博士学位論文

APPLICATION OF SODIUM ALGINATE AS A MEDICAL MATERIAL AIMED TO PREVENT AIR LEAK AND ADHESION

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APPLICATION OF SODIUM ALGINATE AS A MEDICAL MATERIAL AIMED TO PREVENT AIR LEAK AND ADHESION

アルギン酸ナトリウムのエアリークと癒着の防止のための医療材料への応用

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Ⅰ-1. What is alginate?

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Ⅰ-1. What is alginate?

FORMS OF ALGINATE

Alginate is natural polysaccharide extracted from brown algae that has good stability, solubility, viscosity, and biodegradability. It could be purchased in filamentous, granular or powdered forms. We are able to get a various brown seaweeds from all over the world and the seaweeds are converted into the raw material commonly known as sodium alginate. Sodium alginate was used in the various industries including food, textile printing, pharmaceutical, and medical fields. Sodium alginate is safe not only as an ingredient in manufactured foods but also as a hemostat agent. Generally, alginates extracted from different seaweed often have variations in their chemical structure, resulting in different physical properties, for instance about their viscosity (hard gel or weaker gel). Alginates are best used for technical applications regardless of the seaweed species.

STRUCTURE OF ALGINATE

Alginiate is a linear copolymer with homopolymeric blocks of two types of uronic acids; (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively. The residues are linked covalently in different sequences or blocks. The monomers could be constructed as the following three types; homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks) or alternating M and G-residues (MG-blocks).

Uronic acids have carboxyl groups, thus ion-exchange is able to carry out between protons and cations. Divalent cations have a high affinity with the α- L-guluronic acid blocks. Mainly changed cations are Na^{+} and Ca^{2+},

namely sodium alginate (empirical formula is $NaC_6H_7O_6$) and calcium alginate (chemical formula is $C_{12}H_{14}CaO_{12}$), respectively.

USE

The property that alginate could absorb water quickly worls as an additive in dehydrated products and also used for waterproofing and fireproofing fabrics. Alginate is used as a thickening agent for food, drinks, and cosmetics, and as a gelling agent for jellies.

Sodium alginate preparation is used clinically as one of the hemostat. It was reported to superior effect of hemorrhages in the uterocervical area compared to other topical drugs [1]. Other report showed the application of sodium alginate for chronic haemorrhoids, proctosigmoiditis, and chronic anal fissures after surgical interventions in the area of the rectum [2].

Calcium alginate is often used as dermal application incuding skinwound dressings. Heavily exuding wounds mainly for leg, diabetic ulcers and burns.

ALGINATE HYDROGELS

Alginate hydrogel is widely used in tissue engineering, providing a good environment for mesenchymal stem cells (MSCs). The relative simplicity and cell-friendly nature of alginate hydrogel is important for a major biomaterial in cell therapy [3]. Alginate encapsulation has been reported to protect to MSCs when exposed to hypothermic temperatures for a long time (days to weeks) [4, 5].

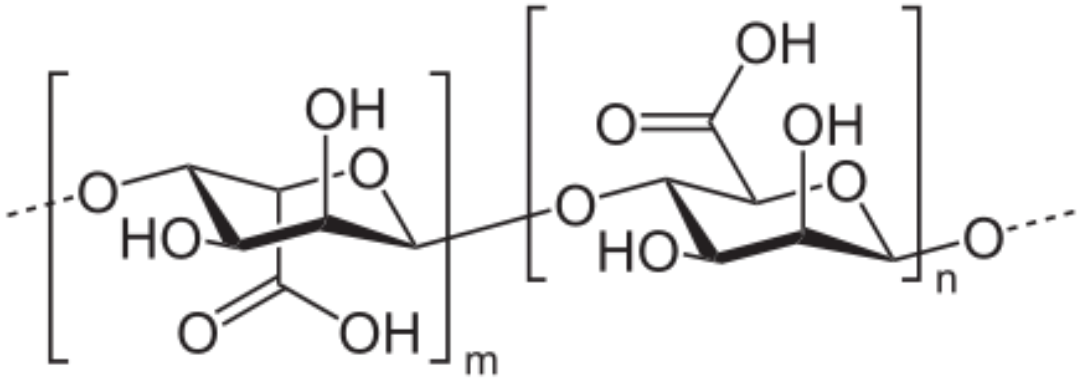

Figure 1. Structural formula of alginate.

Alginiate is a linear copolymer with homopolymeric blocks of two types of uronic acids; (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively. The residues are linked covalently in different sequences or blocks. The monomers could be constructed as the following three types; homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks) or alternating M and G-residues (MG-blocks). Uronic acids have carboxyl groups, thus ion-exchange is able to carry out between protons and cations mainly changed to Na^{+} and Ca^{2+}).

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Ⅰ-2. Properties of PGA

PHYSICAL PROPERTIES

Polyglycolic acid (PGA) is a biodegradable, thermoplastic polymer (aliphatic polyester) and could be prepared from the monomer, glycolic acid, using polycondensation or ring-opening polymerization. Currently PGA and its copolymers, for instance poly (lactic-co-glycolic acid) with lactic acid and so on, are synthesized for absorbable sutures and are being evaluated in the biomedical field [1]. PGA is one of the most frequently used devices during surgeries.

PGA has a glass transition temperature (35 - 40 °C) and a melting tempreture (225 - 230 °C). It also has a high crystallinity (about 46 - 50%), thus it is not soluble in water and most organic solvents such as aceton, excepting highly fluorinated organic solvents such as hexafluoro isopropanol [2].

SYNTHESIS

PGA can be obtained through several different processes starting with different materials. One of the simplest process is polycondensation of glycolic acid, but it is not the most efficient because only low molecular weight products are generated. Briefly speaking about the procedure, glycolic acid is heated at atmospheric pressure and a temperature (about 175 - 185 °C) is maintained until water ceases to distill. A pressure is reduced to 150 mm Hg, keeping the temperature for about two hours, the low molecular weight polyglycolide is obtained [3].

The preferred method for preparing high molecular weight PGA is ring-opening polymerization of glycolide, the cyclic dimer of glycolic acid,

and both solution and melt polymerization methods can be used [2]. The structural formula was shown in Figure 1.

DEGRADATION and INFLAMMATORY REACTION

When exposed to physiological conditions, PGA is degraded by random hydrolysis, not by the digestive enzyme. The degradation product, glycolic acid, is nontoxic to our body. A part of the glycolic acid is also excreted by urine [2].

PGA-made sutures was reported that these sutures loses half of its strength after two weeks and 100% after four weeks. The polymer is completely absorbed in our body within about four to six months [1]. Degradation is faster *in vivo* than *in vitro*, this phenomenon thought to be due to cellular enzymatic activity.

Ikeda et al. examined the strength of PGA non-woven fabric. Breifly speaking, the PGA fabric was soaked into 50 mL of phosphate buffered saline (PBS) (pH 7.4) and maintained at 37°C during 56 days. The residual fabrics were rinsed with water at the each assessment timing, because the PBS would get muddy because of water-soluble PGA oligomers/monomers (degradation products). After drying for 84 hours (Figures 2 shows the photographs), the residual weights was measured at the assessment timing of 7, 14, 28 and 56 days, and the degradation rate was calucurated at every assessment timing. pH levels of the PBS containing the degradation products were also measured at every weeks until 56 days. Figure 3 shows the degradation rate and Figure 4 shows the pH levels.

In addition to the *in vivo* examination, they evaluated the anti-adhesive effect and inflammatory reaction in canin lung model (shown in Figure 5). Briefly, the canine experiment was performed; the PGA fabric only was used and just fixed using adhesive for skin. Under the general anesthesia,

non-pregnant female beagle dogs were fixed in the right lateral decubitus position. Received tracheal intubation and the tube was connected with a respirator, thoracotomy was performed and the inferior lobe of the left lung was exposed. After the surgical procedures, the thoracic incision was closed with two-layered sutures. A drain tube was set through the thoracic wall to decompress in the thoracic cavity, degassing was started. The respirator was stopped when spontaneous breathing was confirmed. Several hours later after the confirmaion, the tube was removed. All dogs were killed humanly with intravenous anesthesia of a lethal dose of sodium pentobarbital at postoperative day 14. The fixed PGA fabrics were removed, and the specimens were fixed in a 10% formalin solution and were sliced into 4 μm sections in thickness. These specimens were evaluated microscopically using hematoxylin-eosin (HE) staining and immunostaining for cytokeratin AE1/AE3, which is used for evaluating the regeneration of pleural mesothelial [4]. Figure 6 shows these microsgraphs at postoperative day 14.

PGA induces a local inflammatory reaction because of local increase of neutrophils and the inflammatory cytokine (for instance interleukin-1α). This results in fibrosis and subsequent tissue adhesion against the PGA implantion site [5]. Several clinical studies of PGA devices also reported its inflammatory foreignbody reactions [6, 7].

USES

There are some PGA-made medical materials; sutures and non-woven fabrics. These materials are absorbable and are degraded in the body by hydrolysis and absorbed as water-soluble monomers over time (about 60 to 90 days).

PGA suture is a braided multifilament which is synthetic and absorbable. Coating with N-laurin and L-lysine contributes it to be extremely smooth, soft and safe for knotting. Before being sold, PGA suture sterilized with ethylene oxide gas. Its color is either violet or undyed. There are many advantages for PGA, such as high initial tensile strength, smooth passage through tissue, and easy handling. It is commonly used during the abdominal and thoracic surgeries.

PGA-made materials developed to implantable medical devices (anastomosis rings, pins, rods, plates and screws [1]) and scaffolds for tissue engineering or controlled drug delivery. PGA-made scaffolds usually obtained through textile technologies in the form of non-woven fabrics. For sinstance, Neoveil® (Gunze, Ltd, Kyoto, Japan) is one of the non-woven fabric whose fiber diameter is 16.2 μm on average and widely used in Japan. This is obtained by two steps of the extrusion process and the needle punch method.

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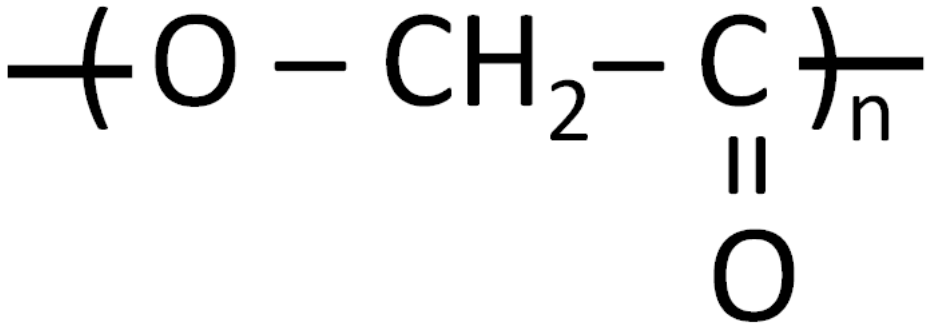


Figure 1. Structural formula of PGA.

PGA is a biodegradable, thermoplastic polymer (aliphatic polyester) and could be prepared from the monomer, glycolic acid, using polycondensation or ring-opening polymerization.

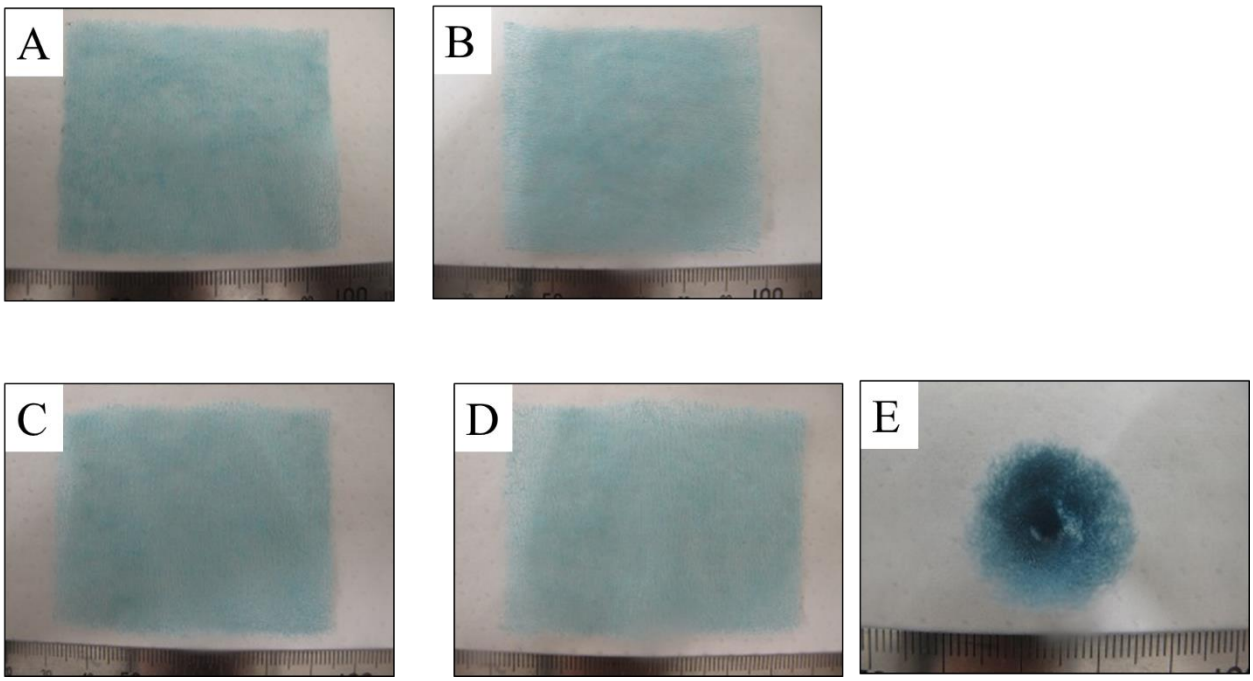
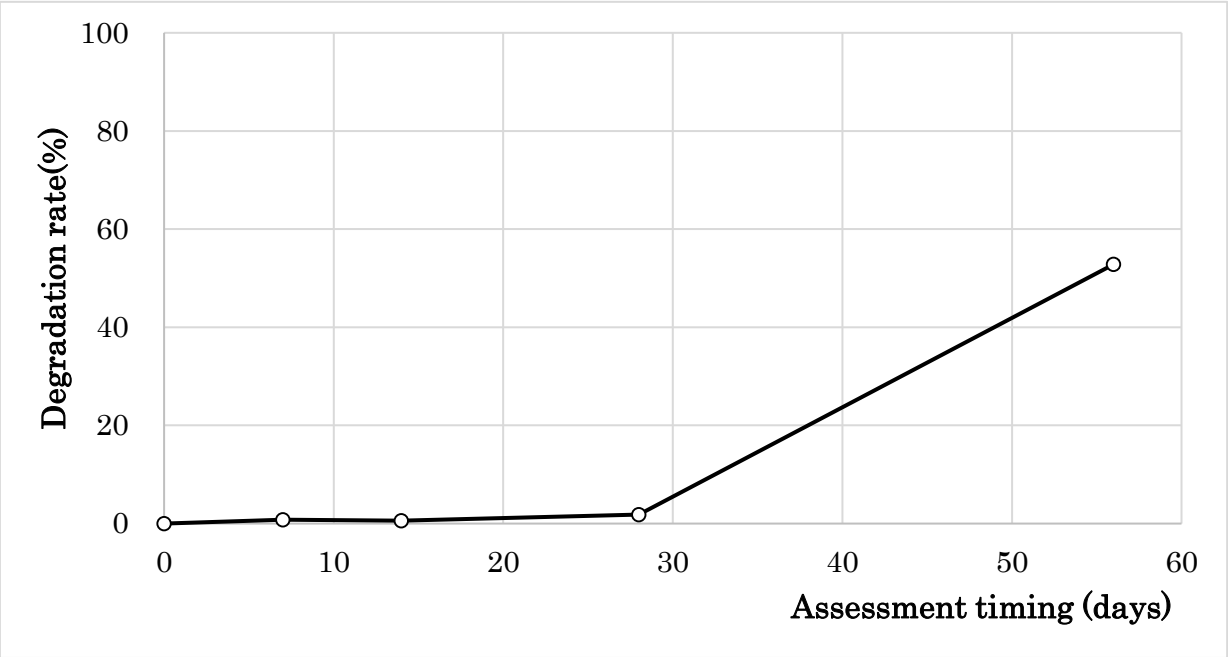


Figure 2. Photographs of the PGA non-woven fabric.
The residual weights was measured at the assessment timing of 7, 14, 28 and 56 days. The photographs show (A) at day 0, (B) day 7, (C) day 14, (D) day 28, and (E) day 56 after degradation, respectively.



Firure 3. The degradation rates of PGA non-woven fabric.
The circle shows the mean degradation rate, and the bar indicates the standard deviation (n=4). Since the value of SD at each point in time is quite small, the bars are not visible in the figure. The degradation rates (mean±SD) were 0.81±0.23, 0.60±0.05, 1.83±0.19 and 52.8±0.76 at the assessment timing of 7, 14, 28, and 56 days, respectively. The degradation rate increased slightly until 28 days, while the rate after 28 days increased largely.

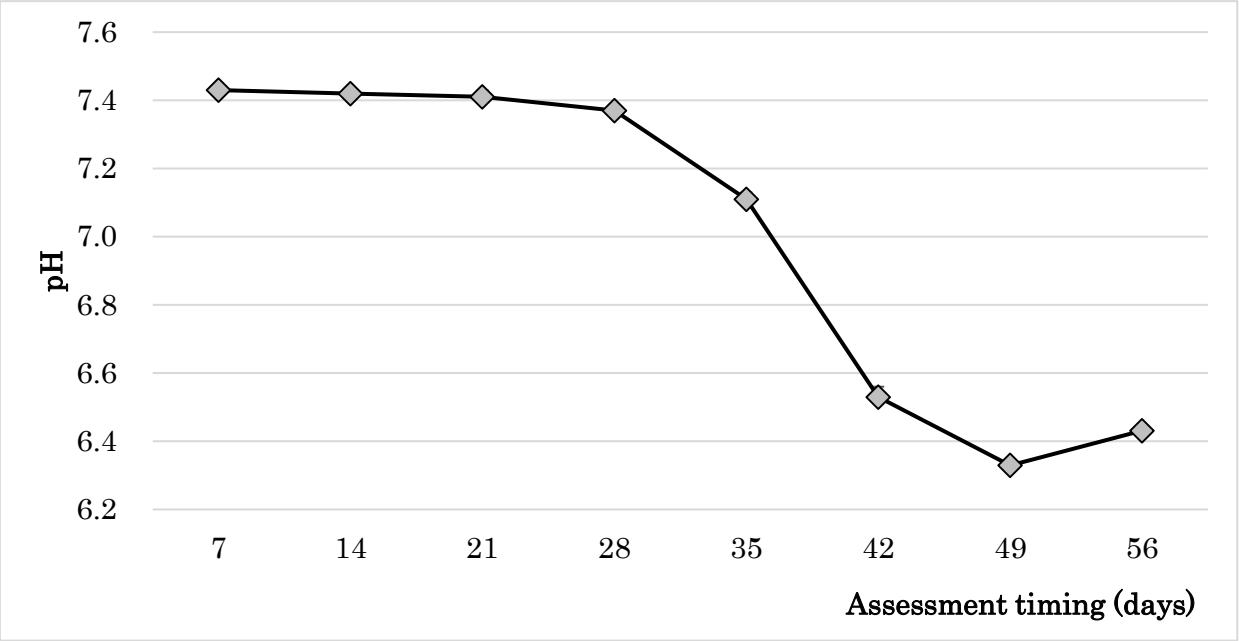


Figure 4. The pH levels of the PBS containing the degradation products. The column shows the mean and the bar indicates the standard deviation (n=4). Since the value of SD at each point in time is quite small, the bars are not visible in the figure. The pH values (means±SD) in the usual fabric group were 7.43±0.01, 7.42±0.01, 7.41±0.02, 7.37±0.00, 7.11±0.02, 6.53±0.03, 6.33±0.01, and 6.43± 0.01 at the assessment timing of 7, 14, 21, 28, 35, 42, and 56 days, respectively.
pH level decreased slightly until 28 days, while pH level after 28 days decreased rapidly by the 49 days and pH level increased a little at 56 days. The lowest level was at 49 days.

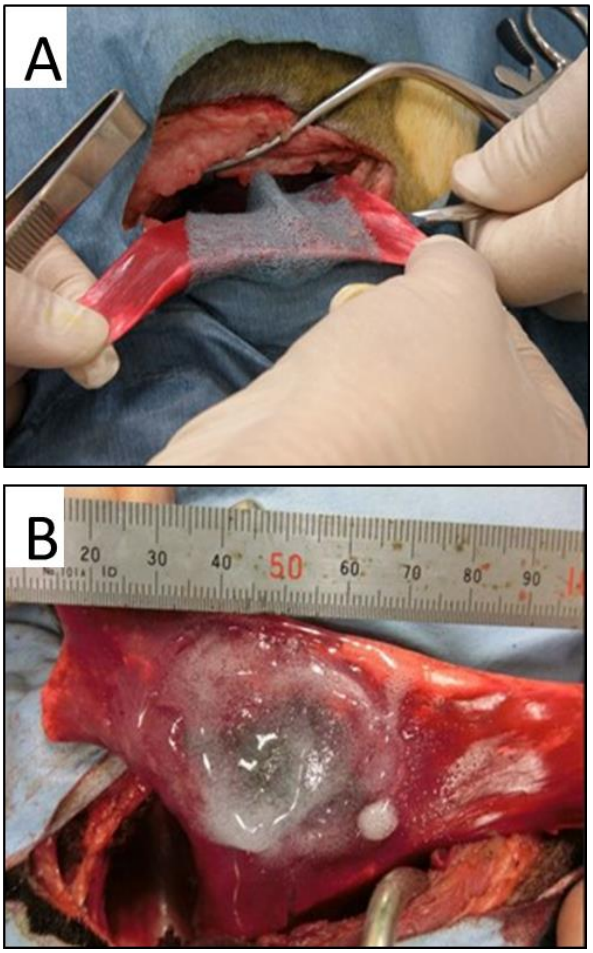


Figure 5. Surgical photographs at the inferior lobe of the left lung.
A shows that PGA non-woven fabric was fixed on the surface using adhesives for skin.
B shows that PGA non-woven fabric was applied to the cauterized area.

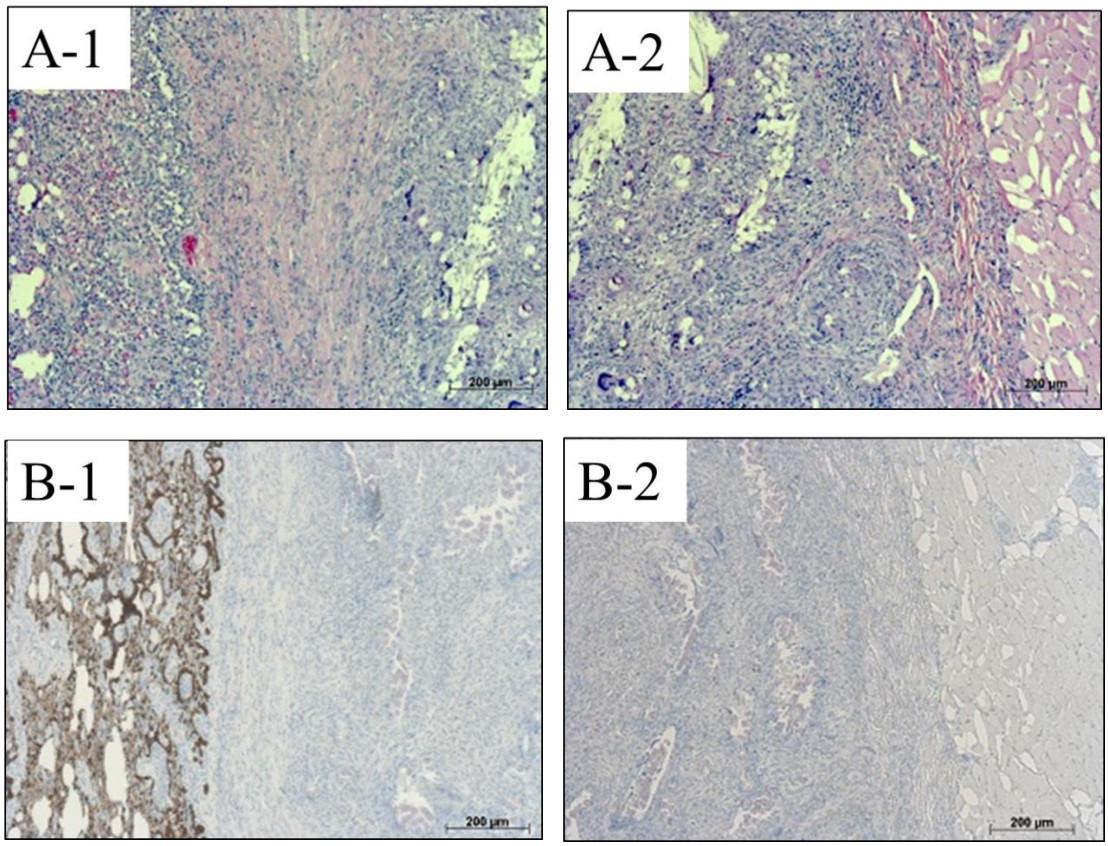


Figure 6. Microsgraphs at postoperative day 14.
A-1 and A-2 were HE staining and B-1 and B-2 were immunostaining for cytokeratin AE1/AE3. All scale bars shows 200 µm.
A-1 and A-2 shows the left is the lung side and the right is the diaphragm side.
The PGA non-woven fabric was adhered strongly to the surface of diaphragm. The surface of diaphragm was found the inflammatory reaction and the inflammatory aea was composed three layers through the surface of the lung to that of the diaphragm. Dense collagen-like substances and fibroblasts were existed at the layer on the lung side. The middle layer was composed of the fabric fibers surrounded by macrophages and fibroblasts. Collagen-like substances and fibroblasts were also exsisted at the layer on the diaphragm side. This layer was relatively thinner than the others. Common finding among the three layers is that inflammatory cells such as

leucocytes and lymphocytes were obserbed and there were no cytokeratin AE1/AE3-positive cells as a result of immnostaining.

Ⅱ.OVERALL SUMMARY

Ⅱ-1. OVERALL SUMMARY IN ENGLISH

Ⅱ-2. 全体の邦文要約

Ⅱ-1. OVERALL SUMMARY IN ENGLISH

BACKGROUND

Sodium alginate was examined for the the effects of preventing pulmonary air leak and postoperative intra-abdominal adhesion in the four studies.

STUDY 1 and STUDY 2: PREVENTIVE EFFECTS OF PULMONARY AIR LEAKS

We focused on the pulmonary air leak in the STUDY 1 and STUDY 2 to apply sodium alginate. Postoperative pulmonary air leaks remain a major cause of morbidity after lung resection. Pulmonary air leaks result in prolonged hospitalization and higher hospital costs. Various strategies have been proposed to manage pulmonary air leaks; however, their outcomes have been inconclusive. Both in the case of suturing or using an automatic suture instrument, it is not easy to suture the fragile tissues without the air leak. The reinforcement is necessary when to suture the fragile tissue.

Polyglycolic acid (PGA) mesh is a widely used biomaterial during various surgeries and is used as a reinforcement for weak tissues. Although PGA mesh was reported to reduce the air leak limitedly, it is hardly to prevent the air leak. Fibrin glue, the conventional agent, is one of the sealing materials generally superior to the other materials, the effect of preventing the air leak is limited. In the case of combination of PGA mesh and fibrin glue, it is insufficient effect and new sealing material is expected to improve the performance.

STUDY 1 and STUDY 2 evaluated the effect of a combination of PGA mesh and sodium alginate on pulmonary air leaks. The experiment model in STUDY 1 was the simplest. The puncture wound was created on

the rat’s lung surface using a needle. After application of each sealant over the wound, the lowest airway pressure that broke the seal was measured and the effect of preventing the air leak was evaluated. STUDY 2 was the experiment canine model closer to clinical operation, namely the thorascopic lung resection. Right middle lobe incision was performed with a linear stapler the lowest airway pressure was measured as the same manner as STUDY 1.

Materials and Methods (STUDY 1 and STUDY 2):

As a new sealing material in STUDY 1, sodium alginate was dissolved in water and sodium alginate solution was used. The alginate solution was partially cross-linked with calcium gluconate and gelled. Four pulmonary sealing materials were evaluated in lung injury: fibrin glue, combination of PGA sheet and fibrin glue, alginate gel, and combination of PGA sheet and alginate gel. With the airway pressure maintained at 20 cmH_2O, a 2-mm deep puncture wound was created on the lung surface using a needle. Lowering the airway pressure to 5 cmH_2O, each sealing material was applied. After application of each sealant, the lowest airway pressure that broke the seal (seal-breaking pressure) was measured. Soap water was sprayed over the sealant in advance. Airway pressure was gradually increased at a rate of 2 cmH_2O/s from 5 cmH_2O, and the minimum seal-breaking pressure was determined by the appearance of a bubble.

Sodium alginate solution was combined with calcium alginate nonwoven fabric and freeze-dried. The freeze-dired alginate turned into a sponge form and used as a new buttress which is usually needed to use a linear stapler in STUDY 2. We designed the new buttresses, including the combination of alginate sponge and PGA mesh, and the conventional ones, namely the combination of fibrin glue and PGA mesh. Right middle lobe incision of the beagle dog was performed with a linear stapler and one of the buttress. Burst pressures were measured under mechanical ventilation management.

Results (STUDY 1 and STUDY 2):

The seal-breaking pressure of alginate gel was as same as that of fibrin glue. The seal-breaking pressure of combination of alginate gel and PGA mesh was significantly greater than the others ($p < 0.01$) in STUDY 1. Burst pressures of alginate sponge was sufficiently effective to prevent the air leak. The combination of alginate sponge and PGA mesh had the best effect compared among the all buttresses in STUDY 2.

STUDY 3 and STUDY 4: PREVENTIVE EFFECTS OF POST-OPERATVE ADHESIONS

STUDY 1 and STUDY 2 evaluated the anti-adhesive effect to postoperative adhesion caused by PGA mesh. Although PGA mesh is a useful biomaterial in the clinical field, the mild acidity of glycolic acid, produced during the non-enzymatic degradation of PGA, causes chronic inflammation. Then the adhesions subsequently occur around the site where the PGA mesh was placed. This adheision has been an issue for a long time.

Generally, intra-abdominal adhesions develop after gynecological, gastroenterological and thoracic surgeries. The postoperative adhesions sometimes cause female infertility, bowel obstructions, and chronic abdominal pain or discomfort, and difficulties with subsequent surgeries, as well as prolonged hospitalization and hospital re-admissions, which could have an impact on both the patient's well-being and healthcare costs. The postoperative adhesions should be prevented as much as possible. The anti-adhesice effect is also important for the sealing material to prevent the air leak.

Based on the past report and recommendation, we used sodium alginate as a gel in STUDY 3 and as a freeze-dried sponge, which could rapidly turn into a gel, in STUDY 4.

Materials and Methods (STUDY 3 and STUDY 4):

The anti-adhesive effects against adhesions caused by PGA mesh; PGA-induced adhesions, using rat model in STUDY 3. PGA mesh was fixed on the parietal peritoneum of the abdomen. Sodium alginate powder was splinked over the PGA mesh. Calcium solution and/or physiological saline was sprayed over the powder. We designed three types of alginate gels which were cross-linked differently. Fibrin glue was sprayed as the same manner as the alginate gel. The above menthoned anti-adhesive materials were set on the PGA mesh and the abdomen was closed. The anti-adhesive effect against the PGA-induced adhesion was compared among the materials. Fifty-six days after the surgery, all rats were killed humanly and the adhesions were evaluated macroscopically by the adhesion scores and microscopically by hematoxylin-eosin staining and immunostaining. Human fibroblasts were cultured on the materials for one week.

STUDY 4 was aimed to improve the operability during surgery of the new alginate material. As a superior material for sealing and anti-adhesiveness, we developed a PGA mesh unified with sodium alginate sponge. It was assessed at 2, 4, and 8 weeks after surgery whether this new form, namely PGA mesh unified with alginate sponge, could prevent the adhesion or not.

