

# Tissue Response to Implanted Ahmed Glaucoma Valve with Adjunctive Amniotic Membrane in Rabbit Eyes

Ji Woong Lee<sup>a,b</sup> Won Young Park<sup>c</sup> Eun Ah Kim<sup>e</sup> Il Han Yun<sup>d</sup>

<sup>a</sup>Department of Ophthalmology, <sup>b</sup>Medical Research Institute and <sup>c</sup>Department of Pathology, Pusan National University Hospital, and <sup>d</sup>Department of Ophthalmology, Busan Paik Hospital, Inje University College of Medicine, Busan, and <sup>e</sup>Department of Ophthalmology, Daegu Fatima Hospital, Daegu, Korea

## Key Words

Ahmed glaucoma valve · Amniotic membrane · Fibrous capsule · Histopathological findings · Myofibroblast transdifferentiation

## Abstract

**Aims:** To investigate the histopathology of the fibrous capsule around Ahmed glaucoma valves (AGVs) implanted with adjunctive amniotic membranes in rabbits. **Methods:** AGV implantation with or without adjunctive amniotic membrane was performed in a single eye of 20 albino rabbits. The upper surface of the AGV body was covered with amniotic membrane in the study group. After 2 months, histology was used to compare the thickness and characteristics of the fibrous capsule, transdifferentiation of myofibroblasts, and density of blood vessels and leukocytes between the study and control groups. **Results:** The fibrous capsule along the roof of the bleb was composed of compact collagen fibers with minimal vascularization in the control group. In contrast, in the study group, the fibrous capsule was looser and had a more disorganized collagen architecture. The thickness of the fibrous capsule and the myofibroblast layer was significantly thinner in the study group than in the control group ( $p < 0.001$ ). The number of CD31-positive blood ves-

sels did not differ between the two groups ( $p = 0.235$ ). CD45-positive inflammatory cells were more frequently observed in the study group than the control group ( $p = 0.001$ ). The groups did not differ in the thickness of the fibrous capsule or myofibroblast layer, or the density of blood vessels and leukocytes along the floor of the bleb. **Conclusions:** Adjunctive amniotic membranes could reduce the risk of encapsulation and aqueous outflow resistance by altering the tissue response to implanted AGVs and subsequent formation of a loose thin capsule.

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## Introduction

The Ahmed glaucoma valve (AGV) is one of the frequently adopted glaucoma drainage implants today to treat refractory glaucoma when conventional filtration surgery has failed. The AGV is also used to treat neovascular glaucoma, uveitic and secondary glaucoma with a wide conjunctival scar from trauma or previous ocular surgery for which the success rate of conventional filtration surgery is low [1]. However, glaucoma implants including AGVs stimulate a foreign body fibrotic response that encapsulates the glaucoma implant body and in-

creases aqueous outflow resistance through the capsule. This can lead to elevated intraocular pressure (IOP) requiring further surgical intervention [2, 3].

Histopathologically, the inner layer of the encapsulated bleb facing the AGV surface consists of compressed collagen fibers characterized by a pronounced transformation of fibroblasts into myofibroblasts [3]. The transdifferentiation of fibroblasts into myofibroblasts is a crucial step in wound healing and scar formation [4] and is characterized by the de novo synthesis of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and the incorporation of  $\alpha$ -SMA into actin stress fibers as part of the contractile apparatus [5]. Myofibroblasts represent an activated fibroblast phenotype. Therefore, enhanced  $\alpha$ -SMA expression indicates the presence of activated fibroblasts with increased synthesis of extracellular matrix proteins, growth factors and integrins [6–8].

Myofibroblast transdifferentiation is regulated by the cytokine transforming growth factor- $\beta$  (TGF- $\beta$ ) [4, 9]. Therefore, downregulation of TGF- $\beta$  signaling is a strategy for preventing scarring during wound healing. A previous study reported that monoclonal antibodies against TGF- $\beta_2$  inhibit conjunctival scarring and improve glaucoma filtration surgery outcome in an animal model [10]. However, a clinical study found that the CAT-152 monoclonal antibody was not effective at preventing trabeculectomy failure [11].

Adjunctive antimetabolites have been used with glaucoma implant surgery to minimize bleb encapsulation. However, mitomycin C (MMC) did not increase the short-term or intermediate success rates of AGV implantation [12]. Rather than improving survival, intraoperative use of MMC was associated with shorter survival 2 years after AGV implantation during the first 2 years of life. This might be because MMC-induced tissue death stimulates a reactive fibrosis around the AGV in very young eyes [13]. In addition, a previous report noted increased postoperative complications such as hypotony and tube exposure in patients who underwent AGV implantation with adjunctive MMC [14].

The amniotic membrane has antiscarring and anti-inflammatory properties [15]. The antifibrotic effect of the amniotic membrane is mediated by downregulating TGF- $\beta$  signaling and myofibroblast differentiation [16, 17]. The amniotic membrane precludes polymorphonuclear cell infiltration and facilitates macrophage apoptosis [15, 18]. The amniotic membrane also exhibits poor immunogenicity and has a high hydraulic conductivity [15, 19].

Although a previous clinical study reported that adjunctive amniotic membrane reduced the risk of bleb encapsulation after AGV implantation [20], the effects of the amniotic membrane on myofibroblast transdifferentiation and extent of fibrosis around the polypropylene valve and reservoir body have not been studied in detail. Here, we compare the histopathological findings of capsules surrounding an adjunctive amniotic membrane and AGVs with those of conventional AGVs in a rabbit model.

