Anti-adhesive Effects of the Newly Developed Two-Layered Gelatin Sheet in Dogs

—Devising of Anti-adhesive Material—

大動物実験における新規ゼラチン 2 層シートの癒着防止効果の検討

—癒着防止材の開発—

CONTENTS

Background and Aim

Background

Postoperative pelvic lesions are significant in the field of gynecologic surgery as they can lead to reduced fertility in relatively young women. Preventing such lesions is important for obstetric and gynecologic care and for government health policy, particularly in Japan, owing to the low birthrate and an aging population. Myoma is extremely common among reproductive-age women. A study reported that incidence increases to 20-50% among women aged 30 years or older, with uterine myoma occurring in 27% of infertile women. (1) Myomectomy is performed if the patient plans to conceive in the future; however, the surgery itself can cause intrauterine adhesions. Endometriosis is a common disease, with a reported incidence of 5-10% among reproductive-age women. Progression of endometriosis can cause pelvic adhesions. Infertility is a complication in 50% of endometriosis cases, with idiopathic infertility accounting for 50% of this infertility. Ascending infections due to *Chlamydia* or other sexually transmitted diseases can also cause pelvic adhesions, which in turn cause infertility and ectopic pregnancies (Figures 1, 2). Given that women now are marrying later in life, surgical treatment for diseases such as uterine myoma and endometriosis is evolving—from removal of the uterus in the past to

preserving the uterus and the patient's fertility, that is, surgical methods are now centered on enabling pregnancy. However, surgery can lead to adhesions, which can cause infertility. (2) Thus, methods to prevent adhesions due to surgery are needed.

Mechanism of adhesions and management of postoperative adhesions

Damage to the local tissue (due to surgery or otherwise) elicits the wound healing process that involves inflammation, proliferation, and remodeling. Inflammation is the migration of inflammatory cells (leukocytes, lymphocytes, macrophages, and so forth) to the damaged tissue, whereas proliferation and remodeling involve the regeneration of tissue, such as the differentiation of fibroblasts into myofibroblasts. If regeneration occurs in a "good" environment, it completes without complications, whereas scars may form if the environment is "bad" (Figure 3). In other words, adhesions are a result of the wound healing process.

Commonly used anti-adhesive agents in gynecologic surgeries are hyaluronic acid carboxymethyl cellulose (Seprafilm®) and oxidized regenerated cellulose (INTERCEED®) membranes. These agents are available as gels; they exert their anti-adhesive action by creating a physical barrier between the wound and the surrounding tissue (Figure 4). However, these agents can sometimes hinder the wound

healing process (Figure 5).

In addition, complications with the use of conventional anti-adhesive agents have been reported. Several studies have shown that Seprafilm® only decreases the severity of adhesion but not its incidence (3, 4), and another study reported that Seprafilm® had no preventive effect against pelvic adhesions in women. (5) Moreover, Seprafilm® should not be used in areas of intestinal anastomosis. INTERCEED® has been reported to have an efficacy superior to Seprafilm®, but it has several limitations. Blood infiltration renders INTERCEED® completely ineffective in preventing adhesions (6-8). INTERCEED® provokes an aggressive leukocyte response, and inflammatory response may enhance adhesion. Finally, these agents are designed to be used in laparotomies and are difficult to maneuver in laparoscopic surgeries.

Aim

The authors developed a new anti-adhesion agent using gelatin that overcomes the aforementioned limitations and (1) has superior anti-adhesion effects, (2) is safe to use, (3) has good maneuverability and tissue adhesiveness, (4) does not inhibit the wound healing process, and (5) is usable in areas of intestinal anastomosis and bleeding. In this study, we designed a variety of configurations that took into consideration the conditions and difficulties associated with gelatin-

based materials. Through study 1, study 2, and study 3, we compared the usefulness of an anti-adhesion agent that can be adapted to numerous clinical situations to that of the conventional agents.

Study 1: Thermally crosslinked gelatin film

We developed a gelatin film that would not hinder wound healing and prevent adhesions. Through physical property tests, cell growth experiments, and experiments in rat and dog models, we confirmed the optimal time for thermal crosslinking and the anti-adhesion effects of the gelatin film. We also examined its safety when used in areas of intestinal anastomosis, which is a contraindication for the conventional agent Seprafilm®.

Study 2: Thermally crosslinked 2-layer gelatin sheet

Based on the results of study 1, we developed a 2-layer sheet comprising the gelatin film described above and a gelatin sponge, to create an anti-adhesive agent that could be used in gynecologic surgeries in which controlling bleeding is difficult, that is, an agent that could also stop local bleeding. The sheets were used in experiments in a dog model, which is considered to closely replicate human conditions, and we compared the hemostatic effects and anti-adhesion effects of the gelatin sheet with those of conventional agents.

Study 3: Thermally crosslinked gelatin flakes

Based on the results of study 2, we developed a powdered anti-adhesion agent that could be used in thoracoscopic and laparoscopic surgeries, which are increasingly being performed. Laparoscopic surgery is speculated to cause fewer adhesions than laparotomy. However, if preserving the patient's fertility is of utmost importance, measures are required to prevent postoperative adhesions in laparoscopic surgeries Therefore, we tested different configurations of gelatin-based materials to obtain a configuration that can be used effectively in laparoscopic surgeries. After performing physical property tests and examining the anti-adhesion effects, we chose the flake morphology. We adjusted the degree of crosslink, particle diameter, and the weight of gelatin used to obtain gelatin flakes, performed physical property tests, and compared the anti-adhesion effects of the gelatin flakes and the conventional agents to determine the optimal conditions for use in thoracoscopic and laparoscopic surgeries.

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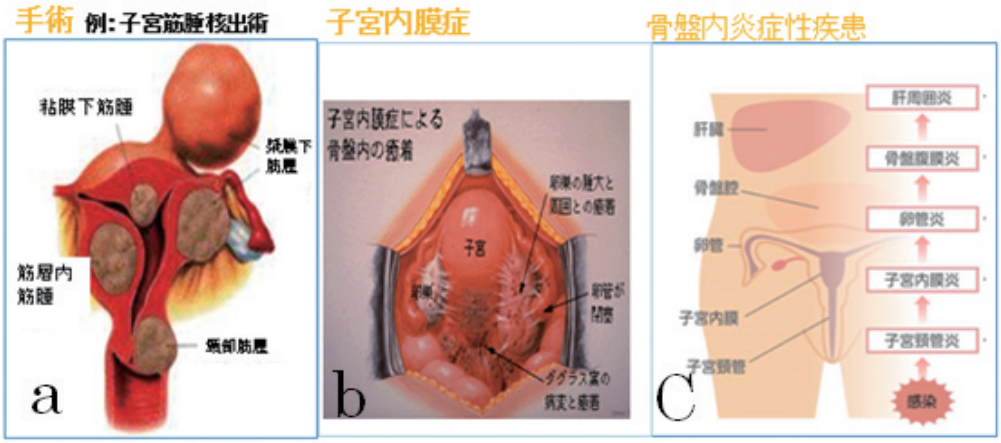

Figure 1. Diseases that cause adhesions

a: Surgery (example: myomectomy)

b: Endometriosis

c: Pelvic inflammatory disease

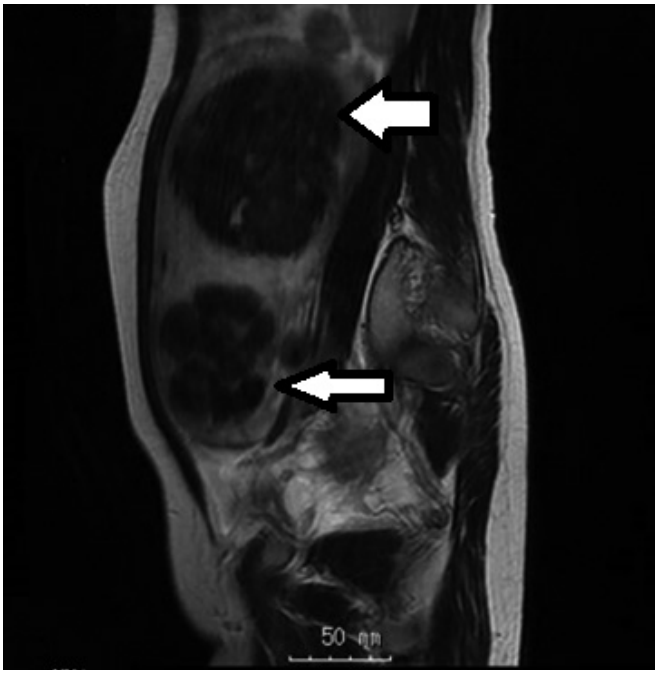

Figure 2. Magnetic resonance image (T2, sagittal section)
White arrow: myoma

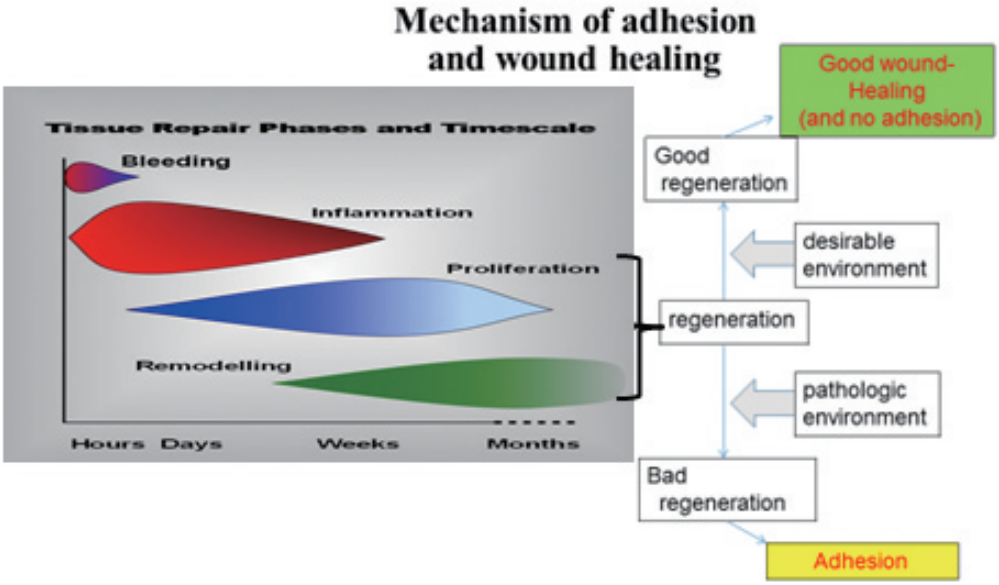

Figure 3. Mechanism of wound healing

The process of wound healing includes inflammation → proliferation → remodeling. Adhesions occur as a result of the wound healing process.

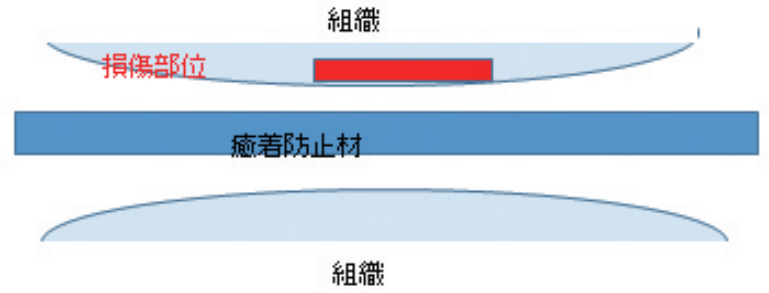

Figure 4. Use of an anti-adhesion agent to prevent postoperative adhesions

A physical barrier is created between the wound and the surrounding tissue.

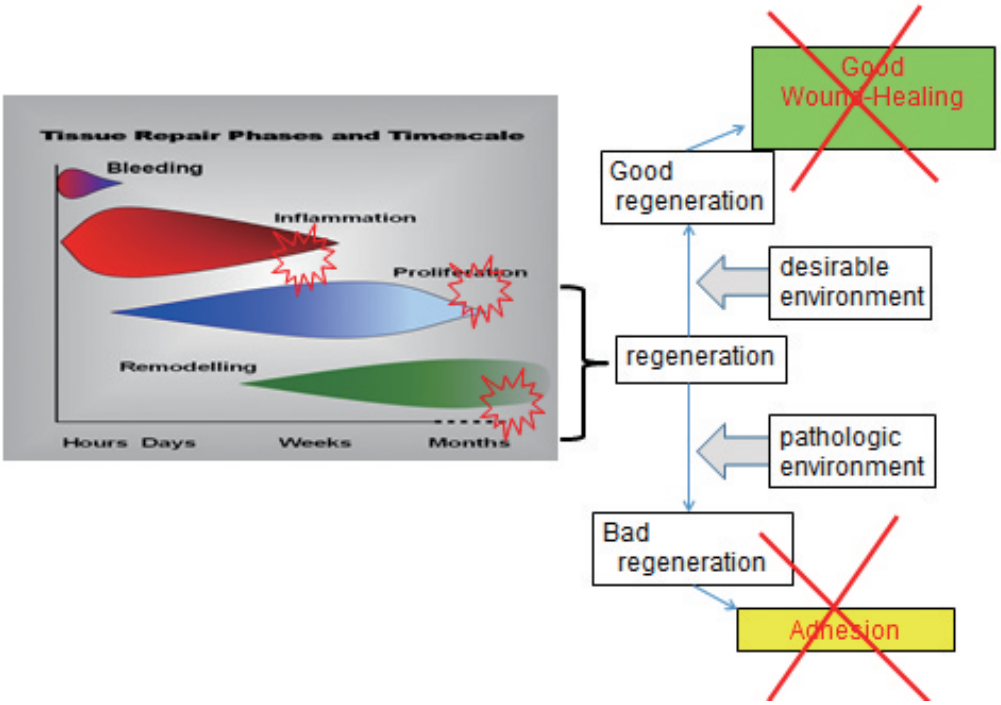

Figure 5. Actions of conventional anti-adhesion agents

These agents have anti-adhesion effects, but can hinder the wound healing process.

STUDY I-1

The Biological Properties and the Extent of Thermal Cross-linking in a Gelatin Film as an Anti-adhesive Material

TABLE OF CONTENTS (STUDY I-1)

ABSTRACT

Background:

In order to prevent postoperative adhesionand the related complications, we developed a thermally cross-linked gelatin film (GFM) and examined the basic biological properties, paying special attention to the relationship between these properties and the extent of cross-linking of the film.

Materials and Methods:

Author developed gelatin films cross-linked thermally for fivedifferent time periods (0, 1, 3, 8and 14 hours) and performed the following tests. Regarding the material characterization of the films, the water content, the water solubility and the enzymatic degradation for collagenase were tested. In an *in vitro* study author conducted to examine the cell growth of fibroblasts cultured on the films. In *in vivo* tests, the films cross-linked for longer time periods (3, 8 and 14 hours) were retained for longer after being implanted into the abdominal cavity in rats.

Results and Conclusions: the degree of cell growth

The water content, the water solubility and the enzymatic degradation for collagenase were closely related to the duration of

thermal cross-linking. In an *in vitro* study the cell growth, except no cross-linked film, was less than that observed in the non-treated group, thus suggesting that such effects of the films on fibroblast cell growth may be related with their anti-adhesive effects. *In vivo* study showed a significant anti-adhesive effect in the rat cecum adhesion models, indicating that the biodegradability and anti-adhesive effects of the GFM depend on the duration of thermal cross-linking.

In order to develop useful and effective anti-adhesive gelatin film, it is very important to optimize duration of the thermal cross-linking.

癒着防止材としてのゼラチンフィルムにおける生物学的特性と熱架橋度に関する検討

要約

背景：

術後の癒着およびそれに付随する合併症を防止するために、熱架橋ゼラチンフィルム（thermarally cross-linked gelatin film: GFM）を開発し、生物学的特性と熱架橋度の関連に着目し、熱架橋ゼラチンフィルムの基礎的な特性を得る実験を施行した。

材料および方法：

０、１、３、８、14時間の異なる時間でゼラチンフィルムに熱架橋を行い、其々に対し以下の実験を施行した。ゼラチンフィルムの特性に関して含水率、水溶性、コラゼナーゼに対する酵素分解実験を行った。また、*in vitro*では熱架橋ゼラチンフィルム上での線維芽細胞の細胞増殖試験を行った。*In vivo*ではラット盲腸擦過モデルを作成し、腹腔内に長時間の熱架橋（３、８、14時間）を施したものを埋植した。

結果および結語：

含水率、水溶性、コラゲナーゼに対する酵素分解は熱架橋時間と密接に関連していた。*In vitro*における細胞増殖試験では未架橋（０

時間）を除いて、コントロール群の様な増殖は認められなかったが、この線維芽細胞に対する影響が熱架橋ゼラチンフィルムの癒着防止効果に関連があると考えられる。*In vivo*では、ラットの盲腸擦過モデルにおいて有意に癒着防止効果が認められ、熱架橋ゼラチンフィルムの生体分解性、癒着防止効果は熱架橋度に依存することが示唆された。

これらの結果により、有用な癒着防止効果を持つ熱架橋ゼラチンフィルムの開発にためには最適な時間で熱架橋を施行することが重要であると考えられる。

INTRODUCTION

Postoperative abdominal adhesion often causes serious complications including chronic abdominal pain, (1) intestinal obstruction, (2) and female infertility. (3, 4) Such adhesion also increases the difficulty of reoperation and associated surgical risks. Inevitably, these unfavorable events raise hospital costs for patients. (5, 6) In a previous report, postoperative abdominal adhesions was found in 55% to 95% of patients at second-look laparoscopy. (7) To prevent post-operative adhesion, various anti-adhesive materials have been developed so far. Among the anti-adhesive materials, placing a mechanical barrier between the injured site and the adjacent tissue is a direct and effective approach for preventing adhesion. (8)

Currently, a cellulose film comprising a combination of hyaluronic acid and carboxymethyl-cellulose is commonly used as an anti-adhesive material in open surgery. (9) This cellulose film has been reported to turn into a gelform within 24 to 48 hours when placed on injured intraperitoneal tissues and remains at the injured site for up to seven days, thus reducing adhesion by acting as a mechanical barrier to separate the adjacent traumatized serosa during the critical period of reperitonealization. (9) Several studies have reported that the use of cellulose film decreases the incidence and/or severity of adhesion, both experimentally and clinically. (9-11) However, cellulose film has

not be found to decrease the incidence of bowel obstruction in clinical trials, although it has been shown to reduce the frequency of severe cases requiring reoperation. (12, 13) In addition, cellulose film has several disadvantages, including poor handling during surgery due to its brittleness and ability to tear easily, and potential risk for leakage at the site of intestinal anastomosis. In fact, several reports have documented that this film is associated with a higher incidence of anastomotic leakage when placed directly on anastomotic sites. (10, 14)

In order to address these problems, we developed a new type of thermally cross-linked gelatin film (GFM) film. Gelatin is a denatured product of collagen, which is the most abundant extracellular matrix protein in mammals and has been recently applied as a scaffold material in the field of regenerative medicine. In our previous report, our novel film showed superior anti-adhesive effects, involving excellent reperitonization without affecting the site of intestinal anastomosis, in comparison with cellulose film. (15) In this study, we examined the biological properties of this GFM, paying special attention to the relationships between these properties and the extent of cross-linking of the film.

MATERIALS AND METHODS

1. Preparation of GFM

Alkali-treated gelatin extracted from porcine skin (type-I collagen, Medigelatin®) with an isoelectric point of 5 was purchased from Nippi Co. Ltd. (Shizuoka, Japan). This alkali-treated gelatin is of medical grade, since the alkaline processing removes the collagen telopeptides that induce major antigenicity (16) and bacterial endotoxins (17), and also in activates contaminated viruses (18) and transmitting agents (prions) causing bovine spongiform encephalopathy (BSE) (19). The gelatin was dissolved in distilled water at a concentration of 2.65%. The gelatin solution was casted on plastic plates and allowed to air dry to obtain films of approximately 30μm in thickness. The gelatin films were cross-linked thermally using a vacuum oven (4VO-250N, As One, Osaka, Japan) at 140℃ for five different time periods: 0 (no cross-linked), 1, 3, 8 or 14 hours (GFM-0, -1, -3, -8, and -14film) (Figure 1a). In preparation for the further examinations, the films were sterilized with ethylene-oxide gas (0.43g/L at 40℃ for four hours).

2. Material Characterization

2.1. Water content of the film

Each 1-cm^2 piece of gelatin film was immersed in distilled water for

one hour. The wet samples were wiped with filter paper to remove excess liquid and weighed. The wet samples were then dried in the vacuum oven at 80℃ for two hours and weighed, and the amount of absorbed water in each piece of film (*Wc*) was calculated according to the following equation: *Wc* (%) = 100 ((*Ww* – *Wd*) / *Ww*), where *Ww* and *Wd* are the weights of the wet and dried samples, respectively. All tests were carried out in quadruplicate. Correlation was evaluated using the Pearson product-moment correlation coefficient. A p value of less than 0.05 was considered to be significant.

2.2. Water solubility of the film

Each 1-cm^2 piece of gelatin film was weighed under air dried conditions and then incubated in distilled water at 37℃ for different time periods (1, 3, 5, or 7 days). The undissolved films were subsequently picked up, dried in the vacuum oven at 80℃ for two hours and weighed. The percentage of remaining undissolved piece of film (*Ws*) was calculated according to the following equation: *Ws* (%) = 100 (*Wu* / *Wi*), where *Wi* is the initial weight of the sample and *Wu* is the weight of the undissolved desiccated sample. All tests were carried out in triplicate.

2.3. Enzymatic degradation of the film for collagenase

Each 4.5-cm^2 piece of gelatin film was weighed under air dried

conditions and then incubated in phosphate buffer saline (pH7.5) containing collagenase type I (1.0 unit/ml, Invitrogen, Grand Is land, NY,, USA) at 37℃ for different time periods (1, 3, and 6 hours). The undigested films were subsequently picked up, dried in the vacuum oven at 80℃ for two hours and weighed. The percentage of remaining undigested piece of film (*Dc*) was calculated according to the following equation: *Dc* (%) = 100 (*Wu / Wi*), where *Wi* is the initial weight of the sample and *Wu* is the weight of the undigested sample. All tests were carried out in quadruplicate.

3. *In Vitro* Examination

3.1. Cell growth tests on the films

Rat fibroblasts were obtained from the subcutaneous tissue of a healthy female Wistar/ST rat (7 weeks of age, 200 g) and maintained via continuous culturing. Human fibroblasts were purchased from Lonza Ltd. (Basel, Switzerland). Both types of cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in an incubator at 37℃ with 5% CO_2 under humid conditions.

Each gelatin film was cut into a circle measuring 15mm in diameter and set on the bottom of a 24-well culture plate (Becton Dickinson Biosciences, Flanklin Lakes, NJ, USA). The cultured rat and human fibroblasts were retrieved in the form of a single cell-suspension at a

concentration of 1.33×10^4 cells/ml. A total of 750μl of the cell suspension, which included 10^4 cells, was poured into each well where the films were settled. Wells without film were used as non-treateds. The cells were then cultured, with regular replacement of the culture medium every two days. At 1, 3, 5 and 7 days after seeding, the number of viable cells in each well was counted using ATP assay with the ATPLite Kit® (PerkinElmer, Waltham, MA, USA). At each time point, three wells for non-treated and each type of film were examined. The number of cells was estimated based on the ATPcount according to the preliminarily standard curve between the ATPcount number and the cell count. Statistical comparisons were made using ANOVA (analysis of variance) among all groups. Post hoc test was done by Scheffe test. A p value of less than 0.05 was considered to be significant.

4. *In Vivo* Examinations

4.1. Animals

Female Wistar/ST rats (8-weeks of age, weighing approximately 200g) were purchased from Shimizu Animal Laboratory (Kyoto, Japan). During the experimental period, the rats were maintained under standard specific pathogen-free conditions (with a light-dark cycle of 12:12hours, mean temperature of 23℃ and mean humidity of 50%). Standard laboratory rodent chow and water were freely

available. The rats were maintained in the laboratory for one week prior to the experiments. All animal care and experimental procedures in this study were approved by the Animal Experimentation Committee of Doshisha University.

4.2. In vivo degradation test

Each gelatin film was cut into a piece measuring 2.0 cm^2 in area and weighed under dry conditions. Forty rats were randomly assigned to five groups consisting of eight rats each: the GFM-0, -1, -3, -8, -14film groups. The rats were anesthetized with isoflurane (Escain®, Mylan, Inc., Osaka, Japan) inhalation and the intraperitoneal administration of sodium pentobarbital (Sommnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 35mg/kg of body weight. Under general anesthesia, a 3-cm-long incision was made along the midline of the abdomen. Three pieces of 2-cm^2 gelatin film per rat were implanted into the abdominal cavity. After closing the incision with 4/0 Prolene® (Ethicon Inc., Somerville, NJ, USA) sutures, all rats were maintained under the standard SPF conditions for one to seven days.

On days 1, 3, 5 or 7 after surgery, two rats in each group were randomly sacrificed via the intraperitoneal injection of a lethal dose of sodium pentobarbital. The abdomen was then reopened, and the residual pieces of film were carefully removed. After being rinsed in distilled water and dried, each piece was measured for weight. When

all pieces of film in a group were found to have completely disappeared, the remaining rats in that group were no longer sacrificed. Finally, the percentage of remaining pieces of gelatin film (*Dr*) was calculated according to the following equation: *Dr* (%) = 100 (*Wr* / *Wi*), where *Wi* is the initial weight of the film sample and *Wr* is the weight of the residual desiccated film sample.

4.3. Anti-adhesive test

Each piece of gelatin film was cut into an oblong shape measuring 2.0 × 3.0 cm in size. Forty-eight rats were randomly assigned to six groups consisting of eight rats each: the GFM-0, -1, -3, -8, -14 film and non-treated (non-treated) groups. Under general anesthesia, a 4-cm-long incision was made at the midline of the abdomen, and a15-mm-diameter area of abrasion was created on the serosal aspect of the anterior wall of the cecum using a dental sanding tip (Sharp-Mini, Ohki Chemical Co, Hiroshima, Japan) until a small blood drops appeared (Figure 1b). Then, another 15-mm-diameter region of abrasion was made on the right lateral internal abdominal wall 2cm from the midline incision on the abdominal wall, directly opposite to the abraded cecum.

In the GFM groups, the abraded cecum was wrapped manually with each type of gelatin film cut in an oblong shape, covering the entire are of abrasion (Figure 1c). The non-treated group received no

treatment. The two abraded surfaces were approximated with 6/0 Prolene® sutures (Ethicon Inc.) before closing the abdomen of all rats in order to induce tight adhesion between the two sites (Figure 1d). All rats were housed carefully until the assessments of adhesion.

Twenty-one days after the procedure, all animals were sacrificed, and the status of the abdominal cavity and abraded sites were observed macroscopically, including each piece of remaining material. The extent and severity of adhesion were graded and scored numerically according to an adhesion grading scale (Adhesion Scores, Table 1) described previously. (15) The evaluation was performed by a researcher blinded to the animal assignments. The statistical comparisons were made by using the Dunn test following the Kruskal-Wallis test. A p value of less than 0.05 was considered to be significant.

After evaluating the adhesion status, the segment with the abraded sites was fixed with 10% buffered formalin, and processed for embedding in paraffin. Transverse sections (3μm) of the segments were stained with hematoxylin and eosin (H/E). Then, histological evaluations, including the status of the adhesion and the healing process or the amount of each film remaining at the abraded sites, were carried out.

RESULTS

1. Material Characterization

1.1. Water content of the film

The water content of each film is shown in Figure 2. Since the film with no cross-linking (GFM-0) dissolved completely immediately after immersion, we could not examine its water content. Nevertheless, as shown in Figure 2, there was a linear correlation between the duration of thermal cross-linking and the water content of the film. The Pearson product-moment correlation coefficient was 0.989 (p<0.05).

