ERECTILE DYSFUNCTION

Cavernous Branched Nerve Regeneration Using Non-Tubular Artificial Nerve Sheets Using Freeze-Dried Alginate Gel Combined With Polyglycolic Acid Mesh in a Rat Model

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ABSTRACT

Introduction: Neuroprotection and neuroregeneration of cavernous nerve plexus by biological/bioengineering solutions may have the potential to maintain erectile function.

Aims: We evaluated the efficacy of a newly developed artificial nerve sheet using freeze-dried alginate (ALG) with polyglycolic acid (PGA) mesh in a rat model. Methods: Bilateral cavernous nerves of male rats were excised to make an approximately 2 mm gap. A piece of the sponge-like freeze-dried sheet created by covalent cross-linking of ALG gel combined with PGA mesh was placed over the gap to cover each stump without any neural anastomosis. We compared erectile functions in the ALG groups with those in the sham group and the bilateral nerve excision group (n = 12, each).

Main Outcome Measures: Main outcome measure was a rat model with cavernous nerve excision.

Results: All rats in the sham group had erection at 63 or 64 days, and mating behavior was confirmed in 10 rats (83.3%) of the sham group at 56 to 62 days. No erection and mating behavior was observed in the excision group. Ten of the 12 (83.3%) rats in the ALG group had a mating behavior and an erection, and the rates of erection and mating behavior were significantly higher in the ALG group than those in the excision group ($P < .01$, $P < .01$, respectively). Using a retrograde FluoroGold, the rate of FluoroGold positive pelvic ganglia proximal to the gap at 61 or 62 days was significantly higher in the ALG group than that in the excision group ($P = .014$).

Conclusion: The results of our animal study have demonstrated that simply filling the cavernous nerve gap using the non-tubular artificial nerve sheets made of ALG with PGA mesh restored erectile function after cavernous nerve excision. **Narita S, Obara T, Ishikawa N, et al. Cavernous Branched Nerve Regeneration Using Non-Tubular Artificial Nerve Sheets Using Freeze-Dried Alginate Gel Combined With Polyglycolic Acid Mesh in a Rat Model. Sex Med 2021;9:100308.**

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Key Words: Erectile Dysfunction; Alginate; Cavernous Nerve; Artificial Nerve; Polyglycolic Acid

INTRODUCTION

Prostate cancer is the most common malignancy in men and the sixth leading cause of cancer-related death

worldwide,[1] and radical prostatectomy is one of the standard treatment options for localized prostate cancer. Due to the excision of neurovascular bundles (NVBs) supplying the cavernous tissues during surgery, erectile dysfunction is a major serious complication after radical prostatectomy.[2] Even with the meticulous cavernous nerve preservation during radical prostatectomy, the functional outcome regarding erectile function has been shown to be unsatisfactory and insufficient.[3] Furthermore, the NVB preservation may be associated with a higher risk of cancer positivity at the surgical margin, which invited a risk to compromise the cancer control.[4] Therefore, neuroprotection and neuroregeneration of the cavernous nerve plexus by biological/bioengineering solutions are expected to overcome the issue.

Tubular artificial nerve materials are used to bridge the damaged peripheral nerves when an end-to-end anastomosis is not possible.[5] However, there are several limitations to the tubular artificial nerve materials, including a need for a device of various diameters depending on the nerve diameter to be regenerated, the inapplicability of the device to damaged sites that are branched into a Y-shape or the plexus type of branched nerves, and the necessity of suturing. In order to overcome these limitations, we developed a novel artificial nerve sheet made of alginate gel (ALG), which is shown to successfully achieve to fill the 50 mm gap in a cat sciatic nerve model.[6] Since it is a sheet, it can be used with nerves of various diameters and with complex branches or plexus such as the cavernous nerve plexus.

Using a cavernous nerve injury model of a rat, we previously showed that the ALG gel sheet regenerated the cavernous nerve and restored erectile function by filling the nerve gap.[7] However, the ALG gel sheet may be too fragile, frail and easily torn, and difficult to apply to cover the nerve gap, such as the excised cavernous nerve with over 4−5 cm in length. By combining polyglycolic acid (PGA) mesh, the ALG sheet is much stronger and more durable. Although we have shown that tubular ALG products with PGA promoted transected nerves to regenerate a peripheral nerve gap in a cat model,[8] there is no study to assess the feasibility and efficacy of ALG with PGA mesh in a model of cavernous nerve injury.