## Materials and Methods

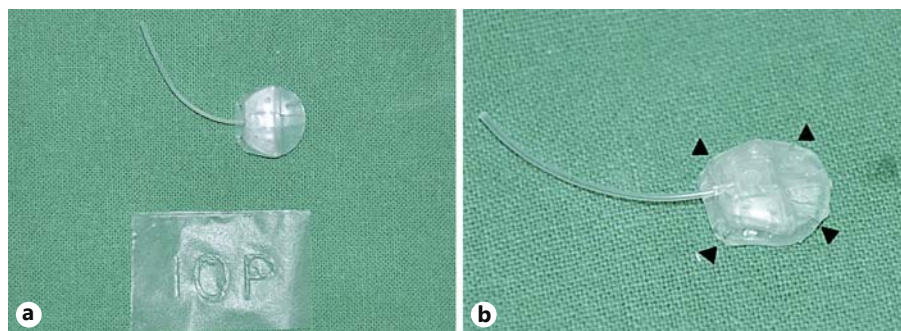
### *Surgical Implantation*

AGV flexible plate implants (model FP8; New World Medical Inc., Rancho Cucamonga, Calif., USA) were used. All animal experiments were carried out in accordance with the guidelines of the Association for Research and Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Pusan National University Animal Care Committee.

Twenty albino New Zealand rabbits, 12–14 weeks old and weighing 2–3 kg, were divided into two groups. The right eye of all rabbits was used for AGV implantation. The study group consisted of 10 eyes that received adjunctive amniotic membranes and AGVs. The dried form of human amniotic membrane (Ambiodry2, IOP Inc., Costa Mesa, Calif., USA) was cut to fit the upper surface of the polypropylene valve/reservoir body of the AGV. The upper surface of the polypropylene valve/reservoir body adjacent to Tenon's capsule and conjunctiva was covered with processed human amniotic membrane with the epithelium/basement membrane side up (fig. 1a). The lower surface of the AGV body adjacent to the sclera was left without amniotic membrane. The amniotic membrane was hydrated with sterile saline solution and secured to the 4 edges of the polypropylene valve/reservoir body to cover the upper surface of the AGV using four 10-0 nylon sutures (fig. 1b). The control group consisted of 10 eyes that underwent conventional AGV implantation without adjunctive amniotic membrane.

The rabbits underwent general anesthesia by intramuscular injection of tiletamine-zolazepam (10 mg/kg; Zoletil 50, Virbac Lab, Carros, France) and xylazine hydrochloride (5 mg/kg; Rompun 2%; Bayer Korea, Seoul, South Korea) and topical anesthesia with proparacaine eyedrops (Alcaine; Alcon, Fort Worth, Tex., USA). The rabbits were prepared with 10% polyvinylpyrrolidone iodine (Besetine solution, Hyundai Pharm. Co. Ltd., Seoul, South Korea), and the lids were secured with a lid speculum. A corneal traction suture was placed 1 mm anterior to the limbus at the 10-o'clock position using an 8-0 Vicryl suture to rotate the eye downward. Conjunctival peritomy was performed at the limbus in the supratemporal quadrant, followed by posterior dissection to separate Tenon's capsule from the globe using Wescott tenotomy scissors. The AGV was primed with balanced salt solution and inserted into the supratemporal quadrant (fig. 2). The end plate was sutured to the underlying sclera with two 10-0 nylon sutures 8 mm from the limbus. The silicone tube was cut 0.5 mm anterior to the limbus using Vannas scissors. A 23-gauge needle was used to enter the anterior chamber 0.25 mm posterior to the limbus. A silicone tube

**Fig. 1. a** Upper surface of the polypropylene valve/reservoir body covered with processed human amniotic membrane. **b** Amniotic membrane secured to the edges of the AGV body using four 10-0 nylon sutures (arrowheads).



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was inserted into the anterior chamber, bevel side up through the needle tract and anchored to the sclera by a 10-0 nylon suture. The conjunctiva was secured to the limbus with an interrupted 8-0 Vicryl suture. At the conclusion of surgery, 3 mg/g ofloxacin ointment (Tarivid; Taejoon Pharmaceuticals, Seoul, South Korea) was applied to the operated eye.

#### Postoperative Care

After surgery, 3 mg/g ofloxacin ointment was applied to the operated eye daily for 7 days. Slit lamp examinations were performed preoperatively and postoperatively at 1, 2, 4 and 8 weeks to determine infection, tube placement, corneal edema and implant or tube erosion. IOP was measured preoperatively and postoperatively at 1, 2, 4 and 8 weeks with a rebound tonometer (ICare, Helsinki, Finland) under topical anesthesia. Three recordings per eye were averaged.

#### Histological Analysis

Animals were sacrificed by barbiturate anesthesia after 2 months. The eyes were enucleated carefully not to disturb the bleb and implant. After enucleation, 10% formalin was injected into the vitreous cavity to fix the eyes, which were immersed in fixative for 24 h. After 24 h, the eyes were dissected, with first incisions passing through the middle of the bleb. One half of the eye was embedded in paraffin and the other was embedded in epoxy resin (Epon) according to the standard protocol.

Paraffin blocks were sectioned at 4  $\mu$ m thickness using a microtome. First sections were used for identification of  $\alpha$ -SMA expression. Second sections were used for CD31 staining, and third sections were used for CD45 staining. Fourth sections were stained with hematoxylin-eosin, and fifth sections were stained with Masson's trichrome for analysis of collagen deposition.