1.2. Water solubility of the film

The degree of water solubility of each film is shown in Figure 3. No GFM-0 or GFM-1 films remained one day after immersion. The percentage of remaining films in other group decreased time-dependently within each group but increased in correlation with the duration of thermal cross-linking across the groups. After seven days, the percentage of remaining GFM-3, -8 and -14 films were 15%, 40% and 70%, respectively.

1.3. Enzymatic degradation of the film for collagenase

The degree of enzymatic degradation of each film for collagenase is

shown in Figure 4. The percentage of remaining films in other group decreased time-dependently within each group but increased in correlation with the duration of thermal cross-linking across the groups.

2. *In Vitro* Examination

2.1 Cell growth test on the films

The results of the cell growth tests including statistical analyses are shown in Figure 5. There were no significant differences in the growth of either type of cell between the GFM-0 film and non-treated groups. However, the level of cell growth on the other films was inferior to that observed in the non-treateds wells for both types of cells. In particular, the growth of rat fibroblasts on the GFM-3, -8 and -14 films and human fibroblasts on GFM-1, -3 and -8 films was significantly decreased compared with that observed in the non-treated group.

3. *In Vivo* Examinations

3.1. In vivo degradation test

There were no morbidities or mortalities associated with either the operation or application of each type of gelatin film. The percentage of the various remaining gelatin films at each time point after implantation is shown in Figure 6. The GFM-0 and GFM-1 films

disappeared completely one day after implantation. In the other GFM groups, the percentage of each remaining film decreased time-dependently. The GFM-3 films disappeared completely five days after implantation, whereas the GFM-8 and GFM-14 films remained beyond seven days.

3.2. Anti-adhesive test

There were no morbidities or mortalities associated with the operation or the application of each type of gelatin film. The adhesion scores for the anti-adhesive test are shown in Figure 7. The scores were significantly lower in the GFM-3, -8, and -14 film groups than in the non-treated group for both the extent and severity. However, there were no significant differences between the three groups (data not shown).

Macroscopically, severe to moderate adhesions were observed between the two surfaces of the cecum and the abdominal walls, mainly in the untreated (non-treated) group, and in the GFM-0 and -1 groups (Figure 8-a, b, c), The microscopic views of these groups showed that the abraded intestine hardly adhered to the abraded abdominal wall with thick fibrous changes of the connective tissue and numerous inflammatory cells such as lymphocytes, and monocytes (Figure 9-a, b, c). In contrast, the GFM-3, -8, and -14 groups showed less adhesion macroscopically (Figure 8-d, e, f).

Microscopically, the abraded sites of these groups showed thick fibrous changes of the connective tissue and inflammatory cells, without any adherence to the abdominal walls (Figure 9-d, e, f). No remaining materials were noted in the GFM-0, -1, -3 and -8 film groups macroscopically. However, in half of the animals treated with the GFM-14 film, broken and shrunken pieces of remaining film with the connective tissues were found at the sites of implantation both macroscopically (Figure 8-f) and microscopically (Figure 9-f).

DISCUSSIONS

It has been previously reported that the biological properties of collagen and gelatin cross-linked with glutaraldehyde (GA) are closely related to the extent of cross-linking with GA. In particular, the report demonstrated a clear correlation between the water content of gelatin cross-linked with GA and the extent of cross-linking, although no such relationships were observed for collagen cross-linked with GA. (20) In this study, we found a linear correlation between the water content of GFM and the duration of thermal cross-linking. This finding indicates that the water content of GFM reflects the extent of cross-linking and may be a useful index showing the biological features of the film. Similarly, the water solubility and

enzymatic degradation for collagenase of the GFM were found to be closely related to the duration of thermal cross-linking, indicating that this property also reflects the extent of thermal cross-linking.

Based on the findings of previous reports, the first five to seven days after peritoneal injury are important for reperitonization. In particular, separating the site of injury from the adjacent tissue during this period is quite critical with respect to the efficacy of anti-adhesive barriers preventing adhesion. (8) Therefore, in this study, we examined the biodegradability of our gelatin films in the abdominal cavity of experimental animals for seven days, and attempted to correlate this feature with the anti-adhesive effects of each type of film in a rat cecum adhesion model.

In the *in vivo* degradation test, the GFM-0 and -1 films were absorbed completely within one day. However, the GFM-3 film remained for at least three days, while the GFM-8 and -14 films continued to be detected beyond seven days. In the anti-adhesive test of the gelatin films, only the GFM-3, -8 and -14 films showed significant anti-adhesive effects in comparison with that observed in non-treated group. These results suggest that the GFM-3, -8 and -14 films acted fully to prevent the development of adhesions, since they remained at the sites of injury for the critical period of reperitonization, effectively separating from these sites from adjacent tissues. In contrast, the GFM-0 and -1 films did not function as

sufficient anti-adhesive barriers since they degraded too quickly. However, it should also be noted that the GFM-14 film remained beyond three weeks after surgery, which it may induce persistent inflammation and further adhesion formation.

In order to investigate the mechanisms underlying the anti-adhesive effects of the GFM, we examined fibroblast cell growth on the gelatin films. Fibroblast proliferation with the consequent excess production of collagen fibers induces adhesion at sites of injury, following the excess production or reduced lysis of fibrin deposits under pathological conditions. (21) Among the GFM evaluated in this study, the level of cell growth on the GFM-0 film was almost equivalent to that observed in the non-treated group for both types of fibroblasts. Since the GFM-0 film dissolved very rapidly within several hours in the water content and solubility test, the fibroblasts seemed to adhere and grow more directly on the culture plate rather than on the GFM-0 film. In contrast, other films (GFM-1, -3, -8 and -14) showed a decreased cell growth, irrespective of the cell type. These films dissolved more gradually in the solubility test. Therefore, it is possible that they lose their scaffold function by dissolving more gradually, thus resulting in a poorer growth of the fibroblasts on the films. Such effects of these films on fibroblast cell growth may play a role in preventing adhesion in addition to acting as a mechanical barrier. Indeed, in the anti-adhesive test, significant anti-adhesive

effects were achieved with the GFM-3, -8 and -14 films, which demonstrated the most inhibited growth of rat fibroblasts. However, further examinations are needed to clarify the detailed mechanisms underlying these observations in order to prevent adhesion with gelatin films.

CONCLUSION

Based on the results of the above experiments, the biological properties, including biodegradability and anti-adhesive effects, of the GFM are closely related to the extent of thermal cross-linking of the films, as determined by the duration of thermal cross-linking. When attempting to develop useful and effective anti-adhesive gelatin films, it is very important to optimize the duration of thermal cross-linking.

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Table 1. The Adhesion Scores

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

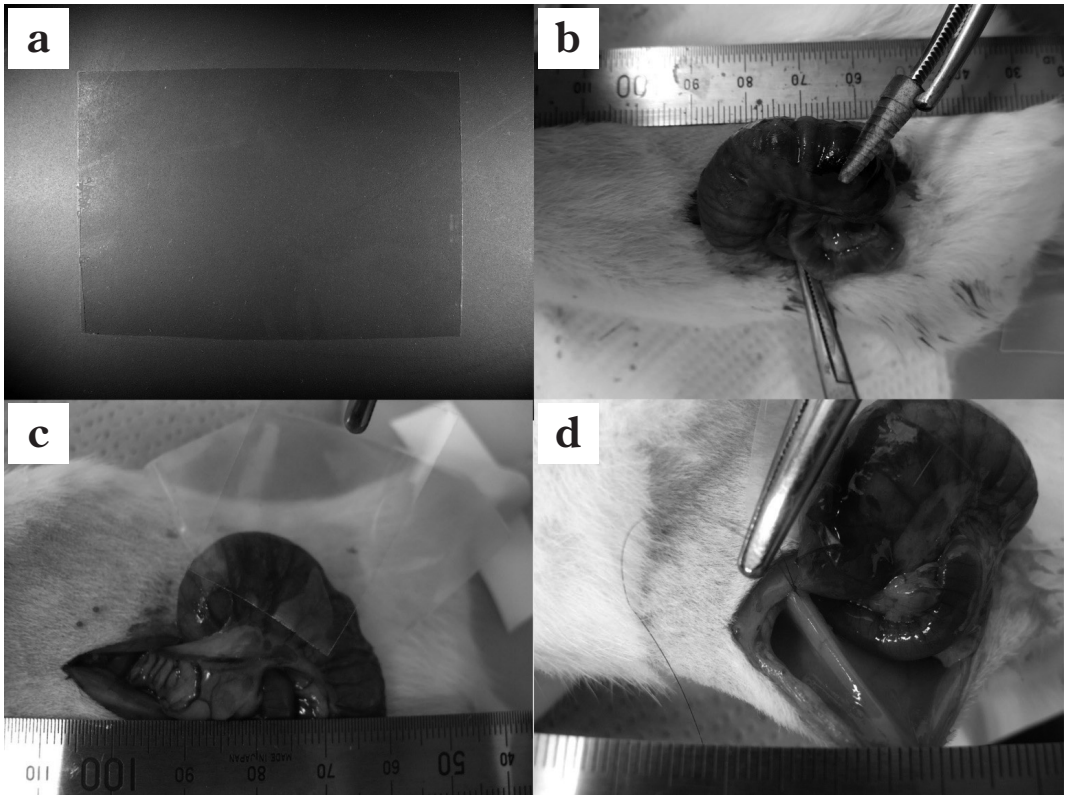

Figure 1. GFM and surgical procedures for anti-adhesive test

a: A view of a representative GFM.

b: An abrasion on the cecum with sanding tip.

c: A wrapping the cecum with thermally cross-linked gelatin film.

d: Two abraded surfaces approximated with sutures.

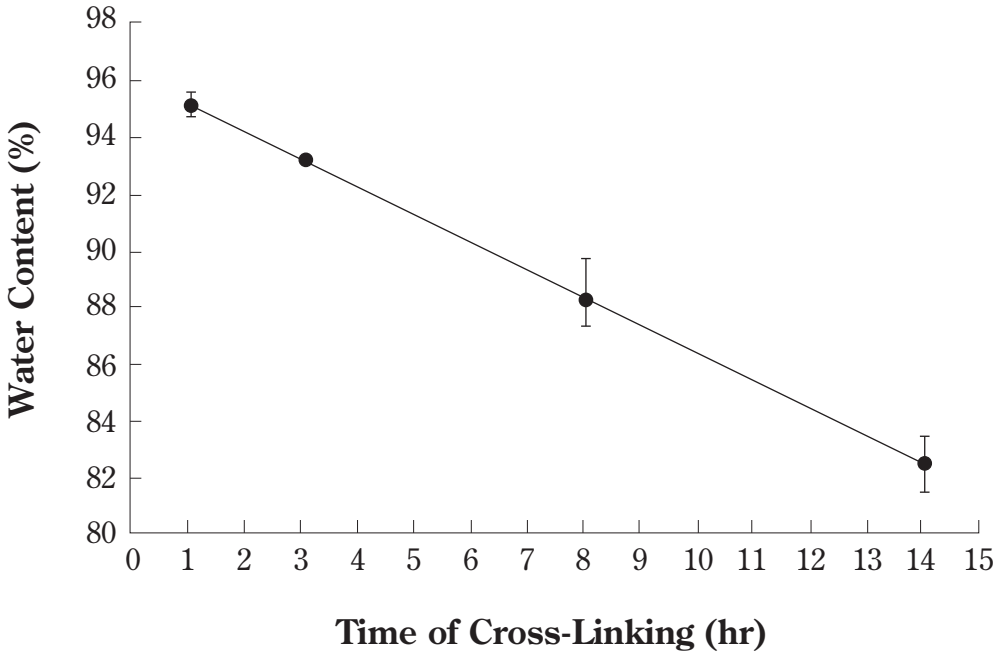

Figure 2. Water content of the GFM

The water content of each gelatin film (*Wc*) was calculated according to the equation: *Wc* (%) = 100 ((*Ww* – *Wd*) / *Ww*), where *Ww* and *Wd* are the weights of the wet and dried samples, respectively. The data are presented as the mean ± SD (n=4).

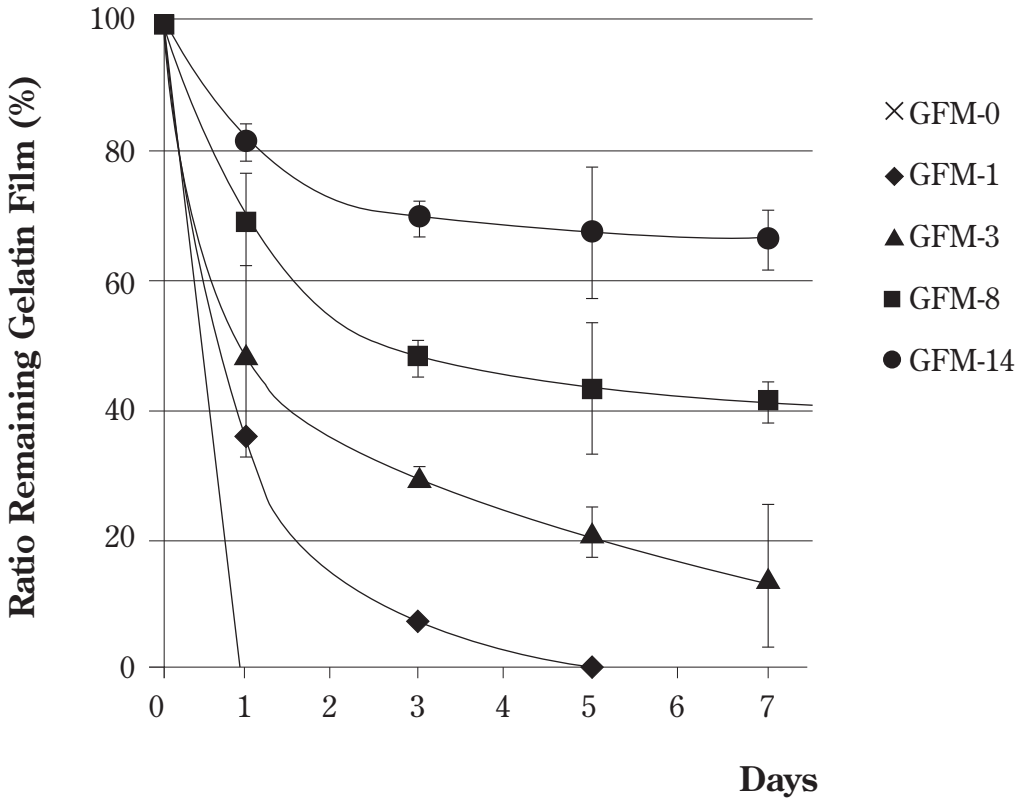

Figure 3. Water solubility of the GFM

The percentage of each remaining undissolved gelatin film (*Ws*) was calculated according to the following equation: *Ws* (%) = 100 (*Wu* / *Wi*), where *Wi* is the initial weight of the sample and *Wu* is the weight of the undissolved desiccated sample. The data are presented as the mean ± SD (n=3).

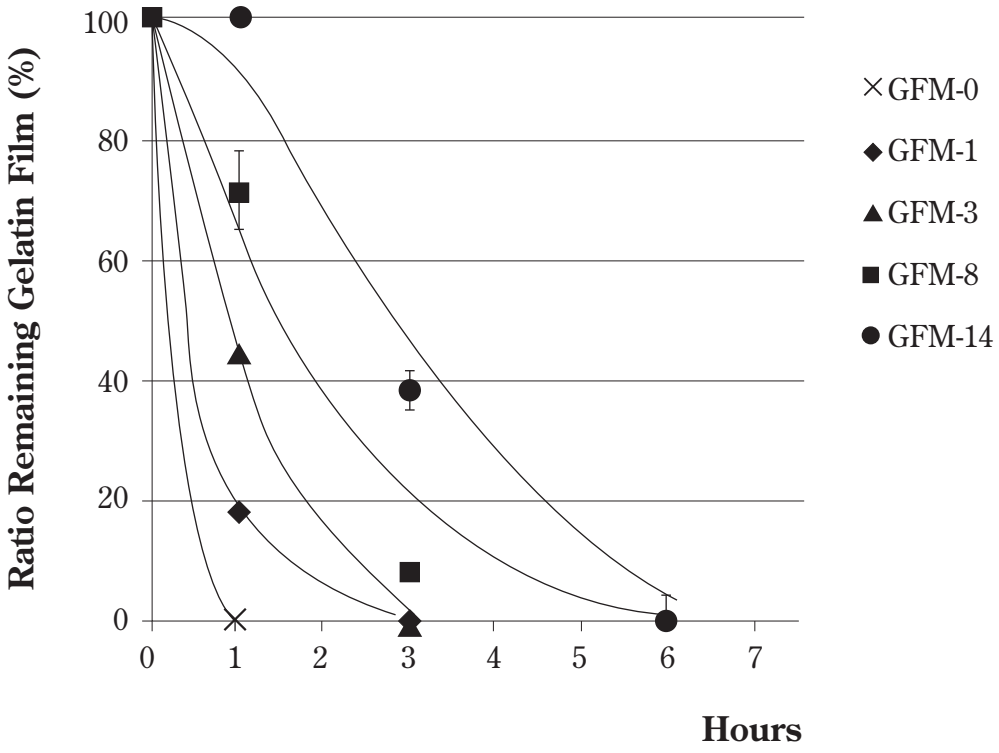

Figure 4. Enzymatic degradation of the GFM for collagenase

The percentage of each remaining undigested piece of film (*Dc*) was calculated according to the following equation: *Dc* (%) = 100 (*Wu* / *Wi*), where *Wi* is the initial weight of the sample and *Wu* is the weight of the undigested sample. The data are presented as the mean ± SD (n=4).

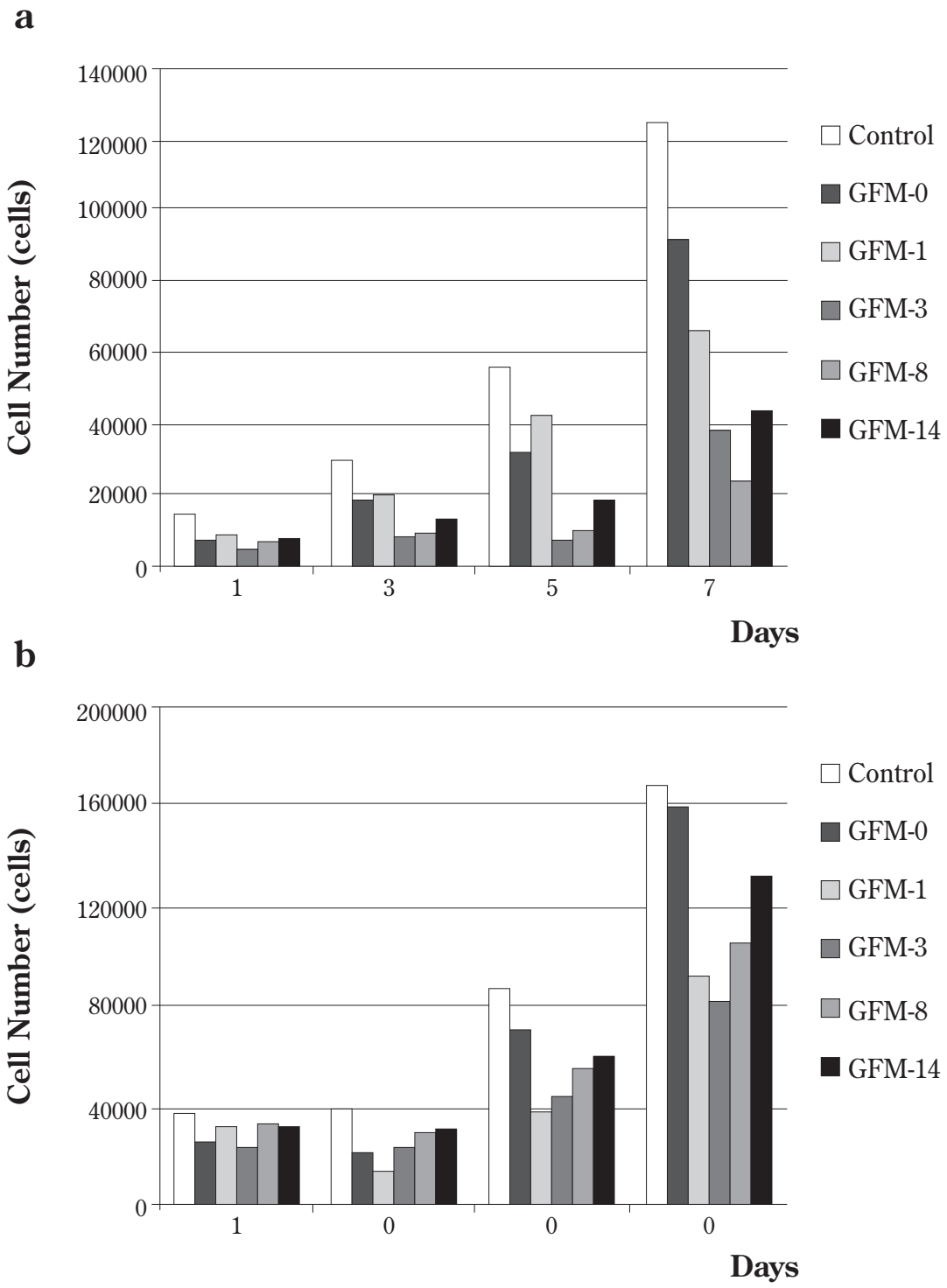

Figure 5. *In vitro* cell growth on the GFM

a: Growth of rat fibroblasts. b: Growth of human fibroblasts.

*: $p<0.05$ **: $p<0.01$

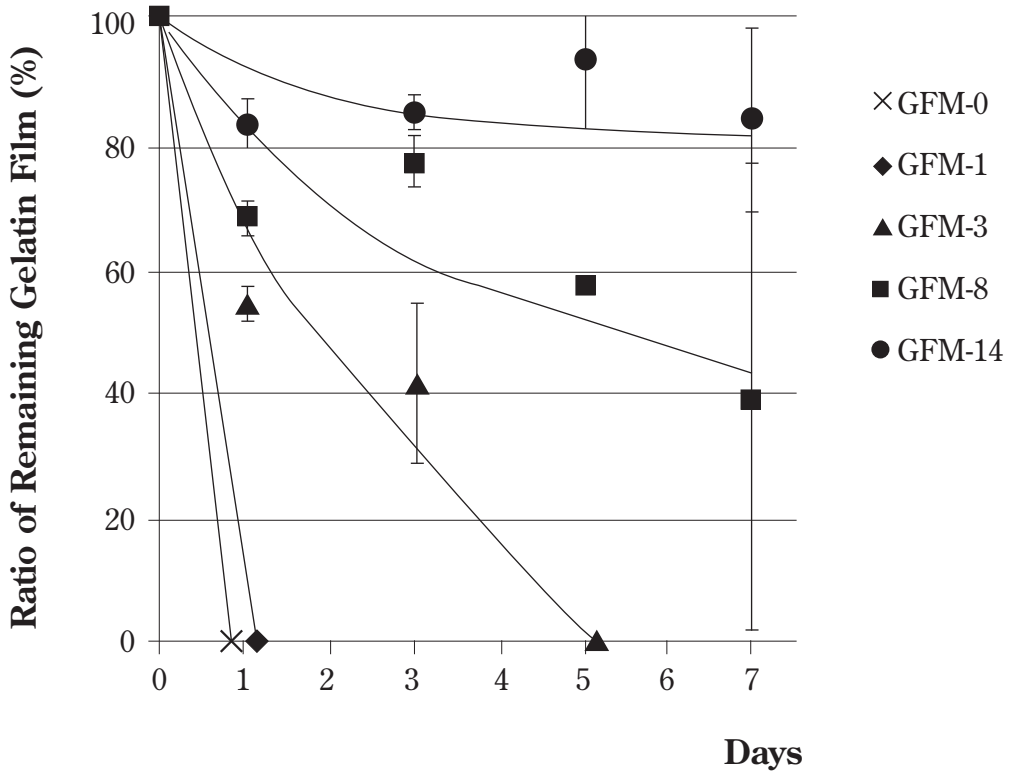

Figure 6. *In vivo* degradation of the GFM

The percentage of remaining pieces of gelatin film in the abdominal cavity of the experimental animals (*Dr*) was calculated according to the following equation: *Dr* (%) = 100 (*Wr* / *Wi*), where *Wi* is the initial weight of the film sample and *Wr* is the weight of the residual desiccated film sample. The data are presented as the mean ± SD (n=6).

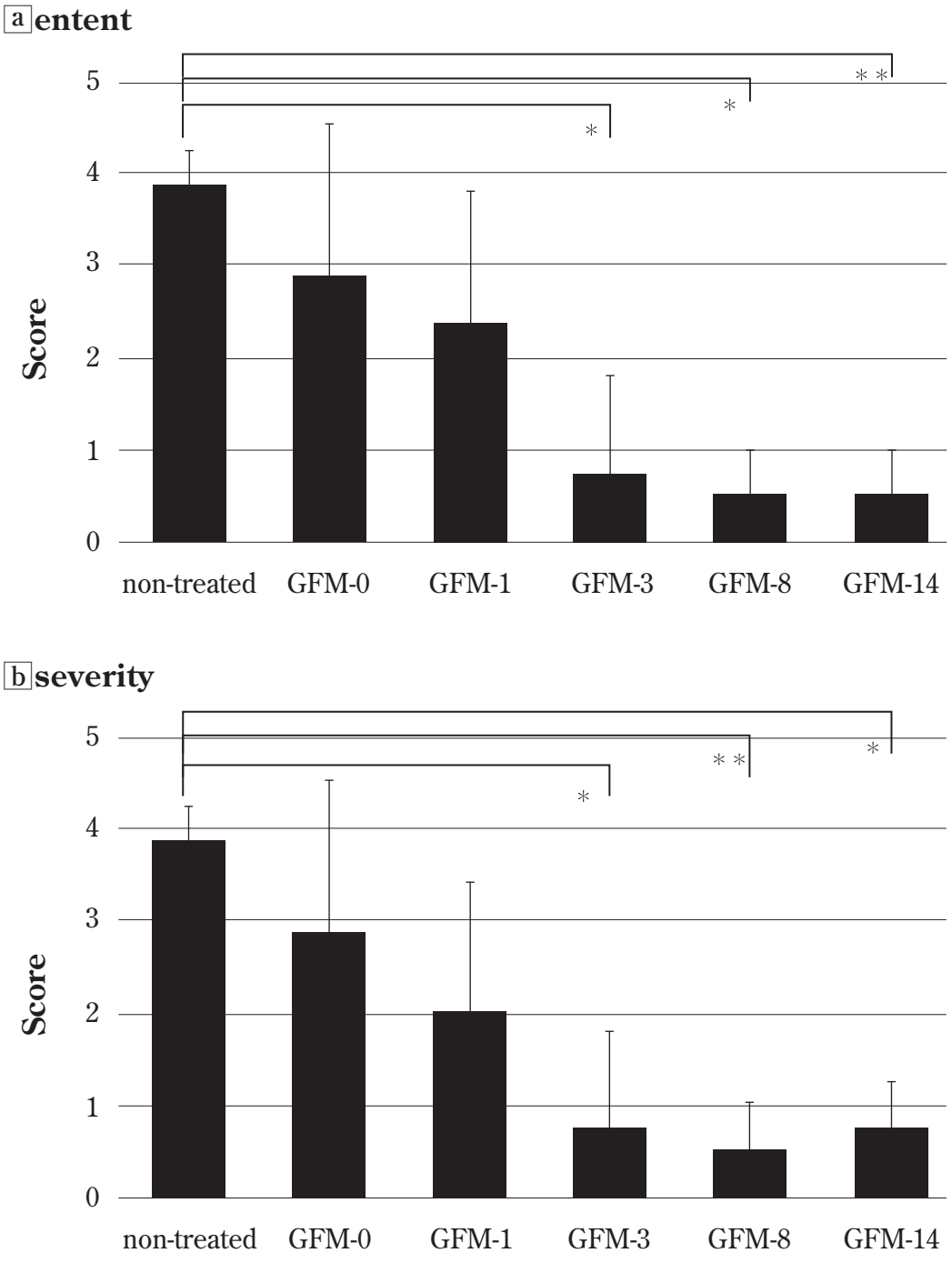

Figure 7. The Adhesion Scores in the Rat Adhesion Model

a: the extent of adhesion　b: the severity of adheision

Significant differences are indicateted by *(p<0.01), **(p<0.001)

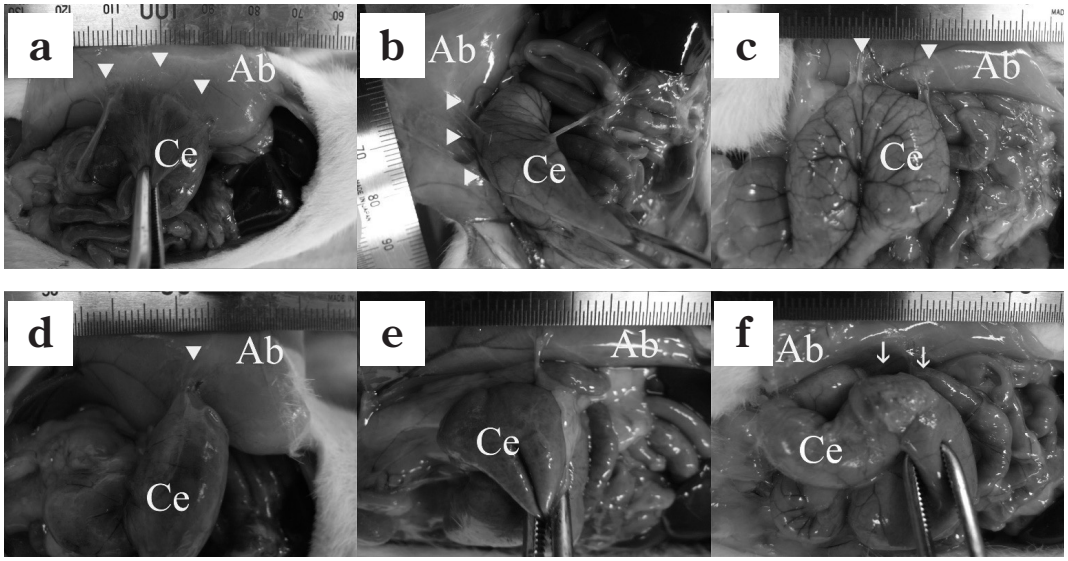

Figure 8. Macroscopic views of anti-adhesive effects of the different GFM

a: Non-treated. b: GFM-0 film. c: GFM-1 film. d: GFM-3 film. e: GFM-8 film. f: GFM-14 film. Ce: cecum. Ab: abdominal wall. White arrowheads indicate the portion of adhesion between the abraded cecum and abdominal wall. White arrows indicate the remaining GFM-14 film with the connective tissues.