Results (STUDY 3 and STUDY 4):

As results of STUDY 3 and STUDY4, sodium alginate had a superior anti-adhesive effect against PGA-induced adhesion. In addition to the adhesion scores of sodium alginate were the lowest significantly among the anti-adhesive materials, microscopic evaluations confirmed that the PGA mesh was covered by a peritoneal layer constructed of well-differentiated mesothelial cells and that the fibroblasts were suppressed to proliferate. Generally speaking, mesothelial cells contribute to tissue regeneration and fibroblasts are promoted to proliferate when imflammation or adhesion is occurred. The fibroblast proliferation was inhibited most on the surface of the alginate *in vitro*.

CONCLUSION

Sodium alginate is safe and superior polysaccharide extracted from seaweed. It was processed to gel form or sponge form in the four studies. The combination of gel or sponge alginate and PGA mesh was a promising alternative material to fibrin glue as a safe and low-cost material for preventing the air leak and adhesion. Therefore the new material, combination of sodium alginate and PGA mesh, was expected to apply as a superior sealing and anti-adhesive material in the clinical fields.

Ⅱ-2. 全体の邦文要約

背景

本研究では、アルギン酸ナトリウムを、肺の空気漏れ（エアリーク）防止と腹腔内の術後癒着防止を目的とした医療材料として臨床応用を行うために、それぞれに対する効果を STUDY 1 から STUDY 4 で検討した。

STUDY 1 ＆ STUDY 2: エアリーク防止効果の検討

STUDY 1 と STUDY 2 では、呼吸器外科領域で課題となるエアリークに焦点を当てた。呼吸器の分野では、肺の損傷部位や縫合閉鎖部位からのエアリークは重篤な罹患状態の原因となり、またエアリーク発生により治療入院期間が延長し医療費の高額化の原因となる場合が多く、疾患治療上も医療経済上も重大な課題となるからである。

この課題に対して、今までも様々なエアリーク防止材が検討されてきた。しかし、未だに確固たる解決策は無い。用手縫合でも自動縫合器使用でも、肺気腫や炎症で脆弱化した肺組織を縫合閉鎖する場合、エアリーク無く縫合することは必ずしも容易では無く、縫合部の組織を補強する組織補強材やエアリーク部位のシーラントなどの医療材料が必要となる。

従来からこの課題に対する様々な医療材料が開発されてきたが、未だにその効果は不十分である。例えば、組織補強材として臨床で広く使用されているポリグリコール酸（PGA）不織布は、ある程度のエアリーク防止効果が認められているが、それでもエアリークを十分に防止することは困難である。また従来製剤のフィブリン糊も、総合的には優れたシーラントではあるものの、その効果は限定的であると言わざるを得ない。また両者の併用もその効果は、臨床

的に十分とは言い難く、エアリーク防止性能の改良向上が望まれる。

STUDY 1 と STUDY 2 では、アルギン酸ナトリウムを PGA 不織布と併用して肺の損傷部位修復を行う場合のエアリーク防止効果を検討した。STUDY 1 では、最も単純な実験モデルであるラットの肺のピンポイント損傷部位からのエアリーク防止効果を、アルギン酸ナトリウム溶液やフィブリン糊を塗布する実験にて検討した。STUDY 2 では、より臨床に近い実験モデルとして、胸腔鏡下肺切除手術を想定し、イヌの肺を用いて自動縫合器で切離縫合を行った場合のエアリーク防止効果を検討した。

材料と方法（STUDY 1 ＆ STUDY 2）:

STUDY 1 では、アルギン酸ナトリウムのエアリーク防止材として、アルギン酸ナトリウムを水溶液にして研究に用いた（以下、アルギン酸溶液とする)。ラットの肺にピンポイントに損傷しエアリークを生ずる状態を作製した。その部位の処置に、アルギン酸溶液と PGA 不織布を併用する場合と、フィブリン糊と PGA 不織布との併用の場合とのエアリーク防止効果と比較検討した。ラットの気道内圧を 20cmH_2O に維持し、針で肺表面に穴を開けてエアリークを惹起した。その後、5 cmH_2O まで気道内圧が下がりエアリークが止まった状態を作製した。アルギン酸溶液と PGA 不織布の併用、またはフィブリン糊と PGA 不織布の併用によりエアリーク防止処置を施し、次に徐々に気道内圧を上昇亢進させて人為的に肺を膨らませた。この気道内圧亢進の過程で処置部からの空気漏れを目視で確認できた時点の、最も低い気道内圧を破裂圧として測定し、それぞれのエアリーク防止材の破裂圧を比較することによりエアリーク防止効果を検討した。

STUDY 2では、アルギン酸のエアリーク防止材として、アルギン酸ナトリウム溶液を凍結乾燥によってスポンジの形状にしたものを用いた（以下、アルギン酸スポンジとする)。アルギン酸スポンジとPGA不織布の併用を含む幾つかのアルギン酸系新規エアリーク防

止材、あるいは従来から使用されているフィブリン糊とPGA不織布の併用を含む複数の従来型エアリーク防止材を作製した。これらを自動縫合器による肺の切除（切離縫合）のエアリーク防止材として用いる実験モデルを用い、各防止材の間でエアリーク防止効果を比較検討した。自動縫合器はヒトの肺の手術用に開発されているので、本実験では、ヒトの肺に近いビーグル犬を用い、イヌの肺を自動縫合器で切離縫合した。破裂圧は人工呼吸器に接続した状態で、その人工呼吸器の回路内気圧を測定して決定した。

結果（STUDY 1 ＆ STUDY 2）:

STUDY 1 の結果は、アルギン酸溶液の単独使用の場合であっても、フィブリン糊と同等の破裂圧であった。アルギン酸溶液を PGA 不織布と併用すると、フィブリン糊と PGA 不織布の併用を含む他すべての群と比べて有意に破裂圧が高かった。STUDY 2 の結果は、アルギン酸スポンジ単独使用において十分なエアリーク防止効果を認め、さらに、アルギン酸スポンジを PGA 不織布と併用することで、フィブリン糊と PGA 不織布の併用を含む従来のすべての製材の単独使用や併用に比較して、有意に高いエアリーク防止効果が認められた。

STUDY 3 ＆ STUDY 4: 術後癒着防止効果の検討

STUDY 3 と STUDY 4 ではアルギン酸ナトリウムを用いて、PGA 不織布惹起性の術後癒着防止効果の検討を行った。PGA 不織布は一般臨床で使用される非常に有用な生体吸収性組織補強材である。しかし、生体内で分解される過程で周囲組織の pH を酸性側に傾かせ、癒着を引き起こすという課題がある。一般的に、消化器や胸部、産婦人科などの手術後に胸腹腔内に癒着が生じると、臓器の機能が妨げられ、時には腸閉塞や慢性腹部痛、女性の不妊の原因となり、癒着が生じた部位の手術の困難性や、入院の長期化が医療費を圧迫するなど社会的影響も大である。この様な理由から術後癒着は可及的に防止することが望ましく、エアリーク防止材でもこの課題は非常に重要である。STUDY 3 では、PGA 不織布にアルギン酸ナトリウムのゲル製材を、STUDY 4 ではスポンジ製材を PGA と併用した場合の、PGA による癒着に対する課題解決について検討した。

材料と方法（STUDY 3 ＆ STUDY 4）:

STUDY 3 では、ラットの腹腔内癒着モデルを用いた。PGA 不織布を腹腔内の壁側腹膜に固定し、その PGA 不織布上にアルギン酸ナトリウム粉末を噴霧した。この上に、カルシウム溶液や生理食塩水を噴霧することで、カルシウム架橋度の異なる 3 種類のアルギン酸ゲルをで覆った状態にした。フィブリン糊も同様に噴霧して、PGA 不織布を覆った状態にした。その後腹壁を縫合閉鎖した。この手術操作の後に PGA により惹起される癒着の防止効果を、アルギン酸ゲルとフィブリン糊との間で比較検討した。術後 56 日後にラットを犠牲死せしめ、肉眼的癒着スコアによる評価とヘマトキシリンーエオシン染色と免疫学的染色標本の顕微鏡的評価により癒着防止効果を評価した。さらに *in vitro* では各癒着防止材上での線維芽細胞の増殖を比較検討した。

STUDY 4 では、手術中の操作性の良さを向上させる目的でエアリーク防止材としての有効性と癒着防止効果を持ちかつ操作性に優れた補強材として、凍結乾燥によりスポンジ化したアルギン酸ナトリウムと PGA 不織布を一体化させた新材形を新規開発した。この形状でも、STUDY3 で検討した場合と同じように癒着防止効果が発揮されるか否かを、術後 2，4，8 週間後に評価を行った。

結果（STUDY 3 ＆ STUDY 4）:

STUDY 3 でも STUDY 4 でも PGA による癒着惹起に対して、アルギン酸ナトリウムの優れた癒着防止効果が認められた。肉眼的スコアによる評価で有意に腹腔内癒着防止効果が高いのみならず、組織学的にも、炎症・癒着発生時に増殖する線維芽細胞の増殖抑制、組織再生に重要な中皮細胞の増殖促進効果が認められた。また *in vitro* の研究において、アルギン酸塩の表面での線維芽細胞の増殖を検討した結果、癒着形成や瘢痕形成に主な役割を果たす線維芽細胞の増殖は、アルギン酸塩の表面では抑制されることが明らかになった。

総合的結論

アルギン酸ナトリウムは、安全性に優れた海藻由来の多糖類である。アルギン酸ナトリウムをゲルやスポンジの材形に加工して PGA 不織布と併用することで、優れた空気漏出防止効果および癒着防止効果を発揮することが可能となった。この結果から、将来的には、アルギン酸と PGA 不織布とを併用したエアリーク防止材は、従来の補強材やシーラントの併用よりもエアリーク防止材効果と癒着防止効果の両面において優れた医療材料として、臨床に応用されることが期待できると考えられる。

Ⅲ. STUDY 1

REDUCTION OF PLUMONARY AIR LEAKS WITH A COMBINATION OF POLYGLYCOLIC ACID SHEET AND ALGINATE GEL IN RATS

ポリグリコール酸シートと アルギン酸ゲルの組合せによる 肺のエアリーク軽減効果の ラットにおける検討

TABLE OF CONTENTS (STUDY 1)

REFERENCES

ABSTRACT (STUDY 1)

Background:

Postoperative air leaks remain a major cause of morbidity after lung resection. Pulmonary air leaks in thoracic surgery result in prolonged hospitalization and higher hospital costs. Various strategies have been proposed to manage pulmonary air leaks; however, their outcomes have been inconclusive. This study evaluated the effect of a combination of polyglycolic acid (PGA) sheet and alginate gel, as a sealant, on pulmonary air leaks in rats. Sealant-inducing pleural adhesion is a concern in the use of sealant materials to prevent pulmonary air leak. Although pleural adhesions are known to prevent pulmonary air leak, those at re-thoracotomies often complicate pulmonary surgeries, making these procedures more time-consuming and hazardous for the patient. It is also important for the sealant to prevent pleural adhesions. According to our previous report, alginate gel was compared to fibrin glue as a sealing material.

Materials and Methods:

Four pulmonary sealing materials were evaluated in lung injury: fibrin glue, combination of PGA sheet and fibrin glue, alginate gel, and combination of PGA sheet and alginate gel. With the airway pressure maintained at 20 cmH_2O, a 2-mm deep puncture wound was created on the lung surface using a needle. Lowering the airway pressure to 5 cmH_2O, each sealing material was applied. After application of each sealant, the lowest airway pressure that broke the seal (seal-breaking pressure) was measured. Soap water was sprayed over the sealant in advance. Airway pressure was gradually increased at a rate of 2 cmH_2O/s from 5 cmH_2O, and the minimum seal-breaking pressure that caused sealing failed was determined by the appearance of a bubble.

Results and Conclusions:

The seal-breaking pressure in each experimental group was fibrin, 10.4 ± 6.8cmH_2O; PGA + fibrin, 13.5 ± 6.5cmH_2O; alginate gel, 10.3 ± 4.9cmH_2O; and PGA + alginate, 35.8 ± 11.9cmH_2O, respectively. The seal-breaking pressure was significantly greater in the PGA + alginate gel group than in the other groups ($p < 0.01$). There were no significant differences among the other three groups. Alginate gel combined with a PGA sheet is a promising alternative to fibrin glue as a safe and low-cost material for air leak prevention in pulmonary surgery.

邦文要約（STUDY 1）

背景：

肺切除後の肺の空気漏れ（エアリーク）は、重篤な罹患状態の原因となる。呼吸器の手術時での肺のエアリークのために、入院期間を延長し、医療費が高額となり、呼吸器外科領域では重要な問題となっている。そのため、エアリークの防止策が様々に検討されてきたが、未だに確固たる解決策は無い。本研究ではラットを用いて、PGA とアルギン酸ゲルの組み合わせがエアリーク防止材（エアリーク防止材）として肺のエアリークを防止する効果があるか検討した。一方、エアリーク防止材により惹起されて肺で癒着が生じることで、肺のエアリークを防止するという目的でエアリーク防止材が使用されることもある。このように、胸腔内の術後癒着はエアリークを防止できると知られているが、再手術が必要となった場合、エアリーク防止のために処置された胸腔内癒着はむしろ、再手術が難しくなり、その結果手術時間の長期化、患者への負担増となる。以上の理由から、エアリーク防止材においても癒着防止効果もある材料が求められる。過去の報告から、癒着防止効果も期待できるエアリーク防止材としてアルギン酸を、従来法のフィブリン糊と比較した。今回は、フィブリン糊、PGA シートとフィブリン糊の組み合わせ、アルギン酸ゲル、PGA シートとアルギン酸ゲルの組み合わせ、以上の 4 種類のエアリーク防止材で検討した。

材料と方法：

気道内圧を 20cmH_2O に維持し、針で肺表面から 2-mm の深さの穴を開けた。5 cmH_2O まで気道内圧が下がったときに、各エアリーク防止材を処置した。シール効果がなくなった時点の最も低い気道内圧（以下、破裂圧とする）を測定した。破裂圧は、PGA シートとアルギン酸ゲルの組み合わせが、他の 3 群と比較して有意に高かった。他の 3 群間では、有意差は認められなかった。

結果および結語：

アルギン酸ナトリウムは安全性に優れた医療材料である。これを呼吸器の手術時のエアリーク防止のために、PGA シートと組み合わせることで、フィブリン糊の代替としての使用が期待される。

INTRODUCTION

Postoperative air leaks remain a major cause of morbidity after lung resection. Pulmonary air leaks in thoracic surgery result in prolonged hospitalization and higher hospital costs [1]. Various strategies have been proposed to manage pulmonary air leaks [2-8]; however, their outcomes have been inconclusive. It is difficult to prevent air leaks by suturing or stapling in cases of severe pulmonary emphysema or at sites near the lung hilum. Moreover, some materials for air leak prevention have limitations relating to safety and cost. Fibrin glue alone or in combination with polyglycolic acid (PGA) have been reported as effective materials for preventing pulmonary air leaks [4, 9]. PGA is biodegradable and functions as a scaffold for tissue regeneration. These materials have been used in thoracotomy or thoracoscopic surgeries and applied on staple lines [7, 10]. PGA sheets alone have been reported to reduce pulmonary air leaks [11, 12], while PGA sheets and fibrin glue in combination showed superior effects to prevent air leaks [13, 14]. However, the effects of conventional materials have been inconclusive thus far [4, 15, 16]. A general concern of fibrin glue is the transmission of blood-borne diseases [17,18]. Moreover, fibrin glue is expensive. In the present study, the sealing effects of alginic acid, which has high safety and low cost, were investigated as a possible alternative for fibrin glue. Few studies have accurately assessed each method in terms of pressure resistance at the time of sealing [19]. In this study, the seal-breaking pressure of this new material was investigated for use in preventing pulmonary air leaks.

MATERIALS and METHODS

Four pulmonary sealing strategies were evaluated in lung injury: 1) fibrin glue, 2) combination of PGA sheet and fibrin glue, 3) alginate gel, and 4) combination of PGA sheet and alginate gel.

1. Materials

1-1 Fibrin glue

Fibrin glue (Beriplast P Combi-Set®, CSL Behring Co., PA, USA) is composed of solutions A (fibrinogen and aprotinin) and B (thrombin and calcium chloride). When solution A and B are mixed for use, the fibrin adhesive mimics physiological fibrin clot formation by the coagulation system.

1-2 PGA sheet

A PGA sheet (Neoveil®, Gunze Ltd., Kyoto, Japan) (Figure 1-1 and 1-2) was cut to 5 mm × 5 mm in size and sterilized with ethylene oxide for 22 hours. The fiber diameter of the PGA sheet (Neoveil®) is 16.1 μm, the average distance between fibers is 27.4 μm, and the average sheet thickness is 0.15 mm. Ethylene oxide gas was removed under conditions of decompression for 1 week.

1-3 Alginate gel

Sodium alginate powder (Alto®, Kaigen Ltd., Osaka, Japan) (molecular weight: 32,000–250,000) was dissolved in saline to prepare 5 w/v% alginate solution. The gelling agent consisted of calcium gluconate solution (Calcicol®, Nichi-Iko Ltd., Toyama, Japan). Sodium alginate solution was partially cross-linked with calcium gluconate and gelled, and the alginate gel acquired high local retentivity.

The application of fibrin and alginate alone and in combination with a PGA sheet to the lung injury site is described in section 2-4.

2. Animal protocol and experimental design.

The animal experiments were approved by the Doshisha University Animal Experimentation Committee. All surgical procedures and anesthesia protocols were conducted in accordance with the Animal Care Guidelines of Doshisha University. During the experimental period, a week of habituation period was set. Non-pregnant 8-week-old female Wistar/ST rats weighing approximately 200 g were used for this study. All the rats were housed separately and maintained under standard specific pathogen-free conditions (a light-dark cycle of 12:12 h, temperature of 20.1–23.5°C, and humidity of 37–65%). Standard laboratory rodent chow and water were freely available. Twenty-four rats were randomly assigned to four experimental groups: 1) fibrin (n = 6), 2) PGA + fibrin (n = 6), 3) alginate gel (n = 6), and 4) PGA + alginate gel (n = 6).

3. Surgical procedure for lung injury

All rats were killed humanely by intraperitoneal injection of pentobarbital sodium at a fatal dose (0.025 mg/g) under isoflurane inhalational anesthesia. Rats were fixed in the dorsal position. Endotracheal intubation with a 16G catheter was carried out, and the catheter was fixed by ligation. Thoracotomy was performed by removing all the ventral side ribs and incising the diaphragm to expose the lung. The tracheal tube was connected to a pressure gauge (testo510®, Testo SE & Co. KGaA, Lenzkirch, Germany) and syringe with a three-way stopcock (Figure 2). With the airway pressure maintained at 20 cmH_2O, a 2-mm-deep puncture wound was created on the surface of the right middle lung lobe using a 23G needle. Bleeding was stopped by astriction. After lowering the airway pressure to 5 cmH_2O, each sealing material was applied (Figure 3).

4. Application of sealing materials (Figure 4)

1) Fibrin glue (fibrin group)

The pleural puncture wound was sealed by instillation of a drop of fibrinogen solution (solution A), followed by simultaneously spraying fibrinogen and thrombin solutions (solution A and B, respectively) (total instillation amount of solution A, 0.1 ml; solution B, 0.1 ml).

2) Combination of a PGA sheet and fibrin glue (PGA + fibrin group)

The pleural puncture wound was sealed by instillation of a drop of fibrinogen solution (solution A), and then a PGA sheet was placed onto the wound with a forceps and pressed by an index finger to absorb the solution. Then, fibrinogen and thrombin solutions were simultaneously sprayed over the PGA sheet (total instillation amount of solution A, 0.1 ml; solution B, 0.1 ml).

3) Alginate gel (alginate group)

The pleural wound was sealed with instillation of 0.025 ml sodium alginate solution followed by five drops of calcium gluconate solution by using a syringe attached to a 26G needle. The same procedure was repeated twice. After a 5-min interval, another 0.025 ml of sodium alginate solution was instilled.

4) Combination of PGA sheet and alginate gel (PGA + alginate group)

The pleural wound was sealed with instillation of 0.025 ml sodium alginate solution, and then a PGA sheet permeated by a drop of calcium gluconate was overlaid. Four drops of calcium gluconate solution were then instilled onto the PGA sheet, followed by spraying with 0.025 ml sodium alginate solution. After a 5-min interval, 5 drops of calcium gluconate followed by 0.025 ml sodium alginate solution were instilled.

5. Measurement of minimum seal-breaking airway pressure

After application of each sealant, it was left motionless for 5 min, and then the lowest airway pressure that broke the seal (seal-breaking pressure) was measured. Soap water was sprayed over the sealant in advance. Airway pressure was gradually increased at a rate of 2 cmH_2O/s from 5 cmH_2O, and the minimum seal-breaking pressure that caused sealing failed was determined by the appearance of a bubble.

6. Statistical analysis

Seal-breaking pressure was compared among the groups by using one-way analysis of variance and Tukey's test. Differences were defined to be statistically significant for p values less than 0.05.

RESULTS

Seal-breaking pressure in each experimental group (mean ± standard deviation) was as follows: 1) fibrin group, 10.4 ± 6.8 cmH_2O; 2) PGA + fibrin group, 13.5 ± 6.5 cmH_2O; 3) alginate group, 10.3 ± 4.9 cmH_2O; 4) PGA + alginate group, 35.8 ± 11.9 cmH_2O. Seal-breaking pressure in the PGA + alginate group was significantly greater than that in the other groups ($p<0.01$). There were no significant differences among the other three groups (Figure 5).

DISCUSSION

In this study, the combination of alginic acid gel and PGA sheet showed excellent effects for the prevention of pulmonary air leaks in rats. In recent years, fibrin glue has been widely used for the prevention of air leaks in pulmonary surgery [11, 16]. However, fibrin glue is problematic in that it is derived from human blood, and thus is associated with a clinical risk of pathogenic infections. Alginate is a naturally derived polysaccharide typically obtained from brown seaweed, and has been safely used for many biomedical applications due to its biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [20]. Consequently, alginate is considered a candidate to replace fibrin glue as a sealant against pulmonary air leaks.

The minimum seal-breaking pressures of fibrin glue and alginate gel alone were approximately 10 cmH_2O in this study, which is insufficient to prevent air leaks during and after pulmonary surgery under mechanical ventilation. By contrast, the substitution of alginic acid gel for fibrin glue in combination with a PGA sheet led to superior air leak prevention. The minimum seal-breaking pressure of the combination of alginate gel and PGA sheet was 35 cmH_2O, which is thought to provide sufficient strength for air leak prevention under forced ventilation by a respirator.

The combination of PGA sheet and fibrin glue has been reported to provide good air leak prevention [12, 15]. The PGA sheet is flexible and follows the shape of the organs, and it retains liquid, allowing it to be combined with a viscous adhesive to close air leaks. However, in the present study, the combination of PGA sheet and fibrin glue did not demonstrate a significant improvement in air leak sealing effects compared to fibrin glue alone. We speculate that there may be two possible reasons for this discrepancy. First, in a previous study on sealing effects with long-term observation of the combination of PGA sheet and fibrin glue, the PGA

sheet functioned as a good scaffold for tissue regeneration to accelerate lung wound healing [21]. The present study did not investigate long-term healing, but only short-term leak prevention, which might have led to the similar results between fibrin alone vs. fibrin + PGA. Rapid wound healing is an important factor for air leak prevention after pulmonary surgery, and alginate is known to promote wound healing as a good scaffold for tissue regeneration [22]. Second, in the assessment of direct and instantaneous sealing effects during surgery, it is possible that differences in procedures to apply fibrinogen, thrombin, and PGA sheets affected this discrepancy. It is considered necessary for a glue or gel to fill the fiber gap of a PGA sheet to show a satisfactory air leak prevention effect. However, it is possible that with the combination of PGA sheet and fibrin glue used in the present experiment, the fibrin glue could not sufficiently fill the fiber gap. Because fibrin glue has poor impregnating ability, solution A was applied the pleural wound in advance, and then solutions A and B were sprayed simultaneously, as in previous reports. Immediately after mixing the two solutions, the fibrin glue develops high adhesiveness and few bubbles remain within the glue. These physical properties may have inhibited fibrin glue permeation into the PGA fiber gap. The 5% sodium alginate solution has low viscosity and turns into highly adhesive gel crosslinked by calcium [22]. Pre-instilled sodium alginate solution in addition to sprayed sodium alginate solution thoroughly permeated the calcium gluconate-dipped PGA sheet. Consequently, the alginate gel filled the fiber gap of the PGA sheet. The sealant was adhered tightly by adding one more layer of alginate gel over the PGA sheet. By applying alginate gel in multiple layers to the lung wound, the sealing effect of the combination of PGA sheet and alginate gel may have been reinforced.

Fibrin glue used alone could easily flow from the application site due to its low retentivity. Sodium alginate solution itself is watery and can be easily handled, but is inadequate as a sealant due to poor local retentivity. To increase pressure resistance and local retentivity, sodium alginate was

gelated by crosslinking with calcium gluconate as described in the MATERIALS and METHODS. As with fibrin glue, the simultaneous use of a PGA sheet allowed the alginate gel to remain at the application site. In this study, the ratio of sodium alginate and calcium gluconate was determined as the concentration that showed superior anti-adhesive effects in our previous study [23]. However, the optimal concentration of sodium alginate solution and dosage of calcium gluconate must be evaluated in further investigations.

Sealant-inducing pleural adhesion is a concern in the use of sealant materials to prevent pulmonary air leak. Although pleural adhesions are known to prevent pulmonary air leak, pleural adhesions at rethoracotomies often complicate pulmonary surgeries, making these procedures more time-consuming and hazardous for the patient [24, 25]. The mild acidity of polyglycolic acid, produced during the non-enzymatic degradation of PGA, causes chronic inflammation, and adhesions subsequently occur around the site where the PGA mesh was placed [21, 25]. Although PGA-induced adhesions have long been considered problematic, PGA continues to be used due to its effectiveness as a biomaterial. Therefore, when assessing sealants for air leak prevention, in addition to their sealing effect, anti-adhesive effects are also desirable. Fibrin glue is often used to prevent adhesion; however, its effectiveness for this purpose is controversial [23, 26, 27]. We previously reported a superior anti-adhesive effect of alginate gel in comparison to fibrin glue in a study of PGA-induced adhesions [23].

The limitations of this study include the fact that it compared the mechanical sealing effect during surgery and not include long-term observation after surgery, and that the healing process of the peritoneal injury was not taken into account due to the short-term nature of the intraoperative observation. Furthermore, this study was conducted using an experimental model in rats, and further investigation in humans is necessary to confirm the results.

CONCLUSION

In conclusion, alginate gel, a safe and low-cost material, is a potential substitute for the use of fibrin glue in combination with a PGA sheet for air leak prevention in pulmonary surgery. The combination of PGA sheet and alginate gel may be useful to close air leaks at sites of pleural injury near the hilar region, where it is difficult to close air leaks by suturing or stapling, due to their ease of handling during application.

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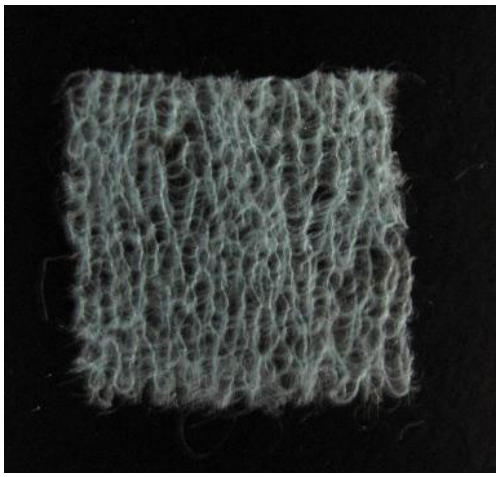

Figure 1-1. A picture of PGA sheet.

A PGA sheet in this study was cut 5 × 5 mm in size and the average sheet thickness is 0.15 mm. The fiber diameter of the PGA is 16.1 μm and the average distance between fibers is 27.4 μm.

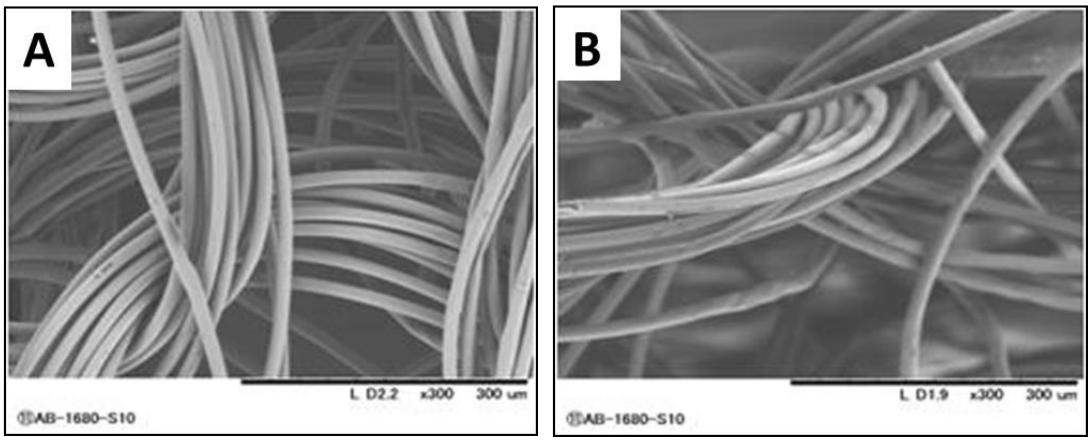

Figure1-2. Scanning electron microscopy (SEM) micrographs which obserbed; (A) the structure of top layer and (B) cross-section surface. All of the fiber diameter and distance were measured by Image J software.

(A): The fiber selection was randomly from among thirty fibers showed in the top layer of SEM micrographs. Each fiber fiameter was measured and the average diameter was calcurated.

We focused on the distance from the fiber-edge to the edge between neighboring fibers. The distance was defined as the size of the fiber-spacing, hereinafter the size was called “spacing-size”.

(B): The spacing-size selection was randomly from among thirty spacing-sizes showed in the cross-section surface of SEM micrographs. The spacing-size was obserbed in light microscope and took images, then measured from them.

As a result of the observation of spacing-size, we found that the structure of PGA was composed of two kinds of locations, namely a "fiber bundle" and a "hole". These locations were constructed by different two spacing-sizes. Such a structure of PGA was considered to be made using its production method. Spacing-sizes of the fiber bundle and the hole was measured in more detail.

The mean of fiber diameter was 16.2 μm, that of spacing-size of the fiber bundle was 5.0 μm, and that of the hole was 825.4 μm. Such structure

elicited a predominantly myofibroblasts response at postoperative day 14 and regenerated dense connective tissue and/or scar at postoperative day 70.