We hypothesized that our non-tubular artificial nerve sheet using the freeze-dried ALG gel combined with PGA mesh has the potential to overcome the limitations of previous artificial nerve materials when applying to cavernous nerve injury. In this study, we evaluated the efficacy of a newly developed non-tubular artificial nerve sheet using freeze-dried ALG gel combined with PGA mesh in a rat model with a defect of cavernous nerve-plexus mimicking erectile dysfunction after prostatectomy in patients with prostate cancer.

MATERIALS AND METHODS

Experimental Animals

Male Crl: CD(SD) rats, weighing 181.6 to 246.8 g and aged 6−7 weeks, were obtained from Japan Charles River Laboratories (Yokohama, Japan). First, we confirmed the early depletion of erectile functions in the ALG group (n = 6, experiment 1). Next, we compared erectile functions and neural regeneration in the ALG group with those in the sham group or the bilateral nerve excision group (excision group) (n = 12 each, experiments 2 and 3). In experiment 2, the rats were randomized to treatment, and all experiments were performed in a blinded manner. All studies pertaining to experiments with animals were approved by the Institutional Review Board (2018-1501).

### Preparation of Alginate Sheet

Water-soluble carbodiimide and ethylenediamine were dissolved well in 1% sodium ALG aqueous solution to develop a transparent gel cross-linked with covalent bonds. The gel was washed with 2.5 mM calcium chloride and 143 mM sodium chloride to remove any residual contaminant and unreacted reagent. The gel was mixed with PGA mesh and freeze-dried to become an ALG sponge. The gross and microscopic appearances of the ALG with PGA mesh are shown in Figure 1. The functional and morphometric analyses of our material using an animal model of peripheral nerve injury were previously reported by one of the authors in this manuscript.[8] These devices were sterilized by 15 kGy electron beam irradiation. The size of the sheet was 6×12 cm, and the sheet contained 2 mg/cm^2 sodium alginate.

Operative Procedure

All operative procedures were conducted using inhalation anesthesia with 2% isoflurane. Surgery was conducted under sterilized conditions, and an antibiotic (enrofloxacin 10 mg/kg

A

B
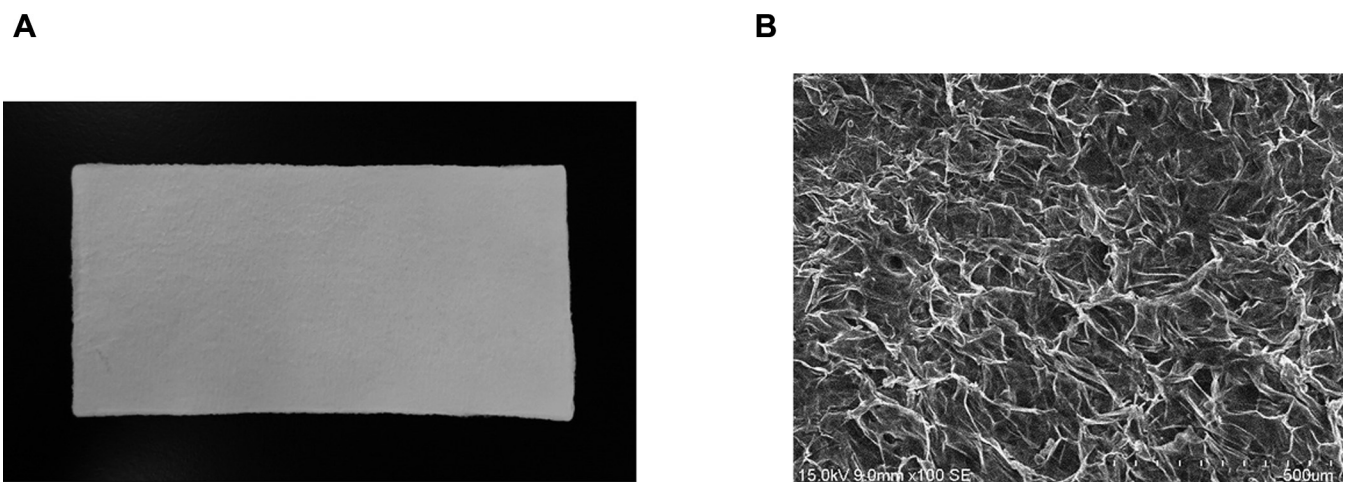
Figure 1. Macroscopic (A) and microscopic (B) views of the artificial nerve sheet using freeze-dried alginate (ALG) with polyglycolic acid (PGA) mesh.
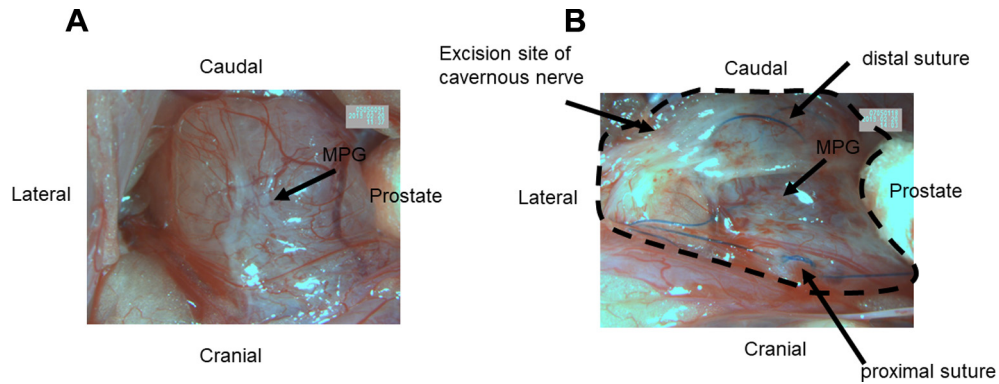
Figure 2. Surgical fields (A) Sham group. (B) Alginate (ALG) group before FluoloGold (FG) staining. 7-0 Nylon suturing was placed proximal and distal to the main pelvic ganglion (MPG). The dotted line shows the region covered by the ALG sheet.