Immunohistochemically, signs of myofibroblast transformation were identified by expression of  $\alpha$ -SMA. The inflammation was analyzed by CD45 staining, and CD31 staining was performed to determine blood vessel density. Samples were incubated overnight with mouse antibody against human SMA (1/50; M0851; Dako, Copenhagen, Denmark), mouse antibody against human CD31 (1/20; M0823; Dako) or mouse antibody against rabbit CD45 (1/50; MCA808; AbD Serotec, Oxford, UK). Bound antibodies were visualized using an amplification kit (REVEAL Polyvalent HRP-DAB Detection System; Spring Bioscience, Pleasanton, Calif., USA). Images were obtained using a microscope (Olympus BX51; Olympus, Tokyo, Japan) with a digital camera (Olympus DP 70; Olympus).



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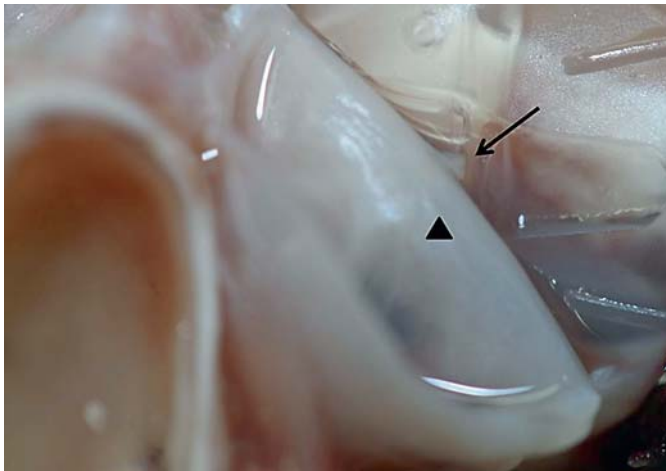
**Fig. 2.** AGV inserted into the supratemporal quadrant of the right eye of a rabbit.

For transmission electron microscopy, tissue specimens were postfixated with 1% osmium tetroxide in phosphate buffer (pH 7.2). Materials were dehydrated with a graded ethyl alcohol series and embedded in epoxy resin (Epon 812 mixture). Thick sections (1  $\mu$ m) were stained with 1% toluidine blue for light microscope examination. Thin sections (50–60 nm) were prepared using an ultramicrotome (Reichert SuperNova; Leica, Vienna, Austria) and double stained with uranyl acetate and lead citrate. Thin sections were also examined with a transmission electron microscope (JEM 1200EX-II; JEOL, Tokyo, Japan).

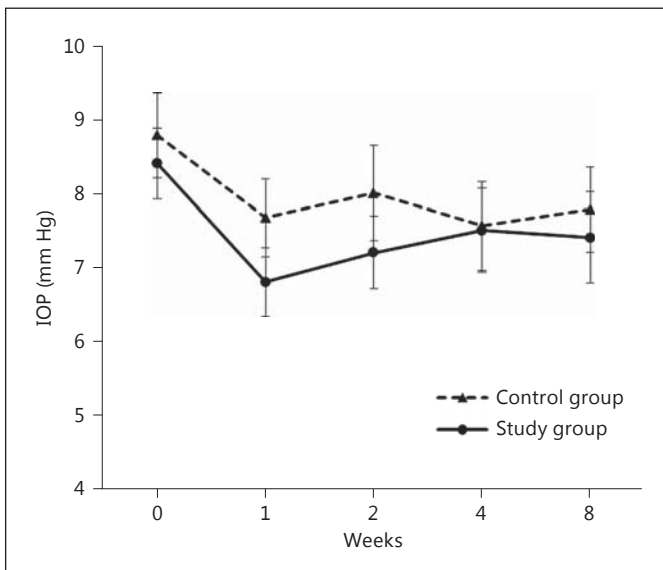
Mean fibrous capsular thickness was determined from photomicrographs of Masson's trichrome-stained sections from rabbit eyes implanted with AGVs. Using a light microscope at a magnification of  $\times 100$ , photographs were taken at 3 different evenly spaced areas in the roof and floor of the bleb, including the full fibrous capsule thickness (fig. 6). The fibrous capsule was defined as the innermost collagen fibers stained blue adjacent to the bleb cavity.

To assess the effect of amniotic membrane on TGF- $\beta$ -induced myofibroblast transdifferentiation and to compare  $\alpha$ -SMA expression between groups, the thickness of the myofibroblast layer was determined by measuring the  $\alpha$ -SMA-positive cell layer in the fibrous capsule. Using a light microscope at a magnification of  $\times 100$ , photographs were taken as described above.





**Fig. 3.** One rabbit eye from the conventional AGV implant group that developed ingrowth of tissue between valve sheets (arrow); arrowhead = roof of the fibrous capsule adjacent to the implant.



**Fig. 4.** IOP measurements were not significantly different between the two groups ( $p = 0.479$ ).

Light microscopy features of the myofibroblast include spindled cell morphology with an abundant matrix [21]. Therefore, to identify the  $\alpha$ -SMA-positive cell layer was thought to be a more efficient method to determine the myofibroblast transdifferentiation than counting the number of  $\alpha$ -SMA-positive cells in the fibrous capsule.

The number of blood vessels in the fibrous capsule was determined by counting CD31-positive cells from photomicrographs of CD31-stained sections. Using a light microscope at a magnification of  $\times 100$ , photographs were taken as described above and the number of blood vessels was counted in each field.