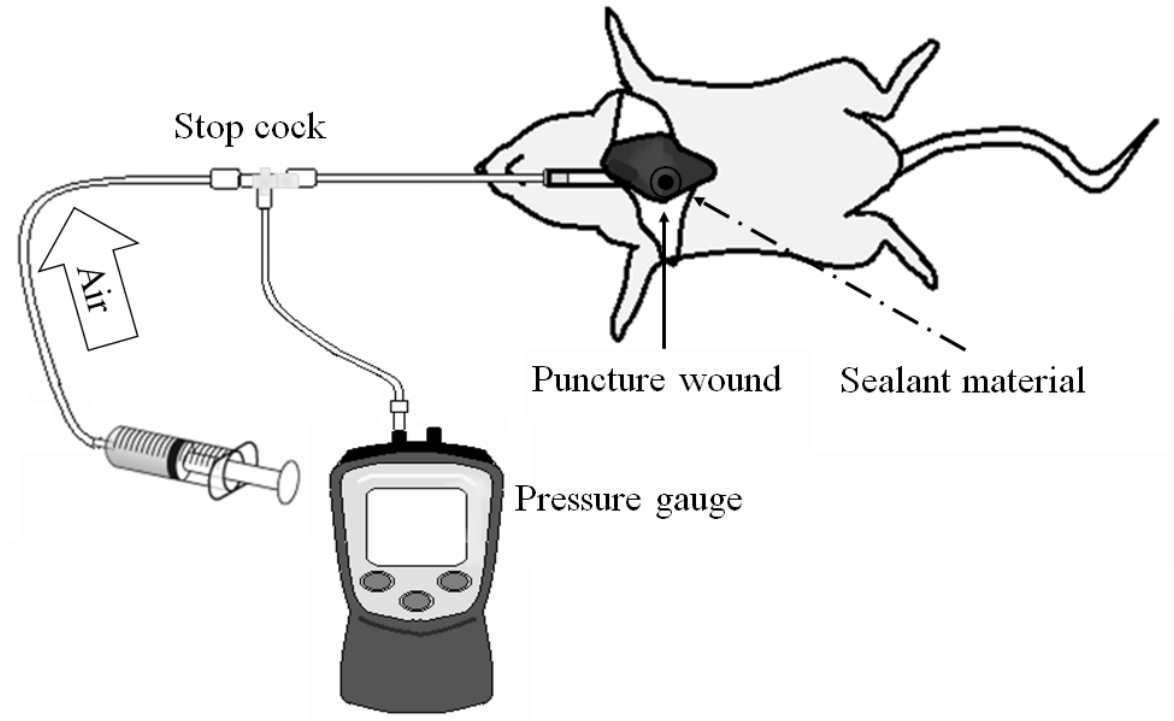

Figure 2. Schema of airway pressure measurement in a rat

All rats were killed humanly by intraperitoneal injection of pentobarbital sodium at a fatal dose under isoflurane inhalational anesthesia. Rats were fixed in the dorsal position. Endotracheal intubation with a 16G catheter was carried out, and the catheter was fixed by ligation. Thoracotomy was performed by removing all the ventral side ribs and incising the diaphragm to expose the lung. The tracheal tube was connected to a pressure gauge (testo510®, Testo SE & Co. KGaA, Lenzkirch, Germany) and syringe with a three-way stopcock. With the airway pressure, a 2-mm-deep puncture wound was created on the surface of the right middle lung lobe using a 23G needle. Bleeding was stopped by astriction. After lowering the airway pressure to 5 cmH_2O, each sealing material was applied.

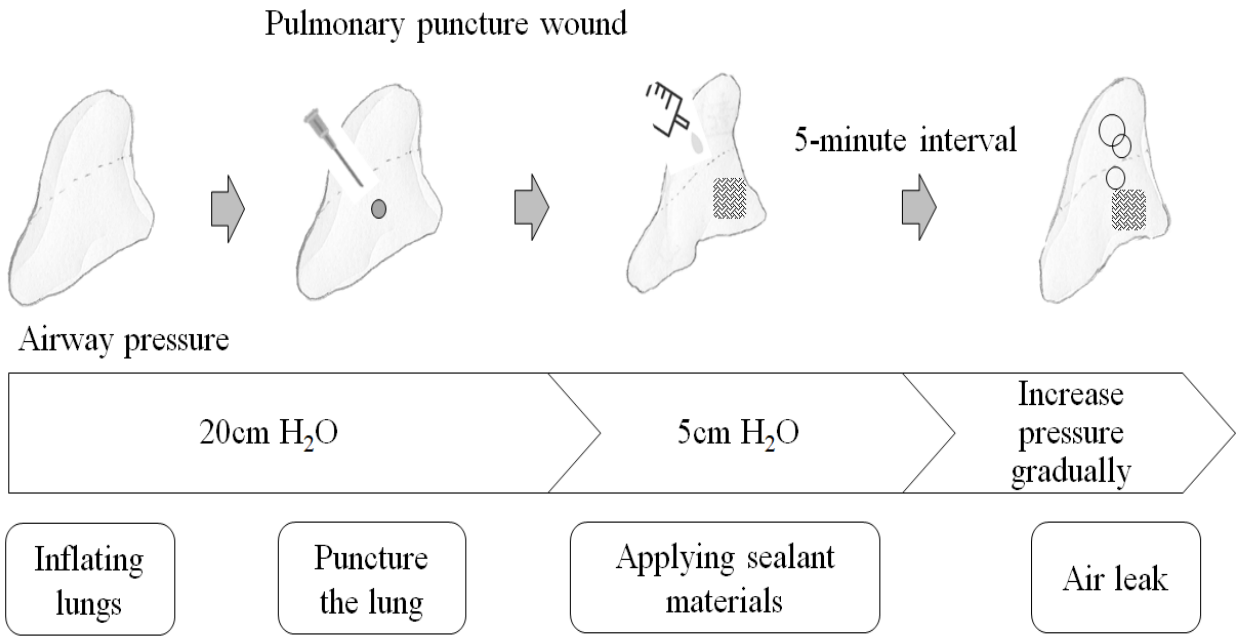

Figure 3. Surgical procedure and pressure measurement
The right middle lung was inflated until 20 cmH_2O and the 2-mm-deep puncture wound on the surface of the lung lobe was created. Without bleeding and after lowering the airway pressure to 5 cmH_2O, each sealing material was applied, and then it was left motionless for 5 min. The lowest airway pressure that broke the seal (seal-breaking pressure) was measured. Soap water was sprayed over the sealant in advance. Airway pressure was gradually increased at a rate of 2 cmH_2O/s from 5 cmH_2O, and the minimum seal-breaking pressure that caused sealing failed was determined by the appearance of a bubble.

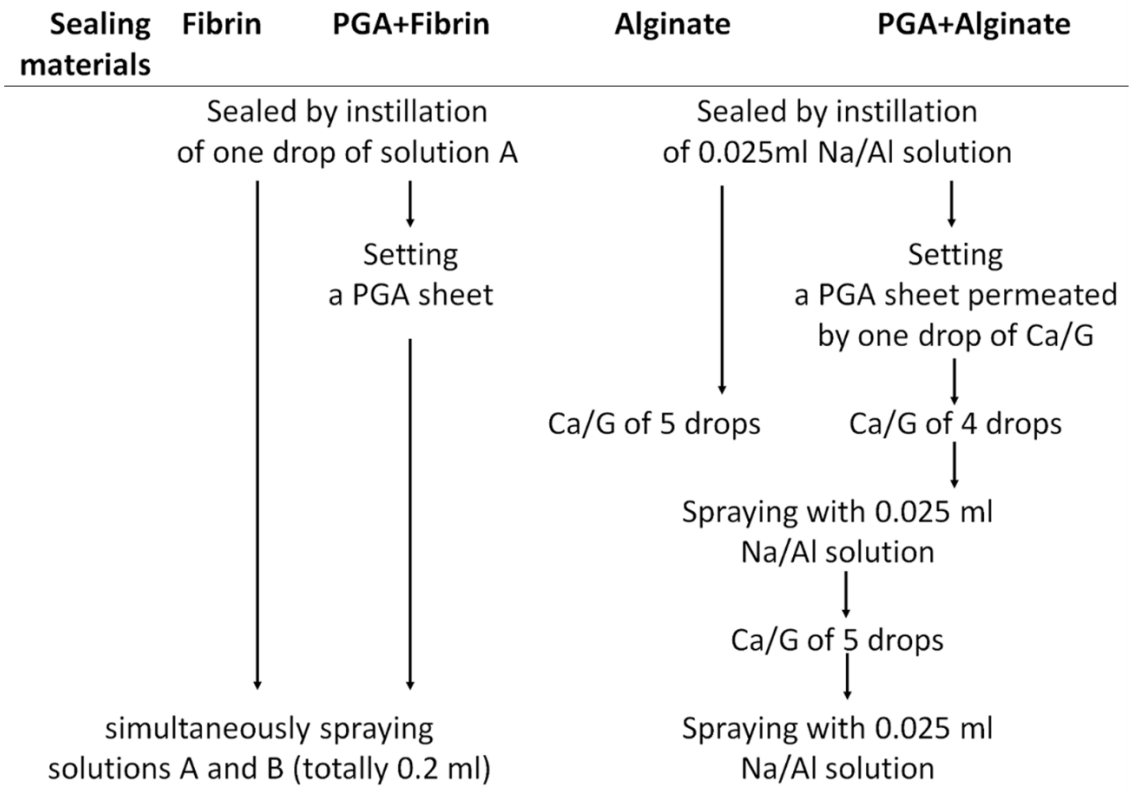

Figure 4. Sealing material application procedures in each experimental group.

Solution A means fibrinogen solution, solution B does thrombin solution, Na/Al does sodium alginate, and Ca/G does calcium gluconate.

Fibrin group: The pleural puncture wound was sealed by instillation of a drop of solution A, followed by simultaneously spraying solutions A and B (total instillation amount of solution A was 0.1 ml and solution B was 0.1 ml).

PGA + fibrin group: The pleural puncture wound was sealed by instillation of a drop of solution A, and then a PGA sheet was placed onto the wound and pressed by an index finger to absorb the solution. Then, solutions A and B were simultaneously sprayed over the PGA sheet (total instillation amount was the same as fibrin group).

Alginate group: The pleural wound was sealed with instillation of 0.025 ml Na/Al solution followed by five drops of Ca/G solution by using a syringe

attached to a 26G needle. The same procedure was repeated twice. After a 5-min interval, another 0.025 ml of Na/Al solution was instilled.
PGA + alginate group: The pleural wound was sealed with instillation of 0.025 ml sodium alginate solution, and then a PGA sheet permeated by a drop of Ca/G was overlaid. Four drops of Ca/G solution were then instilled onto the PGA sheet, followed by spraying with 0.025 ml Na/Al solution. After a 5-min interval, 5 drops of Ca/G followed by 0.025 ml Na/Al solution were instilled.

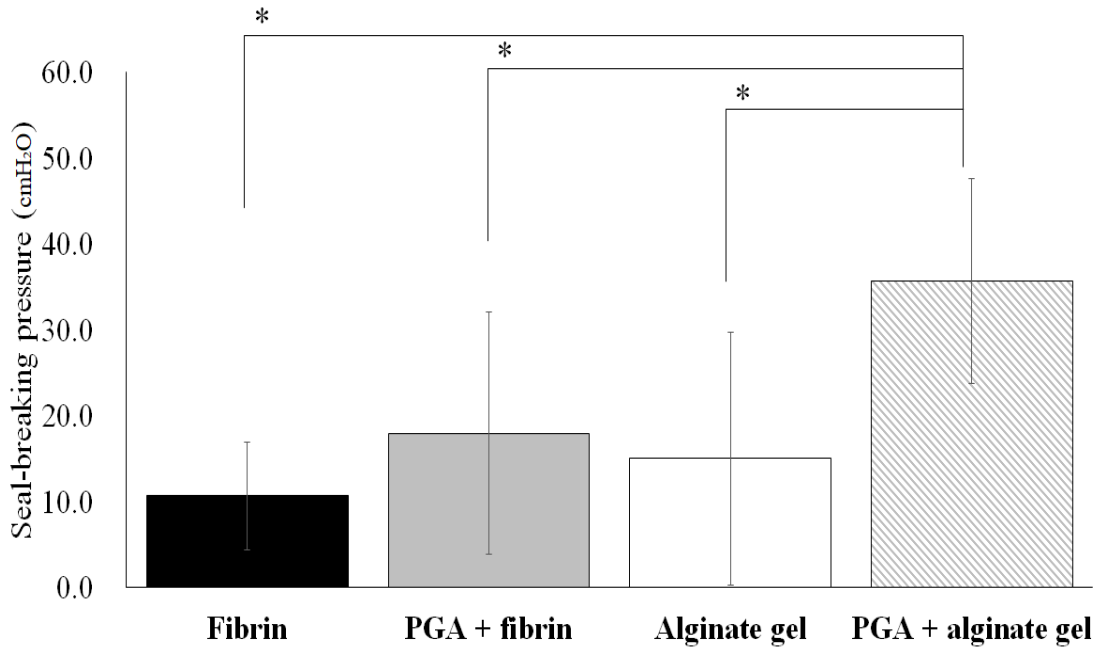

Figure 5. Minimum seal-breaking airway pressure for each sealing material
The column indicates the mean, and the bar shows standard deviation. The asterisk mark means P<0.01.
Seal-breaking pressure was as follows, (1) fibrin group 10.4 ± 6.8 cmH_2O; (2) PGA + fibrin group, 13.5 ± 6.5 cmH_2O; (3) alginate group, 10.3 ± 4.9 cmH_2O; (4) PGA + alginate group, 35.8 ± 11.9 cmH_2O.
Seal-breaking pressure in the PGA + alginate group was significantly greater than that in the other groups ($p<0.01$). There were no significant differences among the other three groups.

Ⅳ. STUDY 2

ALGINATE SPONGE REDUCES THE PULMONARY AIR LEAK AS A NEW BUTTRESS IN OMBINATION WITH AUTO-SUTURING DEVICE

自動縫合器と併用したアルギン酸スポンジの組織補強材は肺のエアリークを軽減する

TABLE OF CONTENTS (STUDY 2)

ABSTRACT (STUDY 2)

Background:

Linear staplers are usually used in the thoracic surgery. The air leak from them is a major issue, thus there are various buttress to prevent the air leak. However the effect of preventing the air leak is not sufficient yet. Therefore, alginate sponge was developed as a new buttress and composed of polyglycolic acid (PGA) mesh.

Materials and methods:

Thirty-three beagle dogs were randomly assigned to 7 groups; no buttress (group A), PGA (group B), fibrin glue (group C), PGA+fibrin glue (group D), polyglycomer sheet (group E), alginate sponge (group F), and PGA+ alginate sponge (group G) groups. The buttresses of from group B to E were the conventional and those of group F and G were the new one. The dogs were underwent thoracotomy. When the right middle lobe was cut with a linear stapler, one of the buttress was performed above described. Spraed the soap water over the lung, airway pressure was measured under mechanical ventilation management. The minimum pressure was defined by the appearance of a bubble as a burst pressure.

Results and conclusion:

Burst pressures were 12.0±6.8 cmH_2O, 31.3±6.6 cmH_2O, 13.9±3.8 cmH_2O, 26.9 ± 2.8 cmH_2O, 24.8 ± 1.8 cmH_2O, 48.5 ± 4.9 cmH_2O, and 54.2 ± 12.4 cmH_2O, respectively from in group A to G. The target pressure was 40 to 50 cmH_2O in this study. The pressure in the group F and G was reached the target and were significantly higher than those of the others ($P < 0.0005$). Alginate sponge should be beneficial to prevent air leak because of its sealing and bolster effects as a conjunction.

邦文要約 (STUDY 2)

背景：

胸部外科手術において、自動縫合器が使用される場合が多いが、自動縫合器を使用して切開した部分からのエアリークは依然として解決されていない。この課題を解決するために、様々なエアリーク防止材が開発されているが、未だに十分な性能の製剤はない。本研究では、新規エアリーク防止材としてアルギン酸スポンジを開発し、これを PGA 不織布との併用によるエアリーク防止効果を、従来方法と比較検討した。

材料と方法：

33匹のビーグル犬を以下の7群に分けた。無処置群（グループA）、PGA群（グループB）、フィブリン糊群（グループC）、PGA+フィブリン糊群（グループD）、ポリグリコーマーシート群（グループE）、アルギン酸スポンジ群（グループF）、PGA+アルギン酸スポンジ群（グループF）である。グループBからEは従来製剤で、グループFとGは新規エアリーク防止材である。開胸後、右中葉切開を行う際に各群のエアリーク防止材を用いた。石鹸水を切開後の肺に噴霧し、人工呼吸器接続下で気道内圧を測定した。気泡確認時の気道内圧を破裂圧とりあえずした。

結果および結語：

破裂圧は、グループ A から順にそれぞれ、12.0_6.8cmH_2O、31.3_6.6cmH_2O、13.9_3.8cmH_2O、26.9±2.8cmH_2O、24.8±1.8cmH_2O、48.5±4.9cmH_2O であった。本研究では 40~50 cmH_2O を破裂圧の目標

値とし、結果、グループ F と G にて達成され、加えて上記 2 群は他群より有意に破裂圧が高かった（P < 0 .0005）

アルギン酸スポンジは、シーリング効果と接着効果の両方に優れるため、非常に有効なエアリーク防止材である。

INTRODUCTION

Linear staplers are indispensable tools in the thoracic surgeries [1]. They have some fine wire staples which are more rigid than the fragile lung tissue. Linear staplers are able to cut and suture the lung simultaneously. Some pinholes were formed by its staples. Whenever the lung is inflated, the lung is often tore and the pinholes are enlarged, and then the air leak occurs. Droghetti and colleagues [2] reported that there was 90% probability of the air leak caused by the staples during a surgery. The air leak occurred during surgeries may increase the risk of serious postoperative complications, such as pneumothorax [3]. The complications always results in delaying the removal of chest drainage tubes [4] and prolonging hospital stay [5].

Various reinforcements have been developed to control and prevent air leak [4-8]. There are mainly two types; namely sealants and bolsters. Singhal and Shrager [9] reviewed currently available reinforcements and considered two points as follows. One was that buttresses had reported to reduce the incidence of air leak and the duration of chest tube drainage and hospital stays became shorter because of their bolster effect, although some reports demonstrated covering the staple line could prevent postoperative air leak. The other was that liquid sealants were developed to improve the effect of postoperative air leak, duration of chest tube drainage, or hospital stay. As a matter of fact, these liquid sealants was not improved neither statistically nor clinically significant.

We developed a new buttress, namely alginate sponge, which has both functions of sealants and bolsters working in conjunction. In a canine model, the burst pressure during surgery of the new buttress was examined and compared it to the conventional one.

MATERIALS and METHODS

1. Preparation of the New Alginate Buttress

The new buttress has two types of layers; the exterior layers are sodium alginate sponge and the midline layer is calcium alginate nonwoven fabric (Figure 1). Calcium alginate nonwoven fabric was put between sodium alginate sponges. Observing by scanning electron microscope (S-2-2380N; Hitachi, Ltd, Tokyo, Japan), the midline layer was 1 mm in width and fiber diameter was 10 mm. The exterior layers were observed honeycomb structure (0.7 mm in width).

Sodium alginate (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in distilled water to 2% (w/v), making sodium alginate solution. Calcium alginate nonwoven fabric (Kaltostat®; ConvaTec, Inc, Skillman, NJ) used in this study is a wound dressing generally used in the clinical fields. A sheet (3.5 × 7 cm) of the fabric was dipped in the alginate solution for 30 seconds. By the dipping process, the sodium alginate changed calcium alginate gel parcially, resulting in forming a mixture of sodium alginate and calcium alginate. This complex was then freeze-dried. Freezing at -80℃ performed for 12 hours and drying at -50℃ did for 12 hours. As a result of these process, the alginate buttress material was made.

2. Animal Experiment

Forty-two beagle dogs were non-pregnant female, 1 - 2 years old, weighing ranging from 9 to 11 kg and were assigned into 7 groups (6 dogs/group) randomly, roughly speaking that no buttress was applied in one group and some sort of buttress was applied in the other 6 groups. The group kinds in regard to the buttress application were as follows; group A: no buttress; group B: PGA; group C: fibrin glue; group D: PGA+fibrin

glue; group E: polyglycomer sheet; group F: alginate sponge; and group G: PGA+ alginate sponge, respectively.

Under general anesthesia with intravenous pentobarbital sodium (25–30 mg/kg intravenously), the dogs were fixed in dorsal position. After endotracheal intubation, thoracotomy was performed the right lung was exposed under mechanical ventilation. The right middle lobe was cut with the linear stapler. Two types of linear stapler was used in this study; one was ENDO-GIA® (Universal Straight R 60: Covidien, Mansfield, Mass) for groups A, B, C, D, F, and G and the other was Duet TRS 60® (Covidien) for group E. Airway pressure was maintained 20 cmH_2O for two minutes. After confirmation of a sufficient expansion of the lung, the jaws of the linear stapler were placed on the lobe over 5.5 cm in length from the peripheral towards the hilum. An air circuit pop-off valve of the ventilator was opened, so that the airway pressure changed depending on the lung condition. When the endotracheal pressure reached 10 cmH_2O (the lung was still expanded), the jaws were closed and clamped for 10 seconds, making an incision of approximately 5.5 cm. Both edges of the incision were stapled with each buttresses, concretely speaking as follows and listed in Figure 2.

Group A (no buttress):

The lung was simply cut with the linear stapler and no buttress was applied.

Group B (PGA):

Polyglycolic acid (PGA) nonwoven fabric sleeves in 0.1 mm thick (Neoveil®, Gunze Ltd, Kyoto, Japan) were covered to the jaws of the linear stapler.

Group C (fibrin glue):

Fibrin glue (Beriplast P Combi-Set®, CSL Behring, Inc, King of Prussia) was sprayed by a pressurized aerosol over the staple closure, precisely 1 mL of fibrinogen solution and 1 mL of thrombin solution were sprayed simultaneously (total spraying volume was 2 mL).

Group D (PGA+fibrin glue):

With covered the PGA sleeves to the jaws, fibrin glue was sprayed in the same manner as group C.

Group E (polyglycomer sheet):

The lung was simply cut with the Duet TRS linear stapler with its pre-attached polyglycomer sheet.

Group F (alginate sponge):

The application process was presented in Figure 3. The lung surface was put between the two alginate buttresses along the cutting line in Figure 3.A. The lung and the alginate buttress were simultaneously stapled and cut (Figure 3.B). After cutting, 2 mL of 0.6% calcium gluconate solution (Wako Pure Chemical Industries) was sprayed over the buttress (Figures 3.C).

Group G (PGA+ alginate sponge):

The lung surface was put between the two alginate buttresses along the cutting line. With covered the PGA sleeves to the jaws, the lung tissue and the alginate buttress were stapled and cut simultaneously. After cutting, the calcium solution (2 mL) was applied with the same method used for group F.

Three minutes (groups C and D) or 30 seconds (groups A, B, E, F, and G) later after the above-mentioned procedures, the airway pressure was raised manually with a bag ventilator at a rate of 0.67 cmH_2O/s. Soap water was sprayed over the buttress in advance. The burst pressure was defined as the airway pressure at which the bubble was confirmed. Thus the airway pressure was raised until the bubble appeared. This canine experiment was approved in advance by the Institutional Animal Care and Use Committee of Doshisha University.

One data of the burst pressure in each groups A, B, E, and F was removed because the lung was damaged without stapling or the bubble was appeared outside the staple line. Thus the number of data was five in groups A, B, E, and F, respectively and that was six in groups C, D, and G, respectively.

3. Statistical Analysis

The data shows mean ± standard deviation (SD). We applied a two-step procedure (step 1 and step 2) to avoid multiplicity issues, because the number of animals were few. In regard to examination about the sensitivity of this study, the no-buttress (group A) was compared to the conventional ones (groups B, C, D, and E) in step 1. Upon obtaining the results of step 1 that a significant difference existed, the new buttresss (groups F and G) were compared to the conventional ones in step 2. Dunnett test (2-sided) was used to analyze the effects of each groups in both steps.

RESULTS

Figure 4 shows the burst pressures of each group. The burst pressures were 12.0 ± 6.8 cmH_2O in group A, 31.3 ± 6.6 cmH_2O in group B, 23.9 ± 3.8 cmH_2O in group C, 26.9 ± 2.7 cmH_2O in group D, 24.8 ± 1.8 cmH_2O in group E, 48.5 ± 4.9 cmH_2O in group F, and 54.2 ± 12.4 cmH_2O in group G, respectively.

As a result of statistical analysis in step 1, the burst pressures of groups B (PGA), C (fibrin glue), D (PGA+fibrin glue), and E (polyglycomer sheet) were significantly higher than that of group A (no buttress) ($P<0.0005$ to $P<0.005$). In step 2, the burst pressures of groups F (alginate sponge) and G (PGA+ alginate sponge) were significantly higher than those of groups B, C, D, and E ($P<0.0005$ for all).

We obserbed carefully how air leak occurred at the stapling site. The bubbles appered mainly from (1): the deep end of the cutting margin, and (2): pinholes formed by the staples. Air leak occurred from both of them without any buttresses in group A. In groups C and D (fibrin glue was used there), the bubbles were obserbed from either (1) or (2). Once air leak occurred, fibrin glue on the lung tissue was peeled off because the bubbles pushed it up, resulting in remaining it removed from the lung tissue during surgery. As for the other groups, the bubbles were obserbed from (1).

DISCUSSION

Several studies have reported that conventional sealing materials (BioGlue [10], Vivostat [8], Coseal [11], TachoSil [12], PleuraSeal [13], and fibrin glue [14]) and buttresses (PGA [15], bovine pericardium [16], and expanded polytetrafluoroethylene [17]) contribute to reduce the postoperative air leak, duration of chest tube drainage, and hospital stay. The physiologic range of airway pressure is less than 25 cmH_2O. During positive end-expiratory pressure ventilation, that's because the inspiratory driving pressure is an attention point ranging from 20 to 24 cmH_2O in order to protect the lung from barotrauma [18] even in cases of acute respiratory distress syndrome. From above-mentioned, the upper limit pressure is set at 20 to 25 cmH_2O under the general anesthesia to prevent barotrauma. Therefore the staple line, used also as a reinforced, is highly recommend to stand the airway pressure of at least 20 to 25 cmH_2O. The burst pressure without any buttress (group A in this study) was significantly lower than in any of the other groups and was also lower than the safe level, namely from 20 to 25 cmH_2O.

Roberson and colleagues [17] examined the effect of stapling with pericardium or expanded polytetrafluoroethylene as a new buttresses. They compared it to that of stapling without any buttresses in canine model. The burst pressure using of these buttresses (11.8 cmH_2O) was increase to that without any materials (9.6 cmH_2O). If we apply this increase to the data obtained for no buttress in our experiment, it suggests that a buttress made of expanded polytetrafluoroethylene or bovine pericardium would have withstood an airway pressure up to 21.6 to 28.8 cmH_2O. Many conventional buttresses withstand pressures in the range of 20 to 25 cmH_2O.

Kawamura and et al. [19] described that a staple line made of PGA nonwoven fabric could withstand until 20 cmH_2O (the airway pressure) as reinforcemet. They also indicated that air leak occurred after severe coughing. Severe coughing would was caused to higher levels of the airway pressure in the postoperative period. With a protective ventilation strategy during positive end-expiratory pressure ventilation, the peak airway pressure is restricted to 40 cmH_2O to protect the alveoli from barotrauma. [17] Furthermore, the incidence of barotrauma is low when peak pressure is kept below 50 cmH_2O. Therefore, we aimed the upper pressure to 40 to 50 cmH_2O. The burst pressures for groups F and G (alginate sponge was used there) were both higher than 40 to 50 cmH_2O, while those of the other groups were lower. This result indicated that application of the alginate sponge as a buttress would be able to withstand the airway pressure when barotrauma occurs.

We considered why the burst pressures of the alginate buttress exceeded those of the conventional ones. Once the alginate buttress is placed on the lung surface, the exterior layer (the alginate sponge) absorbs the moisture and changes into a fluid gel with low viscosity. This fluid gel fills and covers the enlarged pinholes formed by the staples; it also infiltrates into the central layer (calcium alginate nonwoven fabric). It is very important that the central layer includes calcium ion, because alginate sponge only has too low viscosity.

Sodium alginate is a bioabsorbable [20] polymer which has high biocompatibility [21]. It is also cross-linkable with calcium ions, which bind neighboring alginate polymer chains [21]. When calcium ion increases, the fluid sodium alginate gel changes to a firm calcium alginate gel that could work as a sealing material. In regard to the new buttress, the central layer absorbed the moisture 15 to 20 times as much as its own weight in wet conditions. This swollen central layer played a role as a bolster and calcium ion was provided from it. This calcium ions are replaced by sodium ions by the body fluids [22]. The viscosity of the

buttress was changed before and after contacting the body fluids and the firmness was also changed from firm to flexible, but this flexibleness contributed to seal both the cutting margin and the pinholes. The additional calcium gluconate solution was sprayed over the treatment to enhance above-mentioned reaction. The combination of alginate sponge and calcium solution, designed as our new buttress, obtained the function as both sealing and bolster and contributed to sealing more effectively than conventional ones.

When taking a view of the combination of the new alginate buttress and PGA, the each material would contribute to prevent air leak individually not mutually because PGA is also used as a reinforment clinically and alginate sponge showed the effectiveness in group F. Matching with these materials was expected to result in higher values individually and mutually in group G.

Although we predicted that the result of group D (PGA+fibrin glue) would be better than those of group B (PGA) and group C (fibrin glue) the matter was opposite. Two possible reasons are considered. (1) After the PGA nonwoven fabric was covered over the stapler and cut along the staple line, fibrin glue was sprayed over the stapled area in group D. PGA nonwoven fabric itself is a hydrophobic biomaterial; however, the fibrin glue adhered irregularly to the PGA fabric, forming an inhomogeneous layer. This inhomogeneous layer was weak to withstand the air pressure and leaded to the lower burst pressure. (2) The fibrin glue was sprayed over the stapled area at the low pressure (10 cmH_2O). Fibrin glue was let coagulated in three minutes, but the coagulation of fibrin glue would be insufficient tissue adhesive strength or flexibility to prevent the air leak completely. Thus when the airway pressure was increased gradually, it peeled away from the stapled area. As a result, the combination of PGA fabric and fibrin glue (group D) did not mutually performed. In contrast, the combination of PGA fabric and alginate buttress (group G) did

mutually performed. This is because, we thought, the flexibility of the alginate gel contributed.

The safety level was seemed to be high of our new alginate buttress and is expect to be applied clinically because the components of the buttress was already commercially available as surgical materials or clinical drugs. Concretely speaking, calcium gluconate has been used clinically as an intravenous medium (Calcicol®) and calcium alginate nonwoven fabric as a wound dressing (Kaltostat®), respectively. Blair and coworkers [23] reported that Kaltostat® in the body was reabsorbed within 3 months [23]. Sodium alginate has been used clinically as a local hemostat (Alto®), and an antiulcer drug (Alroid-G®).

Virus and prion infections cannot be transmitted generally from plants to animals [24]. The risk of virus infection of alginate is much lower than that of fibrin glue or bovine pericardium [25]. This is because sodium alginate is extracted from brown algae.

CONCLUSION

This experiment suggested that the new buttress could have a superior efficacy to prevent air leak from the staple line. This evaluated the performance of each buttress only in terms of measuring the burst pressure of the staple line during the operation. Further studies should be needed to achieve a more precisely evaluation, namely about the long-term prevention effect of air leak after surgery, chest tube removal time, duration of hospital stay, and associated histologic changes and should be confirm its efficacy and safety. These examination should be needed because air leak occurs postoperatively too. In addition, it is absolutely essential to evaluate the utility of this buttress in patients with emphysema clinically, because the animal experiment performed only against the healthy (non-emphysematous) lungs.

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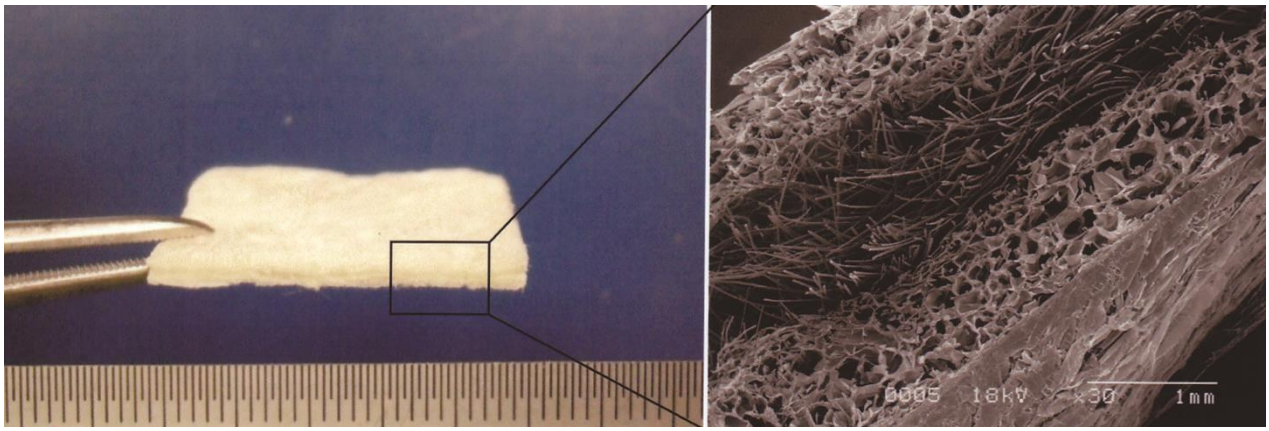

Figure 1. Vertical cross-sectional view of the alginate buttress. (figure courtesy of Ayumi Hashimoto from "*Reduction of air leaks in a canine model of pulmonary resection with a new staple-line buttress*.", J Thorac Cardiovasc Surg. 2011 Aug;142(2): page 368.)