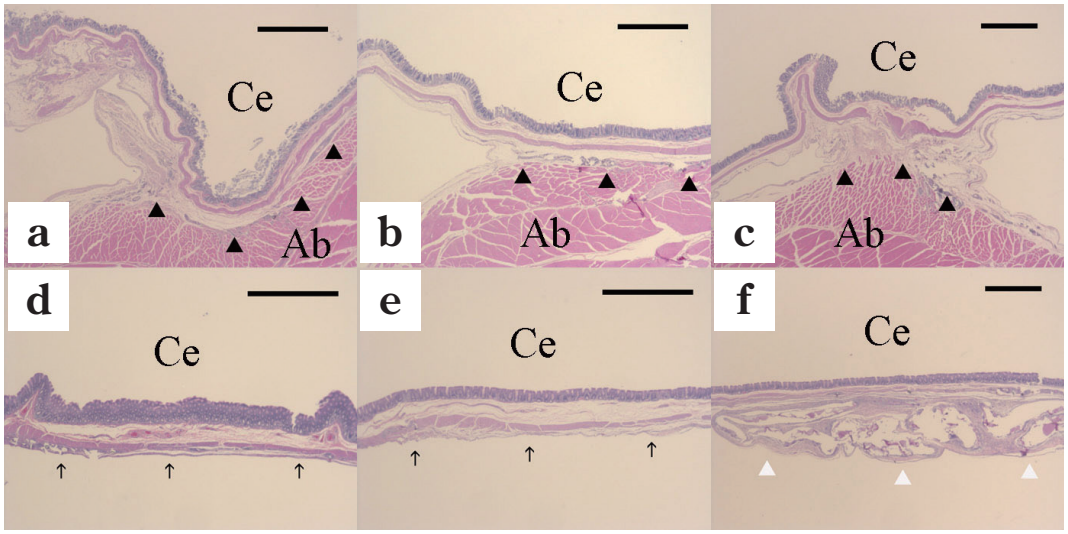

Figure 9. Microscopic views of anti-adhesive effects of the different GFM

a: Non-treated. b: GFM-0 film. c: GFM-1 film. d: GFM-3 film. e: GFM-8 film. f: GFM-14 film. Ce: cecum. Ab: abdominal wall. Black arrowheads indicate the portion of adhesion between the abraded cecum and abdominal wall. Black arrows indicate the abraded sites on the cecum. White arrowheads indicate the pieces of the remaining GFM-14 film with the connective tissues.

STUDY I-2

The anti-adhesive effect and the influence on the intestinal anastomosis of thermally cross-linked gelatin film in canine models

TABLE OF CONTENTS (STUDY I-2)

ABSTRACT

Background:

To generate a more effective and safer anti-adhesive material, we have developed a new type of thermally cross-linked gelatin film (GFM).

Materials and Methods:

We preclinically examined the anti-adhesive efficacy of this film and evaluated the possibility applying the film safely onto fresh intestinal anastomoses, compared with hyaluronate and carboxymethyl-cellulose film (Seprafilm®). Using a canine adhesion model, the degree of adhesion for each film was evaluated by adhesion scoring systems and histological observation.

Next, in a canine anastomosis model, the anastomoses were wrapped directly by each film and the bursting pressures of the anastomoses were examined three and seven days after surgery.

Results:

Only the GFM showed significantly superior anti-adhesive effects compared to the non-treated (no treatment), in particular, exhibiting excellent re-peritonization.

The GFM did not significantly affect either the bursting pressures

or the healing process, compared with the non-treated. However, the Seprafilm® significantly decreased the bursting pressures measured at three days after surgery.

Conclusions:

The thermally cross-linked gelatin film had satisfactory anti-adhesive effects with excellent re-peritonization. It could be safely applied to intestinal anastomoses without decreasing the bursting pressures. The gelatin film is considered to be quite favorable as an anti-adhesive material.

犬の実験モデルにおける熱架橋ゼラチンフィルムの癒着防止効果および腸管吻合部への影響

要約

背景：

術後の癒着およびそれに付随する合併症を防ぐため、より効果的で安全性の高い癒着防止材として熱架橋ゼラチンフィルムを開発した。

材料および方法：

熱架橋ゼラチンフィルムとヒアルロン酸ナトリウム・カルボキシメチルセルロースフィルム（hyaluronate and carboxymethylcellulose film: Seprafilm®）を新鮮な腸管吻合部に貼付し、安全な使用が可能であるか検討した。まず、犬の癒着モデルに対し、癒着スコアによる評価および組織学的評価を行った。また、腸管吻合モデルにおいて、両フィルムを貼付し、術後３、７日後に吻合部の破裂圧を測定した。

結果：

熱架橋ゼラチンフィルムのみがコントロール群と比較して有意な癒着防止効果を認めた。また、腹膜の再生も認めた。

熱架橋ゼラチンフィルムは、コントロール群に比して腸管吻合部

の破裂圧、組織再生の過程において有意な影響は認めなかった。しかし、Seprafilm®では術後3日後の腸管吻合部の破裂圧を有意に低下させた。

結語：

熱架橋ゼラチンフィルムは腹膜再生により優れた癒着防止効果を発揮し、また破裂圧を下げることなく腸管吻合部に安全に使用できると考えられ、癒着防止材として有用であると言える。

INTRODUCTION

Intra-abdominal adhesion formation after abdominal and pelvic surgery is quite common, and is a major cause of considerable post-operative complications. Indeed, adequate ("good") adhesion may be pathophysiologically important for healing the intra-abdominal damages such as at intestinal anastomosis. However, inappropriate ("bad") adhesion often involves serious complications, such as chronic pain, functional intestinal obstruction, and female infertility. In addition, lysis operations for the adhesion often involve difficulties in identifying anatomical features, prolongs the operating time for dissections, and increases the risk of visceral damages and blood transfusion requirements. (1-3)

To prevent intra-abdominal adhesions and the related morbidities, numerous anti-adhesive materials besides careful surgical techniques have been developed, and some of them have been reported to be effective in animal models and in clinical practices. (3) In general, those are divided into two main categories. The first is pharmacological agents, such as antibiotics, steroids, fibrinolytic agents, etc., which reduce inflammatory processes and prevent fibrin deposition. The second is so called "anti-adhesive barriers", which physically prevent direct contact of injured surfaces with surrounding tissues during the critical period of adhesion formation (usually five

to seven days after surgery). (3, 4) This category includes human tissues such as autologous peritoneum, biological solutions such as crystalloids, dextran and hyaluronate, and synthetic solids made of biodegradable or non-degradable materials.

Currently, polysaccharide-based sheet- or gel-type biodegradable anti-adhesive barriers such as a combination of sodium hyaluronate and carboxymethyl-cellulose (Seprafilm®), (5) an oxidized regenerated cellulose, (6) and a form of sodium hyaluronate mixed with phosphate-buffered saline[7] are commercially available and clinically used. Among those anti-adhesive barriers, the Seprafilm® has been most extensively investigated so far. Several studies have reported that Seprafilm® decreased the incidence or severity of adhesions both experimentally and clinically. (5, 8, 9) However, Seprafilm® did not decrease the incidence of bowel obstruction in clinical trials, although it did decrease the incidence of the severe cases that required re-operation. (10, 11) In addition, the Seprafilm® has several drawbacks including its uneasy handling due to its fragility, higher incidences of intestinal anastomotic leakage and the related intra-abdominal abscess or fistula formation in comparison with no treatment. (8, 11, 13) In fact, several clinical studies have warned that wrapping suture or staple lines of the fresh bowels directly with Seprafilm® should be avoided. (12, 13)

To generate a more effective and safer anti-adhesive material to

overcome these problems, we have developed a new thermally cross-linked gelatin film (GFM). In previous reports, UV cross-linked gelatin film showed excellent anti-adhesive effects and involved less inflammatory reaction in comparison with the films composed of poly-vinyl alcohol or expanded-polytetrafluoroethylene (Goretex®). (14, 15) The thermally cross-linked gelatin film have more stable physical properties than those of the UV cross-linked gelatin film, and are less toxic than the gelatin film cross-linked with chemical agents such as formalin (16, 17) In this study, using canine models, we tried to examine preclinically (1) whether the thermally cross-linked gelatin film has satisfactory anti-adhesive efficacy, and (2) whether the film can be applied safely onto fresh intestinal anastomoses without increasing the incidences of leakage, in comparison with the Seprafilm®.

MATERIALS AND METHODS

1. Materials

Medical grade gelatin extracted from porcine skin (type-I collagen, Medigelatin®) with an isoelectric point of 5 was supplied by Nippi Co. Ltd. (Shizuoka, Japan). To prepare the GFM, the gelatin was dissolved in distilled water to a final concentration of 2.65%. Next, the

solution was cast in plastic plates (Kanto Chemical Co.Ltd., Tokyo, Japan), which composed of polystylene measuring 144 × 104 × 16mm in size, and allowed to dry in clean bench with consistent air flow for two overnights at room temperature, yielding a film of approximately 30μm in thickness. When the films were dry, they were easy to take off from the plates (Figure 1A). Thermally cross-linking was introduced by a vacuum oven (AVO-250N, As One, Osaka, Japan) at 140℃ for 3hours. Prior to further processing, we confirmed that this thermally cross-linking condition produced a film which was degraded within 5-7 days in *in vivo* degradation test and had the most effective anti-adhesive properties in rat adhesion models (we are presently preparing to submit the detailed data). Finally, the film was sterilized by ethylene-oxide gas (0.43g/L at 40℃ for 4hours) in preparation for animal experiments.

To test the anti-adhesive effects of GFM and its influences to intestinal anastomosis as preclinical study for, we used the canine adhesion and anastomosis models. Because canine models have more similar organ-size, -structure, and handling during surgery to human ones, compared with murine models. Female beagle dogs less than 2 years of age and weighing 9.9 + 0.9 (mean + SD) kg were purchased from Shimizu Laboratory Animal Supply Co. Ltd. (Kyoto, Japan). All animal care, housing and surgical procedures were in accordance with the institutional guidelines of the Committee for Animal

Research of Doshisha University and Nara Medical University.

2. Canine Adhesion Model

2.1. Surgical procedure

Totally twenty-one beagle dogs were used in this experiment. Under intravenous pentobarbital anesthesia (40mg/kg of body weight), every dog received a 5cm incision at the midline of the lower abdomen. In each dog, the serosal aspect of the terminal ileum (approximately 10cm oral from the ileo-cecal junction) was abraded in a 4 × 4 cm square using sand paper (grit #1000, Daiso Ltd., Tokyo, Japan) with occasional gentle hemostasis by finger compression. Then, the animals were randomized into three groups, with each consisting of seven dogs. In the first group, a GFM measuring 6 × 6 cm was wrapped around the abraded terminal ileum without suturing (GFM group). Similarly, in the second group, the same size Seprafilm® (Genzyme Co., Cambridge, MA) was wrapped around the abraded lesion (Seprafilm® group). According to the product information (available from URL; http//www.genzyme.com), Seprafilm® is a sterile, bioresbsorbable translucent adhesion barrier composed of two anionic polysaccharides, sodium Hyaluronate (HA) and carboxymethyl-cellulose (CMC). Together, these biopolymers have been chemically modified with the active agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC).

In the last group, the dogs received no wrapping (non-treated group). We applied the various treatments on the same days. In all groups, care was taken to keep the time of exteriorization of the intestines within five minutes. After closure of the abdominal walls, prophylactic antibiotics (Penicillin G Potassium, 10,000 units/kg, Meiji Seika Ltd.) were administrated subcutaneously for each dog for three days after surgery. All dogs were kept carefully until the evaluation of the adhesion.

2.2. Evaluation of the adhesion

Three weeks after the operation, the dogs were sacrificed by a lethal dose of pentobarbital. After full abdominal laparotomy, the status of the abdominal cavity and the abraded sites, including the amount of each film remaining, was observed macroscopically. The extent and severity of adhesion were graded and scored numerically according to an adhesion grading scale (Adhesion Scores, Table I) modified from the Adhesion Score of the Surgical Membrane Study Group. (18) The evaluation by the Adhesion Score was performed by a researcher who was blinded to the animal assignments. Statistical comparisons were made by the Dunn test following the Kruskal-Wallis test. A p value of less than 0.05 was considered to be significant for the tests.

2.3. Histological and immunohistochemical analyses

The abraded segments of the dogs used in the above experiments were processed for further histological analyses, as described below. In addition to them, each six additional animals received the same operation and treatments. Then, half of the animals in each group were sacrificed at one and six weeks after the operation to histologically characterize the serosal healing of the abraded terminal ileum over time. After evaluating the adhesion status by adhesion scoring (Table 1), all the abraded ileum sample (covered with GFM, Seprafilm® or no film) at 1, 3, and 6 weeks after the operation were resected and fixed with 10% buffered formalin, and processed for embedding in paraffin. Then, 3μm sections were stained with hematoxylin and eosin (H/E). We used also specimens of the terminal ileum taken from two dogs with no surgery and no treatment for comparison as normal samples.

We carefully observed all samples, paying close attentions to the status of infiltrating inflammatory cells, the formations of granulation tissue and fibrous connective tissue, the regeneration of peritoneal tissue, and the mount of GFM or Seprafilm® remaining at the injured serosal surface. To evaluate the status of re-peritonization and the degree of fibrous connective tissue formation during the healing process at the injured sites, we performed immunohistochemical staining for the mesothelial cell surface marker, HBME-1 (M3505,

1:50 dilution, Dako Corp., Carpinteria, CA), which recognize an antigen at the microvillous surface of mesothelial cell, (19) and Masson's trichrome staining which indicates the degree of the production of collagen fibers. Furthermore, we quantitatively classified the status of inflammatory cells, formation of granulation tissue, formation of connective tissue, and re-peritonization on a scale from 0 to 3, based on the histological scores using a system modified from previous reports (Table 2). (15, 20) The evaluation was carried in blinded manner, and statistical comparisons were made using the Dunn test following the Kruskal-Wallis test in the same manner as were used for the adhesion scores.

3. Canine Anastomosis Model

3.1. Surgical procedure

Totally forty-two beagle dogs were used in this experiment. After one overnight fasting, every dog received a laparotomy according to the same procedure used for the canine adhesion model. Then, the terminal ileum (about 10-15cm from the ileo-cecal junction) was severed completely. After the stool content had been milked out, a standardized end-to-end anastomosis was made with twelve interrupted, inverting sutures of 3-0 poly-glycolic acid (Vicryl, Ethicon Inc., Somerville, NJ, USA). Then, the animals were randomized into three groups, with each consisting of fourteen dogs.

In the first group, the anastomosis sites were covered and wrapped fully by a thermally cross-linked GFM measuring 6 × 6 cm (GFM group). In the second group, the same size Seprafilm® covered the anastomotic site (Seprafilm® group). In the last group, the dogs received no wrapping of the anastomosis (non-treated group). We applied the various treatments on the same days. In all groups, care was taken to keep the time of exteriorization of the intestines within twenty minutes. After closure of the abdominal walls, prophylactic antibiotics (Penicillin G Potassium, 10,000 units/kg, Meiji Seika Ltd.) was administrated subcutaneously for each dog for three days after surgery. All dogs were kept carefully until the following evaluations.

3.2. Evaluation of the bursting pressure

Three and seven days postoperatively, half of the animals in each group were sacrificed using a lethal dose of pentobarbital. The abdominal incision was re-opened, and the status of the abdominal cavity and anastomotic sutures were observed macroscopically. Then, the anastomosis segment, which included 5cm both proximal and distal to the anastomosis line, was cut out, preserving the adhesions. After the intestinal contents were gently removed manually, the bursting pressure of the anastomosis segment was measured with an electronic manometer (testo512, Testo AG., Lenzkirch, Germany) as follows.

Briefly, one end of the anastomosis segment was ligated with a 1-0 silk. The other end was connected to the system used to measure the bursting pressure by a feeding tube. For this purpose, one tip of a three-way stopcock was connected to the feeding tube, the second tip was connected to a 30 ml syringe that continuously insufflated the system at a constant rate, and the third tip was attached to the electronic manometer (Figure 1B). The bursting pressure was measured in a water filled container and recorded as the peak pressure attained before the rupture of the anastomosis, which was indicated by the bubbling of air and an abrupt drop in pressure records. Statistical comparisons were made by the Newman-Keuls test following ANOVA (analysis of variance) for the bursting pressures.

The adhesion status of the anastomosis segment was also evaluated in the same manner as in the adhesion models using the adhesion scores and statistical analyses.

3.3. Histological analyses

After evaluating the bursting pressure and adhesion status, the segment was fixed with 10% buffered formalin, and processed for embedding in paraffin. Transverse sections (3μm) of the segments, including the anastomosis sites, were stained with hematoxylin and eosin (H/E). Then, histological evaluations, including the status of

the healing process of each anastomosis and the amount of each film remaining at the anastomosis site, were carried out in a blinded manner. Furthermore, to evaluate the status of the collagen fiber and connective tissue production, Masson's trichrome staining was performed. Then, we quantitatively classified the status of inflammatory cells and the formation of granulation and connective tissues in the same manner by using the histological scores used in the adhesion models (Table 2), and statistically evaluated the results by the Dunn test following the Kruskal-Wallis test.

RESULTS

1. Canine Adhesion Model

1.1. Adhesion scores in the canine adhesion models

The adhesion scores at three weeks after surgery are shown in Figure 2. There were no mortalities or morbidities associated with the operation or the wrapping of the films. There was no GFM or Seprafilm® remaining in either of the film groups macroscopically. The non-treated group had remarkably higher adhesion scores in terms of both the extent and severity. On the other hand, the GFM group showed the lowest adhesion scores among the three groups. There were significant differences between the non-treated group

and the GFM group in both the extent and severity categories (p<0.01). The adhesion scores of the Seprafilm® group were lower than those of the non-treated group and higher than those of the gelatin group. However, no statistically significant differences were observed either in the non-treated vs. the Seprafilm® groups or in the Seprafilm® vs. the GFM groups.

The adhesion scores including those at one and six weeks after surgery are summarized in Table 3. There were no mortalities or morbidities at one and six weeks after surgery, and macroscopically no GFM and Seprafilm® remaining in the two groups. Similarly to those at three weeks after surgery, the adhesion scores in the non-treated group were highest, whereas the scores in the GFM group were lowest also at one and six weeks in terms of both extent and severity.

1.2. Microscopical observation of the adhesion models

When the injured sites were observed one week after surgery, the non-treated group showed a remarkably thick and dense serosal layer, which included numerous fibroblasts and inflammatory cells such as neutrophils, lymphocytes, and monocytes, forming granulation tissues. These changes were also seen in the adjacent adherent tissues, such as the omentum (Figure 3A, a). In the GFM group, the injured serosal layer was also thickened. However, the

layer was loose and edematous, and contained fewer fibroblasts and inflammatory cells (Figure 3B, b). A thick and dense serosal layer was also seen in the Seprafilm® group. However, the thick serosal layer contained numerous inflammatory cells but fewer fibroblasts (Figure 3C, c). In both the GFM and Seprafilm® groups, there was no film remaining on the tissue. HMBE-1 staining could not demonstrate clearly positive cell in the injured serosal layers of any three groups (data not shown).

At three weeks after surgery, the inflammatory changes at the injured serosal layer seemed to improve relatively in the non-treated group, comparing with those at one week. However, the layer was still thick and dense, and fibrous changes with connective tissue were seen in places (Figure 4A). In the GFM group, the injured serosal layer was much thinner at three weeks than at one week after surgery. The layer had fewer inflammatory cells with sparse fibroblast clusters (Figure 4B). In the Seprafilm® group, the injured serosal layer was also much thinner. However, the layer still contained numerous inflammatory cells (Figure 4C). Furthermore, a layer of clearly HBME-1 positive cells was seen at the outer site of the injured serosal layer in the GFM group (Figure 4b). In physiological condition, the surface lining of the peritoneum consist of mesothelial cells which are highly differentiated with the microvillous structure (21), and HBME-1 antibody recognizes an antigen at the microvillous

surface of mesothelial cell. (19) Therefore, the layer of HBME-1 positive cells indicates that the re-peritonization consisted of structurally and functionally recovered mesothelial cells. In contrast, in the non-treated and Seprafilm® groups, HMBE-1 positive cells were seen only rarely (Figure 4-a, c).

At six weeks after surgery, the non-treated group still showed a relatively thick and dense serosal layer. However, the layer consisted mostly of numerous fibroblasts and connective tissue, while the inflammatory cell infiltration subsided remarkably (Figure 5A). In the GFM group, the injured serosal layer became thinner and similar to normal serosal tissue (Figure 5B and Figure 3d). Because there were few inflammatory cells, and granulation tissue and fibrous changes were sparsely detected. In the Seprafilm® group, the injured serosal layer also became thinner. However, numerous foamy cells still persisted, although the infiltration of acute inflammatory cell such as neutrophils and lymphocytes subsided. The formation of granulation and connective tissues was also mild (Figure 5C). HBME-1 staining revealed a layer of cells stained positively at the outer site of the injured serosal layer mostly in the three groups (data not shown). In terms of Masson's trichrome staining, the non-treated group showed extensive formation of connective tissue, which was stained as blue fibers, in the injured serosal layer (Figure 5a). In the GFM group, the connective tissue formation was mild and the appearance was similar

to that in normal serosal tissue (Figure 5b, d). In the Seprafilm® group, connective tissue formation was also mild. However, it was randomly detected along with cellar components consisting mainly of foamy cell clusters in the serosal layer (Figure 5c).

The histological scores for inflammatory cells, granulation tissue, connective tissue and re-peritonization in the adhesion models are summarized in Figure 6. In comparison with the non-treated group, both the GFM and Seprafilm® groups tended to have lower scores for granulation and connective tissues. However, higher scores for inflammation persisted in the Seprafilm® group, and there was significant difference between the GFM and HA/CMC groups at three weeks. Additionally, the GFM group had higher scores for re-peritonization, especially at three weeks, and these scores were significantly different from both the non-treated and Seprafilm® groups.

2. Canine Anastomosis Model

2.1. Bursting pressures and adhesion scores in the canine anastomosis models

There were no mortalities among any of the groups as of seven days after the surgery. However, peri-anastomotic abscesses due to minor anastomotic leakages were observed in each one case of the Seprafilm® group at three and seven days after surgery, respectively.

No other complications related to the operation or wrapping of the films were observed. At three days after surgery, both the GFM and Seprafilm®s were still present in the gel state at the outer sites of the anastomosis and peri-anastomotic sites in five and six cases, respectively. However, there was no remaining film at seven days after surgery in either group.

The bursting pressures in the three groups are shown in Figure 7 and Figure 6. At three days after surgery, the bursting pressure of animals in the GFM group was slightly higher than that of the non-treated group. However, there was no statistically significant difference between the two groups. In contrast, the bursting pressure of the Seprafilm® group was obviously the lowest among the three groups, and there were significant differences in comparison to both the non-treated group and GFM group ($p<0.05$). At seven days after surgery, the bursting pressure in the Seprafilm® group was lower than that in the other groups. However, there were no significant differences among the three groups.

The adhesion scores of the three groups for the anastomosis models are shown in Figure 9. The GFM group had the lowest adhesion scores in terms of both the extent and severity of the adhesions, especially at three days after surgery. However, there were no significant differences among the three groups in either category at three and seven days after surgery.

2.2. Microscopical observation of the anastomosis models

In the observation of the animals three days after surgery, the non-treated group showed the presence of numerous inflammatory cells and fibroblasts at the anastomotic site and/or the peri-anastomotic sites. Those infiltrating cells were replacing mucosal tissues focally at the anastomotic site (Figure 7A, a). In the GFM group, similar changes were seen in most of the cases. In addition, there was still GFM remaining at the outer sites of the anastomosis and the peri-anastomotic sites (Figure 7B, b). In contrast, the Seprafilm® group seemed to show less inflammatory cell and fibroblast infiltration compared with the other two groups. The mucosal tissues at anastomotic sites still remained nearly intact in most cases. The Seprafilm®s were also retained at the outer sites of the anastomosis and the peri-anastomotic sites at this time point (Figure 7C, c).

At seven days after surgery, the non-treated group showed extensive inflammatory cell infiltration and fibroblast clusters, forming granulation tissues (Figure 9A). Similar changes were seen in the GFM and Seprafilm® groups. There was no remaining GFM or Seprafilm® in either of the groups (Figure 9B, C). Masson's trichrome staining showed extensive connective tissue formation, which was indicated by blue fibers, at the anastomotic sites in the non-treated and GFM groups (Figure 9a, b). However, there seemed to be less connective tissue formation in the Seprafilm® group

(Figure 11c).

The histological scores for inflammatory cells, granulation tissue and connective tissue in the anastomosis models are summarized in Figure 12. In comparison with the non-treated group, the gelatin group had similar scores for inflammatory cells, granulation tissue, and connective tissue at both three and seven days. In contrast, the Seprafilm® group showed lower scores for granulation and connective tissues. In particular, there were significant differences in comparison to the non-treated group in terms of the granulation tissue score at three days and in the connective tissue score at seven days after the operation.