The number of leukocytes in the fibrous capsule was quantified by counting CD45-positive cells from photomicrographs of CD45-stained sections. Using a light microscope set at a magnification of  $\times 200$ , photographs were taken as described above and the number of leukocytes was counted in each field.

Histological sections were examined using light microscopy and transmission electron microscopy by a pathologist masked to the different groups. Three evenly spaced measurements were performed on the roof of the bleb, adjacent to the conjunctiva and Tenon's capsule. Another 3 measurements were performed on the floor of the bleb, adjacent to the sclera (fig. 6). All measurements from the study and control group were averaged to determine mean fibrous thickness, myofibroblast layer thickness, number of blood vessels and leukocytes.

#### Statistical Analysis

Data are presented as means and standard deviations. Data distribution normality was checked using the Kolmogorov-Smirnov test. Continuous variables were compared using Student's *t* test for independent samples. Statistical comparison of IOP curves between groups used repeated measures analysis of variance. Statistical analysis was performed using SPSS 21.0 statistics software for Windows (SPSS Inc., Chicago, Ill., USA).  $p < 0.05$  was considered statistically significant.

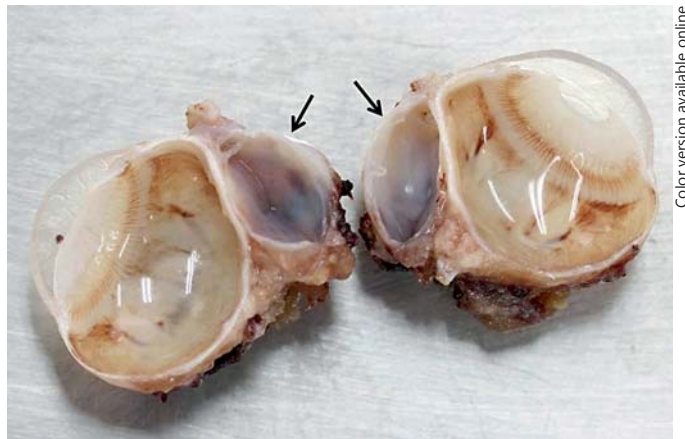
#### Results

Neither group had postoperative intraocular infection, intraocular hemorrhage or unexpected animal death, implant or tube extrusion. However, 1 animal in the control group developed tissue growth into the space between valve sheets (fig. 3). IOP measurements were not significantly different between the two groups ( $p = 0.479$ ; fig. 4). IOP was not significantly different at each time point between the two groups during the entire study period. IOP was  $7.78 \pm 1.72$  mm Hg in the control group versus  $7.40 \pm 1.96$  mm Hg in the study group at 2 months ( $p = 0.662$ ).

All operations in both groups resulted in the formation of a fibrous capsule surrounding the inserted AGV (fig. 5). However, hematoxylin-eosin staining of blebs after conventional AGV implantation revealed that fibrous capsules were thicker along the roof of blebs compared with AGVs with adjunctive amniotic membranes (fig. 6).

Histopathologically, the fibrous capsules along the roofs of blebs were composed of compact collagen fibers with minimal vascularization in the control group (fig. 7a). In contrast to the control group, fibrous capsules associated with the study group were looser with a more disorganized collagen architecture with a more abundant vascular structure (fig. 7b).

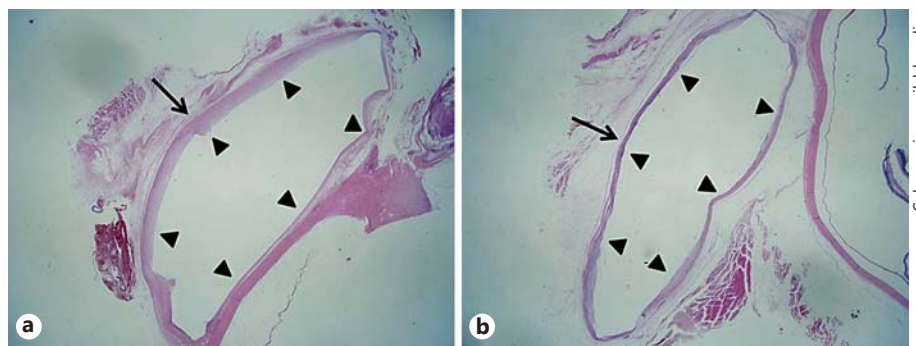
The mean fibrous capsular thickness along the roof of blebs was  $458 \pm 60$   $\mu$ m in the control group and  $280 \pm 28$   $\mu$ m in the study group (fig. 8). The difference between the



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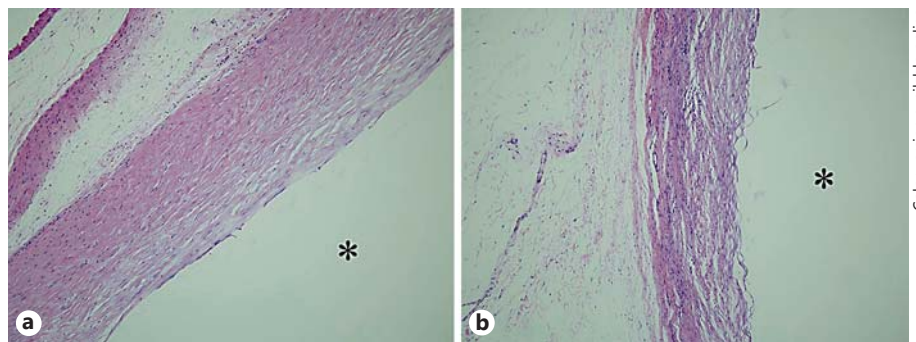
**Fig. 5.** All operations in both groups resulted in the formation of a fibrous capsule surrounding the inserted AGV (arrows).