A shows a macroscopic view.
B shows a scanning electron microscopic view.
How to make the alginate buttress as follows. Sodium alginate solution was made to 2% (w/v). A sheet (3.5 × 7 cm) of the calcium alginate nonwoven fabric was dipped in the alginate solution in short and then freeze-dried. Freezing at -80℃ performed for 12 hours and drying at -50℃ did for 12 hours.
The alginate buttress has two types of layers; the exterior layers are sodium alginate sponge and the midline layer is calcium alginate nonwoven fabric. Calcium alginate nonwoven fabric was put between sodium alginate sponges. Observing by scanning electron microscope, the midline layer was 1 mm in width and fiber diameter was 10 mm. The exterior layers were observed honeycomb structure (0.7 mm in width).

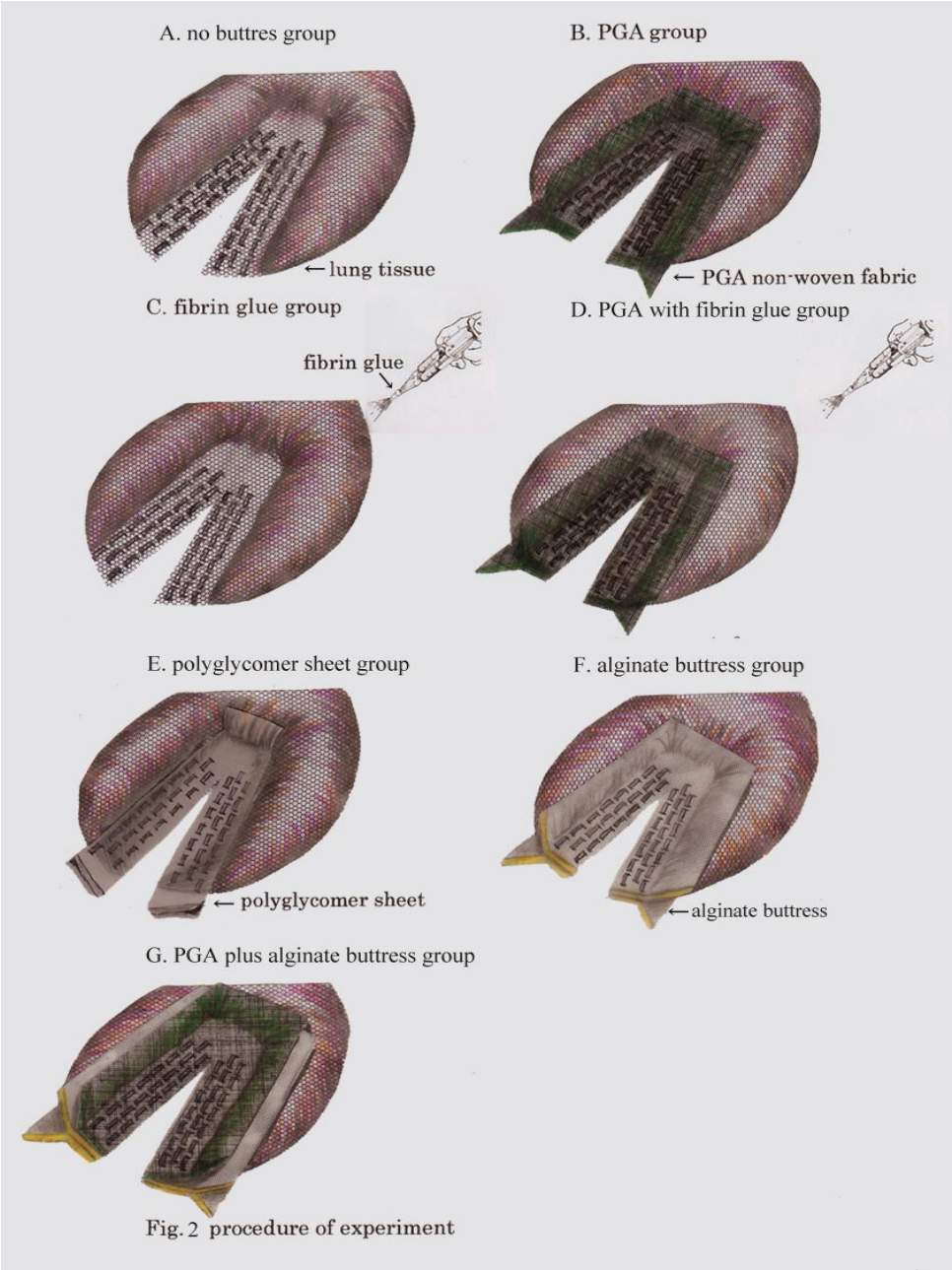

Figure 2. Procedures of experiment. (figure courtesy of Ayumi Hashimoto from "*Reduction of air leaks in a canine model of pulmonary resection with a new staple-line buttress*.", J Thorac Cardiovasc Surg. 2011 Aug;142(2): page 369.)

A, No buttress group. Only the linear stapler (no buttress material) was used to suture and cut the lung tissue. B, Polyglycolic acid (PGA) group. A

linear stapler with its jaws covered with polyglycolic acid nonwoven fabric was used to suture and cut the lung tissue.
C, Fibrin glue group. The linear stapler was used without any buttress material. Then, after the lung tissue had been sutured and cut, the stapled area was sprayed with fibrin glue to seal the stapled closure. D, Polyglycolic acid with fibrin glue group. Polyglycolic acid nonwoven fabric was attached to the jaws of the linear stapler, and the lung tissue was sutured and cut. The stapled area was then sprayed with fibrin glue to seal the stapled closure. E, Polyglycomer sheet group. A linear stapler with attached polyglycomer sheet (Duet TRS; Covidien, Mansfield, Mass) was used to suture and cut the lung tissue. F, Alginate buttress material group. The alginate buttress material was placed on both sides of the cutting line on the lung tissue. The lung tissue, together with the alginate buttress material, was then sutured and cut. After cutting, the stapled area was sprayed with calcium gluconate solution, which changed the sodium alginate fluid gel to a firm gel, to seal the stapled area. G, Polyglycolic acid plus alginate buttress material group. The alginate buttress material was placed on the lung tissue, the polyglycolic acid nonwoven fabric was attached to the linear stapler, and the lung tissue was sutured and cut. After cutting, the stapled area was sprayed with calcium gluconate solution, which changed the sodium alginate fluid gel to a firm gel, to seal the stapled area.

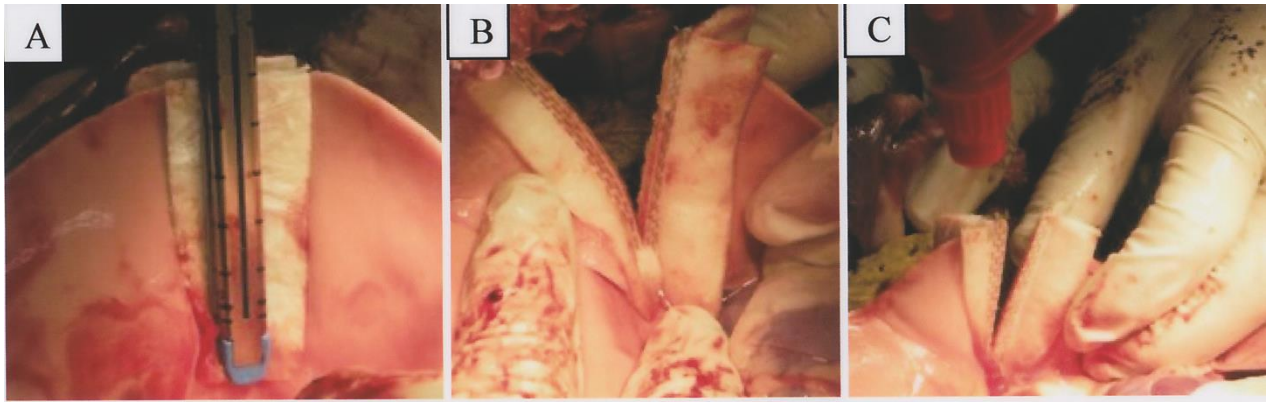

Figure 3. Pictures of the procedure for the alginate buttress group. (figure courtesy of Ayumi Hashimoto from "*Reduction of air leaks in a canine model of pulmonary resection with a new staple-line buttress*.", J Thorac Cardiovasc Surg. 2011 Aug;142(2): page 370.)

A shows that the lung surface was put between the two alginate buttresses along the cutting line.
B shows that the lung and the alginate buttress were simultaneously stapled and cut.
C shows that 2 mL of 0.6% calcium gluconate solution was sprayed over the buttress.

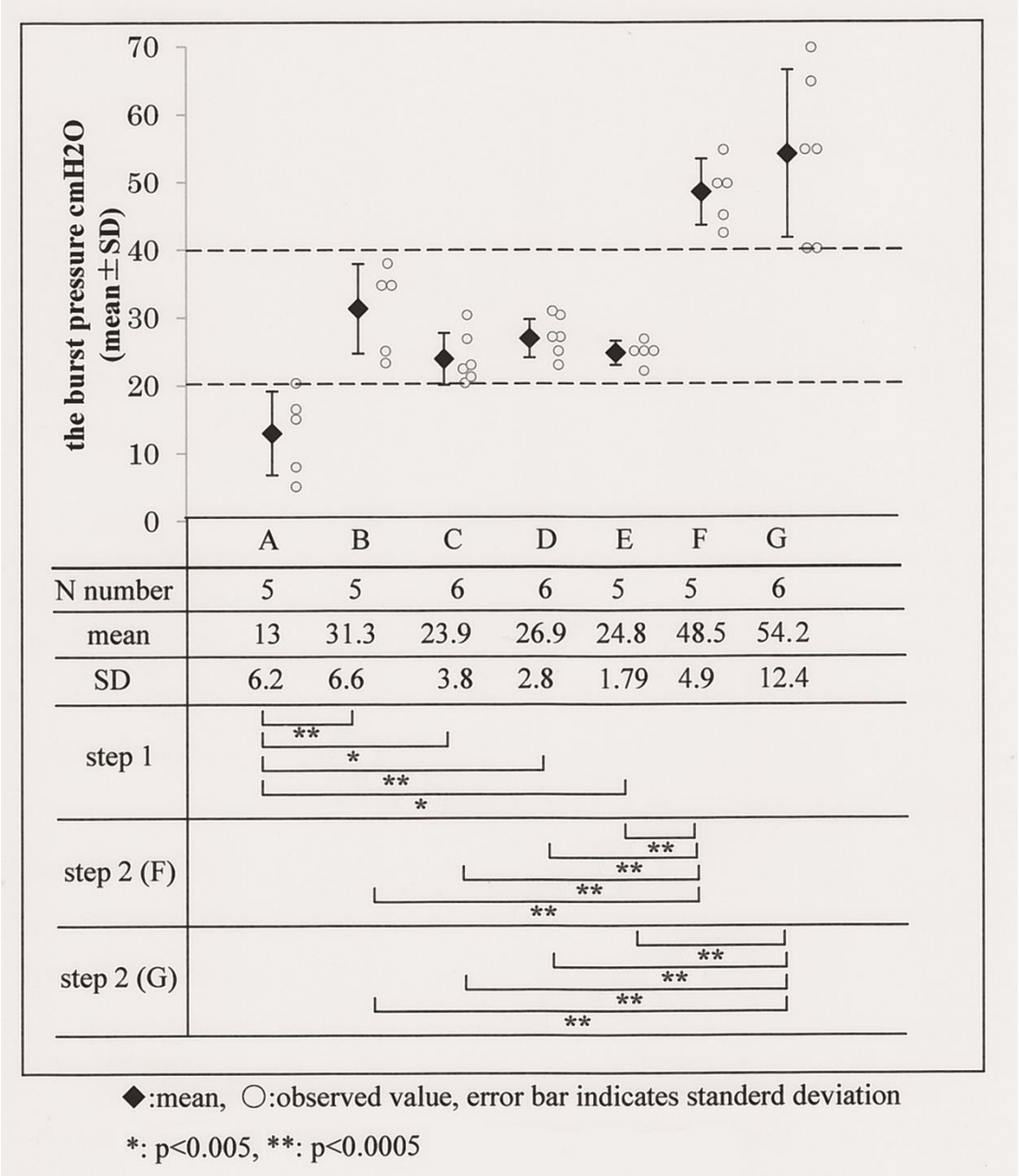

Figure 4. Burst pressures for each experimental group. (figure courtesy of Ayumi Hashimoto from "*Reduction of air leaks in a canine model of pulmonary resection with a new staple-line buttress*.", J Thorac Cardiovasc Surg. 2011 Aug;142(2): page 370.)

All data were indicated mean ± SD (standard deviation). The burst pressures were 12.0 ± 6.8 cmH_2O in group A, 31.3 ± 6.6 cmH_2O in group

B, 23.9 ± 3.8 cmH_2O in group C, 26.9 ± 2.7 cmH_2O in group D, 24.8 ± 1.8 cmH_2O in group E, 48.5 ± 4.9 cmH_2O in group F, and 54.2 ± 12.4 cmH_2O in group G, respectively.

Three minutes (groups C and D) or 30 seconds (groups A, B, E, F, and G) later after the above-mentioned procedures, the airway pressure was raised manually with a bag ventilator at a rate of 0.67 cmH_2O/s. Soap water was sprayed over the buttress in advance. The burst pressure was defined as the airway pressure at which the bubble was confirmed. Thus the airway pressure was raised until the bubble appeared.

We applied a two-step procedure (step 1 and step 2) to avoid multiplicity issues, because the number of animals were few. In regard to examination about the sensitivity of this study, the no-buttress (group A) was compared to the conventional ones (groups B, C, D, and E) in step 1. Upon obtaining the results of step 1 that a significant difference existed, the new buttresss (groups F and G) were compared to the conventional ones in step 2. Dunnett test (2-sided) was used to analyze the effects of each groups in both steps.

Ⅴ. STUDY 3

PREVENTION OF POLYGLYCOLIC ACID (PGA)-INDUCED PERITONEAL ADHESIONS USING ALGINATE IN A RAT MODEL

アルギン酸のポリグリコール酸により惹起される腹膜癒着に対する防止効果の検討

TABLE OF CONTENTS (STUDY 3)

2. Adherent portions and the correlations
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4. Microscopic study of hematoxylin-eosin (HE) stained sections
5. Immunohistochemical study
6. Fibroblast growth on the anti-adhesive materials *in vitro*

DISCUSSION

CONCLUSION

REFERENCES

ABSTRACT (STUDY 3)

Background:

Intra-abdominal adhesions develop after gynecological, gastroenterological and thoracic surgeries. Adhesions sometimes cause female infertility, bowel obstructions, and chronic abdominal pain or discomfort, and difficulties with subsequent surgeries, as well as prolonged hospitalization and hospital re-admissions, which could have an impact on both the patient's well-being and healthcare costs. Therefore, postoperative adhesions should be prevented as much as possible.

There are some reports of the alginate's anti-adhesive effect. Hirasaki et al. reported that it takes a long time for the sodium alginate powder to dissolve in water and turn into a gel. They also reported that the powder causes foreign body reactions, such as the induction of foreign body cells, when dispersed throughout the abdominal cavity, so it's important for alginate to form a gel. Therefore, they recommended that alginate be administered as a gel or a freeze-dried flake, which rapidly turns into a gel. Based on these recommendations, we designed three types of alginate gel, using calcium gluconate solution and changing its volume. Concretely speaking, the three types of alginate gel was made by strongly cross-linked (SL), weakly cross-linked (WL) and non-cross-linked (NL).

Materials and Methods:

The anti-adhesive effects of the three alginate gels was compared to those of fibrin glue (an usually used anti-adhesive material) and no treatment against adhesions caused by polyglycolic acid (PGA) mesh; PGA-induced adhesions, in rat experiments. The anti-adhesive materials were set on the PGA sheet fixed on the parietal peritoneum of the abdomen. Fifty-six days later, the adhesions were evaluated macroscopically by the adhesion scores and microscopically by hematoxylin-eosin staining and

immunostaining. Human fibroblasts and mesothelial cells were cultured on the materials for a week.

Results and Conclusions:

The WL and NL alginate gels have a superior anti-adhesive effect to the fibrin glue and no treatment. A microscopic evaluation confirmed that the PGA sheet was covered by a peritoneal layer constructed of well-differentiated mesothelial cells, and the inflammation was most improved in the NL and WL. The fibroblast growth was inhibited most on the surfaces of the NL and WL. These results suggest that either the WL or NL treatments are suitable for preventing PGA-induced adhesions compared to SL or the conventional treatment.

邦文要約 (STUDY 3)

背景：

腹腔内癒着は消化器や胸部、産婦人科などの手術後に生じる場合がある。術後癒着は女性の不妊、腸閉塞や慢性腹部痛の原因となる。また、癒着が生じた患者の再手術の困難性や、入院の長期化が医療費を圧迫するなど社会的影響も大である。この様な理由から術後の癒着は可及的速やかに防止することが望ましい。アルギン酸は過去にその癒着防止効果が報告されているが、そのひとつで、Hirasaki らがアルギン酸溶液をフリーズドライしたスポンジをフレークにしたものを報告している。その論文ではアルギン酸を癒着防止材で使用する際、アルギン酸がゲル化していることが重要だとある。それを受けて、今回我々は粉末のアルギン酸を生食で溶かした後、カルシウム溶液による強架橋アルギン酸（SL alginate）、弱架橋アルギン酸（WL alginate）、無架橋アルギン酸（NL alginate）という 3 種類のアルギン酸ゲルを作製した。これらが、フィブリン糊と同程度あるいはそれ以上のポリグリコール酸(以下 PGA)惹起性癒着の防止効果があるかどうか、ラットを用いて検討した。

材料および方法：

PGA 不織布を腹腔内の壁側腹膜に固定し、上記の癒着防止材をその上に処置した。術後 56 日後、癒着を肉眼的には癒着スコアを用いて、顕微鏡的にはヘマトキシリンーエオシン染色（HE 染色）と免疫学的染色でそれぞれ評価を行った。さらに、in vitro では各癒着防止材上での線維芽細胞の増殖を検証した。

結果および結語：

WL と NL は、無処置の場合とフィブリン糊処置の場合よりも、癒着防止効果に優れていた。また、顕微鏡下的評価においても、WL と NL では、PGA シートが分化型中皮細胞で構成された腹膜層に被覆されており、炎症反応が最も改善された。線維芽細胞増殖実験において、上記 2 群では細胞増殖が抑えられていた。以上の結果から、弱架橋アルギン酸と無架橋アルギン酸のどちらもが、強架橋アルギン酸や従来の癒着防止材と比べて PGA 惹起性癒着の防止に適していると言える。

INTRODUCTION

Intra-abdominal or intra-thoracic adhesions develop after gastroenterological [1, 2], thoracic [3-6] and gynecological [7-9] surgeries. Adhesions sometimes cause bowel obstructions, chronic abdominal pain or discomfort, female infertility [2-4, 7, 8, 10, 11] and difficulties with subsequent surgeries, as well as prolonged hospitalization and hospital re-admissions, which could have an impact on both the patient's well-being and healthcare costs [11, 12]. Therefore, postoperative adhesions should be prevented as much as possible.

A mesh of polyglycolic acid (PGA) is a widely used biomaterial during gastroenterological [13], thoracic [6, 14-16] and gynecological surgeries [9] and is used, for example, as reinforcement for weak tissues. The mild acidity of glycolic acid, produced during the non-enzymatic degradation of PGA [17], causes chronic inflammation, and adhesions subsequently occur around the site where the PGA mesh was placed [3, 18]. Thus PGA-induced adhesions have been problematic for a long time, but PGA continues to be used because it is a useful biomaterial.

In order to resolve the problems with PGA-induced adhesions, we utilized sodium alginate, which has previously been reported to prevent adhesions [19-21]. The present paper describes, for the first time, animal experiments in which two types of new anti-adhesive materials comprising sodium alginate with and without a small amount of calcium gluconate solution had superior anti-adhesive effects against PGA-induced adhesions to fibrin glue, the conventional anti-adhesive material in the clinical setting [22, 23].

MATERIALS and METHODS

1. Materials

In this study we used a non-woven PGA mesh (trade name Neoveil, Gunze, Japan), fibrin glue (trade name Beriplast P Combi-Set, CSL Behring, King of Prussia, PA, USA), sodium alginate powder (trade name Alto, Kaigen, Japan) with a molecular weight ranging from 32,000 to 250,000 and calcium gluconate solution at a concentration of 8.5% (trade name Calcicol, Nichiiko, Japan). The non-woven PGA mesh was cut into square sheets 15 mm x 15 mm in size (PGA sheets) and sterilized with ethylene oxide for 22 hours, after which ethylene oxide gas was removed under conditions of decompression for one week. Fibrin glue was used according to the manual provided by the manufacturer in the form of a mixture of solutions A (fibrinogen and aprotinin) and B (thrombin and calcium chloride).

2. Placement of alginate gel on the PGA sheet

Hirasaki et al. [19] reported that it takes a long time for powdered sodium alginate to dissolve in water and turn into a gel and that the powder form causes foreign body reactions, such as the induction of foreign body cells, when dispersed throughout the abdominal cavity. Therefore, the author recommended that alginate be administered in the form of a gel or freeze-dried flakes, which rapidly turn into a gel. Based on these recommendations, the alginate was administered as a gel in the present study.

3. Cross-linking by calcium gluconate

We used three calcium ion conditions, specifically 1 ml (rich), 0.1 ml (low) and 0 ml (zero) of calcium solution. Condition 1 (rich): Alginate is strongly cross-linked and turns into an insoluble hard gel [24, 25], which

remains localized over the PGA sheet for a long time and hardly moves away. Therefore, it does not easily disperse throughout the abdominal cavity. Condition 2 (low): Alginate is weakly cross-liked and forms a soft gel [26] that is gradually dissolved in water. The gel remains for a moderate amount of time then gradually moves away from the sheet, thus dispersing slowly throughout the abdominal cavity. Condition 3 (zero): Alginate is not cross-linked and hence turns into a soluble gel [26, 27] that easily moves out from the sheet and is smoothly dispersed throughout the abdominal cavity. We subsequently tested these three types of alginate-based treatments: strongly cross-linked (SL), weakly cross-linked (WL) and non-cross-linked (NL).

4. Animal protocol and experimental design

The present study comprised two parts, experiments 1 and 2. In experiment 1, 40 rats were randomly assigned to five experimental groups of eight rats each: a PGA alone group, fibrin group, SL group, WL group and NL group. The anti-adhesive effects of the three alginate-based treatments against PGA-induced adhesion were compared with those of PGA alone or fibrin glue treatment at a fixed alginate dose of 250 mg/rat. Upon obtaining the results of experiment 1, we decreased the alginate dose in geometric progression (experiment 2): 10, 20, 40, 80 or 160 mg/rat. Sixty rats were randomly assigned to 10 subgroups of six rats each, and the 10 subgroups were randomly divided into two major groups (WL and NL groups) composed of five subgroups. The five subgroups in the WL group (WL subgroups) included a 10W subgroup, 20W subgroup, 40W subgroup, 80W subgroup and 160W subgroup. Similarly, the five subgroups in the NL group (NL subgroups) included a 10N subgroup, 20N subgroup, 40N subgroup, 80N subgroup and 160N subgroup. The anti-adhesive effects and accumulation of ascites fluid were evaluated.

The animal experiments were approved by the Doshisha University Animal Experimentation Committee. All surgical procedures and

anesthesia protocols were conducted in accordance with the Animal Care Guidelines of Doshisha University. During the experimental period, all rats were housed separately and maintained under standard specific pathogen-free conditions (light-dark cycle: 12:12-hours, mean temperature: 23 degrees Celsius and mean humidity: 50%). Standard laboratory rodent chow and water were available *ad libitum*. On the first day of the experiment, the health status of all rats was checked (diarrhea, unusual fur (loss or dirtiness), mucous discharge from the eyes or anus, emaciation). Non-pregnant 8-week-old female Wistar/ST rats weighing 200 g were used for the analyses.

5. Surgical technique

All operations were performed under sterile conditions and all procedures were performed by one surgeon. Rats inhaled diethyl ether (Wako Pure Chemical, Japan), and 5 mg sodium pentobarbital (trade name Sommnopentyl, Kyoritsu Seiyaku, Japan) diluted in 1 ml of physiological saline solution was then administered intraperitoneally. Under general anesthesia, rats fixed in the dorsal position received a 5-cm-long median transabdominal incision. A PGA sheet was fixed at the four corners on the visceral peritoneum of the right lateral abdomen with 7/0 polyvinylidene fluoride monofilament sutures for microsurgery (trade name Asflex, Kono Seisakusyo , Japan)

5.1. Experiment 1

After fixation, no anti-adhesive materials were applied on the PGA sheets in the PGA alone group. Meanwhile, in the fibrin group, a mixture of solutions A and B at a concentration of 0.09 ml each (total: 0.18 ml), as an anti-adhesive barrier, was sprayed over the fixed PGA sheet in order to entirely cover the surface of the sheet.

As for the three alginate groups before fixing the PGA sheet on the peritoneum, 0.1 ml of solution (1) was soaked up into the sheet and 125 mg of sodium alginate powder was subsequently sprinkled onto the sheet. The PGA sheet was fixed on the visceral peritoneum in the same manner as that used in the PGA alone group. Following fixation, 0.45 ml of solution (2) was sprayed over the PGA sheet and 125 mg of sodium alginate powder was sprinkled on the sheet. Finally, 0.45 ml of solution (3) was sprayed on the sheet. The details of the solutions (1-3) are summarized in Table 1.

Following the completion of these procedures, the laparotomy incision was closed using 4/0 polyamide sutures with two-layered sutures, the muscle layer was closed with continuous sutures and the skin was closed

with interrupted sutures. After surgery, all rats were maintained for 56 days under standard SPF conditions.

5.2. Experiment 2.

All operations were performed by the same surgeon who conducted the surgeries in experiment 1. Before fixing the PGA sheet on the peritoneum, 0.1 ml of calcium solution (in the WL subgroups) or physiological saline solution (in the NL subgroups) was soaked up into the PGA sheet, and 10, 20, 40, 80 or 160 mg of sodium alginate powder was then sprinkled on the PGA sheet. The PGA sheet was subsequently fixed on the visceral peritoneum, and, after fixation, 0.9 ml of physiological saline solution was sprayed over the PGA sheet. Following the completion of these procedures, the laparotomy incision was closed in the same manner as in experiment 1.

6. Macroscopic evaluation of the anti-adhesive effects

The macroscopic findings were evaluated by two examiners who were blinded to the rats' treatment. On postoperative day 56, when the PGA had been degraded and partly absorbed by the body [28], the rats were sacrificed by the intraperitoneal injection of a lethal dose of pentobarbital (3.5 mg/kg of body weight). After a full abdominal laparotomy, we observed the status of the adhesion on the PGA sheets macroscopically. We first recorded the extent and severity of adhesion according to an adhesion grading scale (Adhesion score, Table 2 [29]) and subsequently recorded the number of adherent portions, namely, which intra-abdominal portions (tissues and organs) had adhered to the PGA sheet. The total number of adherent portions was compared between the treatment groups.

7. The adherent portions and the correlations among the adherent portions, adhesions scores and the alginate doses

In experiment 2, we investigated the correlations between (1) the

alginate dose and total number of adherent portions, (2) the adhesion scores (extent and severity) and total number of adherent portions and (3) the alginate dose and adhesion scores in the WL and NL subgroups.

8. Evaluation of ascites

We examined the correlation between the volume of ascites and the anti-adhesive effects in experiments 1 and 2. For each of the 90 rats in the WL and NL groups, the volume of ascites was classified into four classes using the ascites score shown in Table 3. A dot was placed on each rat at the point shown by the X- and Y- axes.

9. Microscopic study of hematoxylin-eosin (HE) staining

All rats were subjected to a microscopic study. The peritoneal wall where the PGA sheet had been fixed was surgically removed *en bloc* as a specimen for microscopic examination. Specimens were fixed in 10% formalin solution and were prepared as thin (3 μm in thickness) sections stained with a hematoxylin-eosin (HE) using standard procedures for histological examinations. HE sections were reviewed for all rats.

10. Immunohistochemical study

We selected one section from all five groups in experiment 1 for the immunohistochemical analysis. As a primary antibody, we used an anti-human mesothelial cell antibody (HBME1, Serotec, Japan), which is available for the staining of mesothelial cells [30].

In order to investigate whether or not macrophages were inflammatory or tissue-remodeling, we used an anti-rat CD68 mouse monoclonal antibody (Clone ED1, Serotec), anti-rat CD163 mouse monoclonal antibody (clone ED2, Serotec) and anti-rat CD86 mouse monoclonal antibody as the primary antibodies, which could differentiate inflammatory macrophages (M1 macrophages) [31], tissue-remodeling macrophages (M2a and M2c) [31,32] and both macrophages (M1 and M2b) [32],

respectively. All sections were cut to 3 μm in thickness, were deparaffinized and hydrophilized. Antigen activation was performed by immersing the samples in a warm bath with EDTA at pH 9 (20 minutes at 95 °C) (HBME1), with heat and pressure treatment with 10 mM sodium citrate buffer solution at pH 6.0 (15 minutes at 121°C) (CD68), were not treated (CD163) or which were treated with proteinase K (5 minutes at room temperature) (CD86), respectively. The sections were washed with distilled water and successively treated with 3% H_2O_2 (10 minutes at room temperature). The sections were then washed with distilled water and successively rinsed in 50 mM Tris-HCl buffer, pH 7.6, containing 0.05% tween-20 and 0.15M NaCl (TBST). The sections were incubated overnight at 4°C (HBME1 (1:50 dilution), CD68 (1:1000), CD163 (1:50) and CD86 (1:50), all of which were diluted with ChemMate (Dako, Japan) and were successively rinsed in TBST. After that, the sections for HBME1 and CD68 were incubated with the Envision+ polymer reagent (Dako, Japan). For the CD163 and CD86 staining, the sections were subsequently incubated with a Simple Stain rat MAX-PO (MULTI) kit (Nichirei, Japan), each for 30 minutes at room temperature. After being rinsed in TBST, all sections were incubated with a 3, 3'- diaminobenzidine tetrahydrochloride (DAB+) substrate kit (Dako). After washing, all sections were subjected to counterstaining, dehydration, penetration and mounting.

11. The fibroblast growth on the anti-adhesive materials *in vitro*

Cultured rat fibroblasts, which were established from the subcutaneous tissue under the back skin of a healthy Wistar S/T rat (seven weeks old, 200 g), were retrieved in the form of a single cell suspension with D-MEM medium (Wako Pure Chemical) containing 10% bovine serum. The cell suspension was diluted with the same medium to 1.5 x 10^4cells/ml. This suspension was poured at 100 μl/well into the coated wells in the plates described above so that there were 1.0 x 10^4 human

cells/well (n=4) as a control. The cell suspension was also poured into a humidified incubator with 5% CO_2 at 37 ^{o}C.

For the cell culture, we used 24-well culture plates with wells 15 mm in diameter without a coating (Becton, Dickinson and Company Ltd. Franklin Lakes, USA). The 24 wells were divided into three groups: the SL group, WL group and NL group. Fifty mg of sodium alginate and the calcium gluconate and/or physiological saline solutions were combined in the same proportions by weight. The type and volume of solution are summarized in Table 4. One, three, five and seven days after seeding, the viable cell number in each well was counted with the ATP assay using an ATP Lite Kit (Perkin Elmer, Waltham, USA). For each time point, four wells for each experimental group were examined.

12. Statistical analysis

The statistical analyses were performed using the software program, "StatMate." The adhesion scores (extent and severity) were assessed using the Kruskal-Wallis and Mann-Whitney *U* tests, and the statistical significance of differences in the total number of adherent portions and the correlations between groups was assessed using Pearson's chi-square test (χ^2). A regression analysis was employed to determine the correlation between the anti-adhesive effects and the volume of ascites. Cell proliferation was analyzed using a one-way analysis of variance (ANOVA) and the Tukey test was used as a post hoc-test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

1. Macroscopic findings of the anti-adhesive effects

The adhesion scores (extent and severity) are expressed as the mean ± standard deviation and summarized in Table 5. Figures 1A (extent) and 1B (severity) show the scores for experiment 1 and Figures 2 (A-1 and A-2 for extent) and 2 (B-1 and B-2 for severity) show the scores for experiment 2.