DISCUSSION

Due to the problems of post-operative adhesion-related complications, a wide variety of adjunctive methods to prevent the adhesion formation have been proposed. Among the numerous anti-adhesive methods developed, one of the modalities that has been investigated extensively and has shown the most promising results is the anti-adhesive barrier technique. The ideal anti-adhesive barrier, besides being safe and effective, should be non-inflammatory, non-immunogenic, completely biodegradable, persist throughout the

critical remesothelialization period, stay in place without sutures or staples, remain active in the presence of blood, and also not interfere with wound healing, promote infection, or cause additional adhesion. However, such ideal barriers have not yet been developed, although several anti-adhesive materials, including Seprafilm®, have been commercially available and are being used clinically. (4)

According to a previous report by Burns et al., Seprafilm® turns into a hydrophilic protective gel within 24 to 48 hours after placement and it remains in the peritoneal cavity for up to seven days. During this period, the Seprafilm® reduces adhesion formation by acting as a physical anti-adhesive barrier to separate adjacent traumatized serosal tissues. (5) However, in the present study using canine adhesion models, the Seprafilm® unexpectedly did not show statistically significant anti-adhesive effects compared to the non-treated group, although the adhesion scores of the Seprafilm® seemed to be superior to those of the non-treated group. In contrast, the GFM had significant anti-adhesive effects compared to the non-treated group in terms of both the extent and severity.

Interestingly, in our histologic observations, both the Seprafilm® and GFM had disappeared macro- and microscopically at least one week after surgery. However, the animals treated with the GFM showed excellent re-peritonization consisted of fully restored mesothelial cells at three weeks after surgery, whereas those treated

with the Seprafilm® showed poorer mesothelial cell restoration and prolonged inflammatory reactions with abundant foamy cells beyond three weeks after surgery. It is well-known that mesothelial cell injury and subsequently unveiled submesothelial tissues cause adhesion formation in the pathological condition such as abdominal surgery, trauma and inflammation. In addition, prolonged inflammation also induces adhesion through the suppression of fibrinolysis at the injured peritoneal tissues. (4) Therefore, rapid re-peritonization and reduced inflammatory reaction are thought to be advantageous for anti-adhesion. With regard to the effects of anti-adhesive barriers, not only the physical separation of injured serosal sites, but also the biological properties inducing such effects may contribute to decreased adhesion formation. If so, the differences in the biological properties may be caused by the differences of the components of the anti-adhesive materials. Seprafilm® is a polysaccharide-based material, while the GFM is made from collagen, which forms the most abundant extracellular matrix in human and animal bodies.

In another experiment using the anastomosis models, the GFM did not decrease the burst pressure of the anastomosis at three or seven days after surgery. This result supports that the GFM can be used safely onto intestinal anastomotic sites even when used to wrap the suture lines directly. In contrast, the Seprafilm® significantly decreased the burst pressures of the intestinal anastomosis,

especially at three days after the surgery.

As mentioned in the introduction, several clinical trials indicated that Seprafilm® induced a high incidence of intestinal anastomotic leakage and the related intra-abdominal abscesses or fistula formation. (8, 12, 13) Despite these findings, previous studies using animal models had not demonstrated a decreased bursting pressure of the intestinal anastomosis with use of the Seprafilm® or by wrapping it directly with the Seprafilm®. However, most of those studies were performed in small murine models. (20, 22-25) Only one study by Medina et al. used a rabbit model and showed no differences in the bursting pressure of intestinal anastomosis between the Seprafilm® and non-treated groups when examined at seven and fourteen days after the operation. (26) Clinically, we encounter leakage of the intestinal anastomosis usually within seven days after an operation. Additionally, according to previous animal experiments and our present data, the burst pressures of the anastomosis three days after surgery are obviously lower than those at seven days. (24, 27) Therefore, our data of burst pressures taken at three and seven days after operation in this canine model are considered to be more useful to predict the clinical outcome than previous ones.

In addition, our histological observation of anastomotic sites showed that there were no obvious differences between the gelatin and non-treated groups. This result indicates that the GFM does not

negatively affect the healing process at the anastomosis, and also supports the safety of applying the GFM to an intestinal anastomosis. In contrast, the formation of granulation tissue or connective tissue at anastomotic sites in the Seprafilm® group seemed to be diminished. This indicates the possibility that the Seprafilm®, or its degradation products, may prevent adhesion formation by impairing the healing process at surgical sites as well as by simply physical separating adjacent tissues. In fact, Hamadeh et al. had mentioned the possibility that the CMC solution might curtail fibroblast activities and proliferation, and that this might be the mechanism by which it prevents adhesion. (28)

However, the adhesion scores of the anastomotic sites in the anastomosis models were not significantly different among the three groups at both three and seven days after the operation. Although evaluations were not completed at longer time intervals after surgery in this study, the results indicate that the anti-adhesive effects of the GFM at intestinal anastomotic sites might be diminished. Although the detailed mechanisms remain unclear, several previous reports have suggested that the anti-adhesive effects of Seprafilm® are diminished or overcome under strong inflammatory situations, such as those that occur at intestinal anastomotic sites and in the presence of peritonitis. (10, 25, 26) Similarly, the anti-adhesive effects of the GFM may be attenuated under severe inflammatory conditions and

may be superseded by the subsequent formation of adhesions. Further examinations are needed to determine whether this is the case.

CONCLUSION

The thermally cross-linked GFM had satisfactory anti-adhesive effects and induced excellent re-peritonization. It could also be applied safely to an intestinal anastomosis without decreasing the bursting pressures. Based on these findings, the thermally cross-linked GFM should be considered to be a quite favorable anti-adhesive material.

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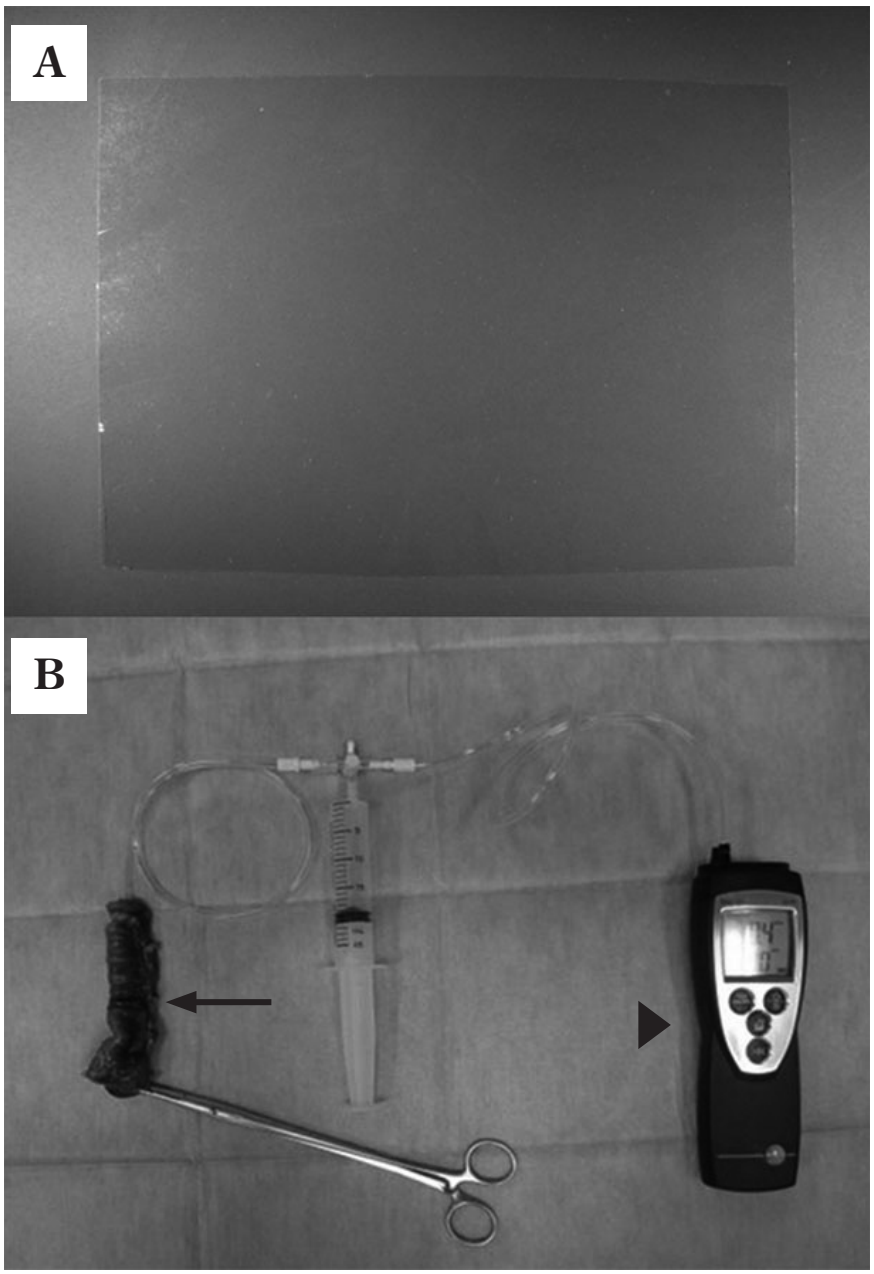

Figure 1. Photographs of the thermally cross-linked GFM (A) and the system used to measure the bursting pressure of the intestinal anastomosis (B). The arrow indicates the resected terminal ileum containing the anastomosis. The arrowhead indicates the electric manometer (B).

Table 1. The Adhesion Scores

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

Table 2. The Histological Scores for Inflammatory Cells, Granulation Tissue, Connective Tissue and Re-peritonization

Category and Description	Score
(Inflammatory cells)	
No infiltration of inflammatory cells such as neutrophils, lymphocytes, and monocytes	0
Sparse infiltration of inflammatory cells	1
Focal infiltration of inflammatory cells	2
Diffuse infiltration of inflammatory cells	3
(Granulation tissue)	
No granulation tissue formation	0
Sparse or focal granulation tissue formation	1
Thin layer of granulation tissue	2
Thick layer of granulation tissue	3
(Connective tissue)	
No fibrous connective tissue formation	0
Sparse or focal connective tissue formation	1
Thin layer of fibrous connective tissue	2
Thick layer of fibrous connective tissue	3
(Re-peritonization)	
No restoration of mesothelial cells	0
Sparse restoration of mesothelial cells	1
Focal restoration of mesothelial cell layer	2
Full restoration of mesothelial cell layer	3

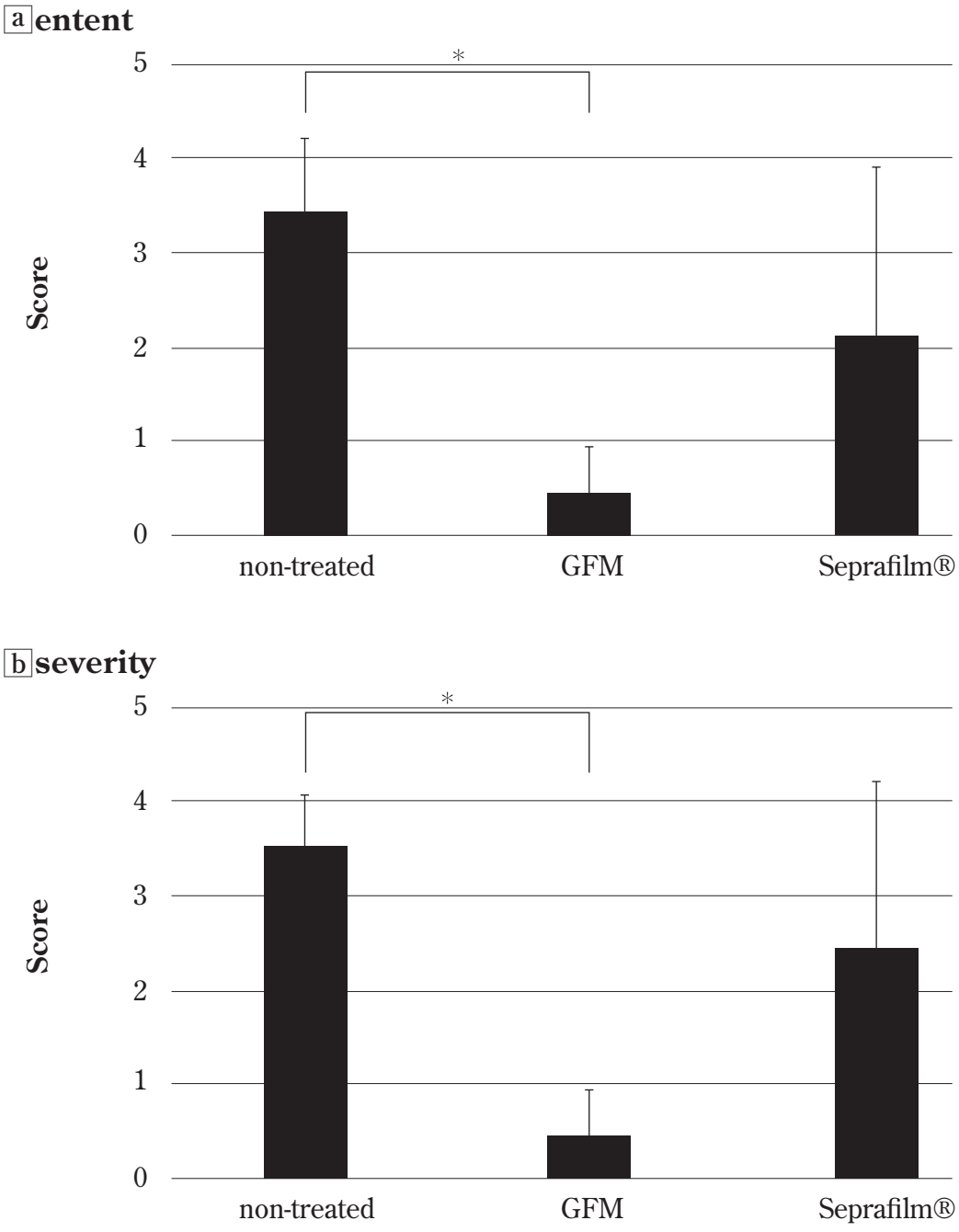

Figure 2. The Adhesion Scores at three weeks after surgery in the Canine Adhesion Models

a: the extent of adhesion b: the severity of adheision
Significant differences are indicateted by * (p<0.01)
n.s.; not significan

Table 3. Summary of the adhesion scores in the Canine Adhesion Models

	1 week (n=3)	3 weeks (n=7)	6 weeks (n=3)
(Extent)			
Non-treated	3.00±0.00	3.43±0.79[a]	3.33±1.15
GFM	0.67±1.15	0.43±0.53[a]	0.00±0.00
Seprafilm®	2.33±2.08	2.14±1.77	1.00±1.00
(Severity)			
Non-treated	1.67±1.15	3.57±0.53[b]	3.67±0.58
GFM	0.67±1.15	0.43±0.53[b]	0.00±0.00
Seprafilm®	1.00±1.00	2.43±1.81	1.33±1.53

Data are presented as the means ± SD.
[a]: $p<0.01$ between the non-treated group versus the GFM group.
[b]: $p<0.01$ between the non-treated group versus the GFM group.

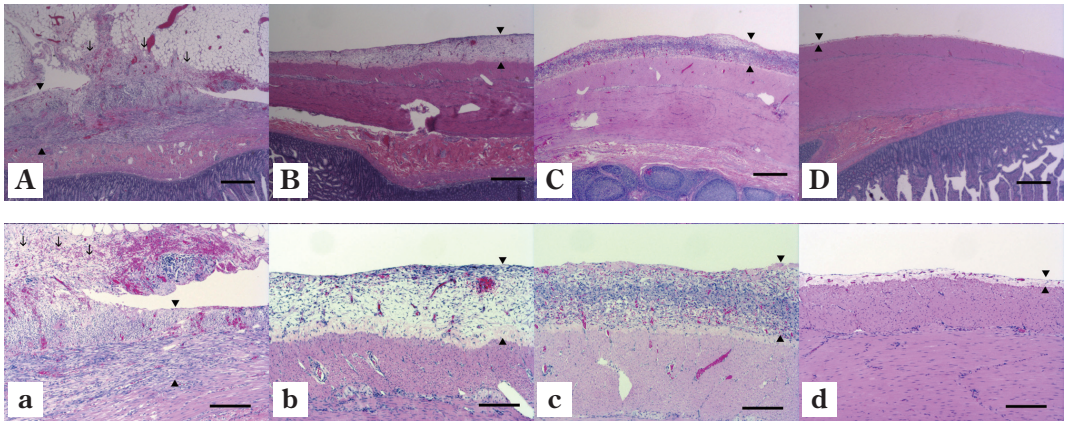

Figure 3. Microscopic views of the abraded sites at one week after surgery. The sites were stained with Hematoxylin and Eosin.

A, a: Non-treated (non-treatment) group. B, b: GFM group. C, c: Seprafilm® group. D, d: Normal dog. (Scale Bars: A, B, C, D: 500μm; a, b, c, d: 200μm) The area between two opposite arrowheads indicates the serosal layer. The arrows indicate numerous fibroblasts and inflammatory cells in the adjacent adherent tissues (omentum) (A, a).

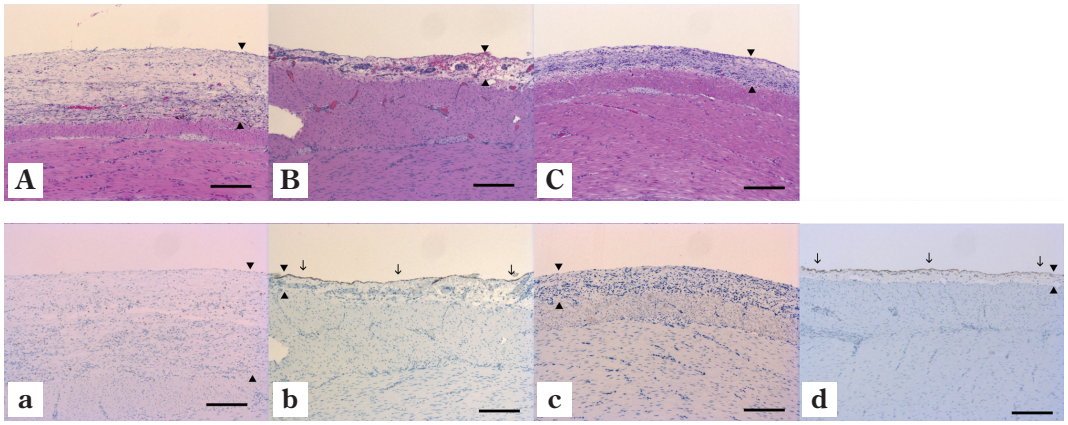

Figure 4. Microscopic views of the abraded sites at three weeks after surgery. The sites were stained with Hematoxylin and Eosin (A, B, C) and HBME-1 (a, b, c, d)

A, a: Non-treated (non-treatment) group. B, b: GFM group. C, c: Seprafilm® group. d: Normal dog. (Scale Bars: 200μm) The area between two opposite arrowheads indicates the serosal layer. The arrows show a layer of clearly HBME-1 positive cells indicating re-peritonization.

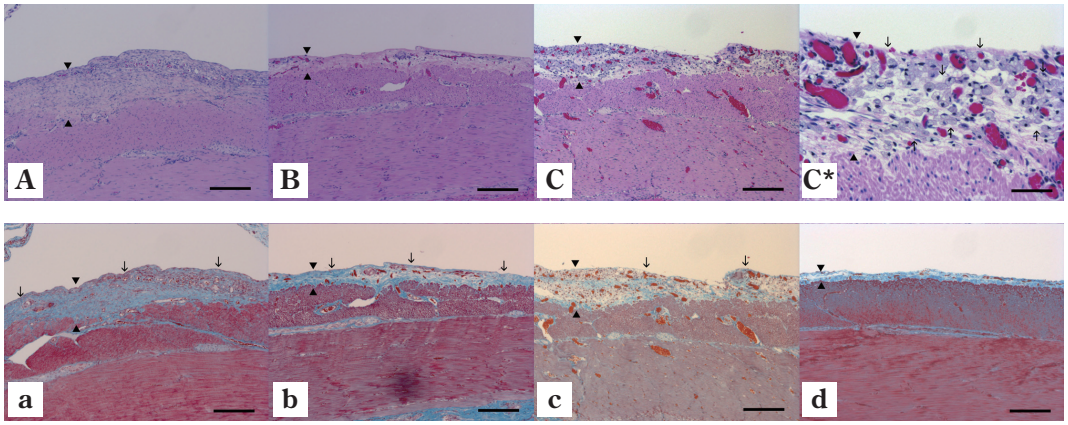

Figure 5. Microscopic views of the abraded sites at three weeks after surgery. The sites were stained with Hematoxylin and Eosin (A, B, C, C*) and Masson's trichrome (a, b, c, d)

A, a: Non-treated (non treatment) group. B, b: GFM group. C, C*, c: Seprafilm® group. d: Normal dog. (Scale Bars: A, B, C, a, b, c, d: 200μm; C*: 50μm) The area between two opposite arrowheads indicates the serosal layer. The arrows indicate foamy cell clusters (C*), and the formation of connective tissue (a, b, c).

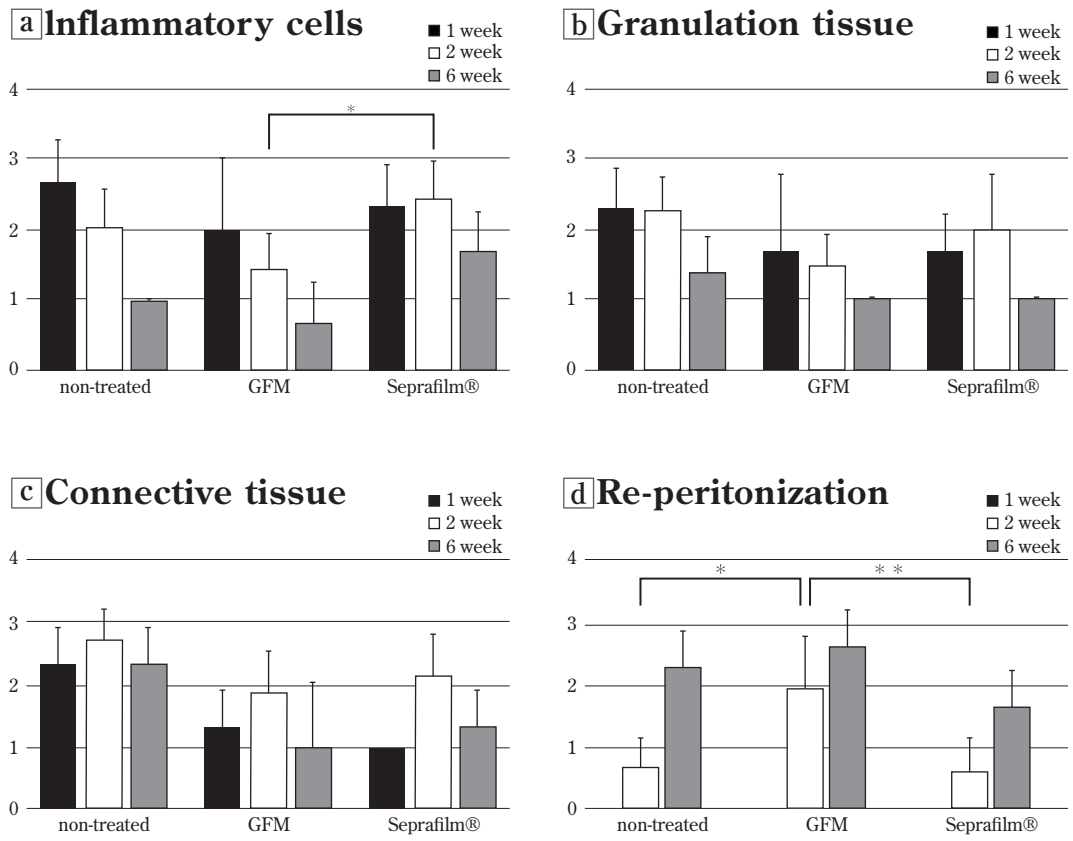

Figure 6. The histological Scores for Inflammatory Cells, Granulation tissue, Connective Tissue and Re-peritonization in the Canine Adhesion Models

a: Inflammatory cells

b: Granulation tissue

c: Connective tissue

d: Re-peritonization

Significant differences are indicateted by *(p<0.05), **(p<0.01)

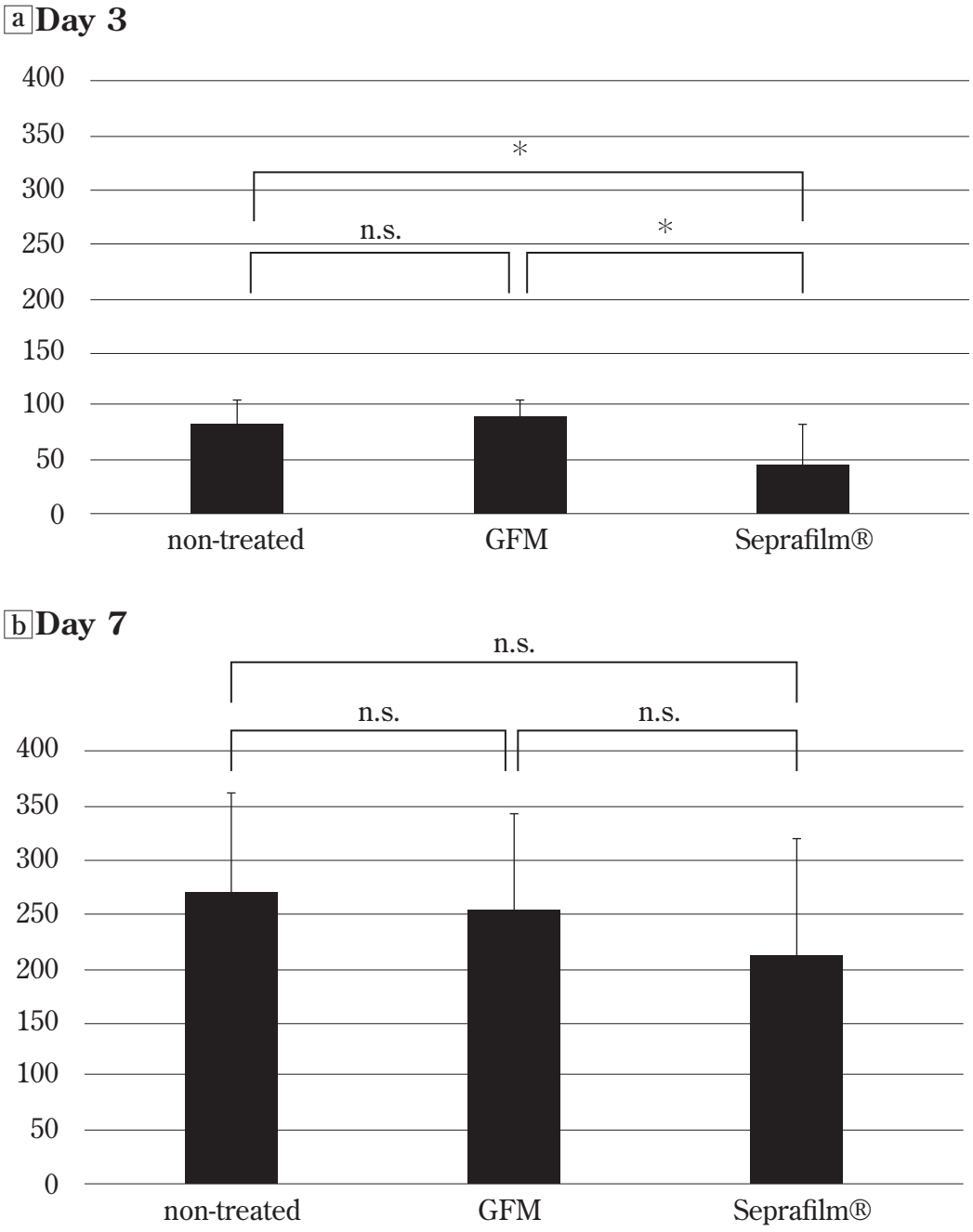

Figure 7. The Bursting Pressure in the Canine Anastomosis Models

a: Day 3 b: Day 7

Significant differences are indicateted by * (p<0.05)

n.s.: not significant

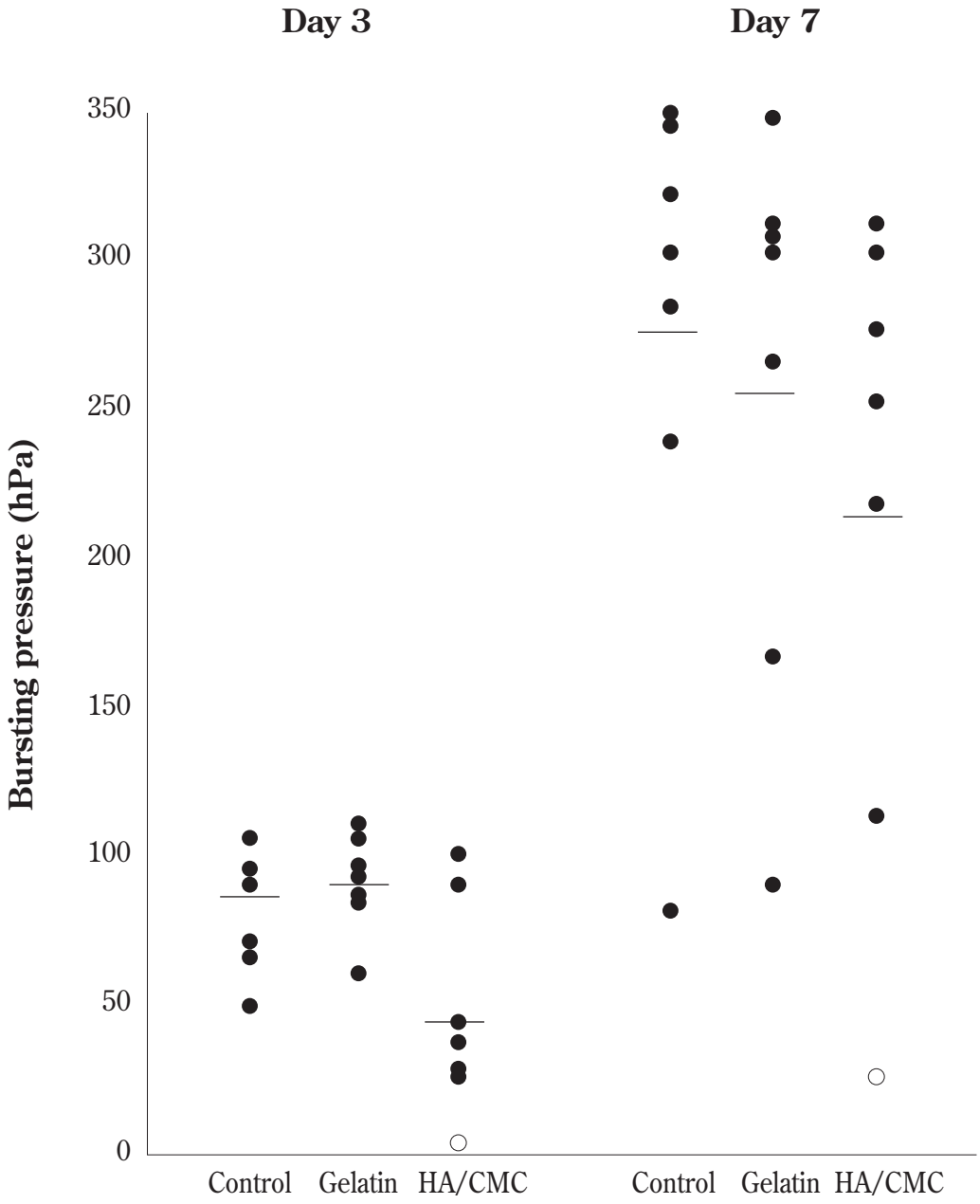

Figure 8. The bursting pressures of the anastomotic sites in dogs treated with GFM, Seprafilm® and non-treatment (non-treated). Horizontal bars represent median values. White dots indicate the cases with anastomotic leakage.