**Fig. 6. a** Hematoxylin-eosin staining of a bleb after conventional AGV implantation without amniotic membrane. **b** Hematoxylin-eosin staining of a bleb after AGV with adjunctive amniotic membrane. The fibrous capsule in the roof of the bleb (arrow) was thinner than in the AGV without amniotic membrane. Photographs were taken in 3 different evenly spaced areas (arrowheads) in the roof and floor of the bleb, including the full thickness of the fibrous capsule. Original magnification  $\times 12.5$ .



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**Fig. 7. a** Hematoxylin-eosin staining of a fibrous capsule after conventional AGV implantation without amniotic membrane. **b** Hematoxylin-eosin staining of a fibrous capsule after AGV with adjunctive amniotic membrane. The fibrous capsule in the roof of the bleb associated with amniotic membrane was looser, with a more disorganized collagen architecture than capsules in the control group; \* = bleb cavity. Original magnification  $\times 100$ .



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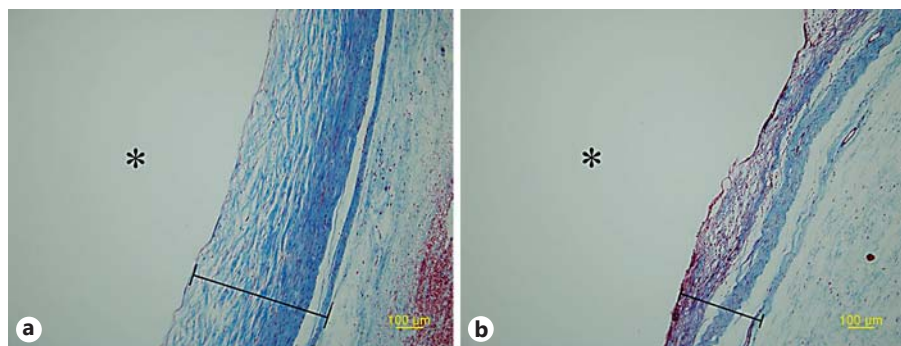
two groups was significant ( $p < 0.001$ ). However, differences in fibrous capsule thickness along the floor of the bleb were not significant ( $p = 0.777$ ). The mean fibrous capsular thickness along the floor of blebs was  $233 \pm 43 \mu\text{m}$  in the control group and  $227 \pm 46 \mu\text{m}$  in the study group (table 1).

In both groups, the inner layer of the fibrous capsule facing the AGV was characterized by transdifferentiation of fibroblasts into myfibroblasts, identified by  $\alpha$ -SMA expression. The thickness of the myfibroblast layer

along the roof of blebs was  $304 \pm 40 \mu\text{m}$  in the control group and  $188 \pm 26 \mu\text{m}$  in the study group (fig. 9), and this difference was significant ( $p < 0.001$ ). However, differences in myfibroblast layer thickness along the floor of blebs were not significant ( $p = 0.168$ ). The thickness of the myfibroblast layer along the floor of blebs was  $75 \pm 11 \mu\text{m}$  in the control group and  $82 \pm 10 \mu\text{m}$  in the study group (table 1).

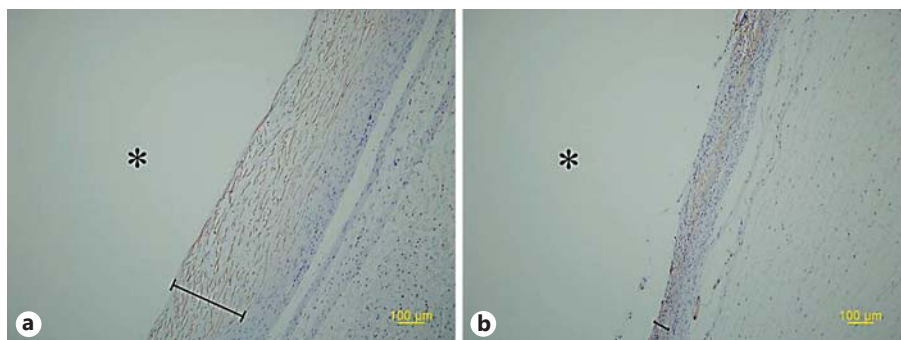
Transmission electron microscopy revealed that both groups had activated myfibroblasts showing a well-de-

**Fig. 8.** Masson's trichrome staining of a fibrous capsule in the roof of the bleb with a decrease in collagen deposition after surgery in the study group (b) compared with the control group (a); \* = bleb cavity. Original magnification  $\times 100$ .



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**Fig. 9.** Immunohistochemical staining for  $\alpha$ -SMA in a bleb roof.  $\alpha$ -SMA expression was reduced in the study group (b) compared with the control group (a). The inner layer of the fibrous capsule facing the AGV showed transdifferentiation of fibroblasts into myofibroblasts, detected as  $\alpha$ -SMA expression; \* = bleb cavity. Original magnification  $\times 100$ .