1.1. Experiment 1

Regarding the extent of adhesion, the scores in the WL and NL groups were significantly different from those in the PGA alone group ($P<0.001$) and the fibrin group ($P<0.01$). In addition, the scores in the SL and fibrin groups were significantly different from those in the PGA alone group ($P<0.001$ - 0.05). As to the severity of adhesion, the scores in the WL and NL groups were significantly different from those in the PGA alone group ($P<0.001$) and the fibrin group ($P<0.01$ - 0.05), the scores in the WL group were significantly different from those in the SL group ($P<0.05$) and the scores in the SL and fibrin groups were significantly different from those in the PGA alone group ($P<0.01$ - 0.05).

1.2. Experiment 2

The adhesion scores decreased as the alginate dose increased in the WL and NL subgroups. With respect to the extent of adhesion, all scores in the WL and NL subgroups were significantly smaller than those in the PGA alone group ($P<0.01$ - 0.05), and the scores in the 80W, 160W, 20N, 40N, 80N and 160N subgroups were significantly smaller ($P<0.05$) than those in the fibrin group. Regarding the severity of adhesion, the scores in the WL and NL subgroups were significantly smaller than those in the PGA alone group ($P<0.01$- 0.05), and the scores in the 160W, 80N and 160N

subgroups were significantly smaller ($P<0.05$) than those in the fibrin group.

2. Adherent portions and the correlations

Table 6 shows the details of the adherent portions. The adherent portions included the omentum, gonadal fat, mesenterium and part of the intestines in experiment 1 and the omentum and gonadal fat in experiment 2.

In experiment 1, the total number of adherent portions was significantly different among the five groups ($p<0.001$), and that observed in the WL and NL groups was significantly different from that noted in the PGA alone group ($p<0.001$) and fibrin group ($p<0.01$). In experiment 2, the total number of adherent portions decreased as the alginate dose increased in both the WL and NL subgroups. In addition, the total number of adherent portions differed significantly ($p<0.001$) among the WL subgroups, while that in the NL subgroups did not. Individually, the total number in the 160W subgroup was significantly different from that observed in the 10W, 20W and 40W subgroups and the number in the 80W subgroup was different from that seen in the 10W subgroup ($p<0.01$ - 0.05).

Figures 3(A) to 3(E) show the correlations between the alginate dose and total number of adherent portions (Figure 3(A)), the adhesion scores and total number of adherent portions (Figure 3(B) for the extent and Figure 3(C) for the severity) and the alginate dose and adhesion scores (Figure 3(D) for the extent and Figure 3(E) for the severity). Except for the correlations between the alginate dose and adhesion scores (extent and severity) in the NL subgroups, the correlation coefficients were all significant ($p<0.01$ - 0.05).

3. Evaluation of ascites

The ascites fluid was examined in the WL and NL groups only. Figures 4 (A) and 4 (B) show the correlations between the ascites score and

adhesion scores (Figure 4(A) for extent and Figure 4(B) for severity). Consequently, the volume of ascites exhibited a strong inverse correlation with both the extent and severity of adhesion.

4. Microscopic study of hematoxylin-eosin (HE) stained sections

The microscopic findings of the HE sections from experiment 1 are summarized in Tables 7A and 7B, and six photomicrographs are shown in Figures 5(A-1) to 5(C-3). Table 7A shows the common changes in the five groups and specific changes in the fibrin and three alginate groups. Common changes had three steps: the first step was that the PGA fibers decreased in size, the second step was that the PGA fibers turned into flakes and third step was that each PGA fiber was covered by macrophages and collagen-like substrates. Thus, a complex was formed by the fibers, macrophages and collagen-like substrates. The specific changes in the fibrin group had two steps: the first step was that a lot of inflammatory cells, mainly lymphocytes, accumulated all over the microscopic visual fields (8/8 rats) and the second step was that huge lymph follicles were found (3/8 rats). This accumulation of lymphocytes was remarkable compared with that in the other groups. The specific change in the three alginate groups was residual alginate form: one form was island formation by the gathering of many macrophages ingesting alginate (IF) and the other form was a "pool" of alginate in a free state (PA). Many of the “pools” were scattered around the PGA fibers. Table 7B shows the amounts of IF and PA. The changes in lymphocyte accumulation, fibroblast infiltration and fibrosis were weak in the three alginate groups, whereas these findings were remarkable in the other two groups. The microscopic findings in experiment 2 were principally similar to those observed in the WL and NL groups in experiment 1. The degree of lymphocyte infiltration, fibroblast growth and collagen fiber accumulation was weak, although ascites accumulation was detected.

5. Immunohistochemical study

Table 8A summarizes immunohistochemical features and Figures 6(A-1 to A-5) represent the microscopic views of HBME-1 staining. HBME-1 was most clearly stained in the single cell layer covering the surface of the tissue over the PGA sheet in the NL group, followed by the fibrin group and the WL group, and the staining was unclear in the PGA alone group and the SL group. The HBME-1 staining was poor on the surface over the lymph follicles in the fibrin group and over the PGA fibers in the PGA alone group.

Figures 6 (B-1 to B-5), 6(C-1 to C-5) and 6(D-1 to D-5) show the typical views of CD68, CD163 and CD86 staining, respectively. The PGA sheet had two different fiber structures: one was a fiber bundle where the PGA fibers were dense; the other was space between the bundles. Macrophages were divided in three groups according to their locations: (1) macrophages between the PGA fibers in the PGA bundles, (2) macrophages in the space between the bundles and (3) macrophages on the surface of the PGA fibers. Table 8B represents the immunohistochemical features using CD68, CD163 and CD86 staining for the macrophage phenotypes (inflammatory or tissue-remodeling). (1) The macrophages in the space between the bundles showed CD163-positive cells most predominantly between the PGA fibers in the NL group, whereas in the SL group, CD163-positive cells were hardly detected. (2) With regard to the macrophages in the space between the bundles, CD68-positive cells and CD86-positive cells were predominant in the SL group, but not in the NL group. (3) As for the macrophages on the surface of the PGA fiber, there were no differences among the groups.

6. Fibroblast growth on the anti-adhesive materials *in vitro*

As shown in Figure 7, the fibroblasts in the three alginate groups did not grow on all alginate gels. The fibroblasts hardly attached to the alginate surface, and the final cell proliferation on day 7 was significantly lower in the NL and WL groups than in the SL group ($p<0.01$).

DISCUSSION

First, as the alginate dose was high, we compared the adhesion scores of the three alginate-based treatments (SL, WL and NL alginate) with those of no treatment (PGA alone) as well as conventional treatment (fibrin glue). The results showed that high doses of the WL and NL treatments had superior anti-adhesive effects to both PGA alone and fibrin glue. Subsequently, as the WL and NL alginate doses were low, we investigated (1) whether a lower alginate dose provides significant anti-adhesive effects against PGA-induced adhesion and (2) which alginate doses show anti-adhesive effects corresponding to those of fibrin glue. The results showed that (1) both WL and NL treatment effectively prevented PGA-induced adhesion, even at the lowest alginate dose, while (2) 80 mg (NL) and 160 mg (WL and NL) of alginate displayed superior anti-adhesive effect to fibrin glue. In brief, the alginates, even at very low doses, exerted strong anti-adhesive effects against PGA-induced adhesion under low- and zero-calcium conditions.

We did not consider the ascites caused by the alginate to be inflammatory based on the size of the alginate molecules and the microscopic findings of inflammation, including inflammatory cell infiltration. Our alginate has a high molecular weight, and such molecules are large enough to prevent the material from passing through the endothelium of blood capillaries under the peritoneum in the peritoneal cavity [33, 34]. Therefore, large molecules are absorbed very gradually into the lymphatic system [34]. The long-term existence of large molecules results in the accumulation of water in the peritoneal cavity. According to the microscopic findings, the degree of inflammation was relatively low in the rats with ascites versus those without. Aksoy et al. [35] showed that the existence of liquid inside the abdomen, or ascites, has a preventive effect against postoperative adhesion caused by peritoneal injury in a rat

experiment. Moreover, the volume of ascites strongly correlates with the anti-adhesive effects. Hence, the ascites detected in our experiments may have contributed to the observed anti-adhesive effects.

In order to detect well-differentiated mesothelial cells, HBME-1 is usually used as a mesothelial cell marker to recognize the microvillus structure of mesothelial cells [30]. After intraperitoneal injury or inflammation, an inflammatory stimulus leads to detach mesothelial cells from the basement membrane and changes their morphology and function [36, 37]. Then, the injured surface is repaired in the tissue repair and remodeling phase. Finally, when adhesion does not occur the injured surface is covered by a repaired peritoneal layer of mesothelial cells. These well-differentiated mesothelial cells are characterized by a microvillus structure [38]. Therefore, the layer of HBME-1-positive cells indicates that there is a peritoneal layer constructed of mesothelial cells that are structurally and functionally well-differentiated. After the peritoneum has been repaired over the injured surface, the injured surface does not adhere. On postoperative day 56, the layer of mesothelial cells was formed clearly on the soft gels well over the PGA sheet (WL and NL groups), but was poorly formed at the free surface of lymph follicles (fibrin group) and at the free surface of residual alginate (SL group). Thus WL and NL treatments showed good anti-adhesive results microscopically, while the SL and fibrin glue treatments showed lesser effects.

Macrophages have recently been divided into two phenotypes; M1 macrophages, which are inflammatory, and M2 macrophage, which are tissue-remodeling [31, 32]. M2 macrophages include at least three subsets (M2a, M2b, and M2c). The M2a and M2c macrophages are involved in tissue-remodeling, while M2b macrophages are inflammatory. M1 and M2b macrophages are characterized by the expression of CD68 and CD86 on their surface, respectively. On the other hand, M2a and M2c macrophages are characterized by the expression of CD163. As the calcium ion concentration decreased, more tissue-remodeling macrophages were

found, whereas fewer inflammatory macrophages were detected. Thus, analyses of the macrophage phenotype and subsets showed that NL treatment had the best “tissue-remodeling” profile and SL treatment had the most inflammatory profile of the three types of alginate-based treatments.

We examined the extent of fibroblast growth on the anti-adhesive materials *in vitro* because fibroblasts induce adhesion. Consequently, the *in vitro* examinations revealed that the fibroblasts hardly adhered to or proliferated on the surface of the soft and soluble gel (WL and NL), while they grew weakly on the hard gel (SL). The above-mentioned properties of the WL and NL treatments likely contributed to their superior anti-adhesive effects compared to the SL alginate and fibrin glue. Doyle et al. [39] isolated human dermal fibroblasts and other cells and investigated the proliferation of these cells in a calcium alginate suspension. The authors subsequently reported that the fibroblasts grew more successfully on the calcium alginate than on the sodium alginate.

In summary, the NL group exhibited the smallest adhesion scores, even when the alginate dose was decreased, with few fibroblasts or inflammatory macrophages on the NL alginate. Moreover, microscopic examinations revealed many tissue-remodeling macrophages and well-differentiated mesothelial cells covering the surface of the PGA sheet. These results show that sodium alginate has the best anti-adhesive effect of all of the tested anti-adhesive materials.

The present paper focused on sodium alginate as an anti-adhesive material, substituting it for fibrin glue, which has well-known anti-adhesive effects, and is used in the clinical setting [22, 23], as well as in animal experiments [40-45]. Some previous reports have shown that alginate has good anti-adhesive effects [20, 21] and great bioabsorbable properties and biocompatibility [24-27, 39, 46-48]. In addition, the risk of infection by alginate has been known to be much lower than that by fibrin glue [27, 49], because alginate is extracted from seaweed. Fibrin glue is derived from human blood, and thus is associated with a clinical risk of pathogenic

infections [48]. For example, human parvovirus B19 cannot be removed from plasma-derived products even by pasteurization at 60ºC for 10 hours [48].

It should be noted that our sodium alginate (trade name Alto) is a clinical hemostatic agent, while the calcium gluconate solution (trade name Calcicol) is a clinical injection used to supplement calcium. We selected only clinically available drugs as alginate-based treatments because surgeons can prepare this new anti-adhesive material at their discretion as needed during surgery, and extensive studies of the safety of these drugs have already been performed.

Our intra-abdominal adhesion model was similar to the experimental rat model established by Junge et al. [51]. We assessed the anti-adhesive effects on postoperative day 56, when the PGA mesh would be degraded into fine fragments and the tensile strength of the PGA mesh would be expected to be 0 [28]. At that point in time, the post-surgical inflammatory reaction had subsided [50]. We thought that this time point would provide a good assessment of the anti-adhesive effects of the treatments, although the anti-adhesive effects of alginate have generally been evaluated at earlier time points [51, 52].

CONCTUSION

The present study showed that two types of newly developed anti-adhesive materials composed of sodium alginate alone and weakly cross-linked alginate with calcium, which can be prepared from clinically available drugs, effectively prevented PGA-induced adhesions. These results suggest that the new anti-adhesive materials would be clinically useful to prevent the adhesions induced by PGA sheets, which are widely used in many clinical fields.

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Table 1. Three alginate groups in experiment 1.

Experimental groups	Solutions		
	1)	2)	3)
SL	Calcium gluconate	Calcium gluconate	Calcium gluconate
WL	Calcium gluconate	Physiological saline	Physiological saline
NL	Physiological saline	Physiological saline	Physiological saline

The details of the solutions (1-3) are summarized.
Before fixing the PGA sheet on the peritoneum, 0.1 ml of solution (1) was soaked up into the sheet and 125 mg of sodium alginate powder was subsequently sprinkled onto the sheet. The PGA sheet was fixed on the visceral peritoneum in the same manner as that used in the PGA alone group. Following fixation, 0.45 ml of solution (2) was sprayed over the PGA sheet and 125 mg of sodium alginate powder was sprinkled on the sheet. Finally, 0.45 ml of solution (3) was sprayed on the sheet.

Table 2. Adhesion score.

Category and Description	Score
Extent	
No involvement	0
≤25% of the site involved	1
≤50% of the site involved	2
≤75% of the site involved	3
≤100% of the site involved	4
Severity	
No adhesion present	0
Adhesions fall apart	1
Adhesions can be lysed with traction	2
Adhesions requiring <50% sharp dissection	3
Adhesions requiring >50% sharp dissection	4

We recorded the extent and severity of adhesion according to an above adhesion grading scale.

Table 3. Ascites score.

Macroscopic accumulation of the ascites	Score
No accumulation	1
Accumulation limited in one side (right or left) gutter of the abdomen	2
Accumulation limited in bilateral gutters of the abdomen	3
Accumulation over the bilateral gutters	4

We examined the correlation between the volume of ascites and the anti-adhesive effects in experiments 1 and 2. For each of the 90 rats in the WL and NL groups, the volume of ascites was classified into four classes using the ascites score.

Table 4. Type and volume of solution(s) sprayed after sprinkling 50 mg of alginate.

Experimental groups	volume of solution	type of solution
SL	0.2 ml	calcium solution
WL	(1) 0.02 ml + (2) 0.18 ml	(1) calcium solution + (2) physiological saline solution
NL	0.2 ml	physiological saline solution

For the cell culture, we used 24-well culture plates with wells 15 mm in diameter without a coating. The 24 wells were divided into three groups: the SL group, WL group and NL group. Fifty mg of sodium alginate and the calcium gluconate and/or physiological saline solutions were combined in the same proportions by weight.
The type and volume of solution are summarized.

Table 5. Adhesion scores (extent and severity).

Experiment No.	Experimental groups	Mean ± SD	
		Extent of adhesion	Severity of adhesion
1	PGA alone group	4.0±0	4.0±0
	fibrin group	2.8±1.8	2.3±1.8
	SL group	1.6±1.7	2.0±1.8
	WL group	0.1±0.4	0.1±0.4
	NL group	0.3±0.7	0.3±0.7
2	10 W subgroup	2.0±1.3	2.5±1.1
	20 W subgroup	2.0±0.6	2.2±0.7
	40 W subgroup	1.0±0	1.5±1.0
	80 W subgroup	0.7±0.8	0.8±1.2
	160 W subgroup	0±0	0±0
	10 N subgroup	1.8±1.5	2.0±1.6
	20 N subgroup	0.7±0.8	1.2±1.2
	40 N subgroup	0.5±0.6	0.8±1.0
	80 N subgroup	0.3±0.5	0.3±0.5
	160 N subgroup	0±0	0±0

The adhesion scores (extent and severity) are expressed as the mean ± standard deviation.

Table 6. Number of adherent portions where the PGA sheet adhered.

Experiment No.	Experimental groups	omentum	gonadal fat	intestine (part)	mesenterium	total
1	PGA group	8/8	5/8	1/8	0/8	14/32
	fibrin group	6/8	4/8	0/8	0/8	10/32
	SL group	4/8	1/8	1/8	1/8	7/32
	WL group	1/8	0/8	0/8	0/8	1/32
	NL group	1/8	0/8	0/8	0/8	1/32
2	10W subgroup	4/6	5/6	0/6	0/6	9/24
	20W subgroup	2/6	6/6	0/6	0/6	8/24
	40W subgroup	5/6	3/6	0/6	0/6	8/24
	80W subgroup	1/6	1/6	0/6	0/6	2/24
	160W subgroup	0/6	0/6	0/6	0/6	0/24
	10N subgroup	4/6	2/6	0/6	0/6	6/24
	20N subgroup	2/6	1/6	0/6	0/6	3/24
	40N subgroup	2/6	1/6	0/6	0/6	3/24
	80N subgroup	2/6	0/6	0/6	0/6	2/24
	160N subgroup	0/6	0/6	0/6	0/6	0/24

Table 6 shows the details of the adherent portions. The adherent portions included the omentum, gonadal fat, mesenterium and part of the intestines in experiment 1 and the omentum and gonadal fat in experiment 2.

Table 7A. Microscopic changes in the five groups in experiment 1.

Common changes in all groups	
PGA fibers decreased in size.	
PGA fibers turned into flakes.	
Each PGA fiber was covered by macrophages and collagen-like substrates.	
Specific changes in each group	
Fibrin group	The three alginate groups
Inflammatory cells, mainly lymphocytes, accumulated. (8/8 rats) Huge lymph follicles were found around PGA fibers. (3/8 rats)	Residual alginate was seen in two forms: (1)Island-formation by gathering of many macrophages ingesting alginate. (IF) (2)"Pool" of alginate in the free state. (PA)

The microscopic findings of the HE sections from experiment 1 are summarized. Table 7A shows the common changes in the five groups and specific changes in the fibrin and three alginate groups. Common changes had three steps: the first step was that the PGA fibers decreased in size, the second step was that the PGA fibers turned into flakes and third step was that each PGA fiber was covered by macrophages and collagen-like substrates.

The specific changes in the fibrin group had two steps: the first step was that a lot of inflammatory cells accumulated all over the microscopic visual fields and the second step was that huge lymph follicles were found.

The specific change in the three alginate groups was residual alginate form: one form was island formation by the gathering of many macrophages ingesting alginate (IF) and the other form was a "pool" of alginate in a free state (PA). Many of the “pools” were scattered around the PGA fibers.

Table 7B. Residual alginates of the three alginate groups in experiment 1.

Experimental groups	IF	PA
SL group	Small amount	Large amount
WL group	Small amount	Moderate amount
NL group	Small amount	Small amount

Table 7B shows the amounts of IF and PA. The changes in lymphocyte accumulation, fibroblast infiltration and fibrosis were weak in the three alginate groups, whereas these findings were remarkable in the other two groups.

Table 8A. Immunohistochemical features on HBME-1 staining of mesothelial cells in experiment 1.

Experimental groups	The single cell layer stained with HBME-1
PGA alone	+
fibrin	++
SL	+
WL	++
NL	+++

HBME-1 stained the single cell layer covering the tissue surface facing the peritoneal cavity. The HBME-1 staining was scored as follows: +++, clearly stained; ++, moderately stained and +, unclear. The HBME-1 staining was poor on the surface over the lymph follicles in the fibrin group and over the PGA fibers in the PGA alone group.

Table 8B. Immunohistochemical features of CD68, CD86 and CD163 staining in three locations in experiment 1.

Experimental groups	(1)			(2)			(3)		
	CD68	CD86	CD163	CD68	CD86	CD163	CD68	CD86	CD163
PGA alone	±	±	○	±	±	╳	○	○	○
fibrin	+	±	○	+	±	╳	○	○	○
SL	+++	+++	╳	+++	+++	╳	○	○	○
WL	++	++	○	++	++	╳	○	○	○
NL	+	+	◎	+	+	╳	○	○	○

The macrophages were stained for CD68, CD86 and CD163 in three locations (1-3). The staining was described as follows: ◎, mostly positive cells; ○, some positive cells and ╳, no positive cells. The visually recognized positive cells were scored as follows: +++, high; ++, medium; +, small; ±, few.

(1): cells between the fibers in the PGA bundles.

(2): cells in the space between the PGA bundles.

(3): cells on the surface of the PGA fiber.

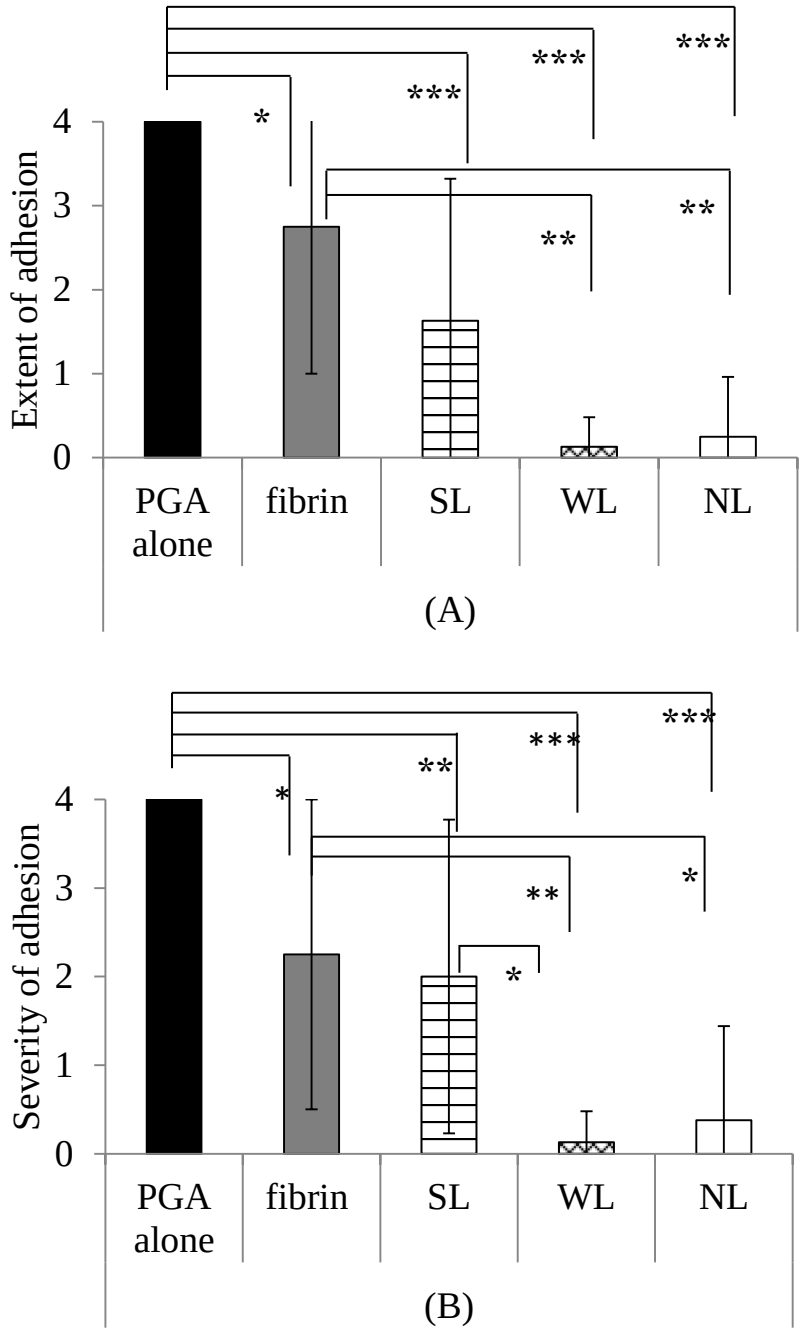


Figure 1. Adhesion scores in experiment 1

(A) Extent and (B) severity of adhesion. The columns indicate the mean scores and the bars indicate the standard deviation. The black column shows the scores in the PGA alone group, the gray column shows the scores in the fibrin group, the horizontally striped column shows the scores

in the SL group, the mesh pattern column shows the scores in the WL group and the white column shows the scores in the NL group. *P* values < 0.05 are marked by an asterisk (*), those <0.01 are marked by double asterisks (**) and those <0.001 are marked by triple asterisks (***).

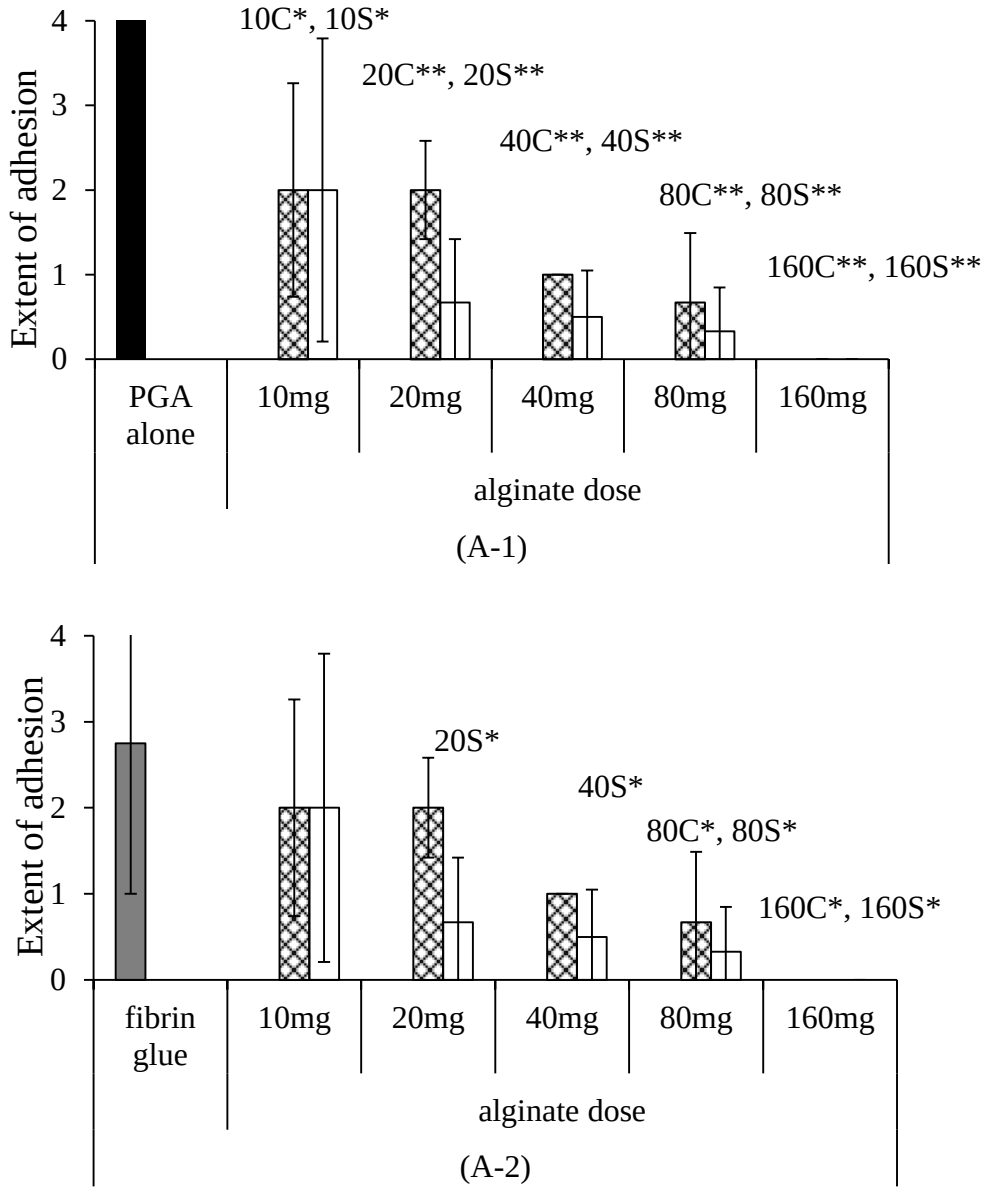


Figure 2 (A-1 and A-2). Extent of adhesion scores in experiment 2
The columns show the mean scores and bars indicate the standard deviation. The black column shows the scores in the PGA alone group, the gray column shows the scores in the fibrin group, the mesh pattern columns show the scores in the five WL subgroups and the white columns show the scores in the five NL subgroups. The extent of adhesion is described in A-1 and A-2. The adhesion scores in the WL and NL groups were compared with those in the PGA alone group (A-1) and the fibrin group (A-2). P

values <0.05 are marked by an asterisk (*) and those <0.01 are marked by double asterisks (**) and shown in the upper right of the graph in capital letters.