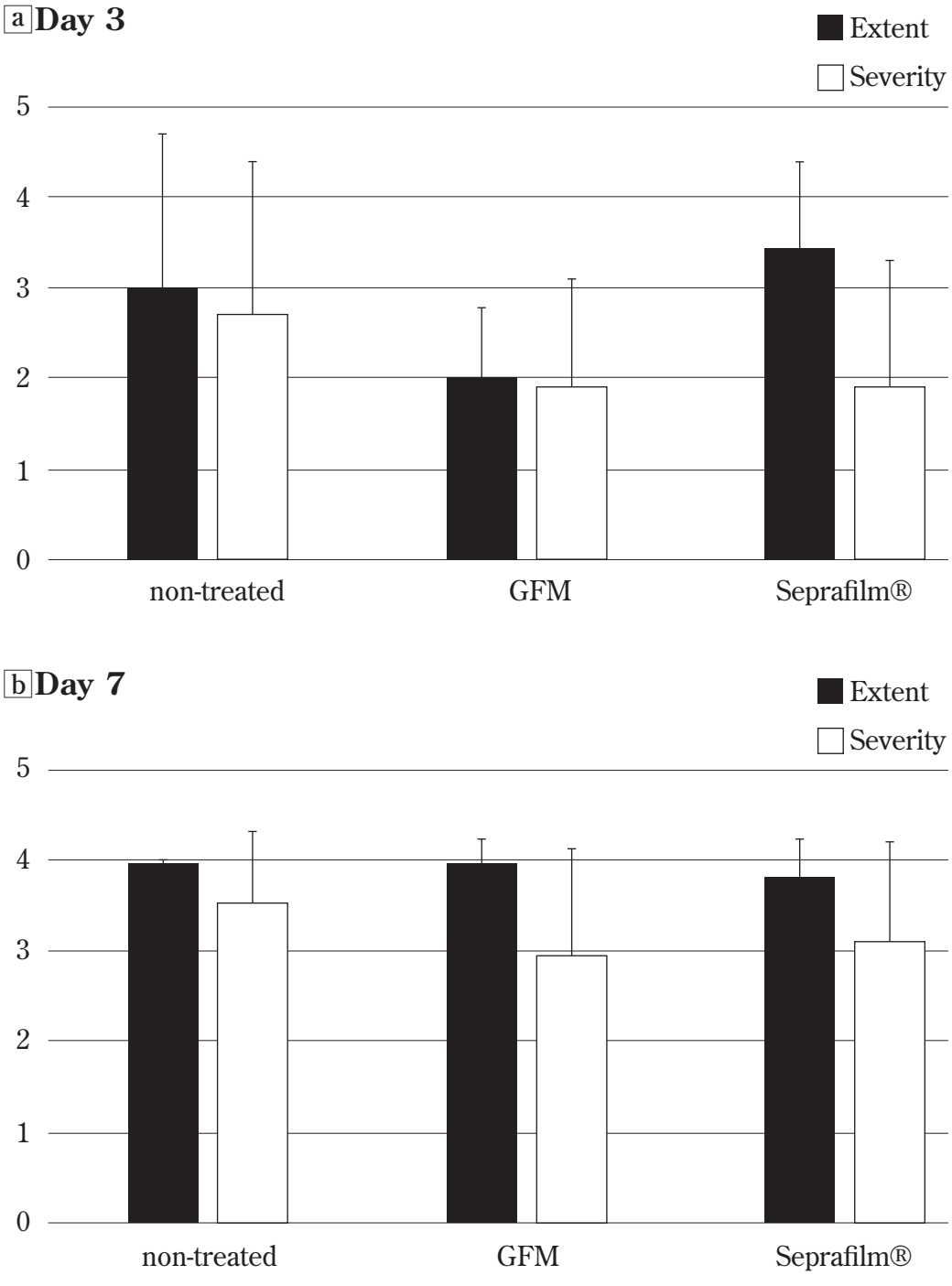

Figure 9. The Adhesion Scores in the Canine Anastomosis Models

a: Day 3 b: Day 7

n.s.: not significant

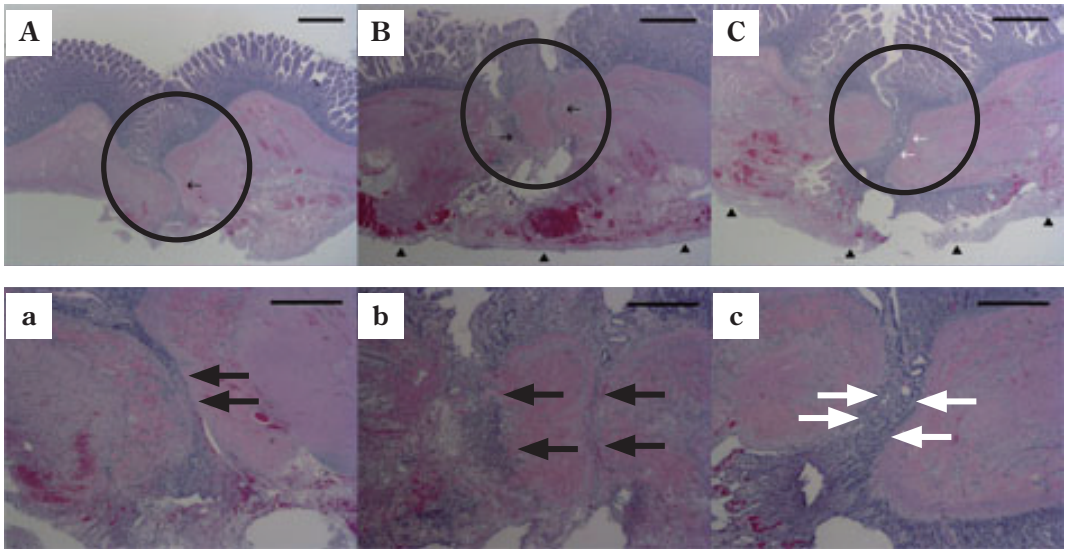

Figure 10. Microscopic views of the anastomotic sites at three days after surgery. The sites were stained with Hematoxylin and Eosin.

A, a: Non-treated (non-treatment) group. B, b: GFM group. C, c: Seprafilm® group. (Scale Bars: A, B, C: 1000μm; a, b, c: 500μm) The arrowheads indicate remaining GFM (B) and Seprafilm® (C). The black arrows indicate numerous inflammatory cells and fibroblasts replacing mucosal tissues (a, b). The white arrows indicate remaining mucosal tissues which are nearly intact (c).

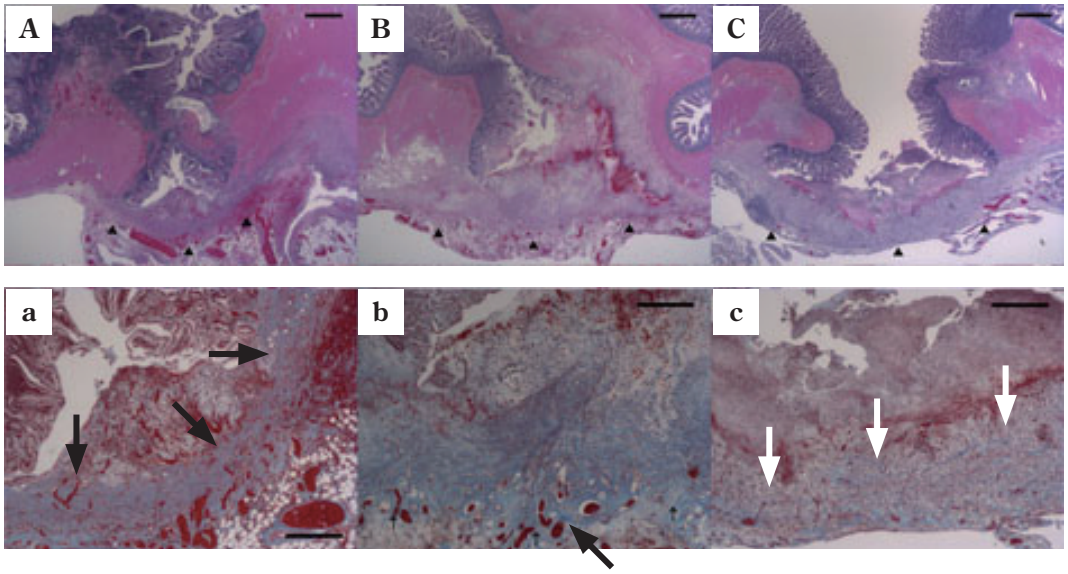

Figure 11. Microscopic views of the anastomotic sites at seven days after surgery. The sites were stained with Hematoxylin and Eosin (A, B, C) and Masson's trichrome (a, b, c)

A, a: Non-treated (non treatment) group. B, b: GFM group. C, c: Seprafilm® group. (Scale Bars: A, B, C: 1000μm; a, b, c: 500μm) The arrowheads indicate massive formation of granulation tissues. (A, B, C). The black arrows indicate extensive formation of connective tissues. (a, b). White arrows indicate less extensive formation of connective tissues (c).

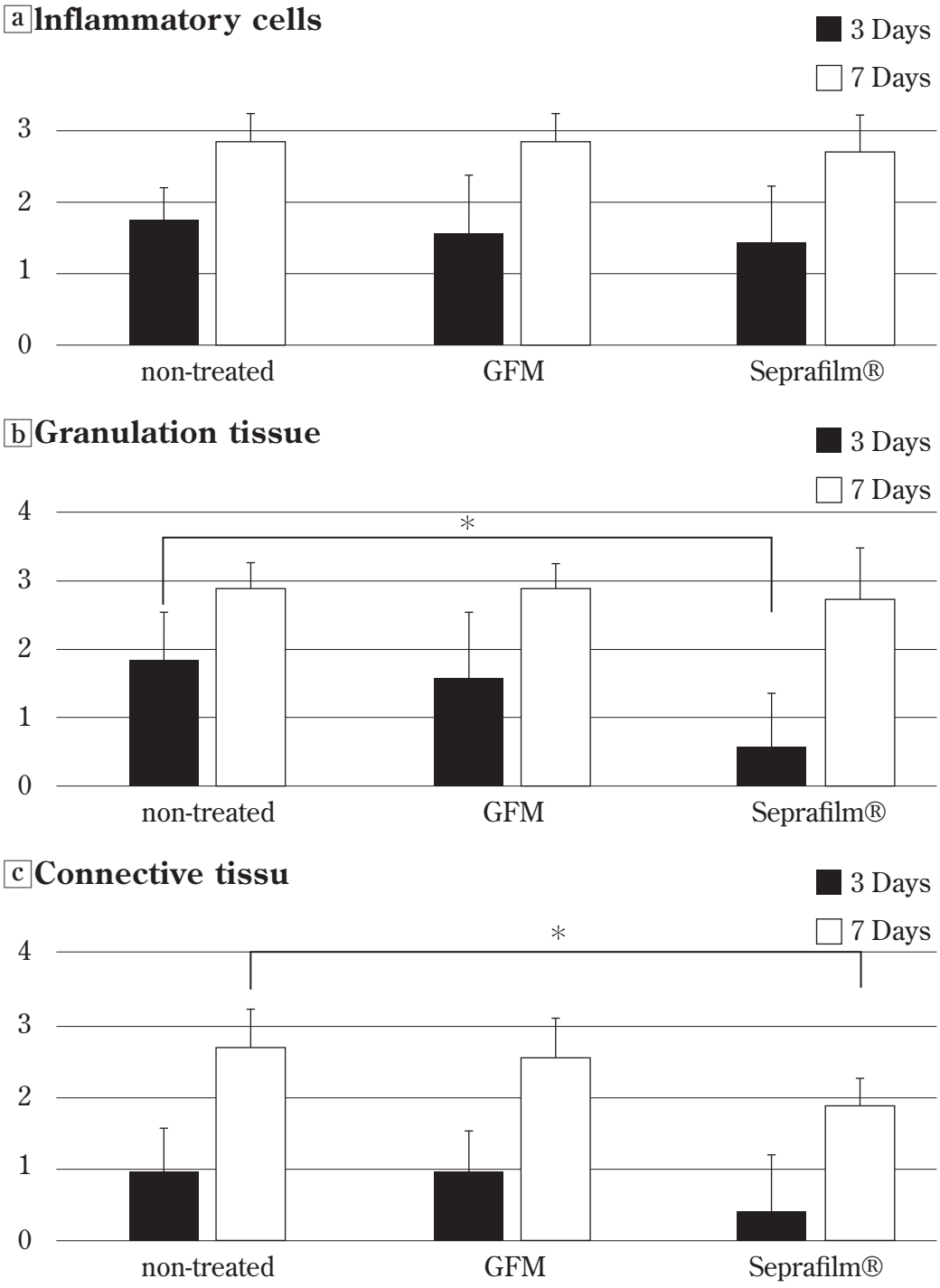

Figure 12. The Histological Scores for Inflammatory Cells, Granulation tissue and Connective Tissue in the Canine Anastomosis Models

a: Inflammatory cells b: Granulation tissue c: Connective tissue
Significant differences are indicateted by * (p<0.05)

STUDY II-1

Hemostatic effects of Two-layered Gelatin Sheet

TABLE OF CONTENTS (STUDY II-1)

ABSTRACT

Background:

Uncontrolled surgical bleeding is associated with increased morbidity, mortality, and hospital cost. Topical hemostatic agents available today have problems controlling hemostatic effects; furthermore, their handling is difficult and they are unsafe.

Methods:

We devised a new hemostatic agent comprising gelatin sponge and film designed to be applied to the bleeding site, thereby creating a topical hemostatic made of gelatin alone. The gelatin was prepared by alkali treatment to eliminate viral activity. Hemostatic effects, surgical handling, and tissue reactions of the materials, namely a two-layered sheet of gelatin, TachoSil®, and gelatin sponge, were evaluated using 21 dogs' spleens.

Results:

The two-layered gelatin sheet and gelatin sponge exhibited superior hemostatic effects (100% hemostasis completed) compared to TachoSil® (0%–17% hemostasis). The gelatin matrix immediately absorbed blood flowing from wounds and activated the autologous components in the absorbed blood that promoted coagulation at the

bleeding site. The two-layered gelatin sheet had the best surgical handling among the evaluated materials. Materials made of gelatin were associated with fewer inflammatory reactions compared to materials of TachoSil®.

Conclusion:

The two-layered sheet of gelatin is a useful topical agent because of its superior hemostatic effects and usability and is associated with a lower risk of transmitting diseases and inflammatory reactions.

ゼラチン2層シートの止血効果の検討

要約

背景：

止血困難な術中の出血は、死亡率、医療費の増加と関連している。現在臨床で使用されている局所止血材は止血効果が十分とは言えず、また操作性、安全性に関しても改善すべき課題がある。

材料および方法：

出血部位に使用するゼラチンスポンジおよびゼラチンフィルムから成る局所止血剤を開発した。ゼラチン２層シート、TachoSil®、ゼラチンスポンジに関して、止血効果、術中の操作性、組織反応を21頭の犬を用い、評価した。

結果：

ゼラチン２層シートおよびゼラチンスポンジは、TachoSil®に比して有意な止血効果を示した。ゼラチン製材は創部からの出血を瞬時に吸収、凝固を促進した。本実験で使用した局所止血材の中で、ゼラチン２層シートが最も術中の操作性に優れていた。ゼラチン製材では、TachoSil®に比べ炎症反応は軽微であった。

結語：

ゼラチン２層シートは、優れた止血効果、操作性、感染および炎症反応の軽減という点において有用な局所止血材であると考えられる。

INTRODUCTION

To non-treated bleeding during surgery, conventional procedures such as ligation, direct compression, electrocauterization, and clipping are used. Topical hemostatic agents are available for treating oozing blood or bleeding from regions difficult to access by conventional methods. Uncontrolled surgical bleeding is associated with increased morbidity and mortality, higher hospital costs, and postoperative adhesions and infections. (1-3) Blood transfusions increase the risk of postoperative complications and have safety issues. Various hemostatic agents have been developed, (3-7) but improvement is needed in terms of efficacy, ease of handling, and safety, especially during laparoscopic surgery.

Among hemostatic materials, TachoSil® (CSL Behring, King of Prussia, PA, USA) is a ready-to-use agent that comprises an equine collagen matrix coated with human fibrinogen and human thrombin. TachoSil® is widely used in many surgical specialties and has proven to be a valuable tool for several indications. (6, 8, 9) TachoSil® has shown clinical superiority in terms of hemostatic efficacy, duration of hospital stay, and postoperative utility for hepatic, cardiac, renal, lung, and pancreatic surgeries compared with conventional surgical procedures. (10-14) However, TachoSil® has problems with intraoperative handling, especially at sites that are difficult to access

and is associated with higher risks of viral infection, other transferable diseases, and allergic reactions resulting from human hemostatic components. (2)

To address the abovementioned problems, hemostatic agents that are safe and easy to handle and have sufficient hemostatic effects are needed. Gelatin-based hemostatic agents with or without fibrin components may be used during surgery. (15-17) However, the hemostatic effects of gelatin matrix are controversial compared to other topical hemostatic agents. (6) Gelatin-based agents without fibrin or thrombin components may be able to solve the problems mentioned above when usability is improved. In the present study, gelatin almost completely eliminated immunogenicity and viral activity by alkali treatment.

The remainder of this article describes the utility of a newly developed topical hemostatic agent, two-layered sheet of gelatin. TachoSil® and the new product were compared in splenic injuries in which hemostasis is not easy to be achieved.

MATERIALS AND METHODS

1. Preparation of hemostatic agents

1.1. Two-layered sheet of gelatin

Low endotoxin gelatin extracted from porcine skins (Type-I collagen, Medigelatin®) with an isoelectric point of 5 was supplied by Nippi Co. Ltd. (Tokyo, Japan). The gelatin was dissolved in distilled water to concentrations of 1.0 and 4.8 wt%. The gelatin 4.8 wt% solution was cast onto a polystyrene petri dish (non-tissue-culture treated; Corning Inc., Tokyo, Japan) and dried overnight on a clean bench at room temperature. The obtained film was slightly cross-linked by exposure to ultraviolet light for 2 min. The gelatin 1.0 wt% solution was cast onto a gelatin film on a petri dish and placed in a deep freezer (MDF-U53V; SANYO Electric Co., Osaka, Japan) at -80℃ for 30 min; it was then freeze dried for 24 h in a vacuum freeze dryer (DRZ350WA; Advantec, Tokyo, Japan) in order to make a gelatin sponge. After freeze drying, a two-layered gelatin sheet composed of gelatin film and sponge layers was removed from the petri dish and dehydrothermally cross-linked in a vacuum oven (DP41; Yamato Scientific Co. Ltd., Tokyo Japan) at 140℃ for 3 h. The two-layered gelatin sheet was cut into square sheets of 30 mm × 30 mm immediately before use. (Figure 1)

1.2. TachoSil®

TachoSil® (CSL Behring, King of Prussia, PA, USA), which is composed of collagen matrix, fibrinogen, and thrombin was used in accordance with the manufacturer's instructions. TachoSil® was cut into square sheets measuring 30 mm × 30 mm immediately before use.

1.3. Gelatin sponge

A gelatin sponge was prepared using the same methods outlined above as a two-layered gelatin sheet without undergoing the process to create a gelatin film (i.e., only a matrix made of sponge layer was used). After freeze drying, the gelatin sponge sheet was removed from the petri dish and dehydrothermally cross-linked in a vacuum oven (DP41; Yamato Scientific Co. Ltd., Tokyo, Japan) at 140℃ for 3 h. The gelatin sponge sheet was cut into square sheets measuring 30 mm × 30 mm immediately before use.

2. Design of animal experiments

The animal experiments performed in this study were approved by the Doshisha University Animal Experimentation Committee. All animal care, housing, and surgical and anesthetic procedures were performed in accordance with the animal care guidelines of the Committee for Animal Research of Doshisha University, Nara Medical

University and European Commission Directive 86/609/EEC for animal experiments.

Twenty-one non-pregnant, female, two-year-old beagles weighing 9.5-10.5 kg were purchased from Shimizu Laboratory Animal Supply Co. Ltd. (Kyoto, Japan). During the experimental period, all dogs were housed separately and maintained under standard conditions (a light–dark cycle of 12:12 h, mean temperature of 23℃, and mean humidity of 50%). Standard laboratory dog chow and water were freely available. Before the study, the dogs were housed in the laboratory for 1 week. On the first day of the experiment, the health condition of all dogs was assessed.

3. Surgical procedure to evaluate the effects of hemostatic agents

All surgeries were performed under sterile conditions, by a team of three persons. The dogs were randomly assigned to one of three groups corresponding to each hemostatic material. Eighteen dogs were anesthetized with intravenous sodium pentobarbital (Somnopentyl® Kyoritsu Seiyaku, Tokyo, Japan) (34 mg/kg). A 12-cm epigastric median incision was made. The surface of the upper or lower part of the spleen was reduced with scissors to 1-2 mm in depth and 20 mm x 10 mm in area and allowed to bleed. Immediately after wiping blood from the spleen with gauze, one of three hemostatic

materials (two-layered gelatin sheet, TachoSil®, gelatin sponge) was applied over the cut surface. Then, the hemostatic material was covered with gauze and digital pressure applied over it for 1 or 5 min; the gauze was removed gently so that immediate bleeding and rebleeding (bleeding after bleeding had stopped) could be observed during a 5-min observation period. The physical status of the dogs remained stable during surgery. To exclude arbitrary procedure by operators or differences of conditions among experimental groups, the operators were blinded to the material being applied until astriction. Random study was scheduled for the experiments. This experiment was performed on six dogs for each hemostatic material. The time of rebleeding was recorded for each material.

Hemostatic effects were evaluated during an observation period of 5 min because the standard range in clinical tests using Duke's method is 1-5 min bleeding time. When blood was observed flowing out of the materials during the observation period, hemostasis was assessed as having been broken (rebleeding) and the time of rebleeding was recorded. (Figure 2) The hemostatic materials were also evaluated for handling and ease of use during surgery.

4. Histological observation of implanted material and surrounding tissues

In order to observe histological change at the site of astriction, we

examined three other dogs. The surfaces of the dogs' spleens were shaved to a size of 1 cm × 1 cm under general anesthesia, and bleeding was confirmed; astriction was performed for a minute with gauze. One of the three hemostatic agents was then applied to the wound and pressed for more than 5 min with gauze over the material. After hemostasis was confirmed, the abdomen was closed. The whole spleens were excised 2 weeks after the initial operation under inhalation anesthesia. The parts of the spleen that had been covered with each hemostatic material were resected, fixed with formalin, and stained with hematoxylin and eosin. The tissue response to the materials and remaining of materials were observed

5. *In vitro* experiments for absorbency and permeability

The absorbency and permeability of each hemostatic material was evaluated with canine blood. Hemostatic materials were cut in 1 cm × 1 cm squares. Single drops of 80 μl blood were placed by pipette on the hemostatic materials from a height of 1 cm. Absorption time was recorded as the time when the droplet disappeared from the material surface. Permeability was recorded as the time when the blood was exuded onto the material base.

6. Statistical analysis

The data are expressed as the mean ± standard deviation. Statistical

analyses were carried out using the Kruskal-Wallis test and the Chi-square test with Stat / Mate III, Windows (ATMS Co., Tokyo, Japan). *P* values less than 0.05 were considered statistically significant.

RESULTS

1. Hemostasis effects in animal experiments

Table 1 shows the results for the three groups of hemostatic agents. In the TachoSil® group, hemostasis was not achieved in three of six spleens after a minute's duration of compression of the bleeding site. In all the other spleens applied with TachoSil®, rebleeding was observed during the 5-min observation period after decompression. With respect to the other two groups, initial hemostasis was achieved after a minute of astriction and rebleeding was not seen in any of the six spleens during the observation period. There were significant differences between the TachoSil® group and the two other groups ($p<0.001$).