subcutaneously) was administered once after surgery. A lower midline abdominal incision was made to expose the pelvic organs in order to visualize pelvic nerves and cavernous nerve plexus under the microscope. Bilateral cavernous nerves of the rats were microscopically excised to form a gap of approximately 2 mm as previously described.[7] Briefly, in the excision and ALG groups, the major pelvic ganglion (MPG) and the cavernous nerve were exposed. The main and ancillary cavernous nerves were then bilaterally excised 1 mm below the MPG, resulting in an approximately 2 mm gap. In the sham rats, the MPG and cavernous nerves were exposed only. In the ALG groups, a piece of freeze-dried ALG gel sponge sheet (5 mm × 7 mm) with PGA mesh was prepared over the gap of the excised cavernous nerves to cover each stump. The stumps were covered by a 7 mm side of the sheets. Mounting behavior, erectile function, and the presence of nerve regeneration were compared with those in the sham group or the excision group. Pictures of surgical fields were shown in Figure 2.

Mounting Behavior Observation

In experiment 1, mounting behavior was assessed at 7 to 13 days after surgery. In experiment 2, mounting behavior was assessed at 56 to 62 days postoperatively. Briefly, a male rat was placed in a cage with a female rat in estrus; the presence of copulatory plug, which is a vaginal secretion used in mating, was visually observed the next day. If the plug was not observed, the presence of the plug continued to be evaluated for a week.

Assessment of Erectile Function

The next week after mounting behavior observation, the erectile function was tested by electric stimulation

(EKH2-1012, 0.5 mA, 1 ms, 10 Hz, 3 seconds) of the cavernous nerve proximal to the excised portion under anesthesia. Electric stimulation was performed by using the EMG/EP Measuring systems (MEB-9402 MB, Nihon Kohden, Tokyo, Japan). After the abdominal cavity was exposed, the excision gap and site for electric stimulation were confirmed under the microscope guided by the marked stitches during the previous operation. Nerve stimulation was allowed up to 3 times. Erectile responses were visually scored based on 4 degrees, including no response (-), penile swelling (+1), penile moving (+2), and rigid penis (+3).

FluoroGold Staining

In experiment 3, the rats at 56 or 57 days after operation were anesthetized, and a retrograde neural tracer FluoroGold (FG) (Fluorochrome, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) was bilaterally injected (4%, 5 μL each) into each penile crus using a 30 G needle. Five days after injection of FG, the pelvis of each rat was visualized under anesthesia, and the rates of FG positive pelvic ganglia proximal to the gap were evaluated by fluorescence microscope (M205FA, Leica Microsystems, Tokyo, Japan).