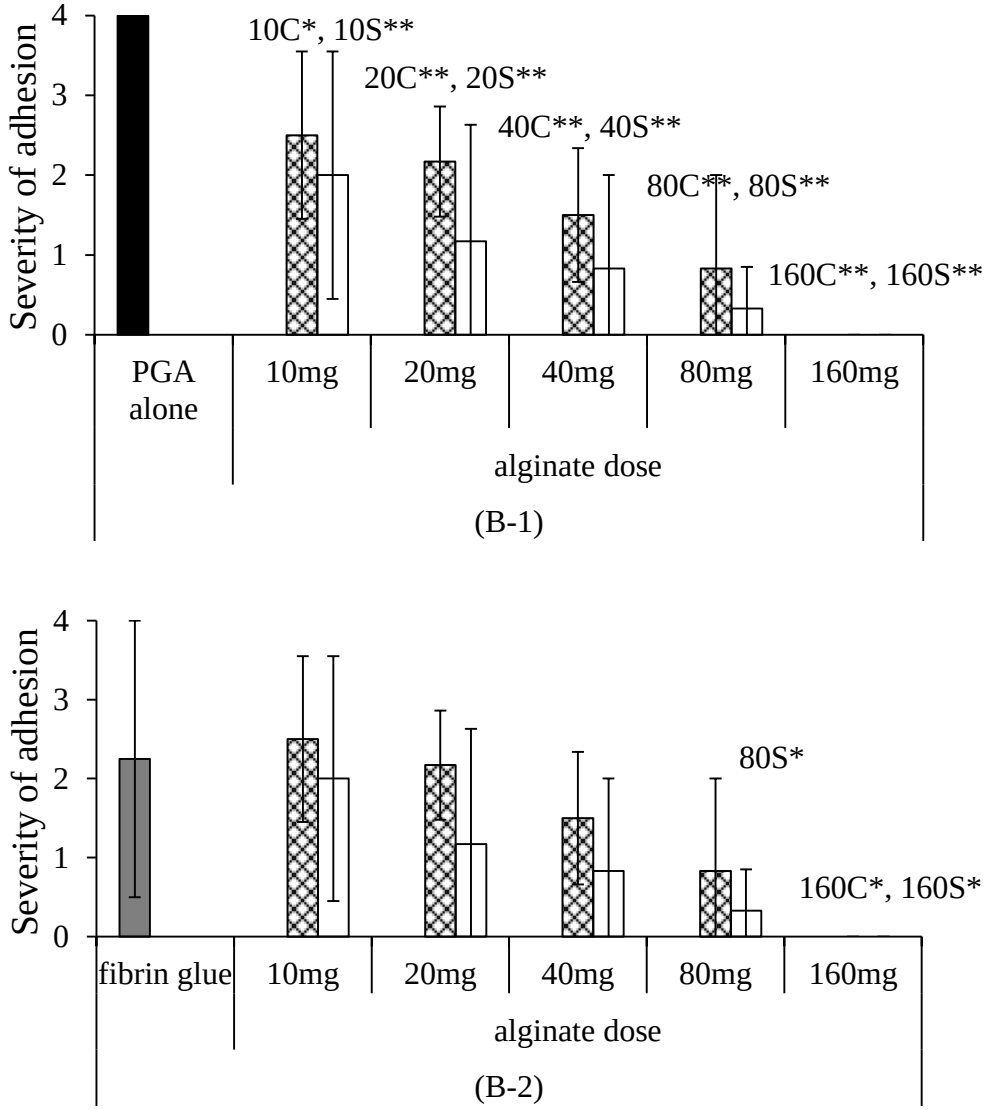
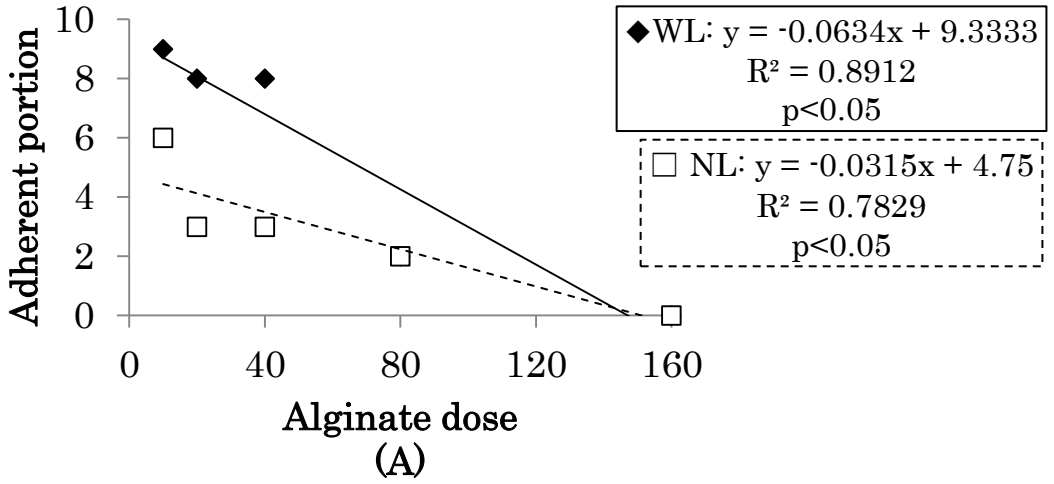
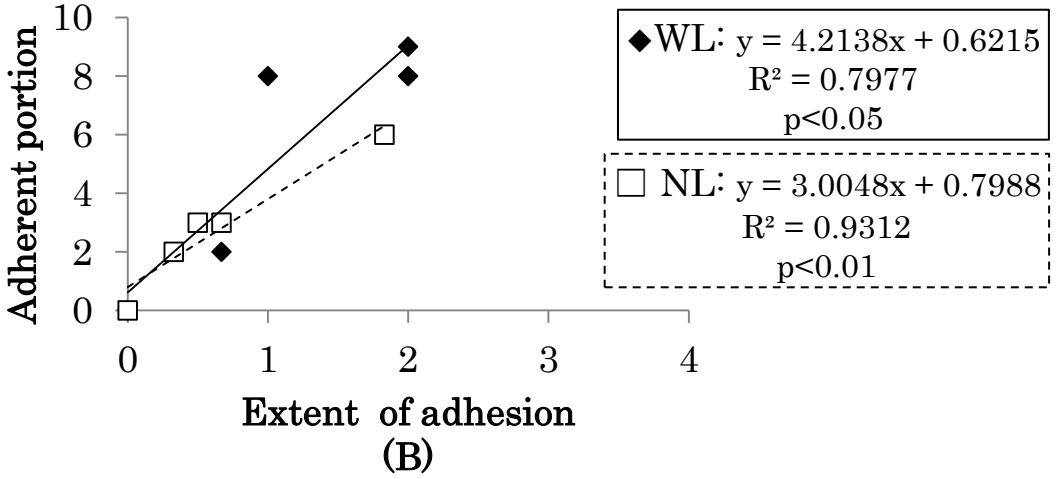


Figure 2 (B-1 and B-2). Severity of adhesion scores in experiment 2
The columns show the mean scores and bars indicate the standard deviation. The black column shows the scores in the PGA alone group, the gray column shows the scores in the fibrin group, the mesh pattern columns show the scores in the five WL subgroups and the white columns show the scores in the five NL subgroups.The severity of adhesions is described in B-1 and B-2. The adhesion scores in the WL and NL groups were compared with those in the PGA alone group (B-1) and the fibrin group (B-2). P values <0.05 are marked by an asterisk (*) and those <0.01 are

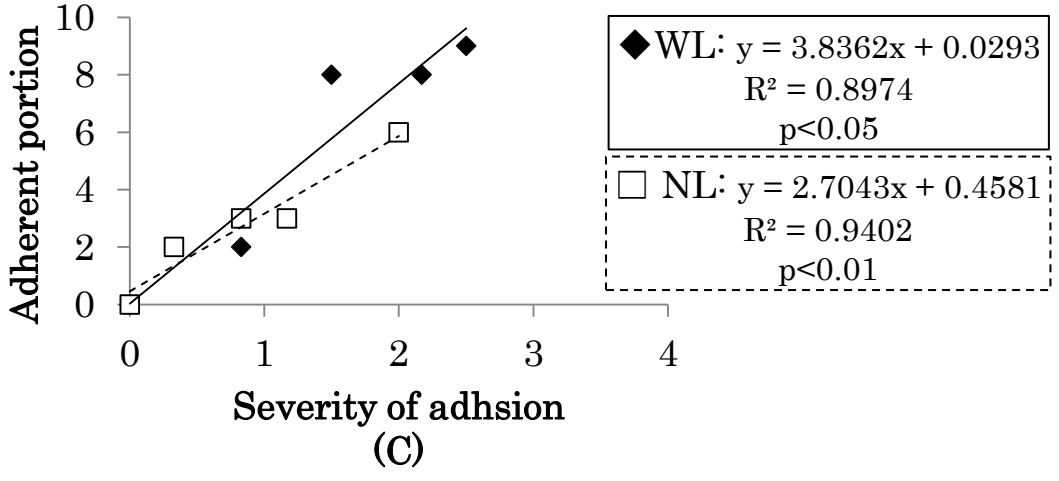
marked by double asterisks (**) and shown in the upper right of the graph in capital letters.


Adherent portion
10
8
6
4
2
0
0
40
80
120
160
Alginate dose
◆WL: y = -0.0634x + 9.3333
R² = 0.8912
p<0.05
□ NL: y = -0.0315x + 4.75
R² = 0.7829
p<0.05

(A)


Adherent portion
10
8
6
4
2
0
0
1
2
3
4
Extent of adhesion
◆WL: y = 4.2138x + 0.6215
R² = 0.7977
p<0.05
□ NL: y = 3.0048x + 0.7988
R² = 0.9312
p<0.01

(B)


Adherent portion
10
8
6
4
2
0
0
1
2
3
4
Severity of adhsion
◆WL: y = 3.8362x + 0.0293
R² = 0.8974
p<0.05
□ NL: y = 2.7043x + 0.4581
R² = 0.9402
p<0.01

(C)

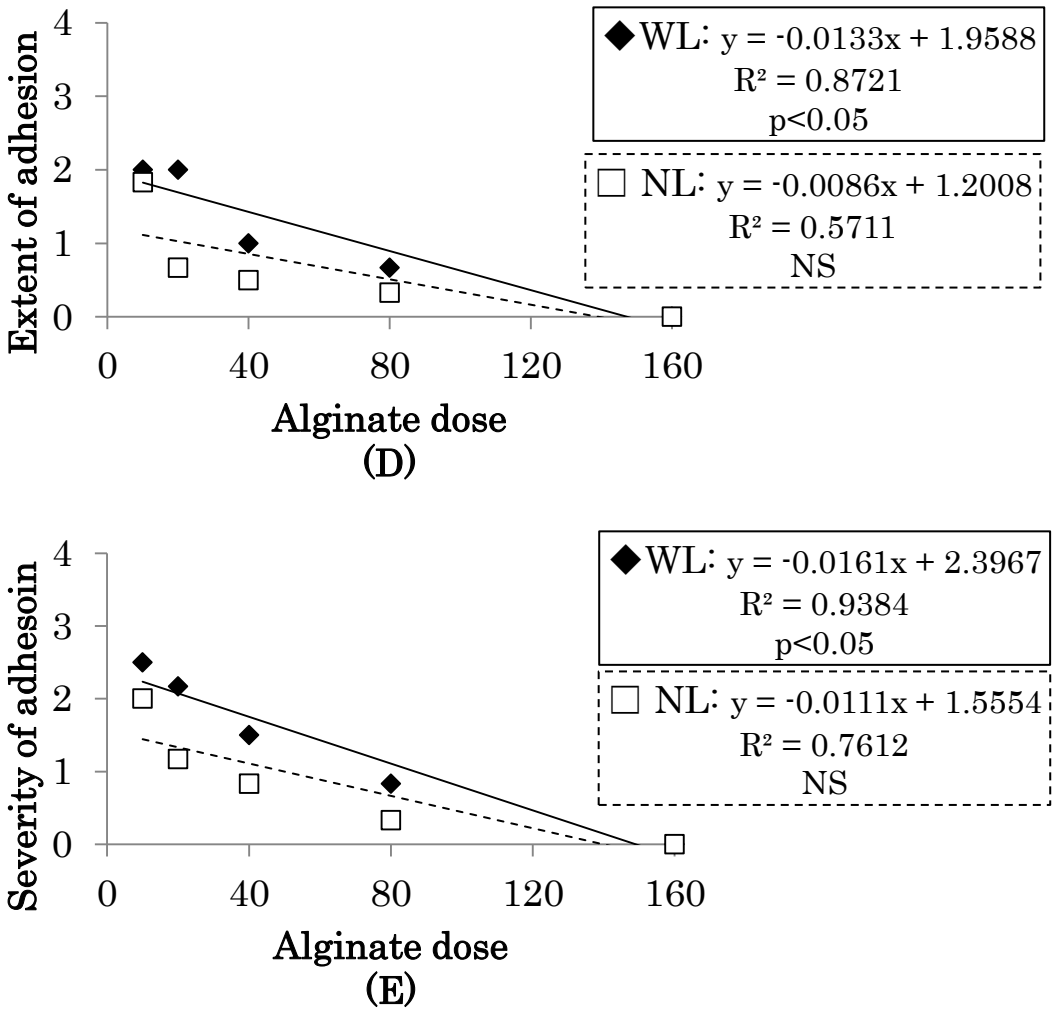


Figure 3. Correlations between the alginate doses, total number of adherent portions and adhesion scores (extent and severity)
The diamonds and squares indicate the WL and NL subgroups, respectively.

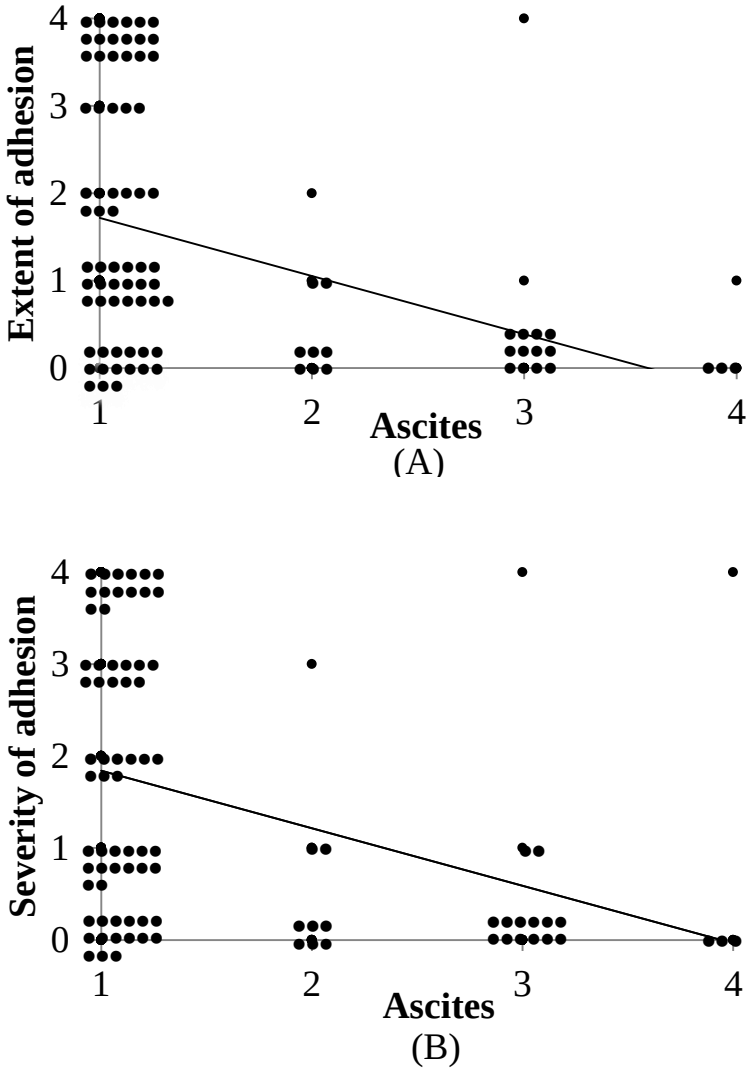
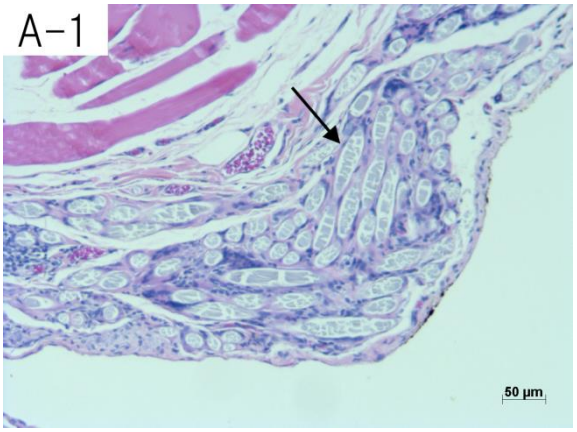
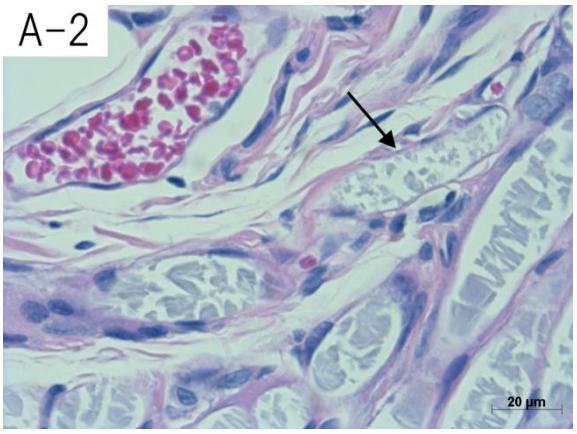
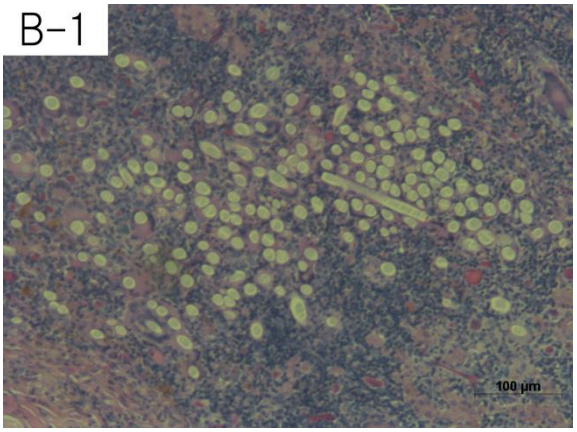


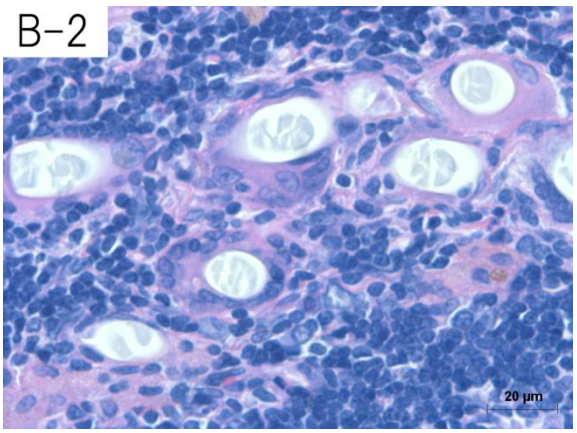
Figure 4. Correlations between the anti-adhesive effects and the volume of ascites.

(A): The correlation is expressed as the equation Y= -0.665X+2.382 (R^2 = 0.1655, p<0.001). (B): The correlation is expressed as the equation Y= -0.6257X+2.4663 (R^2 = 0.1356, p<0.001).


A-1
50 μm


A-2
20 μm


B-1
100 μm


B-2
20 μm

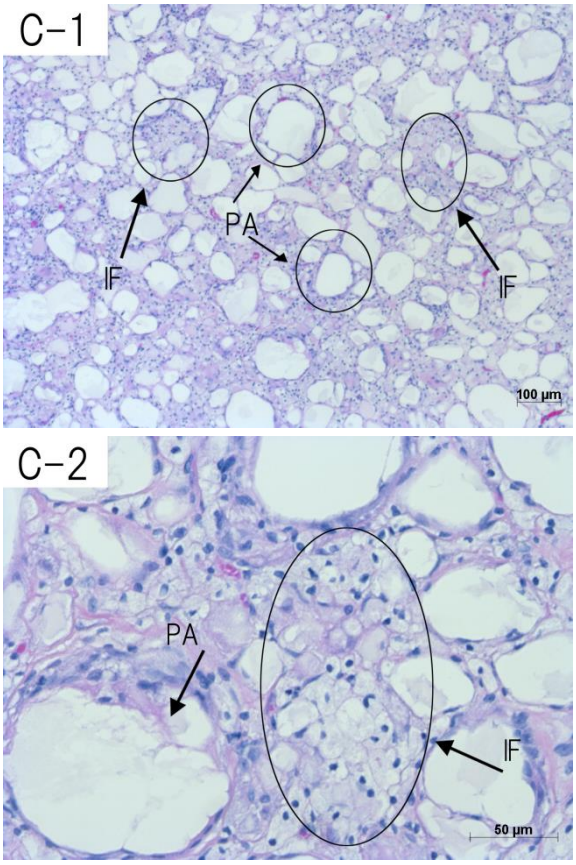
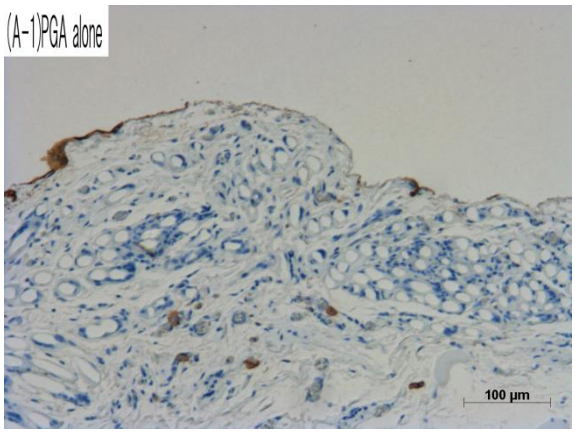
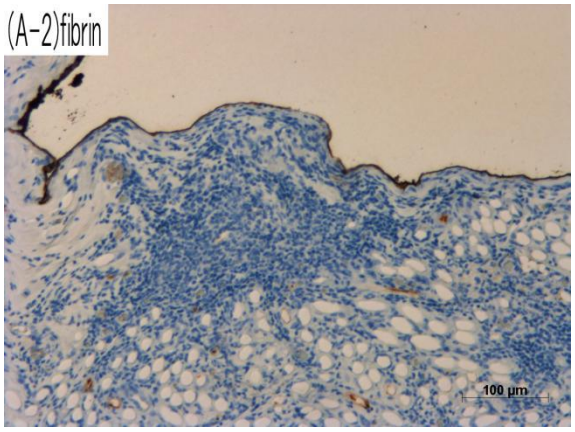
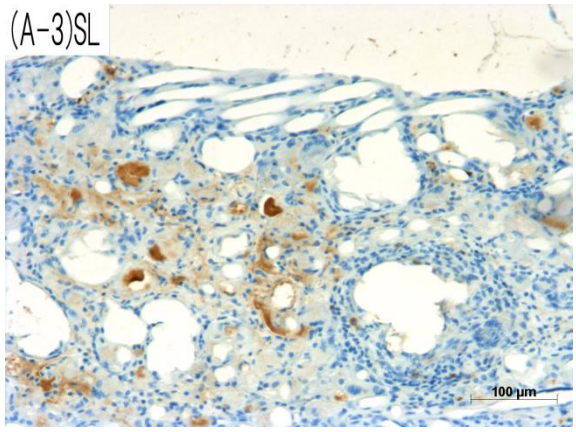


Figure 5. Microscopic findings of HE staining.
A-1 and A-2 show the common changes in the five groups. The PGA fibers decreased in size. The PGA fibers turned into flakes (arrow), with each PGA fiber covered by macrophages and collagen-like materials. B-1 and B-2 show the specific changes in the fibrin group. Inflammatory cells, mainly lymphocytes, had accumulated. Huge lymph follicles were found around the PGA fibers (3/8 rats). C-1 and C-2 show the specific changes in the three alginate groups. There were two forms of residual alginate: island formation (IF) due to the accumulation of many macrophages ingesting alginate, and a "pool" of alginate (PA) in a free state. The scale bars are 50 μm (A-1), 20 μm (A-2), 100 μm (B-1), 50 μm (B-2), 100 μm (C-1) and 50 μm (C-2).


(A-1)PGA alone
100 μm


(A-2)fibrin
100 μm


(A-3)SL
100 μm

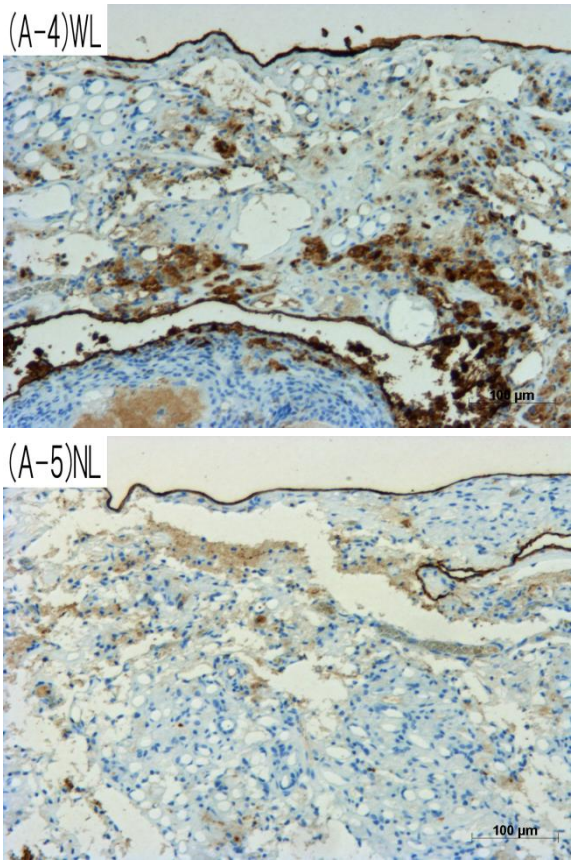
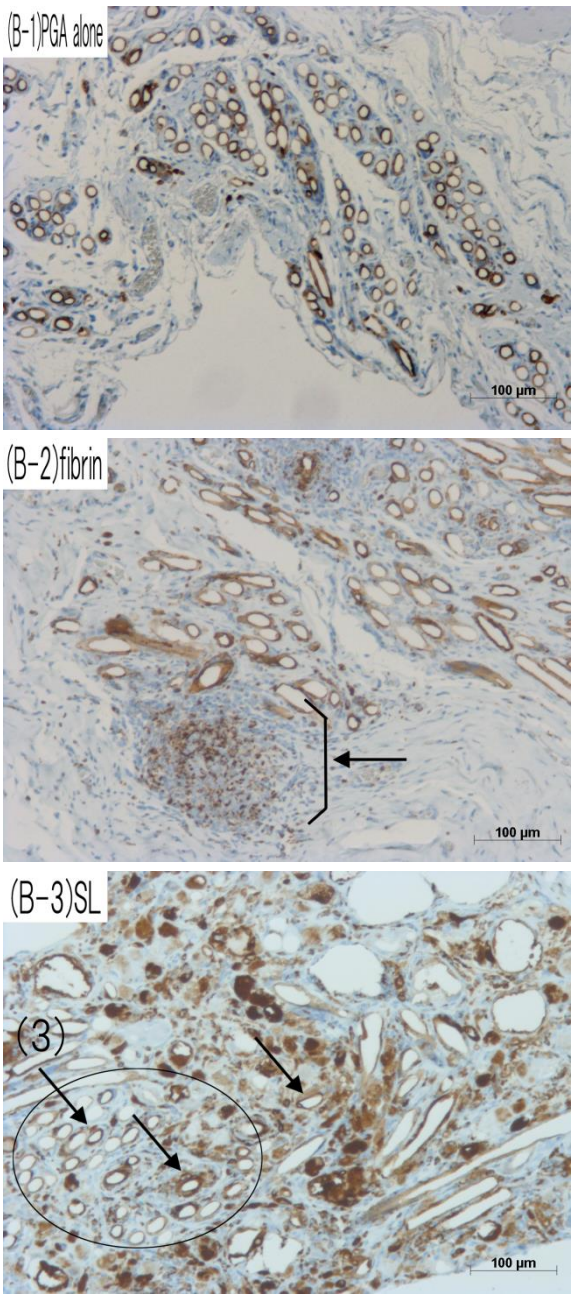


Figure 6. Immunohistochemical staining with HBME-1.
All scale bars are 100 μm.
HBME-1 was most clearly stained in the single cell layer covering the surface of the tissue over the PGA sheet in the NL group (A-5), followed by the fibrin group (A-2)and the WL group (A-4), and the staining was unclear in the PGA alone group (A-1) and the SL group (A-3).
The HBME-1 staining was poor on the surface over the lymph follicles in the fibrin group and over the PGA fibers in the PGA alone group.


(B-1)PGA alone
100 μm
(B-2)fibrin
100 μm
(B-3)SL
(3)
100 μm

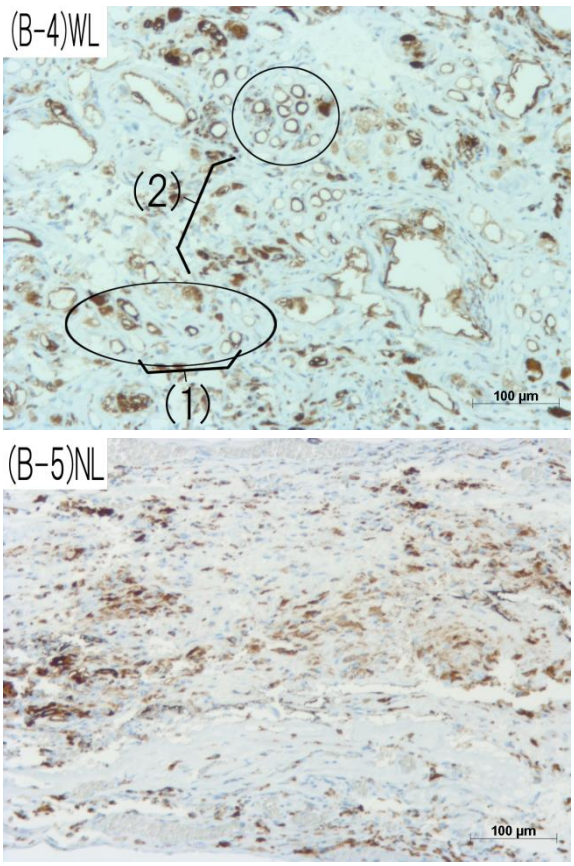
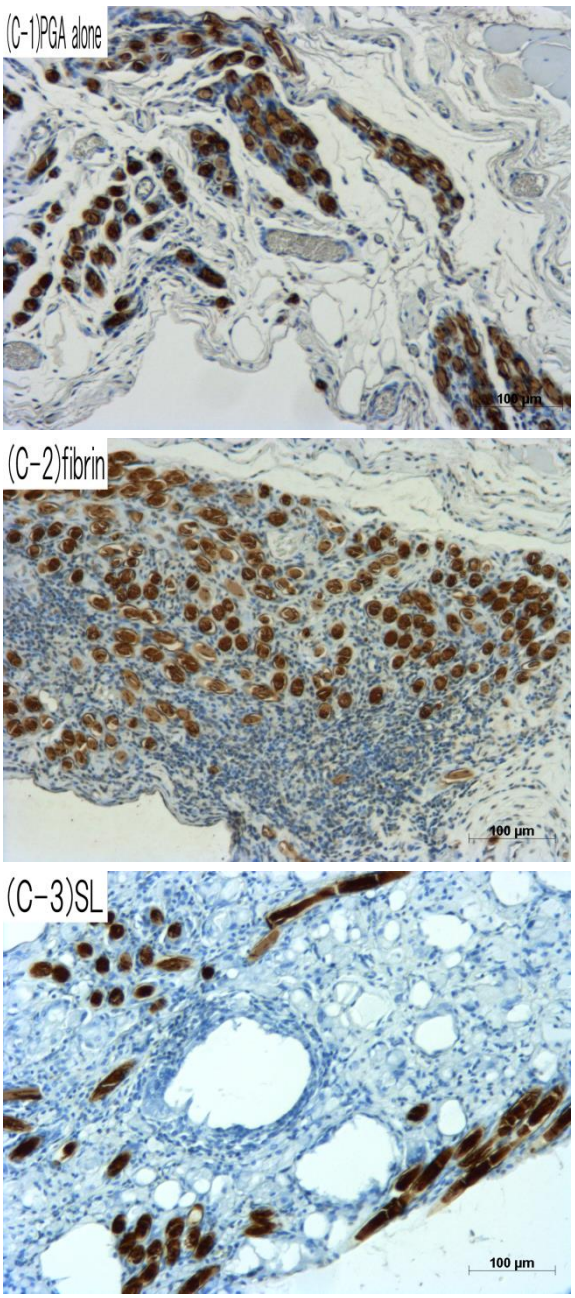


Figure 6. Immunohistochemical staining with CD68.
All scale bars are 100 μm.
(B-2): CD68-positive cells were found at sites of accumulation of lymphocytes (arrow).
(B-3 and B-4): The circles show PGA bundles and (1) – (3) show positive cells; (1) cells between the fibers in the PGA bundles, (2) cells in the space between the PGA bundles, (3) cells at the surface of the PGA fiber.