In all groups, hemostasis was successfully achieved after 5 min of compression. In five of six spleens in the TachoSil® group, rebleeding within 5 min was observed. In gelatin sheet and gelatin sponge groups, there was no rebleeding observed throughout the observation period. (Table 1)

2. Absorbency and permeability of materials *in vitro*

The absorbency of the two-layered gelatin sheet (104 ± 115 s) and the gelatin sponge (277 ± 117 s) were significantly higher than that of TachoSil® (above 360 s; $p < 0.05$). Both the two-layered gelatin sheet and TachoSil® had significantly ($p<0.05$) low permeability (333 ± 61 s, above 360 s, respectively). The gelatin sponge had high permeability. That meant the two-layered gelatin sheet had high absorbency and low permeability. (Figure 3)

3. Usability of the materials during surgery

Blood from the spleen surface infiltrated the TachoSil® and gelatin sponge and reached the compression gauze. The two kinds of gelatin preparations showed good adhesive properties because their sponge layer became an adherent gel after absorbing blood. In contrast, TachoSil® was less adhesive after being soaked in blood and showed no gel formation. The gel layer, composed of gelatin sponge and blood, attached tightly to the compression gauze, but it became difficult to remove the gauze from the material surface in the gelatin sponge group. On the other hand, the gelatin "film" layer was impermeable, and the blood from the wound did not reach the compression gauze. It was easy to detach the gauze without removing the gelatin sheet from the wound. (Figure 4)

4. Histological findings regarding embedded materials

There was strong invasion of inflammatory lymphocytes noted in the injured part of the spleen embedded with TachoSil®. The collagen materials of TachoSil® was still remained in 2 weeks. In the gelatin groups, inflammatory change was absent (Figure 5) and re-epithelization was observed in 2 weeks. The gelatin materials was absorbed almost completely in two weeks. The wound healing in gelatin group was better than in TachoSil® group.

DISCUSSIONS

Our experiments showed that the two-layered gelatin sheet has superiority over TachoSil® in terms of hemostatic effects, utility, and safety in canine experiment.

1. Hemostatic effect

Topical hemostatic agents are used for the non-treated of bleeding during surgery. Among the topical agents available, TachoSil® has been shown to have superior hemostatic effect in various kinds of surgery. (9, 11-14, 20) In contrast, TachoSil® was less effective in sites where a relatively large quantity of bleeding was seen because of weak adhesion to the bleeding site. (9) The hemostatic effect of

gelatin matrix is still controversial, (3, 7, 15, 18, 22) but most of the studies we found showed that gelatin had a poor hemostatic effect compared to TachoSil®. This prompted us to develop a two-layered gelatin sheet to solve these problems.

The physical properties of gelatin matrix contribute to superior hemostatic effects compared to collagen matrix (TachoSil®). In our study, the gelatin sponge layer smoothly and rapidly absorbed blood and activated autologous blood-coagulating components in blood. (Figure 2) The sponge matrix changed to gel, which covered bleeding sites tightly. Moreover, the gelatin film layer of the two-layered gelatin sheet inhibited the permeation of blood, consequently strengthening adhesive bonding to the bleeding site. By contrast, the reduced permeability of the collagen sponge probably permitted continuous bleeding from the site, and subsequent bleeding or oozing disturbed the hemostatic effects of fibrinogen and thrombin because of the blood flowing out. The two-layered gelatin sheet absorbed blood flowing into the material and coagulation occurred quickly, thereby allowing fibrin to bind the sheet and wound tightly. After hemostasis was completed, the hemostatic effects were not necessary. The thickness of the two-layered gelatin sheet was thought to be enough to stop bleeding in this study. On the other hand, TachoSil® absorbed flowing blood more slowly and coagulation inside TachoSil® and on the surface of the material took longer to complete. Regarding the

rebleeding, flexibility and adherence of the materials were thought to be more important than absorbency. The two-layered gelatin sheet's ability to follow the shape of the wound surface is superior to that of TachoSil®. The gelatin sheet can adhere to the tissue tightly after compression, but TachoSil® leaves a small gap after astriction. Rebleeding can occur from a gap between the material and the wound.

In our study, we set the time for manual compression at 1 or 5 min to compare the different hemostatic materials. In past studies, compression time was set at longer than 3 min. Compared with the other materials, the initial hemostasis of TachoSil® took longer and was incomplete, resulting in more frequent instances of rebleeding. The two-layered gelatin sheet required a shorter time to complete hemostasis and the hemostatic effects were so secure that no rebleeding was found in any of the spleens tested. Overall, the hemostatic effect of the two-layered gelatin sheet appears to be superior to TachoSil® in sites where a relatively large quantity of bleeding is seen (e.g., spleen, pelvic floor, blood flow-rich parenchymal organs). (7, 19, 21) In the present study, we evaluated the hemostatic effects of three materials on a bleeding parenchymatous organ where bleeding is notoriously difficult to non-treated. Conventional techniques for hemostasis and TachoSil® are effective in cases where there is a small amount of bleeding and in

sites where bleeding is easy to non-treated. A two-layered gelatin sheet is possible choice for hemostasis in cases where there is a relatively large amount of bleeding.

2. Handling

The nonadhesive property of a gelatin "*film*" layer works more effectively to prevent bleeding immediately after the release of gauze compression. TachoSil® and the gelatin sponge have a permeable layer and thus are disadvantageous for surgical handling because strong adhesion to compression gauze is more likely with the contact of blood and gauze, which may induce rebleeding after detachment. TachoSil® is probably effective in cases where blood does not reach the compression gauze. The two-layered gelatin sheet and TachoSil® present problems for laparoscopic surgery because they are both difficult to place into the abdominal cavity through a port. Gelatin materials must undergo refinements in order to be suitable for laparoscopic surgery. We are reforming gelatin materials for use during laparoscopic surgery.

3. Safety

Hemostatic agents remain in the body after surgery, so that long time safety is very important issue. Excessive inflammatory response of the tissue by hemostatic materials can cause adhesion. Moreover,

long time residual of the material possibly make the risk of infection higher. In the present study histological examination was carried out in order to observe the tissue response to each the material, not to examine the hemostatic effects of each material. Earlier regeneration of the peritoneum and earlier absorption of the material were observed in two-layered gelatin sheet. On the other hand, TachoSil® induced intensive inflammatory response at the implanted site and remained longer than gelatin sheet. Regarding adhesion two-layered gelatin sheet is possibly safer than TachoSil®. (24-26) In our unpublished study (under submission) the two-layered gelatin sheet has anti-adhesive effect due to early regeneration of the peritoneum and mild inflammatory response.

The two-layered gelatin sheet, which is composed of alkaline-treated gelatin, does not carry the same risks as biomaterials (TachoSil®), such as transmission of viral infection and allergic reactions. In fact, the gelatin alkali-treatment process eliminates the risk of transmittable diseases almost completely. (23) Moreover, from the skin of animals no infectivity of Bovine Spongiform Encephalopathy was not detected.

CONCLUSION

We showed that the two-layered gelatin sheet is a more effective, easier to handle, and safer topical hemostatic agent than TachoSil®, which is one of the most popular materials currently in use. The two-layered gelatin sheet is safer than topical agents including fibrin components and/or thrombin in terms of risk of viral transmission and inflammatory reactions. We showed the hemostatic effectiveness of a two-layered gelatin sheet in bleeding dog spleens (an organ where bleeding is difficult to non-treated). The efficacy of newly developed materials should be evaluated by application to other organs (liver, kidney, lung, and vessels) and in humans.

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Table 1. Comparison of the hemostatic effects of TachoSil®, a two-layered gelatin sheet and a gelatin sponge in bleeding dog spleens.

Hemostatic material	Positive for bleeding **(A)**/Total experiments		Positive for **re**bleeding **(B)**/Total experiments		Positive for bleeding **(A + B)**/Total experiments		
Compression time	1 min	5 min	1 min	5 min	1 min	5 min	
Two-layered gelatin sheet	0/6	0/6	0/6	0/6*	0/6*	0/6*	*
TachoSil®	3/6	0/6	3/6	5/6	6/6	5/6	*
Gelatin sponge	0/6	0/6	0/6	0/6*	0/6*	0/6*	

(A) Bleeding just after 1 or 5 minutes of compression
(B) Rebleeding happened during 5-minute observation period after 1 or 5 minutes of compression
*: significantly (p<0.001) different compared to TachoSil®

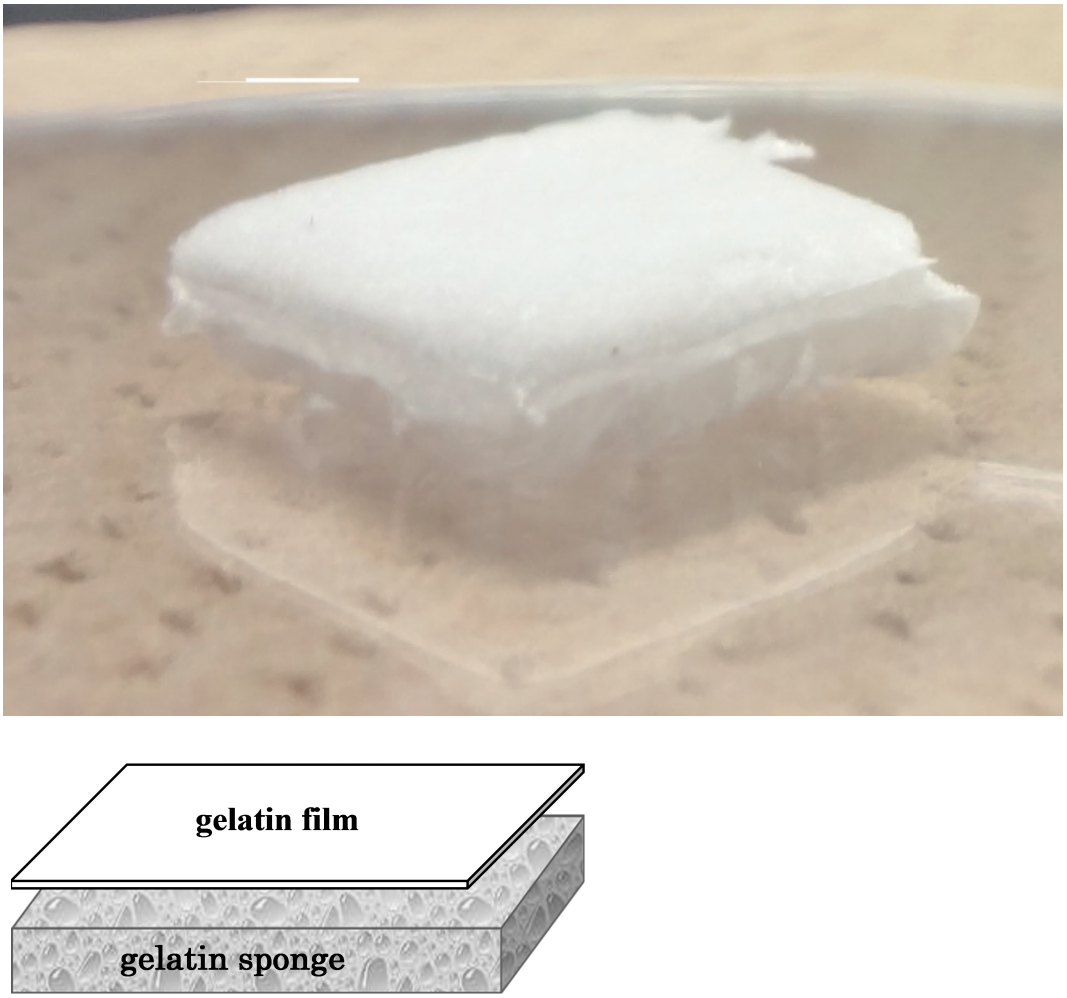

Figure 1. Two-layered gelatin sheet

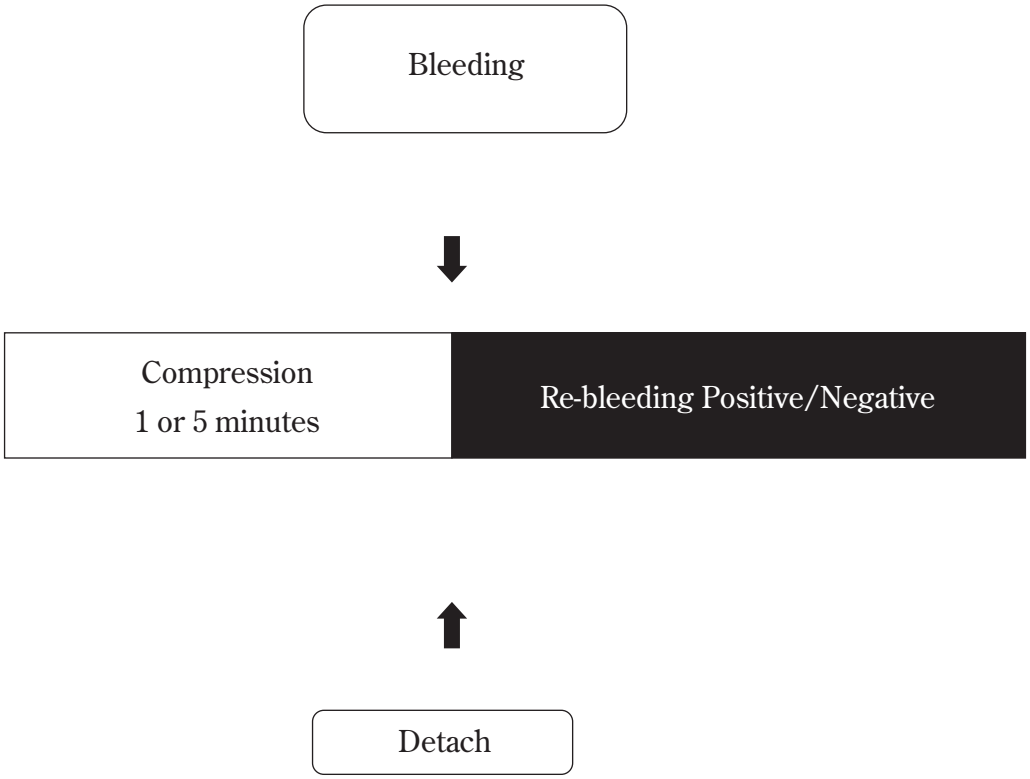

Figure 2. Schematic diagram of the experiment

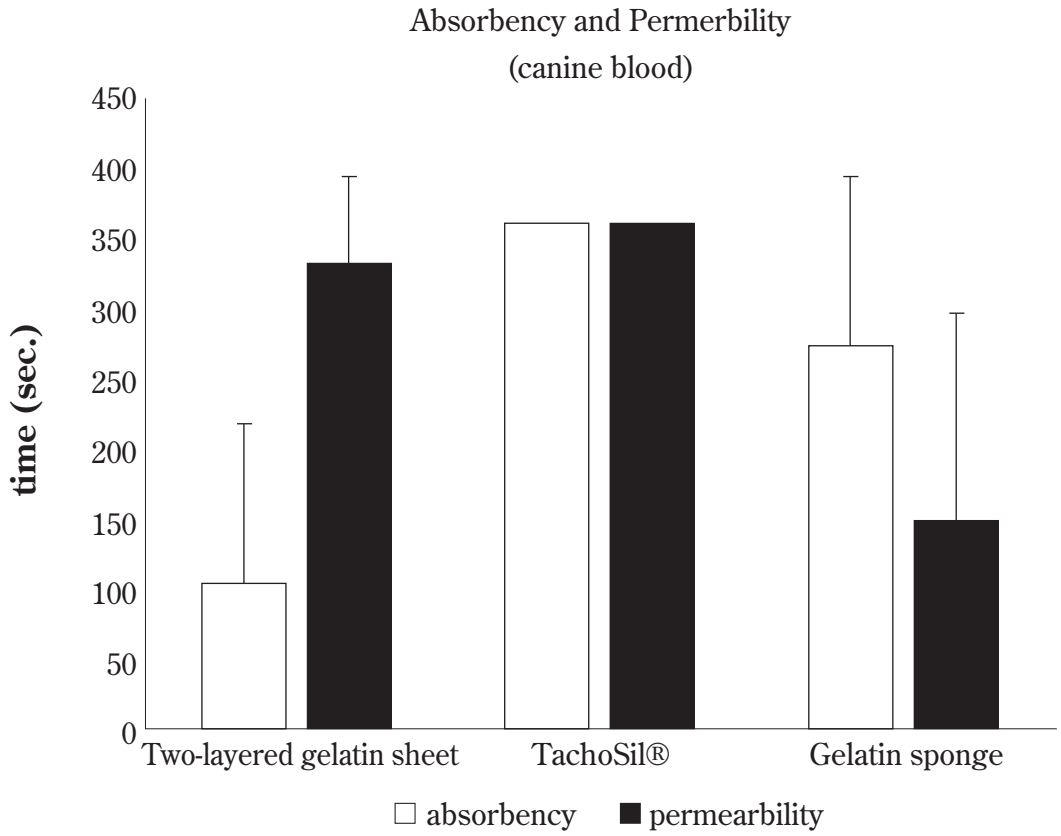

Figure 3. Absorbency and permeability of the materials

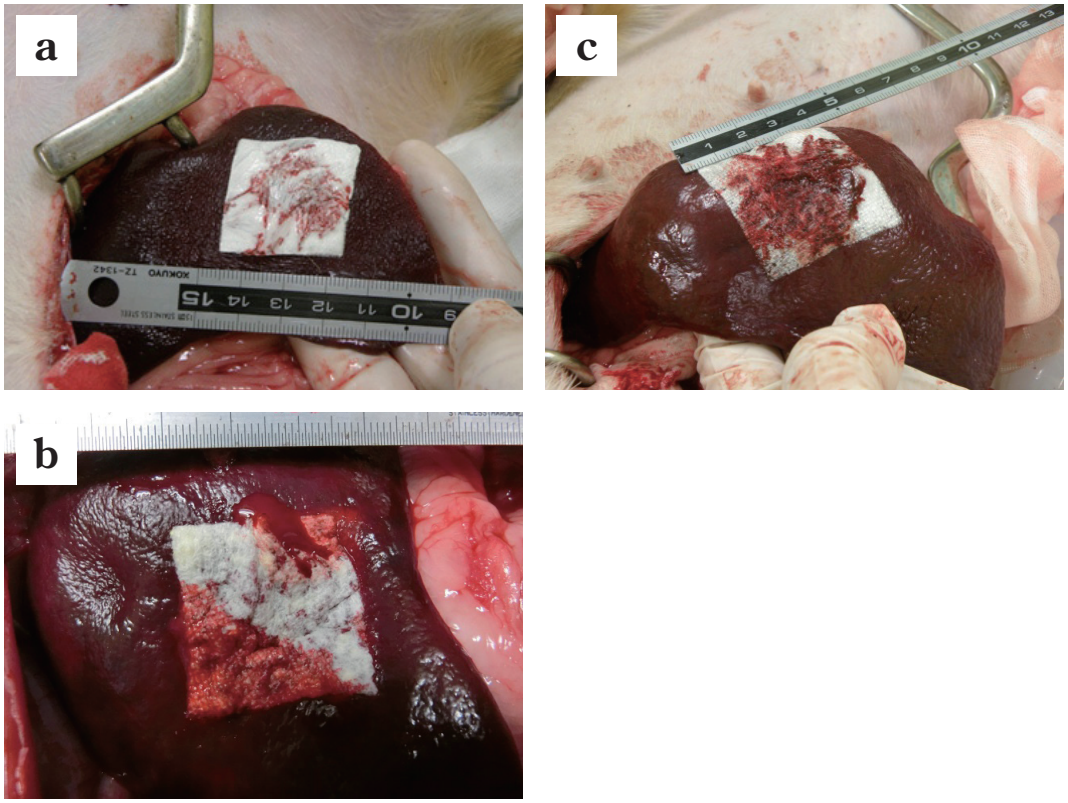

Figure 4. Hemostatic agents set onto the shaved surface of the spleen.

a. Two-layered gelatin sheet; b. TachoSil®; c. Gelatin sponge

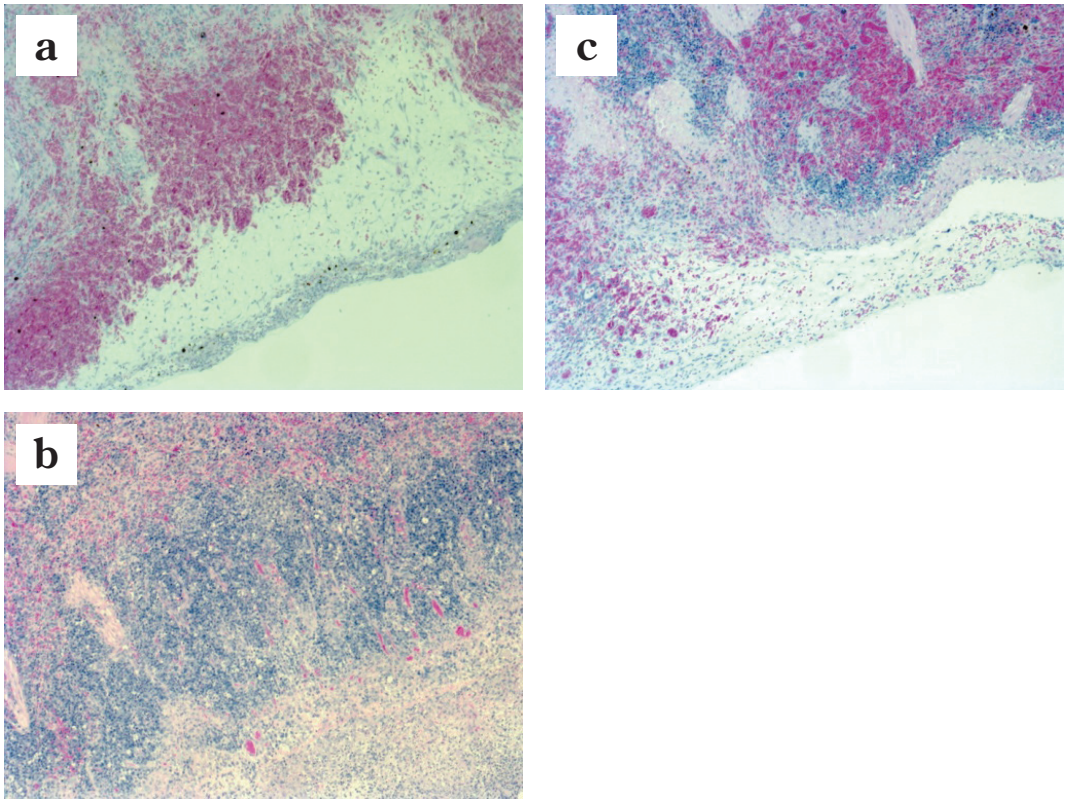

Figure 5. Histologic findings regarding embedded materials

a. Two-layered gelatin sheet; b. TachoSil®; c. Gelatin sponge

STUDY II-2

Anti-adhesive Effects of the Newly Developed Two-layered Gelatin Sheet in Dogs

TABLE OF CONTENTS (STUDY II-2)

ABSTRACT

Background:

The adhesion after pelvic surgery causes infe341rtility, ectopic pregnancy, ileus, or abdominal pain. Any materials available in clinical use have insufficient effects. Anti-adhesive effects of newly developed two-layered gelatin sheet were evaluated compared with conventional agents.

Methods:

Two-layered sheet composed of gelatin film and gelatin sponge, Seprafilm® and INTERCEED® were evaluated for the adhesion prevention in 37 dogs. Anti-adhesive effects were investigated macro- and microscopically with cauterized uterus adhesion model. Cell growth on the materials *in vitro* using human peritoneal mesothelial cells, fibroblasts, and uterine smooth muscle cells were also evaluated.

Results:

The two-layered gelatin sheet had significantly superior anti-adhesive effects to the conventional materials (Seprafilm® and INTERCEED®). A single-cell layer of matured mesothelium was formed in 3 weeks after surgery in the gelatin group. Peritoneum

regeneration in the Seprafilm® and INTERCEED® groups was delayed and incomplete in early phase. Inflammation around materials was week on the gelatin sheet compared to the conventional agents. Cell growth of the three cell lines were proliferated significantly on the gelatin sheet than the others.

Conclusions:

The anti-adhesive effects of two-layered gelatin sheet is superior to the conventional agents in cauterized uterus model. Early regeneration of the peritoneum, weak inflammation, and longtime remain of the material contributes the results. Newly developed two-layered gelatin sheet is considered to be a useful option or anti-adhesive agent in deeply injured and hemorrhagic sites.

大動物実験における
新規ゼラチン2層シートの癒着防止効果の検討

要約

背景：

骨盤内手術の術後癒着は不妊症、異所性妊娠、腸閉塞、腹痛などの原因となり得る。現在臨床で使用されている癒着防止材は効果が十分とは言い難い。新規癒着防止材として開発したゼラチン２層シートの癒着防止効果を従来製材と比較検討した。

材料および方法：

ゼラチンフイルムおよびゼラチンスポンジから成るゼラチン２層シート、Seprafilm®、INTERCEED®を37頭の犬を用い癒着防止効果について比較した。癒着防止効果は子宮を焼灼した癒着モデルを使用し、肉眼的、組織学的に評価した。*In vitro*では、ヒトの腹膜中皮細胞、線維芽細胞、平滑筋細胞を用い、各材料上での細胞増殖試験を行った。

結果：

ゼラチン２層シートは、従来の癒着防止材（Seprafilm®、INTERCEED®）に比して有意に優れた癒着防止を示した。ゼラチン２層シート群では術後３週間で１層の成熟した中皮が形成された

のに対し、Seprafilm®、INTERCEED®の腹膜再生は初期段階では不完全であった。製材周囲の炎症反応はゼラチン２層シートにおいて軽微であり、また、細胞増殖試験でもゼラチン２層シート上では従来製材に比して有意に細胞が増殖した。

結語：

子宮を焼灼した癒着モデルにおいてゼラチン２層シートは従来製材よりも有意に優れた癒着防止効果を示したが、これは、短期間でのの腹膜再生、炎症反応の軽減、材料が長期間残存すること、などに起因すると考えられる。ゼラチン２層シートは深い創部や出血部位に対する癒着防止材として、有用な選択肢であると考えられる。

INTRODUCTION

Adhesions in the intra-pelvic organs such as the uterus, the ovary, and the fallopian tube are common and serious complications following surgeries, endometriosis, and intra-pelvic inflammations. Abdominal surgery induces adhesions at around 90% probability. (1-3) The adhesion causes infertility, ectopic pregnancy, ileus, and abdominal pain. (4, 5) Adhesion accounts for 15-40% of infertility, (1-3) and the pregnancy rate in patients with female infertility after surgery is 38%~ 65%. (4) Various kinds of anti-adhesive agent are proposed to prevent adhesion after surgery, but several controversies such as the effectiveness, usability, and indication of available agents still exist. (5, 6)

Now in gynecological surgery hyaluronic acid carboxymethyl cellulose membrane (Seprafilm®) and Oxidized regenerated cellulose (INTERCEED®) are frequently used. However, those conventional anti-adhesive agents are pointed out some problems. Several studies show Seprafilm® decreases severity of adhesion but not incidence in human, (3, 7) and Seprafilm® had no effects to prevent pelvic adhesion in woman in other reports. (8) INTERCEED® have reported superior efficacy to Seprafilm® in pelvic surgery, but has several limitations. Blood infiltration renders the product completely ineffective in preventing adhesions. (9, 5, 13) INTERCEED® provoke

a large leucocyte response, and inflammatory response may enhance adhesion. (10, 11) We devised a new anti-adhesive agent, named two-layered gelatin sheet composed of thermally cross-linked gelatin lm and sponge. The two-layered gelatin sheet was evaluated for the anti-adhesive effects after pelvic surgery in dogs.

MATERIALS AND METHODS

1. Animal protocol

Animal experiments in this study were approved by the Nara Medical University Experimentation Committee and the Doshisha University Animal Experimentation Committee. All surgical procedures and anesthesia were performed in accordance with the Animal Care Guidelines of Nara Medical University. Thirty seven female beagles (2 years old and weighing 9.9 ± 0.9 (mean ± SD) kg) were purchased from Shimizu Laboratory Animal Supply Co. Ltd. (Kyoto, Japan). Before the study the dogs were housed in the laboratory at least for a week. During the experimental period, all dogs were housed separately and maintained under standard conditions light-dark cycle of 12:12h, mean temperature of 23℃, and mean humidity of 50%. Standard laboratory dog chow and water were available freely. On the experimental day, all dogs were checked for

the condition of their health.