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**Table 1.** Histological results after AGV implantation with or without amniotic membrane

	Roof			Floor		
	control group	study group	p value	control group	study group	p value
Fibrous capsular thickness, $\mu\text{m}$	$458 \pm 60$	$280 \pm 28$	$<0.001$	$233 \pm 43$	$227 \pm 46$	0.777
Myofibroblast layer thickness, $\mu\text{m}$	$304 \pm 40$	$188 \pm 26$	$<0.001$	$75 \pm 11$	$82 \pm 10$	0.168
Blood vessels, n	$7.03 \pm 2.48$	$8.63 \pm 3.29$	0.235	$4.90 \pm 2.34$	$4.80 \pm 2.47$	0.927
Leukocytes, n	$4.00 \pm 1.65$	$15.10 \pm 7.42$	0.001	$2.04 \pm 1.63$	$1.67 \pm 2.13$	0.665

veloped Golgi area, numerous and dilated rough endoplasmic reticulum cisternae, pinocytotic vesicles and bundles of cytoplasmic microfilaments with interspersed dense bodies, arranged parallel to the long axis of the cell beneath the plasma membrane (fig. 10a). Transmission electron microscopy also showed myofibroblasts at the inner surface toward the bleb cavity (fig. 10b).

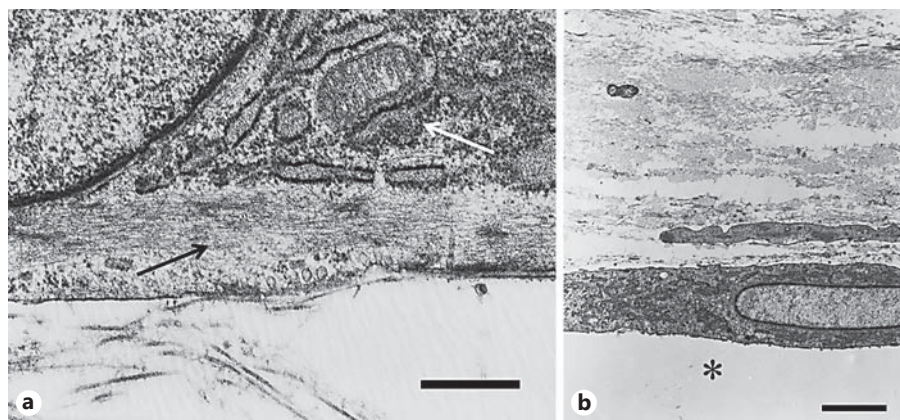
The mean number of blood vessels in fibrous capsules was  $7.03 \pm 2.48$  for the roof and  $4.90 \pm 2.34$  for the floor in the control group, versus  $8.63 \pm 3.29$  for the roof and  $4.80 \pm 2.47$  for the floor in the study group (table 1). The number of CD31-positive blood vessels along the roof

and floor of blebs was not different between the two groups ( $p = 0.235$  for the roof and  $p = 0.927$  for the floor). However, the location and diameter of blood vessels were different between the two groups. In the control group, vessels were mainly in the outer layer of the fibrous capsule and the vessels had a smaller diameter than in the study group. In contrast to the control group, the fibrous capsule along the roof of blebs in the study group had more dilated CD31-positive blood vessels evenly distributed throughout the fibrous capsule (fig. 11).

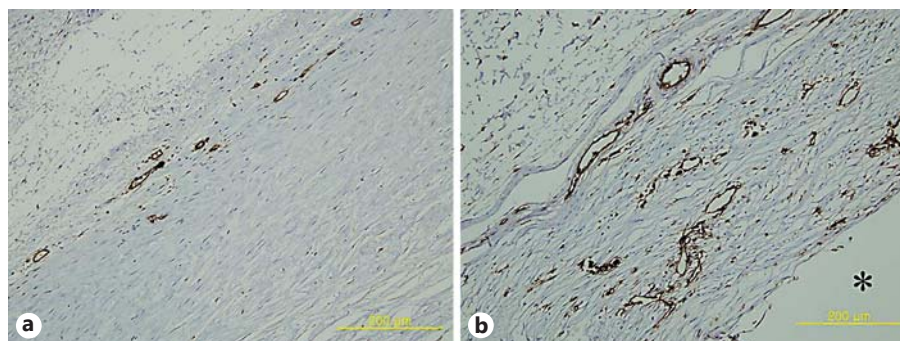
The number of leukocytes in fibrous capsules was  $4.00 \pm 1.65$  for the roof and  $2.04 \pm 1.63$  for the floor in the con-



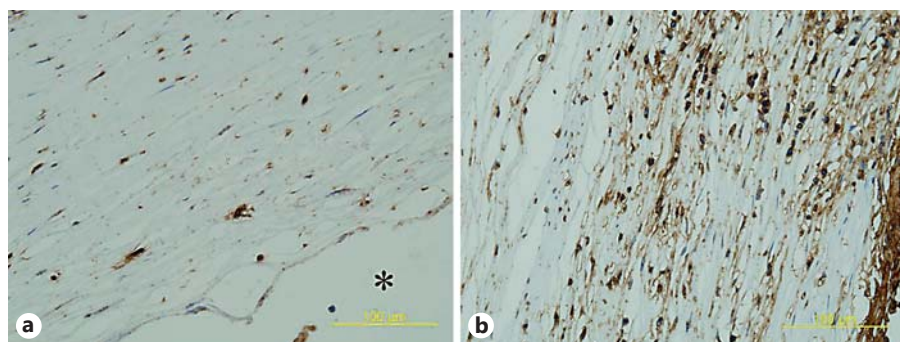
**Fig. 10.** Transmission electron micrographs showing activated myofibroblasts from the control group (a) and study group (b). **a** Dilated rough endoplasmic reticulum (white arrow), pinocytotic vesicles and bundles of cytoplasmic microfilaments (black arrow) with interspersed dense bodies parallel to the long axis of the cell beneath the plasma membrane. Magnification bar = 0.5  $\mu$ m. **b** Myofibroblasts toward the bleb cavity (\*). Magnification bar = 2  $\mu$ m.