Statistics

Statistical analyses were performed using SPSS ver. 24.0 (IBM SPSS Statistics for Windows, IBM Corporation, Armonk, NY, USA). The chi-squared test and Wilcoxon rank-sum test were used to compare differences between the groups. All two-sided P values presented in this report were considered significant when $P < .05$.

Table 1. Presence of copulatory plug in the alginate groups at day 7 to 13 after operation

Group	Total number of rats	Presence of copulatory plug	
		Negative	Positive
Alginate	6	6	0

Table 2. Presence of erectile function in the alginate groups at day 14 after the operation

Group	Total number of rats	Erectile function			
		-	+1	+2	+3
Alginate	6	6	0	0	0

RESULTS

In experiment 1, the depletion of mounting behavior and erectile function at an early period after the operation was observed in all rats in the ALG group (Tables 1 and 2). No rats in the ALG group (n = 6) had a presence of copulatory plug at 7 to 13 days and erectile function at 14 days after the operation, which suggested that erectile function and mating behavior in the ALG group were depleted at an early period after the operation.

In experiment 2, the mounting behavior was visually confirmed in 10 of 12 rats (83.3%) in the sham group at 56 to 62 days (Table 3), and all rats in the sham group had an erection at 63 to 64 days after the operation (Table 4). No erection and mounting behavior were observed in the excision group during the study periods (Tables 3 and 4). The rate of presence of the copulatory plug was significantly lower in the excision group than that in the Sham group ($P < .01$). At 56 to 62 days after the operation, the mounting behavior was confirmed in 10 of 12 (83.3%) rats in the ALG groups, and the rate of presence of the copulatory plug in the ALG group at 56 to 62 days was significantly higher than in the excision group ($P < .01$, Table 3). By electric cavernous nerve stimulation, the rate of erectile function was significantly higher in the Sham group than that in the excision group ($P < .01$, Table 4). The erection was observed in 10 of 12 (83.3%) rats in the ALG groups at 63 or 64 days (Table 4). The rate of erectile function was significantly higher in the ALG group than in the excision group ($P < .01$, Table 4). These results suggested that the ALG group successfully recovered mounting behavior and erectile function after the operation.

In experiment 3, to confirm cavernous nerve regeneration after placing the ALG with PGA, we next assessed the presence of FG positive pelvic ganglia proximal to the gap in each group. All rats in the sham group had FG-positive, whereas no FG-positive were observed in the excision group at 61 or 62 days after the operation (Figure 3 and Table 5). The FG-positive pelvic ganglia were confirmed in 4 of 6 (66.7%) rats in the ALG groups at 61

or 62 days (Table 5). The rate of FG positive pelvic ganglia proximal to the gap in the ALG group at 61 or 62 days was significantly higher than that in the excision group ($P = .014$, Table 5). Taken together, most of the rats in the ALG group had successful nerve regeneration.

DISCUSSION

In this study, we showed that a newly developed non-tubular nerve sheet made of ALG with PGA mesh preserved potency and mating behavior with infiltration of FG positive cells proximal to the excision margin in a rat model of cavernous nerve injury, which provides a potential for novel bioengineering solution to overcome erectile dysfunction in patients with prostate cancer who undergo radical prostatectomy with excision or injury of NVBs. This material potentially overcomes the problems of previous artificial nerve materials, such as the necessity of multiple tube diameters, bioabsorbability of materials, suturing and adequate nutrients and oxygen, bending difficulty, unfit branching sites, and risk of rapid degradation and absorption as previously reported.[9]

Sural nerve grafting is one of the potential options for overcoming the issues of sexual function[10,11]; however, there are several problems such as the extraction of healthy nerves, the sacrifice of healthy functional tissues, and the necessity of microsurgical procedures, which demand a highly meticulous technique and long operation time. Previous studies have reported the advantage of artificial nerve conduits to regenerate peripheral nerves,[6,12] which may also be technically associated, demanding, and time-consuming. Regarding the enhancement strategy of regeneration of cavernous nerve, Patel et al showed that dehydrated human amnion/chorion membrane (dHACM) allograft wrapped around NVBs during robot-assisted radical prostatectomy might be able to accelerate the return to the normal functionality.[13] A recent study reported the potency outcomes in 2 systematically controlled, non-randomized, matched, homogenous patient cohorts consisting of intervention with the placement of dehydrated human amniotic