(C-1)PGA alone
100 μm
(C-2)fibrin
100 μm
(C-3)SL
100 μm

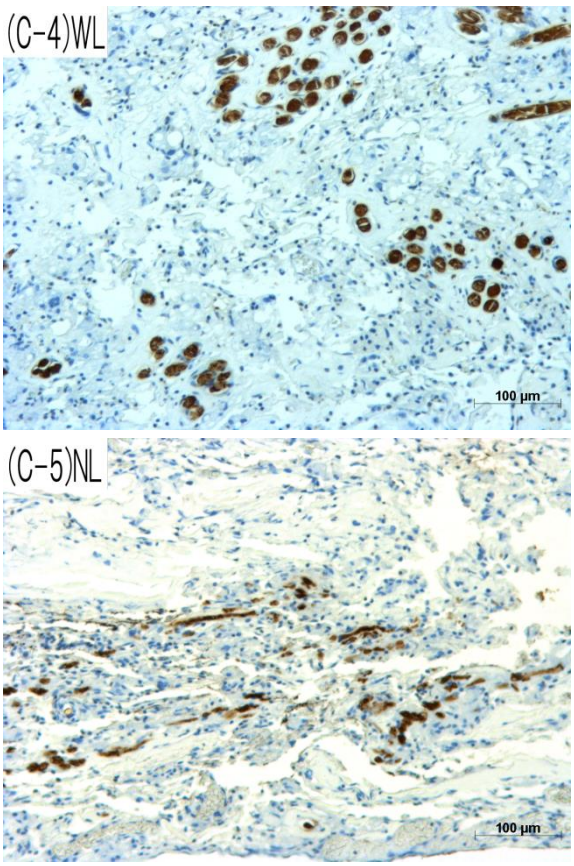
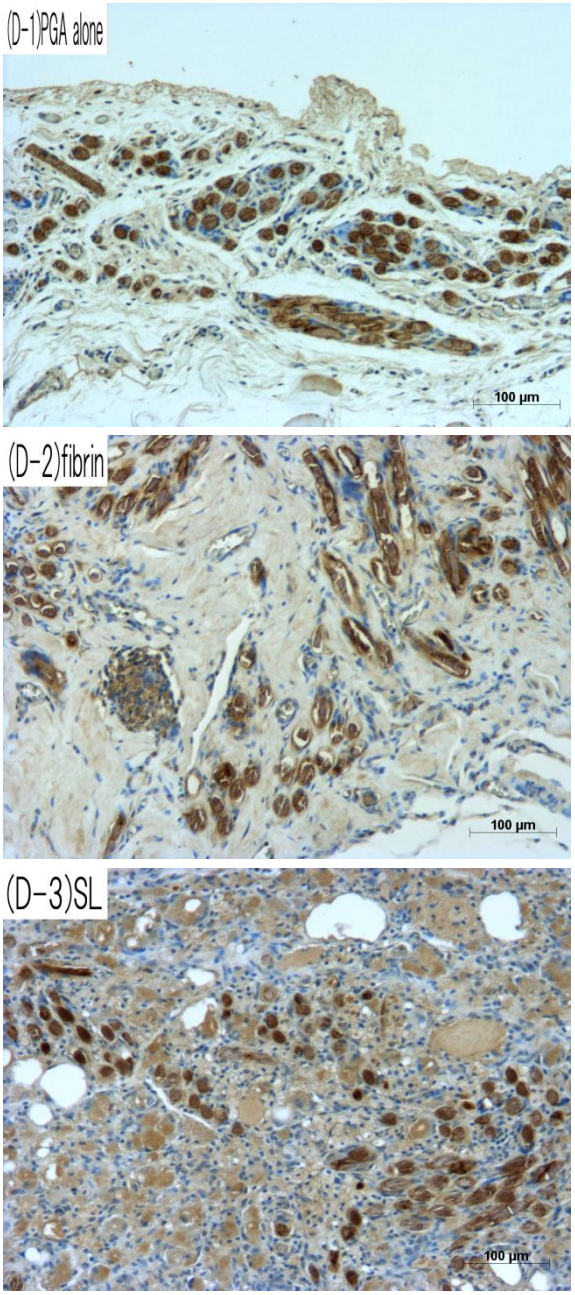


Figure 6. Immunohistochemical staining with CD163.
All scale bars are 100 μm.
(C-3 and C-4):The macrophages in the space between the bundles showed CD163-positive cells most predominantly between the PGA fibers in the NL group, whereas in the SL group, CD163-positive cells were hardly detected.


(D-1)PGA alone
100 μm
(D-2)fibrin
100 μm
(D-3)SL
100 μm

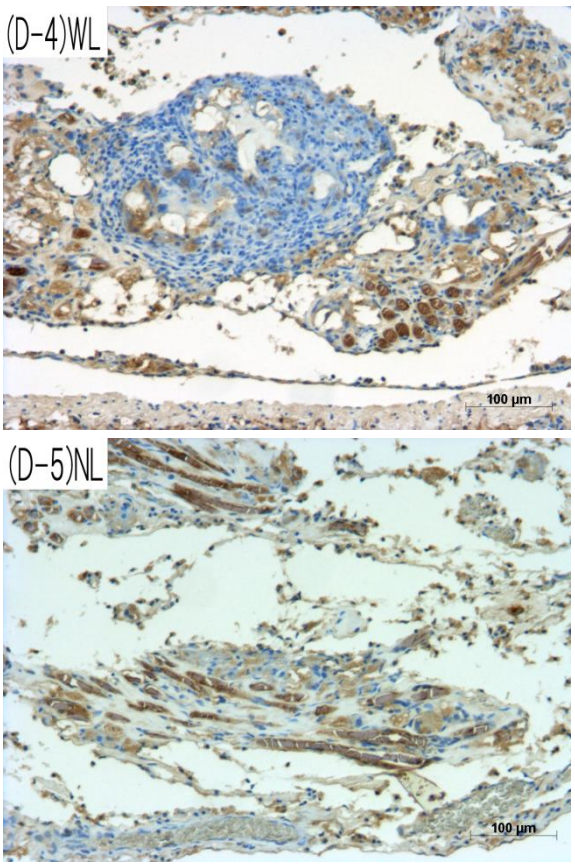


Figure 6. Immunohistochemical staining with CD86.
All scale bars are 100 μm.
With regard to the macrophages in the space between the bundles, CD86-positive cells were predominant in the SL group, but not in the NL group.

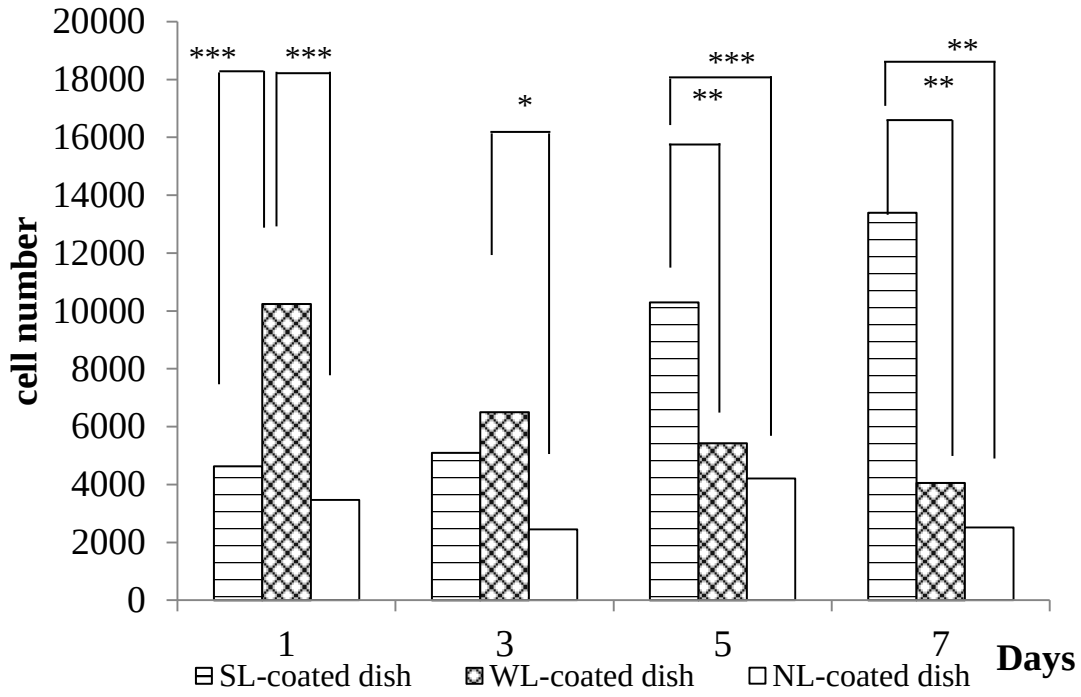


Figure 7. Fibroblast growth on the anti-adhesive materials *in vitro*.
The columns indicate the mean cell number and the bars indicate the standard deviation. The horizontally striped columns, mesh pattern columns and white columns indicate the scores in the SL group, WL group and NL group, respectively. P values <0.05 are marked by an asterisk (*), those <0.01 are marked by double asterisks (**) and those <0.001 are marked by triple asterisks (***).

Ⅵ. STUDY 4

ANTI-ADHESIVE EFFECT OF NEW TYPE OF POLYGLYCOLIC ACID UNIFIED SODIUM ALGINATE

新規材形の一体化型アルギン酸ナトリウム-ポリグリコール酸不織布の癒着防止効果の検討

TABLE OF CONTENTS (STUDY 4)

ABSTRACT (STUDY 4)

Background:

Although polyglycolic acid (PGA) mesh is a reinforcement for injured tissues and used widely during surgeries, PGA may induce the adhesion. We defined the adhesion as “PGA induced adhesion”. PGA induced adhesion could be caused by the inflammations due to foreign body reaction and pH decrease at the injured tissue around the PGA mesh.

Sodium alginate was focused on its anti-adhesive effect and made an alginate sponge. We developed a new PGA unified with the alginate sponge. The aim of this study was to compare the anti-adhesive effects a new PGA to the comventional one.

Materials and Methods:

Ten centimeter square PGA mesh was placed at the bottom a container. Sodium alginate powder was dissolved in saline or calcium gluconate solution, and the alginate solution was poured into the container and was frozen at −80◦C for 30min. The PGA mesh unified with alginate was freeze-dried for 24 h. It’s important that freeze-dried alginate turned into a foam.

Three materials were evaluated in anti-adhesive effect: PGA mesh unified with sodium alginate, and PGA mesh, and PGA mesh unified with calcium alginate. Fifty four rats were divided randomly into three groups: Na-alg group, Ca-alg group, and PGA alone group. The rat was fixed in the dorsal position under general anesthesia. A 4 cm midline incision was made for laparotomy. Each material was fixed onto the peritoneum at the four corners. Adhesion was assessed 2, 4, and 8 weeks after surgery using adhesion score macroscopically.

The abdominal wall with PGA mesh was removed *en bloc* and the specimens were fixed in 10% formalin. Each specimens were stained with hematoxylin eosin and evaluated microscopically and immunologically.

Results and Conclusions:

PGA mesh unified with sodium alginate was the most effective against the adhesion, whereas PGA mesh unified with calcium alginate was less effective. In the sodium alginate group, fibroblasts and collagen fibers around implanted sites were sparse and the material degraded rapidly by macrophage ingestion. Fibroblasts and collagen fibers play a major role in adhesion formation and their excessive proliferation results in postoperative adhesion. Thus, inhibiting their increase is the key in preventing PGA induced adhesion. The reinforcement that is composed of PGA mesh unified with sodium alginate foam strongly inhibited PGA induced adhesion and showed excellent handling during surgery and could be easily applied with a one-step procedure.

邦文要約　(STUDY 4)

背景：

ポリグリコール酸（PGA）不織布は現在臨床使用されている手術材料で、有用な組織補強材である。PGA は生体内で吸収される材料だが、異物反応やその分解時に生じるモノマーが酸性となることが原因で炎症が生じ、結果として PGA 存在部位に癒着が生じることがある。本研究では、組織補強材としての有効性を残しつつ、癒着を惹起しない PGA として、アルギン酸ナトリウム一体化 PGA を新規に開発した。

材料と方法：

アルギン酸ナトリウム一体化 PGA は、10 ㎝四方の PGA 不織布にアルギン酸溶液を浸透させ凍らせた後、凍結乾燥を行い作製した。54 匹のラットを PGA/Na-alg, PGA/Ca-alg, 及び PGA alone の 3 群に分け、腹壁に各材料を縫着し、術後 2，4，8 週間後に評価を行った。評価項目は、癒着スコアを用いた肉眼的評価とヘマトキシリンエオシン染色による組織染色を行い、アルギン酸に浸潤するマクロファージ、線維芽細胞、コラーゲン繊維を組織学的スコアにて評価した。また、免疫学的染色により中皮細胞の再生についても評価を行った。

結果および結語：

アルギン酸ナトリウム一体化 PGA では、どの時点でも他群に比べて有意に癒着スコアが小さく、癒着防止に効果が認められた。局所滞留性を向上させる目的で、カルシウム架橋を行った群（PGA/Ca-alg 群）では、その効果の有意性は限定的であった。

また、組織学的スコアを用いた評価での結果から、アルギン酸ナトリウム一体化 PGA へ浸潤する線維芽細胞とコラーゲン繊維はまばらで、マクロファージの貪食を即座に受けていることが推測された。一般的に、線維芽細胞やコラーゲン繊維が浸潤し、増殖することで癒着が形成されることが知られていので、これらの増殖を抑制することが、PGA 惹起性癒着防止でもキーポイントになる。本研究結果から、アルギン酸ナトリウム一体化 PGA は PGA 惹起性癒着防止に非常に有効であり、術中の操作性の良さの点から臨床応用が十分に期待できる。

INTRODUCTION

Polyglycolic acid (PGA) mesh is useful in the medical fields and the demand is increasing because of its biodegradibility and superior reinforcing effects [1, 2]. The application of biodegradable reinforcement is expanding and increasing in automatic suturing devices as well as surgrical other situations. However, the reinforcement made of PGA sometimes brings about chronic inflammation and adhesion [3–6] due to pathological reactions. PGA is hydrolyzed and turns into a monomer; glycolic acid. Glycolic acid induces adhesion where PGA mesh is placed, hereinafter refereed to as PGA induced adhesion [6]. Adhesion may result in peritoneal complications, female infertility, and difficulties in subsequent surgeries [7–10]. In addition to the physical complications, prolonged hospitalization and hospital readmissions are also serious issue [11], resulting in raising hospital costs [12]. Preventing PGA induced adhesion is an extremely important concern. This study aimed to develop a new PGA reinforcement which was designed not to induce adhesion.

Alginate has been reported the antiadhesive effect [4, 13, 14]. Alginate gel and alginate solution were reported, in our previous report, to prevente PGA induced adhesion effectively [4]. However, the handling are difficult and the retentivity are poor.

In order to solve above described problems, alginate solution was soaked up the PGA mesh and freeze-dried, thus we made an alginate sponge. We developed a new PGA unified with an alginate sponge. As improving its usability during surgery, the usability and anti-adhesive effect were evaluated.

MATERIALS and METHODS

1. Preparation of Materials.

We used the PGA mesh (NEOVEIL®, Gunze, Kyoto, Japan), sodium alginate powder (Alto®, Kaigen, Osaka, Japan), and calcium gluconate solution (Calcicol®, Nichiiko, Toyama, Japan) mainly. Sodium alginate powder has the molecular weight ranging from 32,000 to 250,000. This is available as a hemostatic agent commercially.

There were one control PGA and two types of new PGA unified with alginate as follows.

(I) Control PGA **(**hereinafter called "PGA alone”):

No treatment was performed. (Figure 1(a)).

(II) PGA unified with sodium alginate sponge **(**hereinafter called "PGA/Na-alg”):

Ten centimeter PGA square mesh was placed at the bottom of a silicon-coated square container (10 × 10 × 1 cm). Sodium alginate powder (3.55 g) was dissolved in 96.45g of physiological saline so as to an alginate solution (3.55wt%). All of the solution was poured into the container and soaked up to the PGA mesh, and was frozen at −80℃ for 30min. The frozen PGA mesh unified with the alginate was freeze-dried for 24 h. The freeze-dried alginate turned into a sponge. Figures 1(b1) and 1(b2) show the each side of PGA/Na-alg.

(III) PGA unified with calcium alginate sponge **(**hereinafter called "PGA/Ca-alg”)

Ten centimeter PGA square mesh was placed at the bottom of the container in the same manner as PGA/Na-alg. Calcium gluconate solution (8.5 wt%; 1ml), containing 7.85mg of calcium, was added to 100 g of the

alginate solution (3.55wt%).The alginate solution partially (<5%) cross-linked by Ca^{2+} was poured into the container and soaked up to the PGA mesh, and was frozen in the same manner as PGA/Na-alg. The frozen PGA mesh unified with calcium alginate was freeze-dried for 24 h. Figures 1(c1) and 1(c2) show the each side of PGA/Ca-alg.

All materials were cut 15mm square in size and were sterilized with ethylene oxide gas for 22 h. The gas was removed in a decompression condition for one week.

2. Animal Protocol.

Fifty four rats, Wistar/ST strain, 9-10 weeks old, female, weighing 200 g, were purchased from SHIMIZU Laboratory Supplies Co. (Kyoto, Japan), and were used in this study. During the experimental period, all rats were housed separately and maintained under the standard (light-dark cycle of 12:12 h, temperature of 20.1–23.5℃, and humidity of 37–65%) specific pathogen-free (SPF) conditons. Standard laboratory chow for rodents and water were available freely. The rats were housed in the laboratory for 2 weeks before the experiments. On the day of the experiment, rats werevchecked for the health conditions.

All rats received inhalation anesthesia using isoflurane (Escain, Mairan Pharmaceutical, Osaka, Japan). After the experiments, the lethal dose (75mg/kg of body weight) of sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) was administered into the abdominal cavities. All procedures of surgery and anesthesia were performed in accordance with the Animal Care Guidelines of Doshisha University.

3. Experimental Design.

Fifty four rats were divided randomly into three groups: Na-alg group, Ca-alg group, and PGA alone group. Adhesion was evaluated at 2, 4, and 8 weeks after surgery. Concretely speaking, six rats per group was used at every assessment timing. The anti-adhesive effect against adhesions induced by PGA was evaluated macroscopically and microscopically.

4. Surgical Techniques.

The rats were fixed in the dorsal position under general anesthesia. A 4 cm midline incision was made for laparotomy. Only the PGA mesh was fixed onto the peritoneum with 7-0 polyvinylidene fluoride monofilament sutures (Asflex, Kono Seisakusho Co., Chiba, Japan) at the four corners in the PGA alone group (Figure 2(a)). In regard to the PGA/Na-alg and

PGA/Ca-alg groups, each material was fixed as the same manner as the PGA alone group. (Figures 2(b) and 2(c)). 0.35ml of physiological saline was sprayed entire fabric using 1ml TERUMO syringe (TERUMO, Tokyo, Japan). The laparotomy wound was closed with 4-0 polyamide sutures in two layers.

5. Evaluations of Adhesion.

5.1. Macroscopic Evaluations.

At every assessment timing, the rats received isoflurane inhalation anesthesia and were sacrificed humanely by intra-abdominal administration of a lethal dose (3.5mg/kg of body weight) of sodium pentobarbital. Adhesion between each PGA mesh and intra-abdominal organs was scored into 0–4 macroscopically according to the extent and severity of adhesion. The persons scoring the adhesion were blinded to the treatment. The scoring system was modified from the Adhesion Score of the Surgical Membrane Study Group (Table 1) [4, 15].

5.2. Microscopic Analyses.

The abdominal wall with each material was removed *en bloc*, including the adhering organs and tissues. The specimens were obtained from all rats. All of the specimen were fixed in 10% formalin solution and cut into 4 μm thick slices stained with hematoxylin-eosin (HE). The status of the healing process of the tissues surrounding the materials evaluated histologically in a blinded manner. The status of four points, namely residual alginate, macrophages ingesting alginate, fibroblasts, and collagen fibers, were classified quantitatively using a histological scores in Table 2 [4]. Immunostaining with anti-human mesothelial cell antibody (HBME-1, Serotec, Japan) was performed to evaluate mesothelial regeneration.

6. Statistical Analyses.

Statistical analyses were performed using the software StatMate® (ATMS Co., Ltd., Tokyo, Japan). The assumptions of equality of variances was checked using a Bartlett's test before using an analysis of variance (ANOVA). Paraetric data was analyzed by ANOVA with Tukey's test. The non-parametric Kruskal-Wallis test was used for nonparametric data. The two-sided Mann–Whitney U test was used for the post-hoc analysis. Differences were generally considered statistically significant when p value was <0.05.

RESULTS

1. Macroscopic Evaluations of Adhesion (Table 3).

Severe and wide adhesion to the visceral organs was exhibited in the PGA alone group throughout the assessment timing. The extent and severity of adhesion were limited ($p < 0.01$) significantly throughout the assessment timing in the PGA/Na-alg group. Only the extent of adhesion at 8 weeks after surgery was reduced in the PGA/Ca-alg group ($p < 0.05$).

2. Microscopic Analyses of Adhesion (Table 4).

The results of HE staining was shown in Figure 3 and precise results were referred as follows.

PGA alone (Figures 3(A1) and 3(A2))

Almost all PGA fibers remained at every assessment timing. Some PGA fibers were lost in the process of specimen fixation. They were observed as void spaces or purple staining. It was notable that there were abundant fibroblasts and collagen fibers around the PGA fabrics at all of the assessment timing.

PGA/Na-alg (Figures 3(B1) and 3(B2))

Few fibroblast cells and collagen fibers were found inside and around the PGA/Na-alg at 2 weeks after surgery (Figure 3(B1)). At 8 weeks, they proliferated sparsely (Figure 3(B2)). Although the residuals of sodium alginate sponge remained sparsely at the implanted site, the most of them was ingested by macrophages as early as in 2 weeks.

PGA/Ca-alg (Figures 3(C1) and 3(C2))

Fibroblasts around the PGA fibers proliferate gradually as the weeks went by during the assessment. Collagen fibers surrounding PGA fibers were abundant throughout the assessment timing. Macrophages also ingested the calcium alginate sponge, similar to the PGA/Na-alg group;

however, some residuals of the calcium alginate sponge remained until 4 weeks after surgery.

Figure 4 shows the microscopic findings of HBME-1 staining at 8 weeks. Matured mesothelium was regenerated where the PGA/Na-alg was fixed (Figure 4(b)). On the other hand, no mesothelial layer was formed after 8 weeks in the PGA/Ca-alg group (Figure 4(c)).

DISCUSSION

PGA mesh is a useful tissue reinforcement for surgeries, but PGA may induce adhesion. In this study, we defined the adhesion as a “PGA induced adhesion.” PGA induced adhesion causes some complications; for instance, ileus, abdominal pain, and infertility. The complications should be addressed issues [5, 6, 16, 17]. PGA induced adhesion could be attributed to mainly two types of inflammation due to (1) foreign body reaction and (2) pH decrease at the tissue around the PGA mesh [6]. Regard to the former type of inflammation, biomaterials implanted in the body play an important role as matrixes for cell adhesion and proliferation. In response to the foreign substances, macrophages and fibroblasts migration have been contiued around the materials for over 2 weeks. Referred as the latter one, non-enzymatic degradation of PGA results in acidifying the tissue and the pH decreases around the PGA mesh. The pH decrease may cause local inflammation and induce the fibroblasts migration and collagen fiber proliferation.

We have tested how the pH level in PBS changed influenced by glycolic acid which was producuced when the PGA mesh (NEOVEIL®) was degraded. At 4 weeks pH level decrease started, and the level reached a minimum (pH 6.2) at 7 weeks. pH level increase was noted at 8 weeks (unpublished data). Utilizing the the properties of PGA as a reinforcement, the new PGA material should have the function as a physical barrier at least that induces the cell adhesion to the PGA mesh in order to promote the peritoneum regeneration. The new material is also needed to degrade after tissue regeneratin as soon as possible. PGA in this study induced cell adhesion effectively. Thus, it is an important concern to prevent PGA induced adhesion after surgery.

Alginate is reported the anti-adhesive effect [3, 18–20], and used as a hemostat in the medical field because it is biocompatibility, low toxicity,

and relatively low cost. Alginate is a linear copolymer polysaccharide composed of two types of uronic acids; (1- 4)-linked β-D-mannuronate (M) and C5 epimer α-Lguluronate (G). Uronic acids have carboxyl groups, thus ion-exchange is able to carry out between protons and cations. Divalent cations have a high affinity with the α- L-guluronic acid blocks. Mainly changed cations are Na^+ and Ca^{2+}, namely sodium alginate and calcium alginate, respectively. Sodium alginate has a high water soluble property and is easy to diffuse in the abdominal cavity. On the other hand, calcium alginate has a low solubility, even when it is weakly cross-linked [21]. As a result of this study, fibroblasts and collagen fibers around the material were more abundant in the PGA/Ca-alg group than in the PGA/Na-alg group. This is why calcium aginate would be able to substitute a matrix for cell adhesion and prolifereation of fibroblasts and collagen fibers because of its insolubility. Moreover calcium ion dispersed through the abdominal cavity would activate prothrombin and accelerate fibrin formation. Supersaxo et al. [22] reported that high molecular weight >16,000 substances are absorbed and drained mainly by the lymphatics. That's why alginate in this study is considered to be absorbed by the lymphatics.

A new PGA reinforcement composed of alginate should be easy to use during surgery based on our previous study [4]. The previous study made alginate gels composed of alginate powder, and calcium or saline solution. These alginate evaluated the anti-adhesive effects to the PGA induced adhesion among the alginates cross-linked with or without calcium. The anti-adhesive effect was different depending on the degree of Ca^{2+} cross-linking. Weakly cross-linked algiante showed a better effect to prevent PGA induced adhesion than strongly cross-linked one. The alginate gels were shown to be good retentivity in the PGA mesh. On the other hand, no cross-linked alginate prevented most strongly compared to Ca^{2+} cross-linked ones, but it had poor retentivity. Although these alginate gels have good anti-adhesive effect, they were difficult to use during surgery.

To solve the problem, we developed a new PGA mesh unified with alginate sponge and compared the anti-adhesive effect among the materials. PGA without any treatment was formed adhesion strongly, whose result was similar to our previous report. PGA/Na-alg showed the strongest anti-adhesive effect from early assessment timing, while PGA/Ca-alg showed the moderate effect after 8 weeks (long assessment timing). However, there were no notably significant effect compared in the earlier to long timing.

Early mesothelium regeneration and reduction of local inflammation around PGA may contribute to the anti-adhesive effect. Mesothelial cell started to proliferat in the 2 weeks, and mesothelial layer matured in 8 weeks in the PGA/Na-alg group. Mature mesothelium did not forme yet in 8 weeks in the PGA/Ca-alg group. That's why PGA/Na-alg was considered to be sutable for tissue regeneration and physical barrier. On the other hand, the few mesothelium was regenerated in the PGA alone group.

In regards to the fibroblasts and collagen fibers, these proliferation was observed early in the assessment timing and increased further as time went by in the PGA alone group. Fibroblasts and collagen fibers were scarce in the PGA/Na-alg group (Figure 3), while these proliferation was moderate in the PGA/Ca-alg group. Doyle et al. reported that calcium alginate could lead to fibroblasts proliferation more easily than sodium alginate [23]. It is one of the important factor that suppression of fibroblast and collagen fiber proliferation. Excessive fibroblasts and collagen fibers contribute to form the postoperative adhesion. That's why it is key point to inhibit their increase in order to prevent PGA induced adhesion. Histological study evaluated the fibroblast and collagen fibers proliferation. In particular, collagen fibers should be evaluated quantitatively and the result would be useful to consider how to prevent the PGA induced adhesion. The evaluation of the level of hydroxyproline at the adhesion site or the peritoneal surface would help to track collagen fiber synthesis. Although it was not indicated whether the antiadhesive effects and collagen

suppression were clearly related, we should evaluate hydroxyproline level quantitatively at the injured site in the further study.

Regarding alginate absorbing behavior, sodium alginate was ingested by macrophages from the early time and few residues were obserbed, while calcium alginate was less investigated than sodium alginate and the residues of calcium alginate were higher. The difference of the residual amount may be contribute partly to the solubility. Alginate should be degradate as soon as possible after the completion of mesothelial regeneration to avoid long-term xenobiotic reactions, and rapid degradatoion would be an advantage. The effect of the new reinforcement, PGA unified with alginate sponge, should be applied and evaluated in the human surgeries.

CONCLUSION

The new reinforcement, PGA unified with sodium alginate sponge, reduced PGA induced adhesion strongly. Because of the superior handling in this experiment, it would be applied easily with a one-step procedure during surgery and the clinical use is expected in the future.

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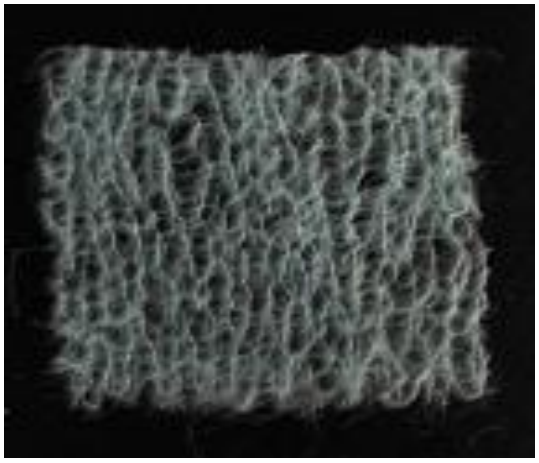

(a1)

(b1)

(b2)

(c1)

(c2)

Figure 1. Picutures of PGA mesh. (figure courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 2)

(a): PGA mesh: No treatment was performed
(b1, b2): PGA unified with sodium alginate foam (PGA/Na-alg)
PGA square mesh was placed at the bottom of a square container. Sodium alginate powder was dissolved in physiological saline (making an alginate solution). The solution was poured into the container and soaked up to the PGA mesh, and was frozen at −80℃for 30min. The frozen PGA mesh unified with the alginate was freeze-dried for 24 h. The freeze-dried alginate turned into a sponge.
(c1, c2): PGA unified with calcium alginate sponge. (PGA/Ca-alg)
PGA square mesh was placed at the bottom of the container in the same manner as PGA/Na-alg. Calcium gluconate solution was added to the alginate solution (making a calcium alginate solution). It was poured into the container and soaked up to the PGA mesh, and was frozen in the same manner as PGA/Na-alg. The frozen PGA mesh unified with calcium alginate was freeze-dried for 24 h.

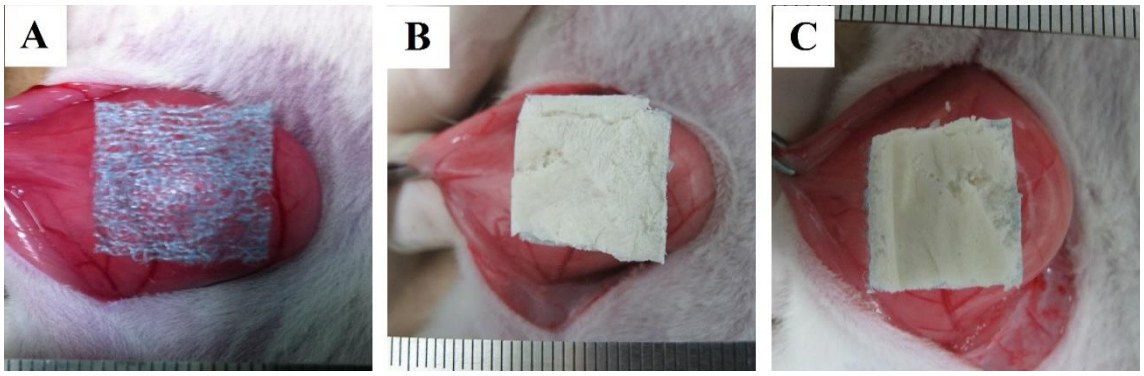

Figure 2. Each material was fixed onto the peritoneum of the right lateral abdominal wall. (figure courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 3)

(A) PGA alone, (B) PGA/Na-alg, (C) PGA/Ca-alg.

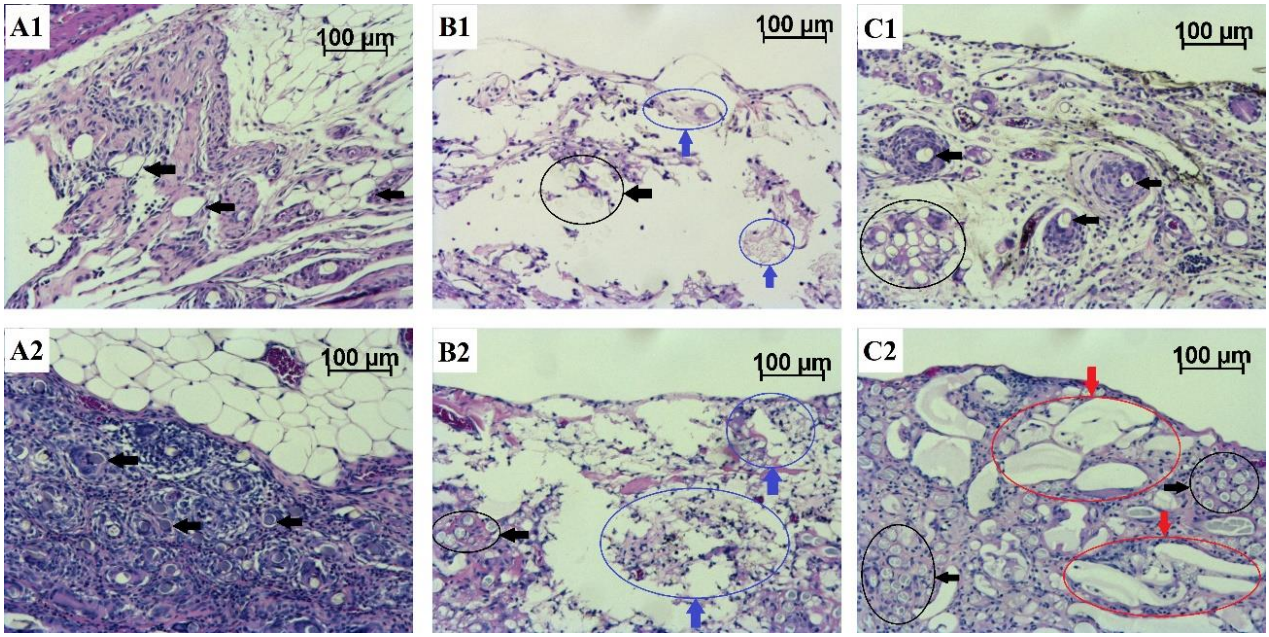

Figure 3. Microscopic findings of the explanted tissues surrounding the materials (HE staining):
(A) PGA mesh at 2 weeks (A1) and 8 weeks (A2).
(B) PGA/Na-alg at 2 weeks (B1) and 8 weeks (B2).
(C) PGA/Ca-alg at 2 weeks (C1) and 8 weeks (C2). Black arrows
(figure courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 5)

Arrows (←) show PGA fibers. Blue arrows show macrophages ingesting alginate.
Red arrows show alginate surrounded by fibroblasts and collagen fibers.