**Fig. 11.** Immunohistochemical staining for CD31. Original magnification  $\times 100$ . **a** Control group. **b** Fibrous capsule in the roof of the bleb associated with amniotic membrane with numerous dilated CD31-positive blood vessels in the inner layer in the study group; \* = bleb cavity.



**Fig. 12.** Microscopy of the CD45 immunostaining of bleb roofs with increased infiltration of CD45-positive leukocytes in the study group (b) compared with the control group (a); \* = bleb cavity. Original magnification  $\times 200$ .



control group, versus  $15.10 \pm 7.42$  (roof) and  $1.67 \pm 2.13$  (floor) in the study group (table 1). CD45-positive inflammatory cells were more frequently observed along the roof of blebs in the study group than in the control group ( $p = 0.001$ ; fig. 12). No difference in the number of CD45-positive inflammatory cells was detected along the floor of blebs between the two groups ( $p = 0.665$ ). Few CD45-positive inflammatory cells were observed in the control group.

## Discussion

This study evaluated the effects of amniotic membranes on myofibroblast transdifferentiation and the extent of fibrosis around the polypropylene valve/reservoir body of AGVs. Histological analysis revealed formation of fibrous capsules surrounding inserted AGVs in both groups. In contrast to the thick, dense fibrous capsules in the control group, fibrous capsules over the AGV in the group with adjunctive amniotic membranes were thin

and loose. While activated myofibroblasts were visible using ultrastructural techniques in both groups, the thickness of the myofibroblast layer identified by  $\alpha$ -SMA expression was thinner in the study group than in the control group.

The significantly reduced  $\alpha$ -SMA expression might explain the thin and loose fibrous capsules associated with the amniotic membrane. Our results correspond with those of an earlier study that reported that levels of TGF- $\beta_1$ , - $\beta_2$ , - $\beta_3$  and TGF- $\beta$  type II receptor transcripts and TGF- $\beta_1$ , - $\beta_2$  proteins were reduced in cultured fibroblasts when in contact with an amniotic membrane [16]. As a result of amniotic membrane suppression of the TGF- $\beta$  signaling system, subsequent fibroblast differentiation, as identified by  $\alpha$ -SMA expression, was also suppressed [16]. This may explain the formation of a loose and thin fibrous capsule over the implant when amniotic membrane covered it, and may further result in antiscarring effects in turn.

Our results also agree with a previous study in which the conjunctival flap was replaced with amniotic membrane in filtration surgery in rabbits [22]. Less fibroblast outgrowth occurred from amniotic membrane transplantation explants compared with unoperated conjunctiva [22]. Numbers of fibroblasts and macrophages around trabeculectomy sites in rabbits were significantly reduced by amniotic membrane [23].

Several human clinical studies reported encouraging results from using amniotic membranes in trabeculectomy [24–27]. Fujishima et al. [24] noticed that amniotic membrane transplantation in trabeculectomy with MMC reduced IOP in patients at high risk for surgical failure. Sheha et al. [25] reported synergetic beneficial effects of MMC and amniotic membrane for refractory glaucoma. Drolsum et al. [26] reported promising results for amnion-shielded trabeculectomy in patients with severely refractory glaucoma who had previously undergone 2 or more regular trabeculectomies with MMC.

Amniotic membrane has a high hydraulic conductivity and is semipermeable to water [19] and may not increase outflow resistance when applied to the upper surface of the AGV body. Amniotic membrane also has anti-inflammatory properties including facilitating macrophage apoptosis [15, 18].

These findings are applicable to glaucoma shunt surgery in humans. A previous study in humans reported that no eyes developed encapsulation after AGV implantation with adjunctive amniotic membrane [20], but did not discuss the histopathological findings of a bleb around the AGV. Our histology results indicated that the reason

for the favorable outcome of the previous study might be the alteration of fibrous capsule characteristics by suppression of myofibroblast transdifferentiation and fibrosis by the amniotic membrane.

Our histological analysis revealed thick, dense and avascular fibrous capsules surrounding the AGVs in the control group. In the control group, the inner layer of the fibrous capsule was compact collagen fibers characterized by transdifferentiation of fibroblasts into myofibroblasts, and the outer layer of the fibrous capsule was vascularized. These results were consistent with those of Thieme et al. [3], who reported histopathological findings of encapsulated blebs around AGVs.

In contrast to the control group, fibrous capsules in the study group were looser with a more disorganized collagen architecture. Capsules also had more dilated vessels throughout the fibrous capsule. These results were in agreement with previous studies with histopathological findings for a glaucoma drainage device modulated by various adjunctive treatments [28–30]. Park et al. [28] evaluated the effects in rabbits of topical cyclosporine on the function of filtering blebs after glaucoma drainage implant surgery. The fibrous capsule in their study group was thinner and looser than their control group. Flow resistance through the implant capsule was significantly lower in the study group [28]. DeCroos et al. [29] reported that encapsulating tissue was altered by an expanded polytetrafluoroethylene membrane in AGV surgery, led to greater vascular tissue response and a thinner fibrous capsule compared with a standard glaucoma implant plate. Sahiner et al. [30] examined histological findings in rabbits with fibrous capsules around a slow-release antifibrotic drug-coated glaucoma device. The fibrous capsules associated with the drug-coated implant were thinner than in the control group [30].