Table 3. Comparison of presence of copulatory plug among the experimental groups at day 56 to 62 after the operation

Group	Total number of rats	Presence of copulatory plug		
		Negative	Positive	
Sham	12	2	10	
Excision group (Control)	12	12	0	$P < .01$ (vs Sham)
Alginate	12	2	10	$P < .01$ (vs Control)

Table 4. Comparison of presence of erectile function among the experimental groups at day 63 or 64 after the operation

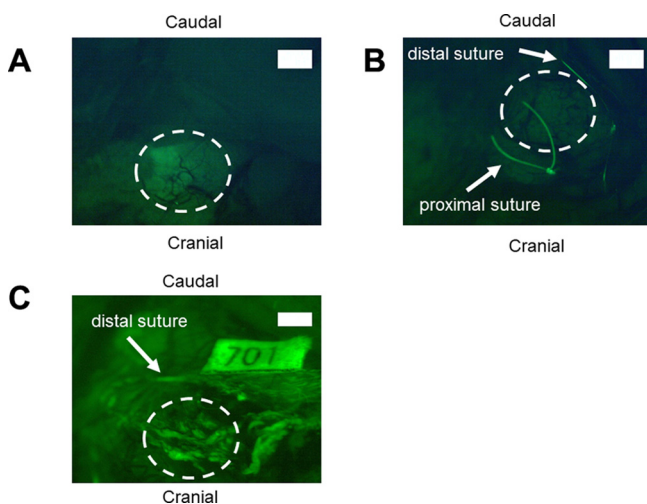| Group	Total number of rats	Erectile function				
		-	+1	+2	+3	
Sham	12	0	0	7	5	
Excision group (Control)	12	12	0	0	0	$P < .01$ (vs Sham)
Alginate	12	2	9	1	0	$P < .01$ (vs Control)

membrane around NVBs during radical prostatectomy or without (control).[14] The study concluded that the intervention with the placement of dehydrated human amniotic membrane was an independent significant ($P < .001$) predictor of achieving potency at 1 year and was 3.86 times (95% CI 2.43−6.13) more likely to achieve potency compared with the control.[14] Although this material has the potential to be a promising option for nerve-regeneration in patients who underwent prostatectomy, we cannot ignore a limitation such as a supply problem associated with human resources.

Matsui et al demonstrated that the initial 3 patients were treated with robot-assisted laparoscopic radical prostatectomy with cavernous nerve reconstruction using Nerbridge, which is a medical grade collagen with a polyglycolic acid.[15] All patients received wide ipsilateral resection of NVB, and nerve grafting was conducted at the side. They concluded that the application of conduit was technically feasible, and one patient achieved a very early recovery of potency within 1 month. However, the device needs to be sutured on distal and proximal site with the mean operative time of 40 min. In addition, it may be difficult to identify the stump of excised cavernous nerve because the

Figure 3. FG imaging of the pelvic ganglia in each group. The rats at 56 or 57 days after operation were anesthetized, and a retro-grade neural tracer FluoroGold (FG) (Fluorochrome) was bilaterally injected (4%, 5 μL each) into each penile crus. A, sham group, B, excision group, C, ALG group. 7-0 Nylon suturing was placed proximal and distal of the main pelvic ganglia in the 2 surgical groups. The white circles indicated the area of the major pelvic ganglia.

cavernous nerve consists of the plexus type of fine nerve fibers.[16,17] Therefore, novel materials without the need for suturing resected ends are warranted to further enhance the nerve regeneration in a more robust manner. Regarding the current material, we have already assessed the handling through the laparoscopic ports and the durability and feasibility in suturing in the pelvic space of the current material by a porcine model and confirmed its feasibility in the laparoscopic pelvic surgery (data not shown), which allows us to proceed with a clinical trial of robot-assisted laparoscopic prostatectomy.