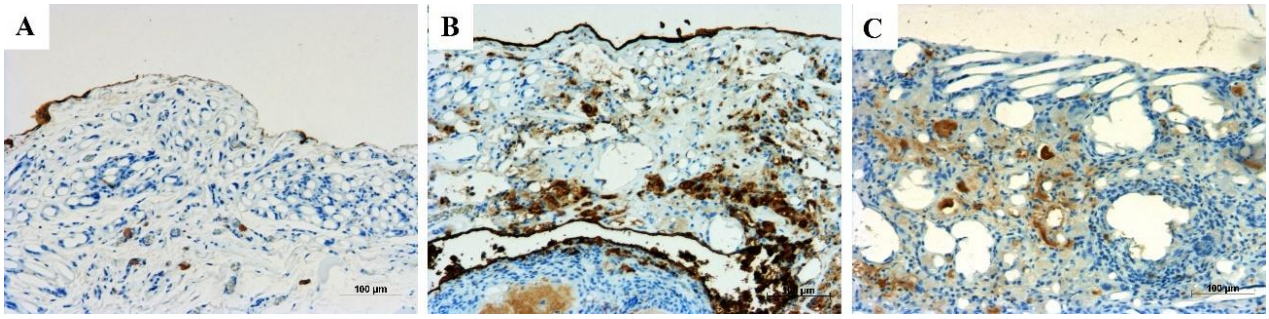

Figure 4. Macroscopic findings of immunostaining (HBME-1) at 8 weeks. (figure courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 6)

(A) PGA mesh, (B) PGA/Na-alg, (C) PGA/Ca-alg.

Table 1. Adhesion score. (table courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 3)

Category and description score	Score
Extent	
No involvement	0
≤ 25% of the site involved	1
≤ 50% of the site involved	2
≤ 75% of the site involved	3
≤ 100% of the site involved	4
Severity	
No adhesion present	0
Adhesions fall apart	1
Adhesions can be lysed with traction	2
Adhesions requiring < 50% sharp dissection	3
Adhesions requiring > 50% sharp dissection	4

Adhesion between each PGA mesh and intra-abdominal organs was scored into 0–4 macroscopically according to the extent and severity of adhesion.

Table 2. The scores by histological findings. (table courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 4)

Category and description	Score
Residual alginate	
Sparse residual alginate	±
Focal residual alginate	+
Diffuse residual alginate	++
Macrophages ingesting alginate	
No alginate ingestion	—
Sparse macrophages ingesting alginate	±
Focal macrophages ingesting alginate	+
Diffuse macrophages ingesting alginate	++
Fibroblasts	
Sparse fibroblasts	—
Focal fibroblasts between PGA fibers	±
Diffuse fibroblasts between PGA fibers	+
Diffuse fibroblasts and focal connective tissue formation with collagen fibers	++
Diffuse fibroblasts and diffuse connective tissue formation with collagen fibers	+++
Collagen fibers	
Sparse collagen fibers	—
Focal collagen fibers between PGA fibers	±
Diffuse collagen fibers between PGA fibers	+
Diffuse collagen fibers and diffuse connective tissue formation with fibroblasts	++
Diffuse collagen fibers and focal connective tissue formation with fibroblasts	+++

The status of four points, namely residual alginate, macrophages ingesting alginate, fibroblasts, and collagen fibers, were classified quantitatively using a histological scores.

Table 3. Adhesion scores (extent and severity) at 2, 4, and 8 weeks after surgery. (table courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 4)

Experimental group	Extent of adhesion		
	2 weeks	4 weeks	8 weeks
PGA alone	2.67 ± 1.03	3.67 ± 0.52	4.00 ± 0
Na-alg	0.67 ± 0.52 *	0.50 ± 0.84 **	0.17 ± 0.41 **
Ca-alg	2.00 ± 1.26	2.50 ± 1.64	1.17 ± 1.83 *

Experimental group	Severity of adhesion		
	2 weeks	4 weeks	8 weeks
PGA alone	3.00 ± 0.89	3.00 ± 0.89	3.83 ± 0.41
Na-alg	1.00 ± 0.89*,††	0.5 ± 0.84**	0.17 ± 0.41**
Ca-alg	3.17 ± 0.41	2.50 ± 1.76	1.17 ± 1.83

The data was expressed mean ± SD. In regard to the comparison with PGA alone, *P* values < 0.05 are marked by an asterisk (∗), those <0.01 are marked by double asterisks (∗∗).
In regard to the comparison with PGA/ Ca-alg, *P* values < 0. 01 is marked by double crosses (††).

Table 4. Summary of the scores by histological findings at 2, 4, and 8 weeks after surgery. (table courtesy of Shinichiro Morita from "*Newly Developed Polyglycolic Acid Reinforcement Unified with Sodium Alginate to Prevent Adhesion.*", Biomed Res Int. 2018 Apr 3;2018:4515949: page 5)

Experimental groups	Category	2 weeks	4 weeks	8 weeks
PGA/Na-alg	Residual alginate	+	+	±
	Macrophages ingesting alginate	++	++	+
	Fibroblasts	-	±	+
	Collagen fibers	-	±	+
PGA/Ca-alg	Residual alginate	+++	++	+
	Macrophages ingesting alginate	++	+	±
	Fibroblasts	+	++	+++
	Collagen fibers	++	+++	+++

The HE staining was scored as follows: +++, clearly stained; ++, moderately stained and +, little stained; ±, sparsely stained; -, unclearly stained.

Ⅶ. CONCLUSION THROUTGHOUT THE PAPER

CONCLUSION THROUTGHOUT THE PAPER

Sodium alginate currently in clinical use is a safe seaweed-derived polysaccharide. In this study, we developed a material that is used in combination with PGA nonwoven fabric by processing this sodium alginate into a gel or sponge material (new alginate material), as a medical material to be used during surgery for the purpose of preventing air leak from suture closure site.

In the first half of this study, STUDY 1 and STUDY 2 focused on the air leak of lung injury site, which is a problem in plumonary surgery, and developed sodium alginate as a tissue reinforcing material widely used in clinical field. The new alginate material was compared with the combination of fibrin glue and PGA nonwoven fabric, which are representative conventional sealants. As a result, the new alginate material showed excellent air leakage prevention effect compared with the conventional sealants.

Although PGA nonwoven fabric is a useful biomaterial in the clinical field, the mild acidity of glycolic acid, produced during the non-enzymatic degradation of PGA, causes chronic inflammation. STUDY 3 and STUDY 4 in the second half of this study examined solving the adhesion induced by PGA (PGA-induced adhesion) using new alginate material. As a result, it was found that PGA-induced adhesion can be prevented by new alginate material.

Based on these results of STUDY 1 - 4, in the future, the new material using sodium alginate and a PGA nonwoven fabric in combination is superior in terms of both the effect of preventing air leakage and adhesion as compared with conventional reinforcement and sealant. Thus this new alginate material was suggested to be applied as a medical material.

Ⅷ. APPENDIX

EVALUATION OF CHITOSAN SPONGE HEMATOSTAT FOR LOCAL BLEEDING COMPARED WITH ALGINATE SPONGE IN ANIMAL EXPERIMENT

局所出血に対する止血効果のキトサンスポンジのアルギン酸スポンジと比較した動物実験での評価

The appendix-paper described the alginate formulation. This is cited from *Science and Engineering Review of Doshisha University* and partially rewritten, because this study was co-worked by Ikeda and Matoba.

TABLE OF CONTENTS

ABSTRACT (APPENDIX)

Background:

Astriction is a basic hemostatic practice and highly effective for bleeding during surgery, but the hemostat should be needed in the case of heavy bleeding. Because uncontrollable bleeding could result in life-threatening complications, an accurate hemostasis is needed during surgery. The conventional hemostats have a trouble to allergy reaction or infection risk. Chitosan and sodium alginate, extracted from crabs and seaweed, were focused because they have less risk of allergy reaction and infection. We developed two novel local hemostats, formed a sponge, made from chitosan and sodium alginate and compared both of the tissue adhesivity and hemostatic effect.

Materials and Methods:

Chitosan and sodium alginate were dissolved in water and made solution. They were poured into a dish and frozen at -80 ºC for 3 days, and then freeze-dried for 4 days. After freeze-drying, they formed sponge.

The tissue adhesivity of these sponges was evaluated by their maximal shear stress using a porcine skin in *ex vivo* experiment. The measurement of the shear force was performed 5 or 6 times. Sixty rats were killed humanly and fixed in the dorsal position. The mesentery was exposed, and the hemorrhage volume was measured where mesenteric blood vessel was cut with scissors in a rat experiment.

Results and conclusion:

The maximal shear stress in the chitosan sponge was significantly larger than that in the alginate one. The hemorrhage volume of chitosan was less than that of alginate. These results suggest that the hemostatic effect of chitosan sponge was more effective because the positive-charged

amino groups of chitosan drew the blood platelets, which was negative-charged, and promoted a platelet aggregation action. In addition, the sponge formation was contributed to the good hemostatic effect because of its easy handling and good tissue adhesivity.

邦文要約 (APPENDIX)

背景：

通常、術中に出血が生じた場合、圧迫止血にて止血できることが多い。だが、圧迫止血での止血が困難な場合は止血剤が必要となる。出血は可及的速やかに止血する必要があるので、止血剤は止血能力が十分かつ速やかであることが求められる。現在、臨床で使用されている止血剤はアレルギー反応や感染のリスクが問題となっている。本研究では、感染リスクの低い海藻類から採取されるアルギン酸と甲殻類から採取されるキトサンを使って新規止血剤を開発した。結果、キトサンを用いた止血剤が最も止血効果があることを報告する。

材料と方法：

キトサン溶液とアルギン酸溶液をそれぞれ−80℃で凍結し、真空乾燥にかけてスポンジを作製した。それらの組織接着性と止血効果を比較した。

組織接着性は、ブタの皮膚を用いて最大せん断応力測定し検討した。動物実験では腸間膜を切開し、各材料をあてて、止血できなかった出血量を測定し比較した。

結果および結語：

キトサンはアルギン酸よりもせん断応力が高く、出血量が少ないことから、止血材により適していることがわかった。これは、キトサンがアルギン酸と違い、正電荷アミノ酸が多く、凝固反応を促進していると推測される。また、スポンジであることで、操作が容易で出血部へよく接着することも高止血効果に寄与していると考えられる。

INTRODUCTION

Astriction is a basic hemostatic practice and highly effective for bleeding during surgery, but the hemostat should be needed in the case of heavy bleeding. Because uncontrollable bleeding could result in life-threatening complications, an accurate hemostasis is needed during surgery. Collagen-preparation [1] and fibrin glue [2] are the conventional local hemostatis, but they have a trouble to allergy reaction or infection risk. Collagen-preparation would promote local infection and be induced the allergic reaction, such as anaphylaxis, by the foreign proteins because it was originated from animals [3]. Thus, careful follow-up observation should be needed. Fibrin glue is be deeply concerned risks of serious infection such as hepatitis virus and human immunodeficiency virus (HIV), because of derived from human blood [4].

Chitosan and sodium alginate, extracted from crabs [5] and seaweed [7], were focused because they have less risk of allergy reaction and infection [6], expecially chitosan was expected a better hemostatic effect because it was reported a better hemostatic effect than fibrin glue [5]. We developed two novel local hemostats, formed a sponge, made from chitosan and sodium alginate and compared both of the tissue adhesivity and hemostatic effect.

This study shows that chitosan sponge has superior hemostatic effect and tissue-adhesion intensity to alginate one.

MATERIALS and METHODS

1. *Preparation of hemostatic materials*

We got chitosan (Kimica Chitosan, Kimica Corporation, Tokyo, Japan), 99.7% acetic acid (Acetic Acid, Wako Pure Chemical Industries, Ltd, Osaka, Japan), and sodium alginate (Sodium Alginate, Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Three gram of chitosan was dissolved in 300 ml distilled water (Otsuka Distilled Water, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan), and then 1.5 ml of 99.7% acetic acid was added in order to dissolve chitosan, resulting in 0.01 g/ml of chitosan solution. We made the chitosan solusion into a sponge as follows. Chitosan solution was poured into a petri dish, frozen at −80ºC for 3 days, and freeze-dried at a pressure of less than 13.3 Pa for 4 days with a freeze-dryer (FZ-2.5, Asahi Life Science Co., Ltd, Saitama, Japan). After freeze-drying, chitosan sponges were obtained. Four gram of sodium alginate was dissolved in 400 ml distilled water, resulting in 0.01 g/ml alginate solution. We made the alginate solution into a sponge as the same manner as chitosan sponges.

The sponges were cut into a 2.0 cm square (0.03 g in weight) (Figure 1).

2. *Ex vivo examination*

2.1 *Shear force between the hemostatic material and porcine skins*

Fresh porcine skins were obtained from a local slaughterhouse. The porcine skins were thoroughly washed and shaved finely. They were cut into a 2.0 cm square, removing the fat fully. The epidermal side of the skin was fixed firmly to a board by cyanoacrylate. One chitosan or alginate sponge were pinched between two dermal sides of the skins (Figure 2), and load of 50 g/cm^2 was applied for 2 minutes. The shear force was measured using a tensile machine (CPU gauge: MODEL-9500, TEST STAND: MODEL-1356, Aikoh Engineering Co., Ltd, Osaka, Japan) at a separation rate of 80 mm/min. The maximal shear force was determined

as the maximal load when the chitosan or alginate sponge was divided from the porcine skin. The measurement was attempted 6 times. All data was available in the alginate group but one deta was exlued in the chitosan group because the fixation between the porcine skin and the fixed board came loose. This result was presented as the maximal shear stress (gf/cm^2).

3. *In vivo examination*

3.1 *Experimental design and animal protocol*

The animal experiments were approved by Doshisha University Animal Experimentation Committee. All the surgical procedures and anesthesia were performed in accordance with the animal care guidelines of Doshisha University. During the experimental period, a week of habituation period was set. Sixteen 8-10-week-old female Wistar/ST rats (180-230 g in weight) were housed separately and maintained under standard specific pathogen-free conditions (a light-dark cycle of 12:12 h, temperature of 20.1–23.5°C, and humidity of 37–65%). Standard laboratory rodent chow and water were freely available. After the heath check, they were randomly assigned to two groups; the chitosan group and alginate group.

3.2 *Surgical technique*

All operations were performed under sterile conditions. The rats were given isoflurane (Escain®, Mylan, Inc., Osaka, Japan) inhalation anesthesia. Sodium pentobarbital (4.5 mg, Sommnopentyl®, Kyoritsu Seiyaku, Inc., Tokyo, Japan) was dissolved in one ml of physiological saline. One ml of pentobarbital aqueous solution was administrated intraperitoneally to every rat with a tuberculin syringe and a 23G injection needle.

Under the general anesthetic, the rats were fixed in the dorsal position. A linear median incision was performed and the mesentery was exposed. A parafilm sheet was laid under the mesentery. The primary branch of the mesenteric blood vessel was cut and the 4 hemastatic materials in each group was put on the bleeding. When to stop bleeding, a gauze was covered over the hematostatic materials (Figure 3). Astriction was performed with a finger at 160 g for 5 minutes. We measured the

weight of the hemostatic materials and the gauze including the blood before and after the experiment. The amount of bleeding was defined as the difference.

4. *Statistical Analysis*

The amount of bleeding was compared by Student's *t*-test. Differences were defined to be statistically significant for p values less than 0.05.

RESULTS

1. *Ex vivo examination*

1.1 *Maximal shear stress of chitosan and alginate sponges*

The results of the maximal shear stress test are showed in Figure 4. The maximal shear stress in the chitosan and the alginate group were 139.75 ± 12.309 gf/cm^2 and 64.83 ± 28.768 gf/cm^2, respectively. The maximal shear stress in the chitosan group was significantly ($p<0.01$) larger than that in the alginate group.

2. *In vivo examination*

2.1 *Hemostatic effect of chitosan and alginate sponges*

The hemostatic results are showed in Figure 5. The amount of bleeding in the chitosan and the alginate group were 0.195 ± 0.080 g and 1.589 ± 0.315 g, respectively. The amount of bleeding in the chitosan group was significantly ($p<0.01$) smaller than that in the alginate group.

DISCUSSION

Chitosan is a polysaccharide containing partially N-deacetylated chitin, which is a linear homopolymer of 1,4-β-linked N-acetyl-D-glucosamine [8]. We developed a novel local hemostatic material using chitosan. The reason whe we chose chitosan because the the positive charged amino groups draw the negative charged blood platelets and, resulting in promoting a platelet aggregation action [5].

Chitosan was prepared in the form of a sponge rather than powder, sheet, and liquid form. There are three following problems in the conventional form. First as for the powder form, collagen-preparation is difficult to handle during a surgery. It adheres to the wet glove surface, tweezers and sites other than the hemostatic region [3]. As the alginate powder, in addition to the difficulty of handling, alginate powder was reported to activate foreign-body reaction because it was hard to dissolve in water, resulting in remaining on the surface of tissues for a long time [9]. Second as for the sheet form, it is too hard to fit onto the bleeding site although handling is easier than powder [3]. Third as for the liquid form, spraying a liquid has good points that it does not adhere to gloves and that it is the simplest to handle. However, it is difficult for the surgeon to target and attach correctly to the bleeding site [3]. Chitosan was thought to have these problems similarly when it is made into the hemostatic material. Therefore, chitosan was made into the form of sponge. In fact, the chitosan sponge absorbs blood smoothly, fits well to the bleeding surface, and stops the bleeding when the surgeons presses it with thier fingers from above, as the standard hemostatic maneuver.

Sodium alginate was chosen as the control to compare with the chitosan sponge in this experiment. Alginate is an anionic polysaccharide containing β-D-mannuronate and its C5 epimer, α-L-glucuronate [8]. The hemostatic effect of sodium alginate involve in adhesion and covering the bleeding site, promotion of fibrin formation and a wound-healing facilitatory effect [7]. A 5% solution of

sodium alginate has beneficial effect on gastrointestinal bleeding or reflux esophagitis [7]. Therefore, alginate is a recently developed hemostatic material.

The current experiment showed that the amount of bleeding in the chitosan group was significantly smaller than that in the alginate group. This result suggests that the chitosan sponge has a superior hemostatic effect than the alginate one.

Strong tissue-adhesion is one of the major factors that contribute to a strong hemostatic effect [4]. Therefore, in this experiment, the tissue-adhesion was also compared by measuring the maximal shear stress of the chitosan sponge compared to the alginate one. As a result, the maximal shear stress in the chitosan group was significantly greater than that in the alginate in this study. This suggests that chitosan may provide a higher-performance hemostatic effect due to stronger tissue-adhesion than the alginate.

Chitosan has other good characteristics distinguished from collagen or fibrin. Chitosan composed of a polysaccharide is expected to have very low risks of allergic reactions in comparison with collagen composed of the other mammal proteins [3,6,8]. The chitosan in the current experiment originated from crabs, thus the risks of viral infections are also much lower than that with collagen [3,5,6]. Fibrin glue, the other commonly used hemostatic material, also has risks of infection by human pathogenic agents because it is human blood product [4].

CONCLUSION

Chitosan sponge has a stronger hemostatic effect with stronger tissue-adhesion in comparison with an alginate one. It is also important to make a new hematostat as a sponge form.

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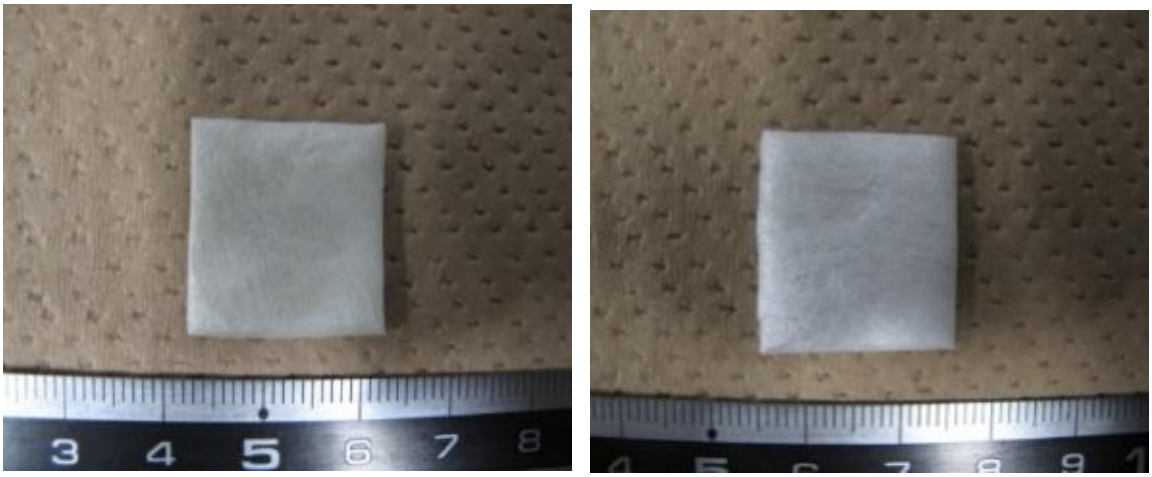


(a) (b)

Figure 1. Photographs of the square-shaped sheets. (figure courtesy of Junki Ikeda from "*Changes in granulation-formation induced by different fiber-diameters and fiber-spacing in scaffolds for tissue-regeneration : preliminary study*.", The science and engineering review of Doshisha University. 2012 Apr; 53(1): page 50)

(a) chitosan sponge
(b) alginate sponge.

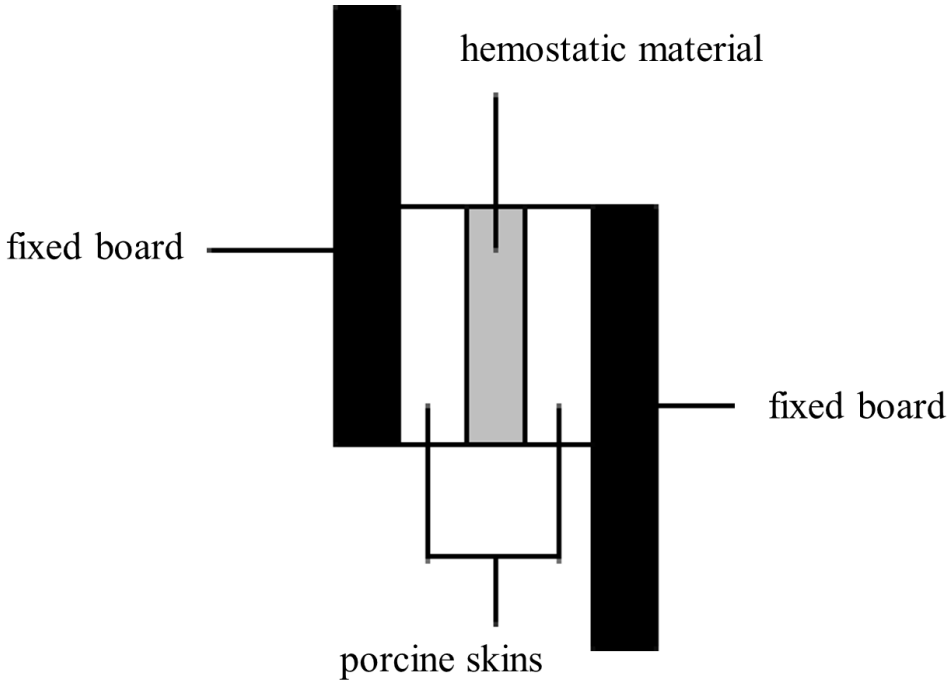
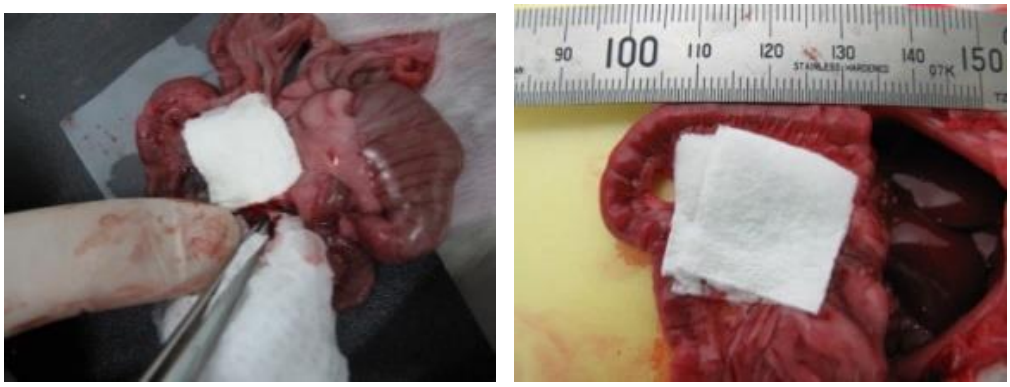


Figure 2. Measurement of shear force. (figure courtesy of Junki Ikeda from "*Changes in granulation-formation induced by different fiber-diameters and fiber-spacing in scaffolds for tissue-regeneration : preliminary study*.", The science and engineering review of Doshisha University. 2012 Apr; 53(1): page 50)

One chitosan or alginate sponge were pinched between two dermal sides of the skins.



(a) (b)

Figure 3. Photographs of hemostatic procedures. (figure courtesy of Junki Ikeda from "*Changes in granulation-formation induced by different fiber-diameters and fiber-spacing in scaffolds for tissue-regeneration : preliminary study*.", The science and engineering review of Doshisha University. 2012 Apr; 53(1): page 51)

(a) The square-shaped sheet of chitosan sponge.
(b) The square-shaped sheet of Alginate sponge.
The sheets were placed on the bleeding point of the mesentery for hemostasis.

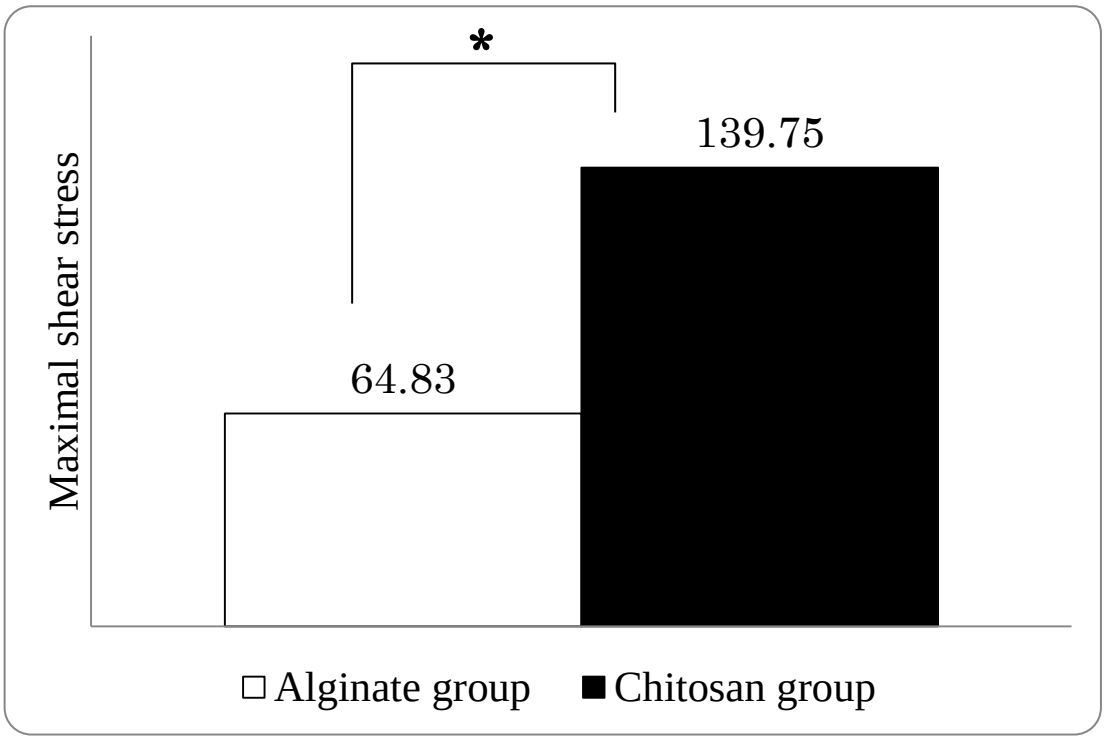


Figure 4. Maximal shear stress in the chitosan and the alginate groups. (figure courtesy of Junki Ikeda from "*Changes in granulation-formation induced by different fiber-diameters and fiber-spacing in scaffolds for tissue-regeneration : preliminary study*.", The science and engineering review of Doshisha University. 2012 Apr; 53(1): page 51)

The column indicates the mean, and the bar shows SD. The asterisk mark means $P<0.01$

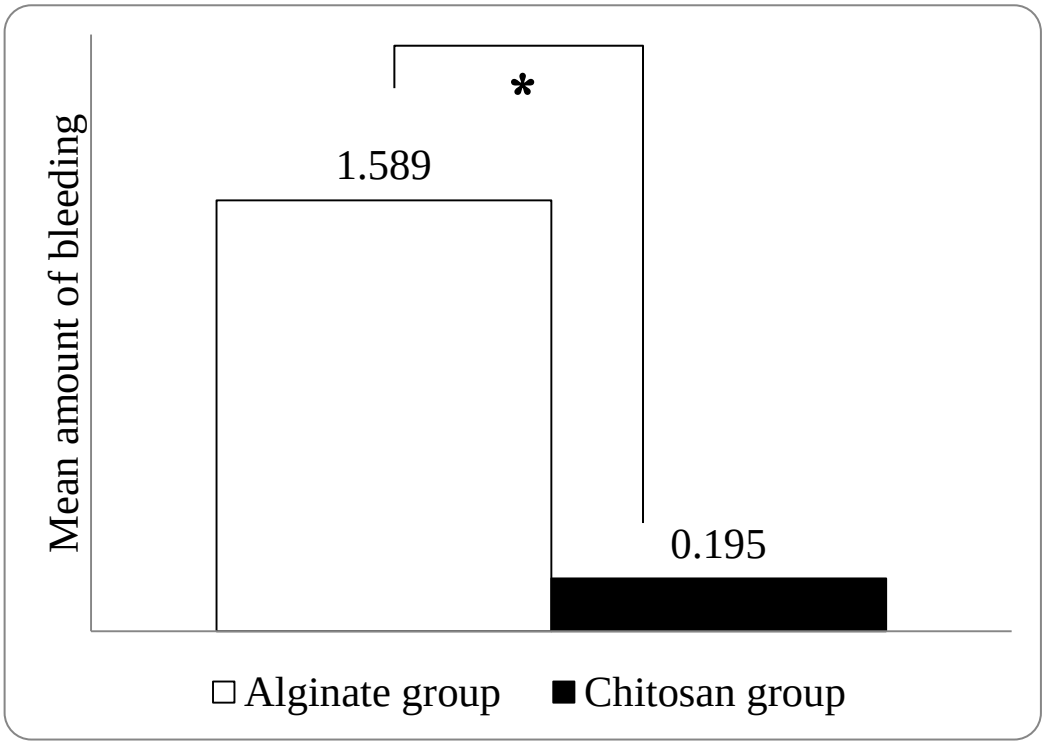


Figure 5. Amount of bleeding in the chitosan and the alginate groups. (figure courtesy of Junki Ikeda from "*Changes in granulation-formation induced by different fiber-diameters and fiber-spacing in scaffolds for tissue-regeneration : preliminary study*.", The science and engineering review of Doshisha University. 2012 Apr; 53(1): page 52)

The column indicates the mean, and the bar shows SD. The asterisk mark means $P<0.01$