We found no differences in the number of CD31-positive blood vessels between the two groups. However, the location and diameter of blood vessels were different between the groups. Instead of small-diameter blood vessels in the outer layer in the control group, the study group showed dilated blood vessels throughout the fibrous capsule wall. Reports on the effects of amniotic membrane on angiogenesis are controversial. The amniotic membrane is reported to have antiangiogenic properties [15, 31]. However, a previous study reported that the amniotic membrane has both angiogenic and antiangiogenic properties [32]. Amniotic mesenchymal cells secrete several angiogenic factors such as interleukin (IL)-6, IL-8, growth-related oncogene, monocyte chemoattractant protein-1 and intravascular adhesion molecules [32].



The possibility that the postoperative inflammation in the study group had not subsided and influenced the diameter of blood vessels cannot be excluded. Kassem et al. [33] reported that significant foreign body inflammation was observed in rabbit eyes 6 weeks after cryopreserved human amniotic membrane implantation. We presumed that the inflammatory response observed 2 months after surgery in our study group was related to the xenogenic nature of human amniotic membrane used in rabbits. Therefore, longer-term studies are needed to confirm the stability of xenogenic inflammation induced by human amniotic membrane used in rabbits.

However, the difference in the blood vessel diameter might be not merely influenced by inflammation, but also result from the difference in collagen architecture between groups. In the control group, the diameter of blood vessels might be smaller due to strong capsular compression. More disorganized and loose connective tissue observed in the study group might produce less compression to the blood vessels in the fibrous capsule compared with the control group and result in more dilated blood vessels subsequently.

In a hypertensive phase after AGV implantation, a formation of a dense fibrous capsule separates the aqueous humor from the conjunctival blood vessels and is associated with IOP elevation [30]. A previous histopathological study reported that little vasculature may further impair the transportation of fluid out of the cyst or through the cyst wall in the encapsulated bleb formed after AGV implantation [3]. Another experimental study reported that the thinner and more vascular capsule formed by a modified AGV with expanded polytetrafluoroethylene may result in decreased resistance to aqueous egress compared with avascular fibrous encapsulation [29]. Therefore, in the study group, the dilated blood vessels located throughout a thin and loose fibrous capsule might be more effective for fluid transport through the capsular wall than in the control group [3, 29, 30].

In our study group, CD45-positive inflammatory cells were more frequently observed in the roofs of the blebs associated with amniotic membrane than in our control group. Our results are not in agreement with previous studies that reported that amniotic membrane transplantation had anti-inflammatory effects in humans [34].

However, findings similar to ours were reported in a previous study in rabbits in which the conjunctival flap was replaced with human amniotic membrane [22]. Therefore, we hypothesize that the inflammatory response in our study group was because the human amniotic membrane we used was xenogenic in rabbits [22].

The first limitation of this study is to use human amniotic membrane in an animal model. A previous experimental study demonstrated an immunosuppressive property of dry human amniotic membrane using a splenocyte proliferation assay, in which mouse splenocytes were harvested from Balb/c mice [35]. Cryopreserved human amniotic membrane was transplanted to the limbal area of rats in another experimental study. Although some CD4- and CD8-positive T cells surrounded the transplanted amniotic membrane, the response was mild [36].

In contrast to the previous studies, a significant inflammatory reaction was observed in our study group. However, amniotic membrane significantly reduced the thickness of the fibrous capsule and  $\alpha$ -SMA expression in spite of its failure to prevent inflammation in this study. These findings are in agreement with a previous animal study [33]. Kassem et al. [33] noticed that human amniotic membrane was effective in reducing postoperative extraocular muscle adhesion in rabbits, although significantly more foreign body inflammation was found in amniotic membrane eyes compared with control eyes. In addition, Tseng et al. [37] reported that the antiscarring action of amniotic membrane is not caused indirectly by suppressing inflammation but by directly suppressing TGF- $\beta$  signaling at the transcriptional level. The second limitation is the lack of evaluation of fibrous capsule function [28]. Flow resistance through the fibrous capsule was not measured. The third limitation is that only the upper surface of the AGV body facing Tenon's capsule and conjunctiva was covered with amniotic membrane. In a previous clinical study also, only the upper surface of the shunt plate was covered with amniotic membrane similar to our study [20]. Therefore, we chose to implant AGVs with amniotic membrane in a design similar to the human clinical study.

Although the present study revealed that amniotic membrane was effective in reducing the thickness of the fibrous capsule and  $\alpha$ -SMA expression, IOP in the study group was not significantly different from that in the control group at each time point. A first explanation for our findings is that IOP measurement may not be accurate in the rabbit due to its thin cornea [30]. A second explanation is that rabbits did not have increased IOP because they did not develop glaucoma in this study [38]. These results are consistent with data suggesting that IOP may not correlate with scarring in a subconjunctival wound healing animal model [29, 38, 39].

Our results indicated that the tissue response around the AGV with adjunctive amniotic membrane was different from the response from conventional AGVs. A thin, loose vascularized tissue was observed when implanted

with adjunctive amniotic membrane, instead of the usual thick, dense and avascular capsule, which may decrease outflow resistance and improve long-term IOP control [28–30]. In conclusion, the adjunctive amniotic membrane in glaucoma implant surgery could reduce the risk of encapsulation and thereby decrease aqueous outflow resistance by altering the tissue response, and may enhance the efficacy of the surgery.

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