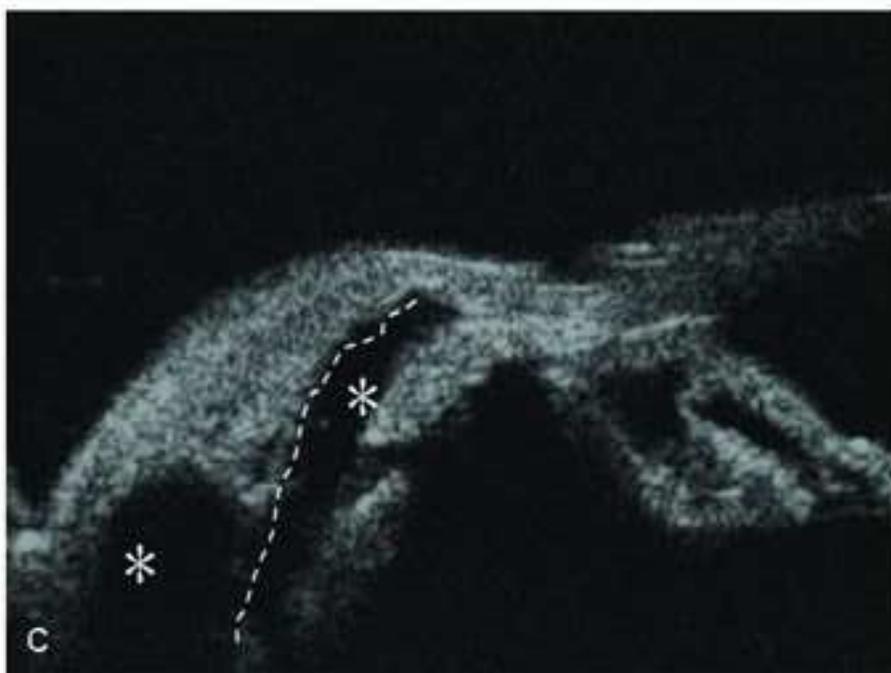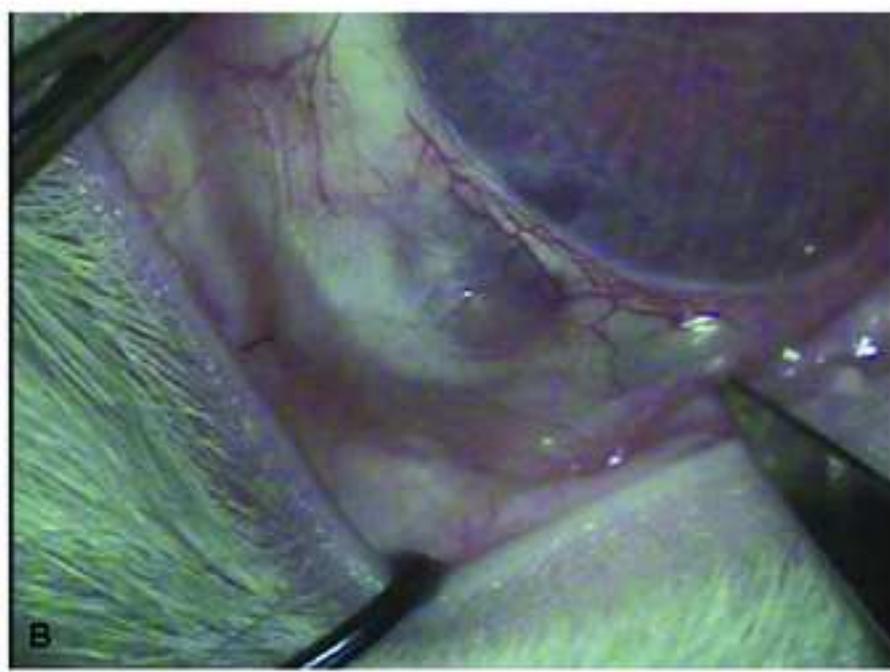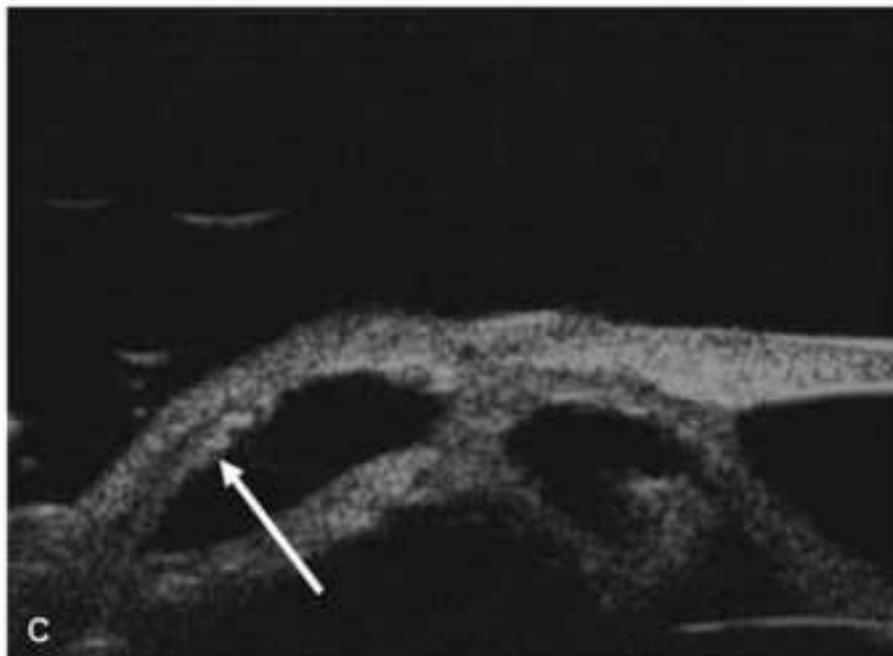[Click here to download high resolution image](#)
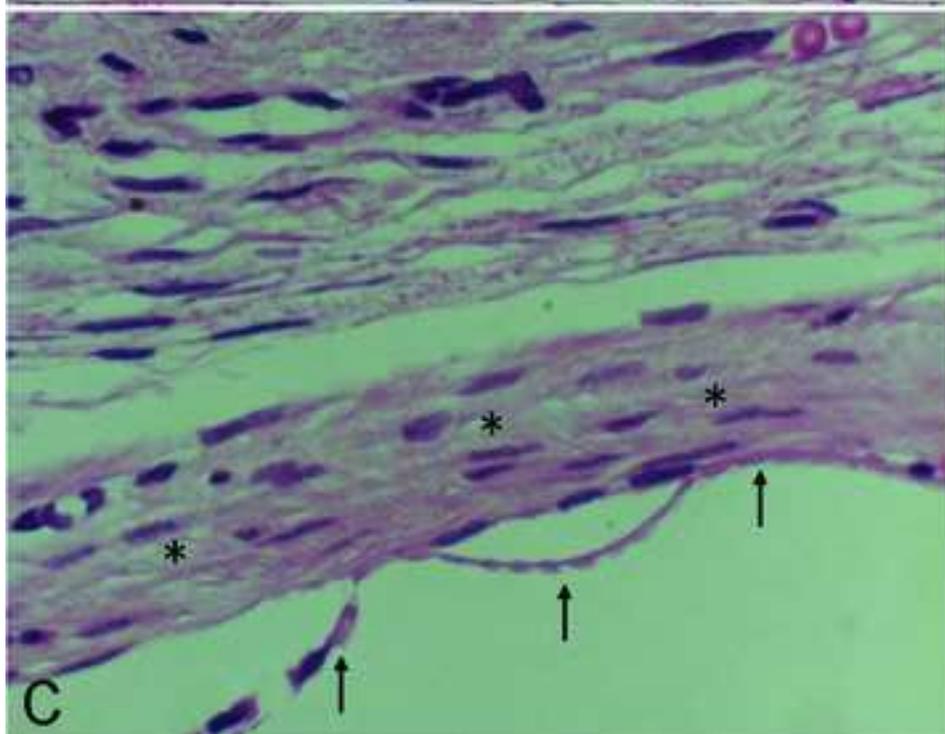
Figure 4
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A