2. Preparation of anti-adhesive agents

2.1. Two-layered gelatin sheet

Low endotoxin gelatin extracted from porcine skins (type-I collagen, Medigelatin®, Nippi Co. Ltd. Tokyo, Japan) was dissolved in distilled water into the concentrations of 1.0 and 4.8 wt%, respectively. The 4.8 wt% gelatin solution was casted onto a polystyrene Petri dish (not treated for tissue-culture, Corning Inc., Tokyo, Japan; Non treated surface dish can avoid the gelatin sheet to detach to the plastic and better detach the sheet.) and dried at room temperature overnight. A gelatin film was processed. The gelatin film was cross-linked by UV light for 2 min. The 1.0 wt% gelatin solution was casted onto gelatin film on the Petri dish, and frozen in a deep freezer (MDF-U538, SANYO, Osaka, Japan) at -80℃ for 30min. It was freeze-dried for 24h in a vacuum freeze-dryer (DRZ350WA, ADVANTEC, Tokyo, Japan) and the gelatin solution (1.0 wt%) should turn into sponge. The gelatin sponge was formed over the gelatin film. A two-layered gelatin sheet composed of a gelatin film (10μm thickness) and a sponge layer (1mm thickness) was taken out from the Petri dish and cross-linked dehydrothermally in a vacuum oven (DP41, Yamato Scientific Co. Ltd., Tokyo Japan) at 140℃ for 3h. The sheet was sterilized by ethylene-oxide gas (0.43g/L at 40℃ for 4h) for

animal experiments.

2.2. Seprafilm®

Seprafilm® (HA/CMC) (Genzyme Co., Cambridge, MA, USA) which is widely used anti-adhesive material is composed of two anionic polysaccharides, sodium hyaluronic acid (HA) and carboxymethyl-cellulose (CMC).

2.3. INTERCEED®

INTERCEED® (TC7) (Ethicon, CA, USA) made of oxidized regenerated cellulose is commonly used in gynecological surgery. INTERCEED® is an absorbable knitted fabric.

3. Surgical procedure

All procedures were performed responsible for one team of two gynecologists and two surgeons blinded to the experimental assignment. Operations were done under sterile conditions. Under intravenous pentobarbital (40mg/kg of body weight) anesthesia, the uterus, the ovaries, the fallopian tubes were recognized to be normal after 5cm midline incision at the lower abdomen. One side of the uterus horns was cauterized 40mm long near circumferentially, reaching the surface of the muscle layer with electric scalpel (Hyfrecator®2000, CONMED, NY, USA). Hemostasis was confirmed

after astriction.

28 dogs were divided into 4 groups at random: 1) non-treated group, 2) two-layered gelatin sheet group, 3) Seprafilm® group, 4) INTERCEED® group. Besides the evaluation of adhesions 9 dogs were taken the same treatment at both sides of the uterus horns for histological evaluation. Each anti-adhesive agent was applied onto the cauterized site of the uterus horn (two-layered gelatin sheet, Seprafilm®, or INTERCEED®, 50 × 75 mm in size), wrapping the site entirely without suturing. The sponge side of the two-layered gelatin sheet was placed onto the traumatized tissue. After these procedures the wound was closed by layers. All dogs were bred under the standard conditions, and killed humanely with intravenous pentobarbital (100mg/kg of body weight) on 3weeks or 6weeks after the surgery.

The 28 dogs killed on day 42 were subjected to macroscopic and microscopic evaluations of adhesion, and the 9 dogs (3 dogs in each treated group) were subjected to the only microscopic evaluation of adhesion 3 weeks after surgery.

4. Evaluation of adhesion

At autopsy dogs received full abdominal midline incision, and adhesion of the cauterized site of the uterus to the other abdominal organs was evaluated macroscopically. A score was put on the degree

of 1-4 regarding extent or severity of adhesion, (Table 1) which is modified from the Adhesion Score of the Surgical Membrane Study Group. (12) The evaluation was performed by two doctors (one gynecologist and one surgeon) who were blinded to the animal and treatment assignments.

5. Histological analyses

The cauterized sites of the uterus were analyzed microscopically. These specimens were fixed with 10% buffered formalin, and processed for embedding in paraffin and stained with hematoxylin and eosin (H/E). The examiners were blinded to the materials in the histological evaluation.

6. Cell Growth

6.1. Preparation of materials

Two layered gelatin sheet, Seprafilm®, and INTERCEED® were cut into 15 mm diameter circles. INTERCEED® is constituted of mesh structure. On two-layered gelatin sheet two groups were set that sheets were placed film side up and sponge side up. In order the materials covered the bottoms of the wells completely, two sheets of INTERCEED® were stacked to make stitches alternative. The materials covered the bottoms of the wells completely. The wells in the non-treated group were not covered by any materials.

6.2. Cell lines

Human mesothelial cells (MSE-F, Zen-Bio Inc., Research Triangle Park, NC, USA) were cultured in mesothelial cell growth medium (MSO-1, Zen-Bio). Human fibroblasts (NHDF-AD-Der Fibroblasts FGM-2, Lonza Japan, Tokyo, Japan) were maintained in Dulbecco's modified Eagle medium (D-MEM, Wako Pure Chemical Industries, Ltd. Osaka, Japan) containing 10% fetal bovine serum. Human uterine smooth muscle cells (HUt-SMC) (PromoCell, Heidelberg, Germany) was maintained in the medium suggested by the vendor (PromoCell,) with 5% fetal bovine serum, 0.5% epidermal growth factor, basic fibroblast growth factor (BFGF) and Insulin (maintenance medium). The cells were cultivated in an incubator at 37℃ with 5% CO_2 under humid conditions for 7 days. Twenty four-well ultra-low attachment surface plates (CORNING, NY, USA) for the materials, and 24-well culture plates without any coating (Becton, Dickinson and Company Ltd. Franklin Lakes, NJ, USA) for the non-treated were used. The cultured cells were harvested in the form of a single cell-suspension. The cell suspension containing 1.0×10^4 cells/well was poured into the wells. After 7 days cultivation viable cells' number in each well was counted with the ATP assay using ATPlite Kit (Perkin Elmer, Inc. Waltham, MA, USA).

7. Statistical analyses

Anti-adhesive effects were analyzed using Kruskal-Wallis H test and Man-Whitney V test with Bonferroni correction. One-way analysis of variance (ANOVA) and Tukey test as a post hoc-test for the cell growth. *P* value of <0.05 was determined to be statistically significant.

RESULTS

1. Evaluation of the adhesion

Adhesion scores at 6 weeks after surgery were shown in Figure 1a, 1b. With regard to the extent of adhesion, the scores (means ± SD, n = 7 in each group) were 3.6 ± 1.0 in the non-treated group, 0.3 ± 0.7 in the gelatin sheet group, 2.9 ± 1.4 in the Seprafilm® group, and 2.7 ± 1.3 in the INTERCEED® group, respectively. The score in the gelatin sheet group was significantly (p<0.05) lower than the score in the non-treated group. Among the other 3 groups there were no significant differences in the extent of adhesion scores. The Seprafilm® group and INTERCEED® group showed no significant anti-adhesive effects compared to the non-treated group.

For the severity of adhesion, (Figure 1b) the scores were 3.6 ± 0.5 in the non-treated group, 0.3 ± 0.7 in the gelatin sheet group, 2.7 ± 1.3 in the Seprafilm® group, and 2.1 ± 1.4 in the INTERCEED® group,

respectively. The score in the gelatin sheet group was significantly (p<0.05) lower than the score in the non-treated group. Among the other 3 groups there were no significant differences in adhesion severity. The Seprafilm® group and INTERCEED® group showed no significant anti-adhesive effects compared to the non-treated group. There were no mortalities or major morbidities associated with the operation or the application of the anti-adhesive materials.

2. Histological analyses (Table 2)

2.1. Three weeks after surgery

Upon autopsy 3 weeks after surgery gelatin remained and covered the cauterized site. On the free abdominal cavity side of the gelatin sheet, a single-cell layer of matured (complete formation of single layer of mesothelium over the whole range of injured sites) mesothelium and sub-mesothelial tissue were formed (Figure 2a) in all dogs of two-layered gelatin sheet group. On the opposite side granulation was grown on the cauterized surface. (Figure 2a) In the Seprafilm® group abundant foamy cells of macrophages ingesting Seprafilm® were found around the cauterized surface, (Figure 2b) little amount of the material of Seprafilm® remained. Mesothelial cells layer was not formed. (Figure 2b) In the INTERCEED® group poorly matured (incomplete or scattered formation of mesothelium over the range of injured sites) mesothelial cells layer was formed

(Figure 2c) in all dogs. A large number of foamy cells of macrophages ingesting INTERCEED® were found around the cauterized surface, (Figure 2c) and INTERCEED® was almost absorbed.

2.2. Six weeks after surgery

In the gelatin group on 42 days after surgery, the cauterized surface was completely covered by matured mesothelium accompanied with sub-mesothelial layer. Macrophages were scarce, and inflammation of the implanted sites were subsequently improved. The material remained little (Figure 3a). In the Seprafilm® group injured surface was covered by poorly matured mesothelium. There were abundant foamy cells ingesting Seprafilm®, and the material was ingested almost completely but still remained. Greater omentum adhered to the granulation around the Seprafilm® (Figure 3b). In the INTERCEED® group relatively matured mesothelial layer was formed in all dogs, and there were abundant macrophages ingesting INTERCEED®. The material was observed abundant in the phagocytes (Figure 3c).

3. Cell growth

On Seprafilm® or INTERCEED® peritoneal mesothelial cells, fibroblasts, or uterine smooth muscle cells did not proliferate, and the number of seeded cells decreased. On the both surfaces of gelatin

sheet (film, sponge) all kinds of the cells proliferated significantly ($p < 0.01$) compared with Seprafilm® or INTERCEED® (Figure 4a, b, c).

DISCUSSIONS

To prevent adhesions anti-adhesive agents should act for the barriers those avoid direct contact to adjacent organs. The followings are important factors for anti-adhesive agents. 1) Materials should stay in the same place and keep to be a barrier until complete regeneration of the injured peritoneum. 2) Materials should not disturb the regeneration of the peritoneum. 3) It is desirable materials themselves have hemostatic effects because abundant fibrin formation accelerates adhesion at bleeding site. 4) Materials should be absorbed and disappear after the regeneration of the peritoneum to avoid inflammation.

We developed two-layered gelatin sheet to equip the characters above mentioned. Two-layered gelatin sheet was most effective to prevent adhesion after uterine surgery regarding the extent and the severity of adhesion in this study. Seprafilm® and INTERCEED® had tendencies to prevent adhesion compared with non-treated group, but the effects of them were so unstable that no significant differences from the non-treated group were noted. The anti-adhesive effects of

Seprafilm® or INTERCEED® for the pelvic surgeries are controversial. (1, 2, 15)

The factors to show the differences among the materials were considered. It is important to prevent adhesion for the injured organs to keep away from the adjacent organs. The peritoneum prevents fibrin attachment which is the first step of adhesion. Early regeneration of the peritoneum of the injured site is one of the most important factor for adhesion prevention.

In the group treated with two-layered gelatin sheet matured single layered mesothelium was completely regenerated at 3 weeks after surgery. On the other hand, in the Seprafilm® group the formation of the mesothelium layer was not observed at 3 weeks after surgery. In the INTERCEED® group mesothelial cell layer was formed to small extent, and most part of injured surface was explored at 3 weeks, but relatively matured mesothelial cell layer covered injured surfaces almost completely at 6 weeks after surgery. Rapid regeneration of the peritoneum was considered to contribute subsequently the prevention of adhesion. (13, 14) As for INTERCEED® another anti-adhesion study showed rapid regeneration of the peritoneum. (11) The regeneration of the peritoneum was suspected to be delayed because the tissue damage was stronger in the cauterized uterus model than the other study.

To explore the background of the histological findings of cell

growth (#2 factor above mentioned) on each material was observed *in vitro*. Growth of all kinds of cells was proliferated most in gelatin film and sponge group. According to the results Seprafilm® and INTERCEED® restrained cell growth and then disturbed tissue repair and regeneration. On the contrary relatively better cell proliferation on gelatin film supposed to be the important factor for better tissue repair.

The period when materials remain on the injured site to act as the barriers against adhesion between the adjacent tissues is the important factor (#1, #4 factors above mentioned). Almost all gelatin remained on the site at 3 weeks after surgery, but disappeared at 6 weeks. Seprafilm® and INTERCEED® were ingested by macrophages and almost completely disappeared at 3 weeks after surgery. Postoperative adhesion is known to initiate within a week after surgery. (14) In cauterized uterine model local inflammation was so strong that regeneration of the peritoneum was incomplete at even 3 weeks after surgery in Seprafilm® or INTERCEED® group. It is considered that the progress of adhesion continued until the completion of the peritoneum regeneration. It is most likely seen for anti-adhesive agents to keep the barrier function over a month after the surgery such as injury of the peritoneum and other organs reaches wide and deep like the operation for gynecological malignant tumor.

Local hemorrhage is the important factor (#3 factor above mentioned) for the prevention of adhesion. Local bleeding, insufficient hemostasis, or re-bleeding after surgery attenuates the anti-adhesive effects of INTERCEED®. (15) Seprafilm® also has no hemostatic effect. Secure hemostasis of the operatively injured site is the key for the anti-adhesive agents to produce an effect. It was observed during the experiment that gelatin sheet absorbed flowing blood and blood coagulation completed rapidly. As for the handling of the materials at bleeding site, two-layered gelatin sheet could easily attach and stay on the bleeding site, but Seprafilm® and INTERCEED® could hardly stay on the site. This character of two-layered gelatin sheet has an advantage for anti-adhesive agent. The surgery of the gynecological malignant tumor requires extensive dissection, and the injury of the tissues is wide and reaches in deep tissues. At the same time a large quantity of bleeding often become the problem, too. Two-layered gelatin sheet had extensive hemostatic effects (in print). (16)

In the present study there are some limitations. This study was conducted with canine model, and only the observation of cauterized uterus. Evaluation of the effects in humane abdominal surgery is necessary. We have plans to advance the investigation. Moreover, to apply laparoscopic surgery, we are developing a new form of gelatin available for it.

In conclusions, the anti-adhesive effects of two-layered gelatin sheet is superior to the conventional agents in cauterized uterus model. Early regeneration of the peritoneum, weak inflammation, and longtime remain of the material contributes the results. Newly developed two-layered gelatin sheet is considered to be a useful option or anti-adhesive agent in deeply injured and hemorrhagic sites.

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Table 1. Adhesion Score

Category and Description	Score
(Extent of site involvement)	
None	0
≤25%	1
≤50%	2
≤75%	3
≤100%	4
(Severity)	
No adhesion	0
Adhesion falls apart	1
Adhesion lysed with traction	2
Adhesion required ≤50% sharp dissection	3
Adhesion required >50% sharp dissection	4

The degree of adhesion was scored by criteria of 0-4 for the extent or severity of the adhesion

Table 2. Histological findings of the site around materials

	Gelatin sheet		Seprafilm®		INTERCEED®	
WEEKS	3w	6w	3w	6w	3w	6w
EPITHELIUM REGENERATION	Yes	Yes	No	Yes	Yes	Yes
LAYER FORMATION OF MESOTHELIUM	Matured Single layer (Fig. 2a, 3a)		No	Immature	Poorly matured layer (Fig. 2c)	Matured Single layer (Fig.3c)
INFLAMMATION	Mild	Improved	Abundant macrophage Rich granulation		Abundant macrophage Rich granulation	
REMAINS OF MATERIAL	Fully remained	Little remained	Almost Ingested	Ingested	Almost Ingested	Ingested

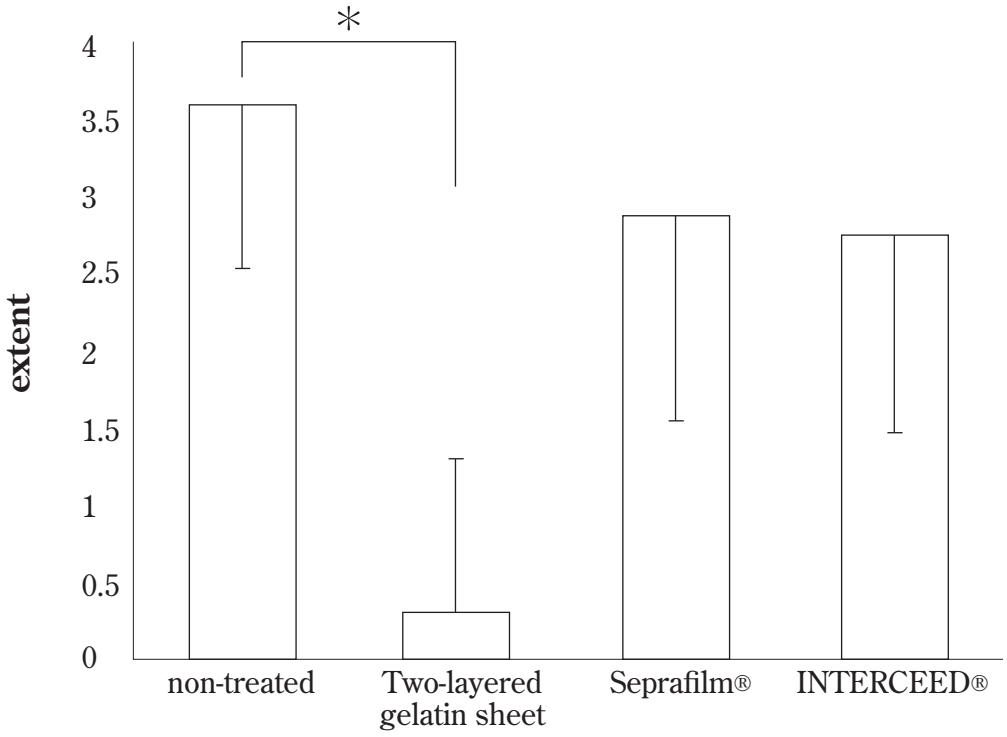

Figure 1a. The adhesion scores of the anti-adhesive agents (the extent of adhesion)

Two-layered gelatin sheet showed significant ($p<0.05$) less score than non-treated group.

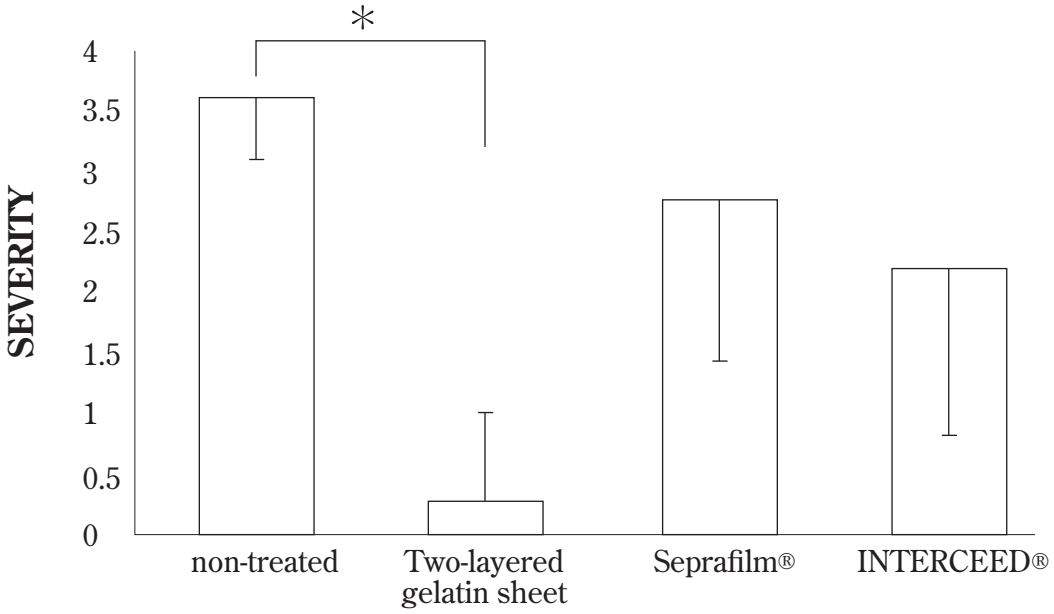

Figure 1b. The adhesion scores of the anti-adhesive agents (the severity of adhesion)

Two-layered gelatin sheet showed significant ($p<0.05$) less score than non-treated group. Gelatin sheet showed no significant ($p<0.05$) differences compared to Seprafilm® or INTERCEED®.

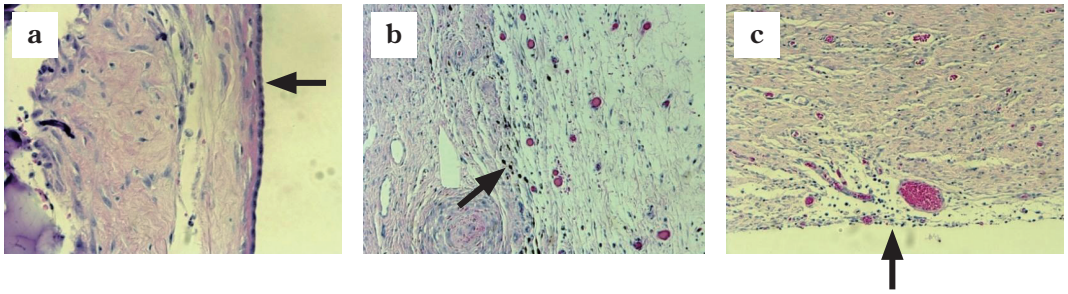

Figure 2. Histological findings 3 weeks after surgery.

a: two-layered gelatin sheet. b: Seprafilm®. c: INTERCEED®.

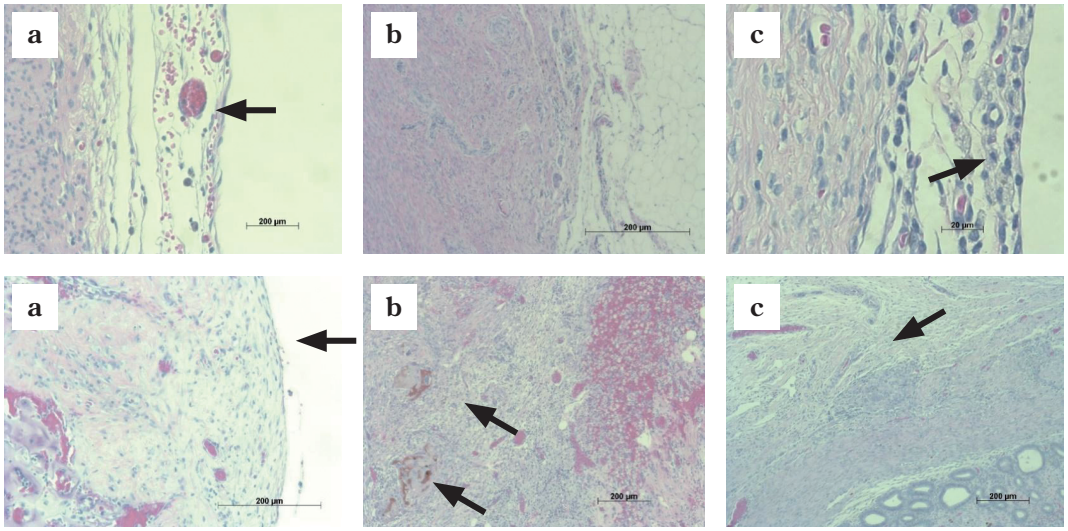

Figure 3. Histological findings 6 weeks after surgery.

a: two-layered gelatin sheet. b: Seprafilm®. c: INTERCEED®.

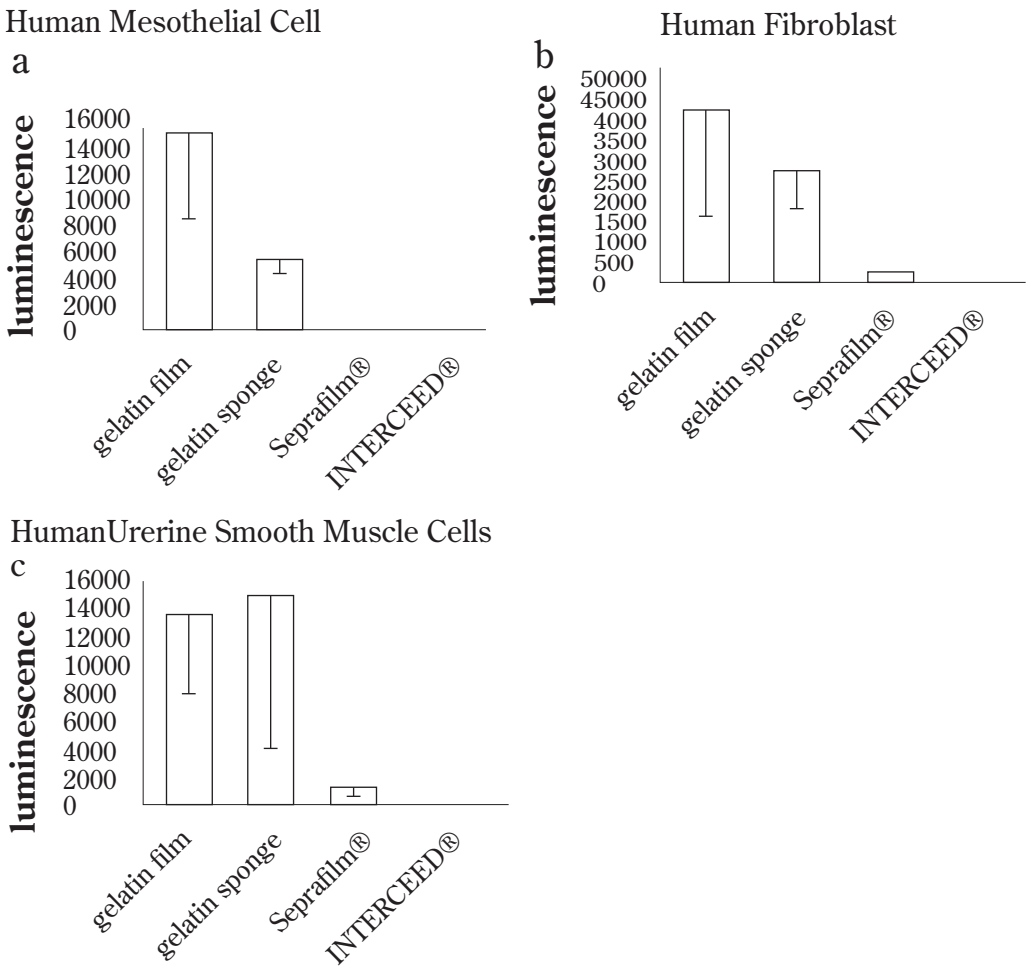

Figure 4. Cell growth on the materials (1week).

a: human mesothelial cells.

b: humane fibroblasts.

c: human uterine smooth muscle cells. Gelatin film and sponge groups showed significantly (p<0.01) richer cell growth than Seprafilm® or INTERCEED® group.

STUDY III-1

Anti-adhesive Effects of Gelatin Powders with Different Particle Forms

TABLE OF CONTENTS (STUDY III-1)

ABSTRACT

Background:

To overcome the problems associated with the use of Seprafilm®, a commonly used anti-adhesive material, we have developed a powdered anti-adhesive material made of thermally cross-linked gelatin. We prepared three kinds of powdered gelatins by varying the particle form. We examined the anti-adhesive effects and the fundamental properties of the powdered gelatins, and investigated how the latter affects the former.

Materials and Methods:

Three kinds of powdered gelatins were prepared for the evaluation of the anti-adhesive effects using the rat cecal adhesion model. Another powdered gelatin was added to the groups in the experiments regarding the fundamental properties for comparison. We observed the particle and the gel, and examined the solubility and the ability to absorb water.