Sodium ALG and chitosan are marine polysaccharides, and many studies have shown that the both have good biocompati-bility and biodegradability,[18−20] indicating that the materials are suitable as biomaterials to enhance the recovery of damaged tissue.[21] The phase II study to evaluate the potency recovery with the use of chitosan membrane application on the NVBs showed that the chitosan membrane application achieved a higher potency rate at 1 and 2 months after bilateral and unilateral nerve-sparing prostatectomy.[22] The same group also assessed the functional characteristics of the chitosan membrane, including neurogenerative effect, modifying effect, and tissue reaction using the preclinical models.[23] In the study, they confirmed that the functional effects of an implanted material using a grasping test of a rat median nerve model.[23] In contrast, we successfully developed the cavernous nerve damage model in a rat and confirmed the effectiveness of ALG with PGA mesh in restoring the erectile function after cavernous nerve excision, suggesting that the efficacy of our novel material in enhancing the autonomic nerve regeneration along with somatic nerve regeneration.

We have previously demonstrated that the ALG sheets regenerated the human digital nerve to regain its sensory func-tion for patients, and this material was reliable enough for clinical application after peripheral nerve damage.[9] Compared to other commercially available artificial nerves, several advantages of the ALG has been reported in the previous literature.[9] We also revealed that both tubular ALG products with PGA and non-tubular ALG promoted the transected nerves to regenerate a 50 mm peripheral nerve gap in a cat.[8] Given that the ALG sheets used in our previous study of the cavernous nerve injury model had a handling problem due to fragile and frail material, we improved an ALG sheet by mixing with PGA mesh. The com-bination of ALG with PGA mesh enhanced the strength of the material, which could be sutured to nerve stump if necessary, and achieved optimal fabric durability to be inserted from endoscopic

Table 5. Comparison of FG positive pelvic ganglia proximal to the gap among the experimental groups at day 61 or 62 after the operation

Group	Total number of rats	FG staining at the proximal to the gap		
		Negative	Positive	
Sham	12	0	6	
Excision group (Control)	12	6	0	P < .01 (vs Sham)
Alginate	12	2	4	P = .014 (vs Control)

Abbreviation: FG, FluoroGold.

ports. The present results, taken together, are an important step for the clinical application of this material to apply patients with prostate cancer treated with endoscopic radical prostatectomy in an era of robot-assisted surgery.

There are some limitations to the study. We could not reveal the dosage effect of ALG sheets. We previously performed a pilot study to identify the appropriate concentration of ALG and thickness of the ALG sheet with PGA mesh applied in this study (data not shown); however, there were no consistent results regarding the recovery rate in the mounting behavior, erectile function, and FG staining among the various concentrations and thicknesses of the ALG sheet. Although these results might be caused by the complexity of the erection mechanism and/or the technically demanding experimental procedure, we speculated that the optimal ALG concentration and thickness of the ALG sheet were not fully delineated and clarified yet. Therefore, we may have to define further the ideal ALG concentration, and the thickness of the ALG sheet in animal models to secure the enhancement of the recovery of erection. Second, the changes in morphology, biochemistry, and FG positivity were not evaluated in the present cavernous nerve model because morphometric analyses of this material were previously performed using peripheral nerve models.[24] In addition, the present study lacks the quantification data on positive FG-stained neuronal cell bodies and erectile function in FG-positive or negative rats because of technical difficulty. However, our previous study revealed that the mean number of total FG-positive cells in rats treated with the prototype of the ALG gel was not significantly different from that in the sham group.[7] In addition, the visual assessment of erections is inferior to real-time measurement via pressure transducer normalized to the mean arterial pressure. Furthermore, in a clinical setting, a study may be required to explore the best location and timing of placement of the ALG sheet in patients who undergo prostatectomy. Now, we are planning a clinical phase I/II study in humans undergoing robotic radical prostatectomy to evaluate the feasibility, durability, and safety along with preliminary efficacy of the ALG sheet.

CONCLUSIONS

The results of our animal study have demonstrated that filling the cavernous nerve gap using a non-tubular artificial nerve sheet made of ALG with PGA mesh restored the erectile function after cavernous nerve excision. The application of the ALG sheet in human settings, especially for patients undergoing radical prostatectomy, is strongly expected although further studies are required to define the optimal concentration and thickness of the ALG sheet and to explore the location and timing of the ALG sheet placement clinically.

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STATEMENT OF AUTHORSHIP

Shintaro Narita: Data collection, manuscript writing. Takashi Obara: rat experiments, Namiko Ishikawa: supervision. Yoshihisa Suzuki, Tomonori Habuchi: manuscript writing and editing, supervision. All authors had read and approved the final manuscript.

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