B

Figure 5
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Figure 6
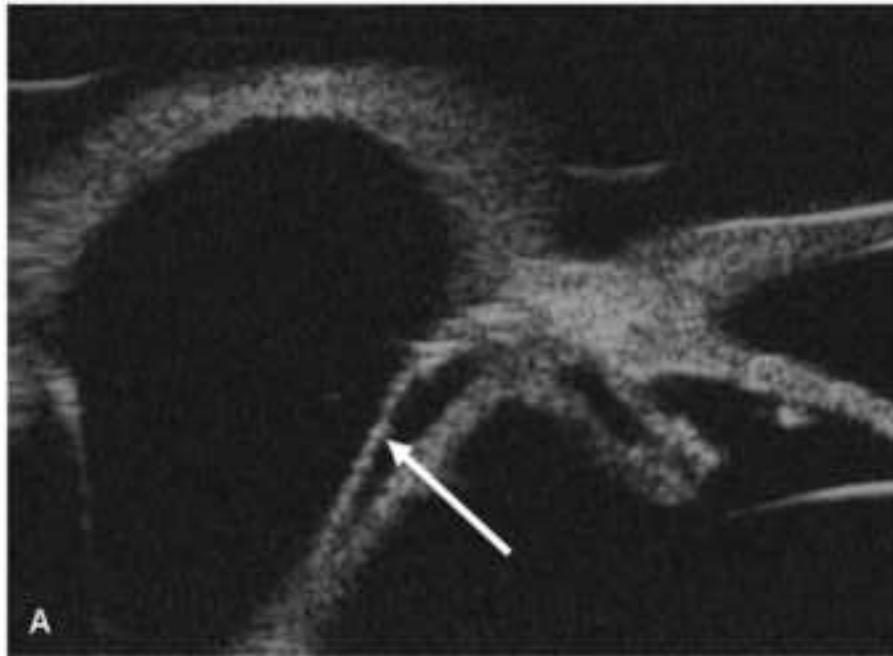**Click here to download high resolution image**

Figure 7
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