Result:

In the evaluation of the anti-adhesive effects, two powdered gelatins showed a similar anti-adhesive effect to Seprafilm® and the other showed a slightly lower effect. In the experiments regarding the

fundamental properties, the powdered gelatins showed different properties depending on its particle form.

Discussions:

The compact arrangement of gelatin in the gel and its ability to absorb water immediately can be considered to be the reason why the two powdered gelatins showed a similar anti-adhesive effect to Seprafilm®. In addition, the powder form can be considered to provide a high handle ability. AS the conclusion, the fundamental properties largely affect the anti-adhesive effect, and powdered gelatin has the potential to be a superior anti-adhesive material to Seprafilm® when the appropriate particle form is used.

粒子形状の異なるゼラチンパウダーによる癒着防止効果の検討

要約

背景：

現在、臨床で広く使用されている癒着防止材であるSeprafilm®の課題を解決するべく、熱架橋ゼラチンで構成されたゼラチンパウダーを開発した。3種類の形状のゼラチンパウダーを作成し、癒着防止効果および各々のゼラチンパウダーの基本的特性について検討した。

材料および方法：

ラットの盲腸擦過癒着モデルに対し、3種類のゼラチンパウダーを用い、癒着防止効果を評価した。比較のため、別の形状のゼラチンパウダーを加え、生物学的特性に関して検討した。

結果：

癒着防止効果実験においてゼラチンパウダーはSeprafilm®に近い癒着防止効果、若しくは、わずかに低いという結果となった。特性試験では、ゼラチンパウダーの形状により異なる結果を得た。

結語：

Seprafilm®に近い癒着防止効果を得た要因はゼラチンの速やかなゲル化と水溶性にあると考えられる。さらに、ゼラチンパウダーは操作性にも優れる。生物学的特性は癒着防止効果に大きく寄与すると考えられ、Seprafilm®よりすぐれた癒着防止効果を得るには最適な形状を検討する必要がある。

INTRODUCTION

The postsurgical adhesion, which occurs between tissues after surgeries, appears in more than 90 % of surgeries. It often causes serious complications, such as chronic abdominal pain, functional intestinal obstruction, female infertility and technical difficulty in the second surgery. (1) The film made of a mixture of carboxymethyl cellulose and hyaluronate sodium (Seprafilm®, KAKEN Pharmaceutical Co. Ltd., Tokyo, Japan) is commonly used to prevent the postsurgical adhesion in the abdominal cavity. It absorbs surrounding moisture and turns into gel after the placement onto a wound tissue in the abdominal cavity. It remains at the site for approximately seven days and acts as a barrier to prevent the postsurgical adhesion. (2)

Seprafilm®, however, has a slight cytotoxicity. (3, 4) Even a small amount of the film remaining after wound healing was completed holds the possibility to cause an extra adhesion because of its cytotoxicity and the following inflammation. (2)

Seprafilm® also has a tendency to split in handling. Its fragility requires extra technique of surgeons. Especially, it is very difficult to use Seprafilm® under a laparoscope. (2)

To overcome these problems, we have developed the powdered anti-adhesive material made of thermally cross-linked acid gelatin

(Gelatin-flakeballs). (5, 6) Its particles have a ruffled surface shape and the cross-linked part swells like Seprafilm® immediately after the placement. Gelatin is highly biocompatible (7) and the powder form provides a high handle ability even under a laparoscope.

As the next stage, we varied its particle form and examined the anti-adhesive effects of the new powdered gelatins. We also examined the fundamental properties of them to investigate how they affect the anti-adhesive effect.

MATERIALS AND METHODS

1. Preparation of the powdered gelatins

1.1. Gelatin-flakeballs

Acid gelatin with an isoelectric point of 5 (Medigelatin, Nippi Co. Ltd., Shizuoka, Japan) was dissolved in distilled water (Otsuka distilled water, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) to obtain4.8wt% solution. The solution was frothed with a homogenizer (Excel Auto, Nippon Seiki Co. Ltd., Tokyo, Japan), flowed into an aluminum tray (160 mm × 120mm), frozen in a freezer (ULTRA LOW, SANYO Electric Co. Ltd., Osaka, Japan) at -80℃ for approximately 30 minutes and freeze-dried with a freeze dryer (TF 10-80 A TA, Takara, Tokyo, Japan) for approximately 24 hours to obtain spongiform

gelatin.

Thermal cross-linking was introduced to the spongiform gelatin with a vacuum oven (AVO-310NS-D, As One Co. Ltd., Osaka, Japan) at 140℃ for 5 hours. The cross-linked spongiform gelatin was pulverized with a coffee mill (KHN22-5889, Kohnanshouji Co. Ltc., Osaka, Japan) into powder and passed through two meshes (TESTING SIEVE (sieve size 150 × 60, aperture 0.5 mm and 1 mm), TOKYO SCREEN Co. Ltd., Tokyo, Japan). We defined the powder which passed through the mesh with 1 mm apertures and remained on the mesh with 0.5mm apertures as Gelatin-flakeballs (Flakeballs, below).

1.2. Gelatin-flakes

We obtained another powdered gelatin in the same method as Flakeballs except that the concentration of the solution was 1wt% and the frothing was not introduced. We defined the powdered gelatin as Gelatin-flakes (Flakes, below).

1.3. Gelatin-polyhedra

The same kind of gelatin was dissolved in distilled water to obtain 26wt% solution. The solution was air-dried to obtain film-form gelatin. Thermal cross-linking, pulverizing and screening were introduced to the film-form gelatin in the same method as Flakeballs and Flakes. We defined the powdered gelatin as Gelatin-polyhedra (Polyhedra,

below).

1.4. Gelatin-spheres

As a comparative material, we added another powdered gelatin in the experiments for the fundamental properties. Thermal cross-linking in the same method as Flakeballs, Flakes and Polyhedra was introduced to a bead-form gelatin provided by Nippi Co. Ltd. The particle size was 500-850μmand the kind of gelatin was the same. We defined the powdered gelatin as Gelatin-spheres (Spheres, below).

2. Evaluation of the anti-adhesive effects of the powdered gelatins

2.1. Animals

Female Wistar / ST rats, at the age of 8weeks and weighing approximately 200g, were purchased from Shimizu Animal Laboratory (Kyoto, Japan). During the experimental period, the rats were maintained under the standard specific pathogen-free (SPF) condition (a light-dark cycle of 12:12hours, a mean temperature of 23°C, and a mean humidity of 50%). Standard laboratory rodent food and water were provided freely. Before the experiment, the rats were maintained in the laboratory for one week. On the experimental day, the rats were checked for their overall health condition. The animal care protocol and the following animal experiment performed in this

research were approved by the Animal Experimentation Committee of Doshisha University. All of the surgical procedures were performed under a sterile condition and by one surgeon.

2.2. Surgical procedures used to create the rat adhesion model

To examine the anti-adhesive effects of the powdered gelatins or observe the status of the powdered gelatins applied to the experimental animals, we used the rat cecal abrasion model designed to create adhesion between the rat's cecum and abdominal wall. (8) The common process of the experiment was as follows (Figure 1):

The rats were anesthetized using isoflurane (Escain®, Mylan Inc., Osaka, Japan) inhalation. Then, sodium pentobarbital (Sommnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) was administered intraperitonealy at a dose of 35mg/kg of the body weight with a tuberculin syringe and a 23-G injection needle. Under the above-described general anesthesia, the rats were fixed in the dorsal position. A 4-cm-long midline incision was made on the abdominal wall. The serosal surface of the cecum was abraded 10 mm in diameter with a dental sanding paper (Dental grinding material, Sharp Mini (size: microfine), Ohki Chemical Industry Co. Ltd.) until small blood drops appeared. Another 10-mm-diameter abrasion was made on the right lateral internal abdominal wall directly opposite to the abraded cecum.

Next, 40 mg of the powdered gelatins were placed onto the sites of the abraded ceca. The non-treated group (no anti-adhesive material) and the Seprafilm® group (2 cm × 3 cm of Seprafilm®) were added to the experimental groups. The two abraded surfaces were approximated with a 7-0 prolene suture (monofilament, Asflex, Kono Seisakusho Co. Ltd., Chiba, Japan) to induce adhesion since the cecum floats freely in the abdominal cavity. The laparotomy incision was closed with a 4-0 nylon suture (Natsume Co, Tokyo, Japan). In the non-treated group, no powdered gelatin was placed and the laparotomy incision was closed with the 4-0 prolene suture after the two abraded surfaces were approximated.

2.3. Scoring of the adhesions

The rats were euthanized by lethal doses of pentobarbital3 weeks after the surgery. The abdomen was opened again, and the adhesion was scored macroscopically according to the adhesion grading scale shown in Table 1. (9) The scoring of the adhesions was performed by one researcher who was blinded to the animal assignment. The Dunn test following the Kruskal-Wallis test was used for the statistical processing.

3. Macroscopical and microscopical observation of the powdered gelatins

The particles of the powdered gelatins were observed macroscopically and microscopically. Two scanning electron microscopes (Miniscope TM-1000, Hitachi-High-Technologies Co., Tokyo, Japan for Flakeballs and JMS-6701F, Nippon Denshi Co. Ltd., Tokyo, Japan for the others) were used in the microscopical observation.

4. Microscopical observation of the gels

40 mg of each powdered gelatin was placed between two slices of chicken breast meat. The powdered gelatins were immersed in distilled water for 3 hours. After the immersion, the gels derived from the powdered gelatins were fixed with formalin for 1 week and cut vertically to the faces. After air-drying, the cross section of the gels was observed microscopically. A stereo microscope was used in the observation.

5. Measurement of the fraction of cross-linked gelatin and the degree of swelling

The powdered gelatins were dried in a vacuum oven (AVO-250N, As One Co. Ltd., Osaka, Japan) at room temperature under vacuum overnight. Approximately 30 mg of the powdered gelatins were put in

meshes (Cell Strainer (size 40 μm), Japanese Becton, Dickinson and Company, Tokyo, Japan) and the weights were precisely measured (W*s*). The powdered gelatins were immersed in distilled water in an oven (WFO-500W, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 37℃ for 3 hours and the weights of the gels derived from the powdered gelatins (cross-linked part) were measured (W*w*). The gels remaining in the Cell Strainers were dried in the vacuum oven at 60℃ under vacuum overnight and the weights of the dried gels were measured (W*d*). The fraction of cross-linked gelatin and the degree of swelling were calculated. The values of the fraction of cross-linked gelatin and the degree of swelling are shown by the expressions below respectively.

Fraction of cross-linked gelatin (%) = (W*d* / W*s*) × 100

Degree of swelling (%) = (W*w* – W*d*) / W*d* × 100

The tukey test was used for the statistical processing.

6. Measurement of the speeds of dissolving and swelling

To measure the speeds of dissolving and swelling, the same method as the measurement of the fraction of cross-linked gelatin and the degree of swelling was used except that the measurement of W*w* was conducted after three time periods of immersion (3 seconds, 3 minutes and 3 hours). The ratio of the remaining weight and the degree of swelling at each time period of immersion were calculated. The values of the ratio of the remaining weight and the degree of

swelling are shown by the expressions below respectively.

Ratio of the remaining weight (%) = (Wd / Ws) × 100

Degree of swelling (%) = (Ww – Wd) / Wd × 100

RESULTS

1. Evaluation of the anti-adhesive effects of the powdered gelatins

The adhesion scores are shown in Figure 2. No significant difference was found. The statuses of the powdered gelatins applied on the sites are shown in Figure 1.

2. Macroscopical and microscopical observation of the powdered gelatins

The macroscopical forms of the powdered gelatins are shown in Figure 3. All the powdered gelatins are composed of the same kind of gelatin and have almost the same size of particles. However, the weight of each particle is different among the powdered gelatins. The particles of Flakeballs and Flakes have a small weight, while those of Polyhedra and Spheres have a large weight. Also, the hardness of particles is different among the powdered gelatins. Flakeballs and Flakes have soft particles, while Polyhedra and spheres have hard

particles.

The microscopical forms of the powdered gelatins are shown in Figure 4. The particles of Flakeballs and Flakes have a ruffled surface shape, while those of Polyhedra and Spheres have a smooth surface shape.

3. Microscopical observation of the gels

The appearances of the gels are shown in Figure 5. Flakeballs, Flakes and Polyhedra formed bonds among the swollen particles, while Spheres did not form strong bonds among the swollen particles. Accordingly, Flakeballs, Flakes and Polyhedraturned into one integrated mass of gel and showed the complete covering of the site, while Spheres held space among the swollen particles and did not cover the site completely. The thickness of the gels derived from Flakes (approximately 2 mm) and Polyhedra (approximately 2 mm) was larger than those of the gels derived from Flakeballs (approximately 0.5 mm) and spheres (approximately 1.5 mm).

The inner structures of the gels are shown in Figure 6. Flakes showed the compact arrangement of gelatin throughout the gel. Flakeballs also showed it in the part close to the surface of the gel. Polyhedra showed small spaces within the gel.

4. Measurement of the fraction of cross-linked gelatin and the degree of swelling

The values of the fraction of cross-linked gelatin are shown in Figure 7. Though the time period of cross-linking was the same (5 hours), the values of the fraction of cross-linked gelatin were different.

The values of the degree of swelling are shown in Figure 8. The values of the degree of swelling were different according to the values of the fraction of cross-linked gelatin. There was a negative correlation between the fraction of cross-linked gelatin and the degree of swelling.

5. Measurement of the speeds of dissolving and swelling

The values of the ratio of the remaining weight after 3 seconds, 3 minutes and 3 hours of immersion are shown in Figure 9. The values of the ratio of the remaining weight were not largely different among the three time periods of immersion in Flakeballs, Flakes and Spheres, while that of Polyhedra rapidly decreased after 3 minutes of immersion.

The values of the degree of swelling after 3 seconds, 3 minutes and 3 hours of immersion are shown in Figure 10. The values of the degree of swelling were not largely different among the three time periods of immersion in Flakeballs, Flakes and Spheres, while that of

Polyhedra rapidly increased after 3 minutes of immersion.

DISCUSSIONS

In the evaluation of the anti-adhesive effects, Flakeballs and Flakes showed the similar adhesion score to Seprafilm®. However, Flakes showed the slightly higher adhesion score in extent. Polyhedra showed the slightly higher adhesion score than the others in both extent and severity. To discuss what caused the difference, let us discuss how the fundamental properties were first.

Because of the difference in the weight of each particle, the number of particles in the same weight of particles is different among the powdered gelatins. Flakeballs and Flakes have a large number of light particles, while Polyhedra and Spheres have a small number of heavy particles. That is, even though the weight is the same, the apparent volume is different among the powdered gelatins. Flakeballs and Flakes have a large apparent volume, while Polyhedra and Spheres have a small apparent volume.

The particles of Flakeballs and Flakes easily get squashed. It seems that the softness of the particles caused them to turn into the thin gels with an even surface. On the other hand, the particles of Polyhedra keep their forms against the external pressure. It seems

that the form of each particle appeared in the surface of the gel because of the hardness of the particles. As a result, the gel with an uneven surface was formed. It is also suggested that its high absorption of water and space among the small gels derived from each particle discussed below caused the gel to be thick. The particles of Spheres have an almost complete sphere form. It seems that the sphere form made the particle surface area which touches the surface of other particles very small and accordingly bonds between the particles less than the others. As a result, the bottom face was not covered completely.

To search for the most suitable gel form, we need to consider how adhesion occurs. Adhesion occurs in the process of wound healing. Fibrin and collagen play important roles in wound healing. In a wound tissue, fibrinogen exudes from vessels with white blood cells and turns into fibrin. They cause inflammation to protect the wound tissue from a further damage. Growth of fibroblasts and collagen synthesis by them follow the exudation of fibrinogen and while blood cells to fill up the lost tissue. This is called granulation tissue. In this process, fibrin deposit formed by inflammatory stimuli causes an early adhesion between opposing tissue surfaces (2) and a late adhesion by collagen follows. Growth of granulation tissue under this situation causes the adhesion between the two tissue surfaces.

Therefore, it is necessary for anti-adhesive materials to have the

ability to cover a wound tissue enough to prevent fibrinogen and collagen from passing through them. That is, unless fibrinogen and collagen pass through the anti-adhesive material and the opposing tissue surfaces are linked by them, separately formed granulation tissue or regenerated mesotheliumon the both surfaces prevent a further adhesion even after the anti-adhesive material was degraded, regardless of whether fibrin deposition or collagen synthesis has occurred within each side or not. Therefore, powdered gelatin which forms a gel with the compact arrangement of gelatin is suitable for an anti-adhesive material. The gel derived from Spheres obviously lacked the compactness of gelatin. Each particle just swelled independently and no integrated mass of the gel was formed. The gel derived from Polyhedra also lacked it although one integrated mass of the gel was formed. The mass was composed of small masses of the gel and it held small spaces among the masses. The gel derived from Flakes held the compactness of gelatin throughout the gel. The gel derived from Flakeballs also held it in the part close to the surface of the gel. The compactness of gelatin in the gels of Flakeballs and Flakes can be considered to have caused the similar anti-adhesive effect to Seprafilm®.

In general, gel has inter-molecular bonds among three-dimensionally arranged molecules and water molecules are located in the space among the molecules. In the case of thermally cross-linked

gelatin gel, in addition to the space among gelatin molecules, the space where the uncross-linked part was located also holds water molecules. The uncross-linked part dissolves to water and water molecules enter the space. Also, the number of cross-links in the cross-linked part is subject to the extent of the cross-linking.

It seems that water molecules held in the space where the uncross-linked part had been located and the space among cross-linked gelatin molecules caused the negative correlation between the fraction of cross-linked gelatin and the degree of swelling. That is, as the extent of cross-linking increases, the amount of the space where the uncross-linked part was located and the space among the cross-linked gelatin molecules decreases. The similar result was shown in the past research on Flakeballs. (5)

As shown by the negative correlation of the degree of swelling to the fraction of cross-linked gelatin, the fraction of cross-linked gelatin is an important factor which determines the properties of cross-linked gelatin, as well as the indicator of the extent of cross-linking.

Flakeballs and Flakes dissolved to water immediately to their minimum ratio of the remaining weight in comparison. It seems that it is because the particles of Flakeballs and Flakes have a large specific surface area and they were attacked by a large number of water molecules. On the other hand, the particles of Polyhedra and Spheres have a small specific surface area and they did not dissolve

to water immediately.

Flakeballs and Flakes swelled immediately to their maximum degree of swelling. Flakeballs and Flakes have space within each particle and it seems that the space pulled and held water immediately. On the other hand, the speed of swelling was slow in Polyhedra and Spheres. It seems that it is because each particle of them does not have a space to pull and hold water immediately. Immediate swelling can be considered to be important for immediate covering of the whole wound tissue.

The fundamental properties discussed above seems to have complicatedly affected the anti-adhesive effect, in which Flakeballs and Flakes showed the similar adhesion score to Seprafilm®, while Polyhedra showed the slightly higher score. It seems that the pattern of the arrangement of gelatin in the gel and the degree and speed of swelling especially affected the adhesion score. The compact arrangement of gelatin and the immediate swelling observed in Flakeballs and Flakes can be considered to be suitable properties for anti-adhesive materials. In addition, however, the high degree of swelling shown in Flakes seems to have caused the slightly extensive adhesion compared with Flakeballs. Flakes absorbed more blood than Flakeballs and fibrinogen included in the blood can be considered to have caused the slightly higher score in extent.

CONCLUSION

Flakeballs and Flakes have the compact arrangement of gelatin in the gel and the ability to absorb water immediately. Because of these two properties, Flakeballs and Flakes have a similar anti-adhesive effect to Seprafilm®. In addition to a similar anti-adhesive effect, they have the merit of powder form. Therefore, Flakeballs and Flakes have the potential to be a superior anti-adhesive material to Seprafilm®.

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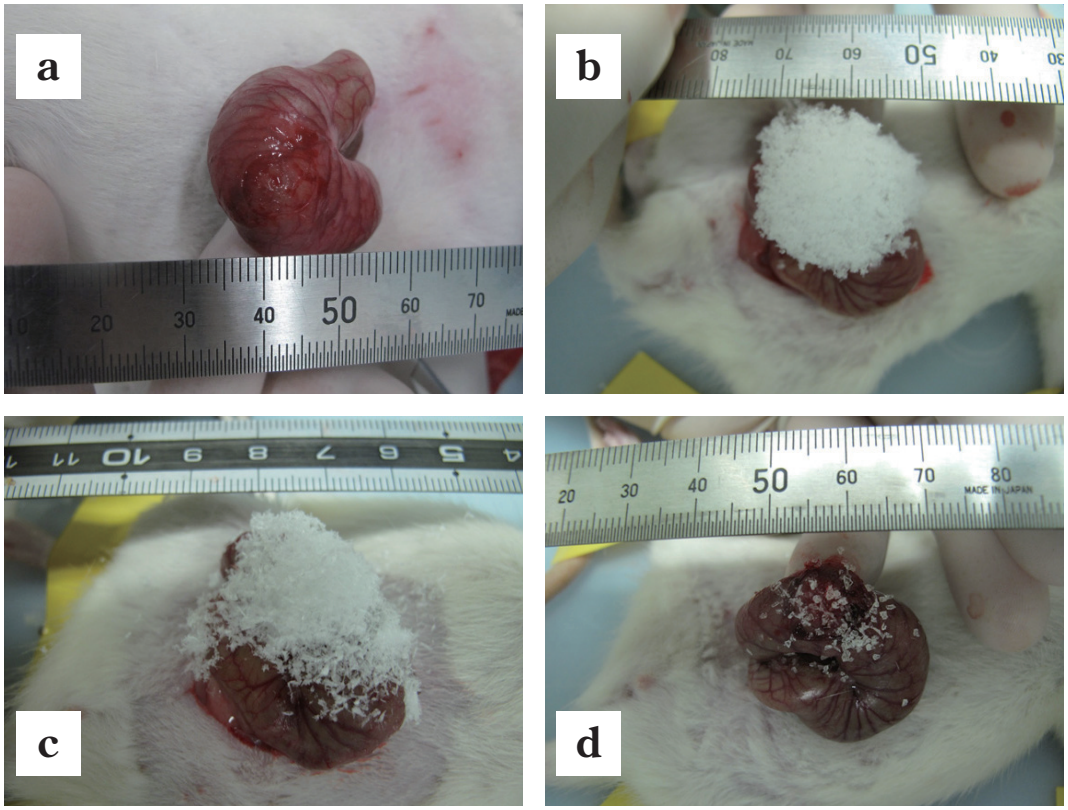

Figure 1. The procedures used to create the rat adhesion model

a: The abrasion made on the cecum. b: Flakeballs placed onto the abraded cecum. c: Flakes placed onto the abraded cecum. d: Polyhedra placed onto the abraded cecum

Table 1. The adhesion grading scale

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 adhesions 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

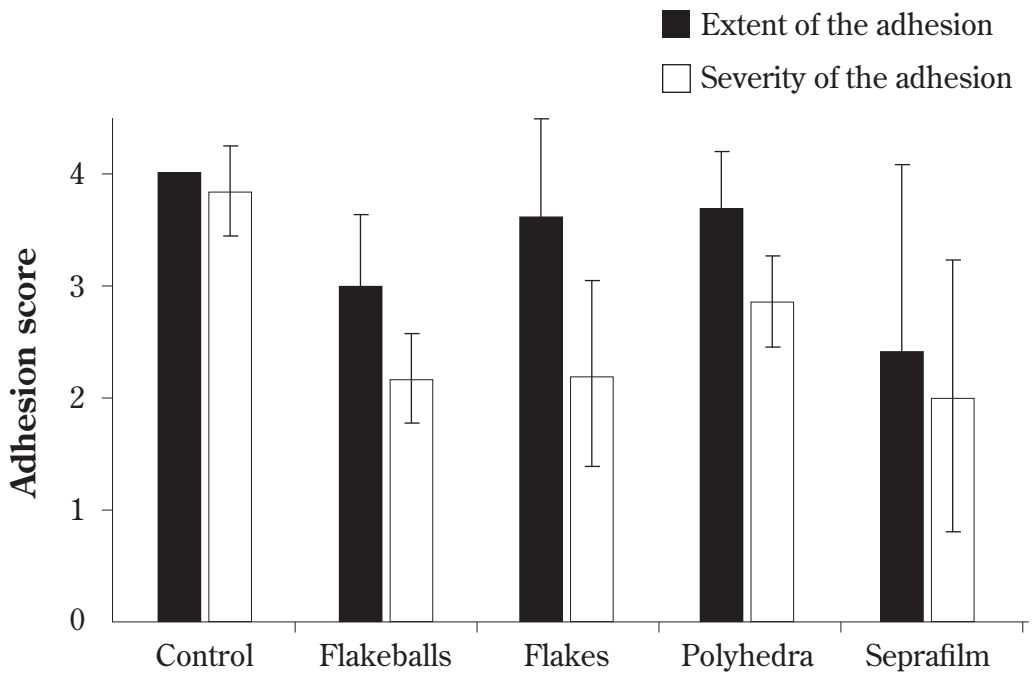

Figure 2. The adhesion scores

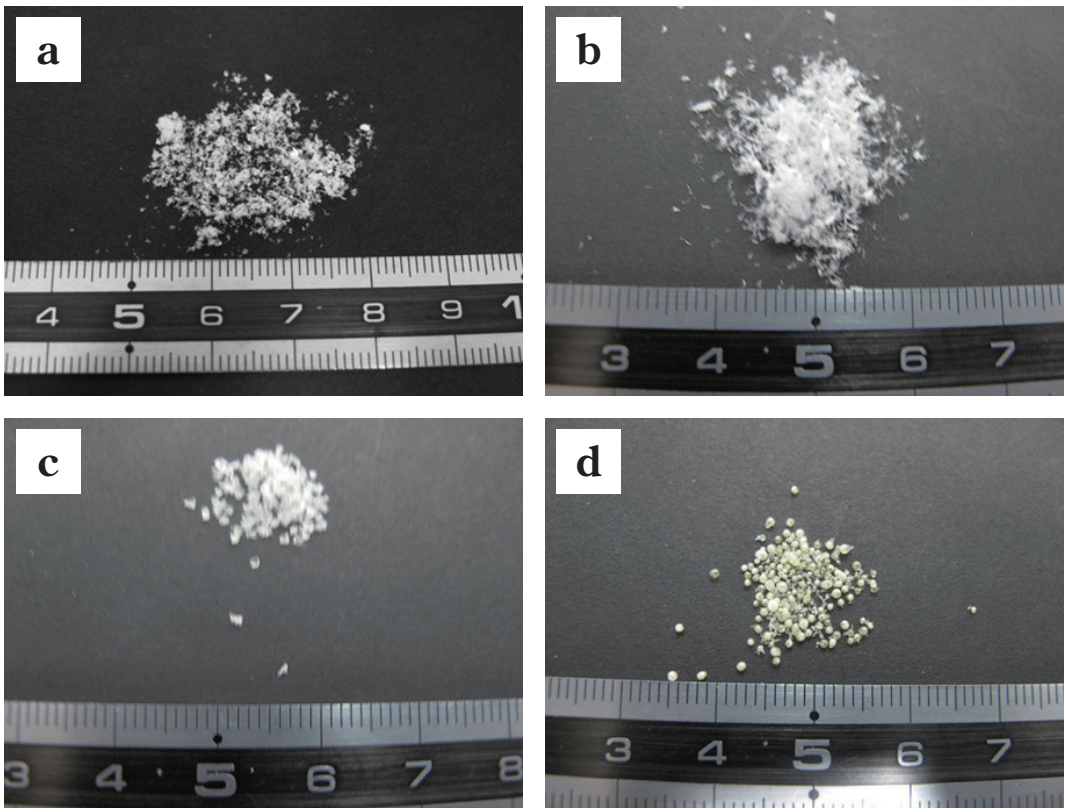

Figure 3. The macroscopical forms of the powdered gelatins

a: Flakeballs. b: Flakes. c: Polyhedra. d: Spheres.

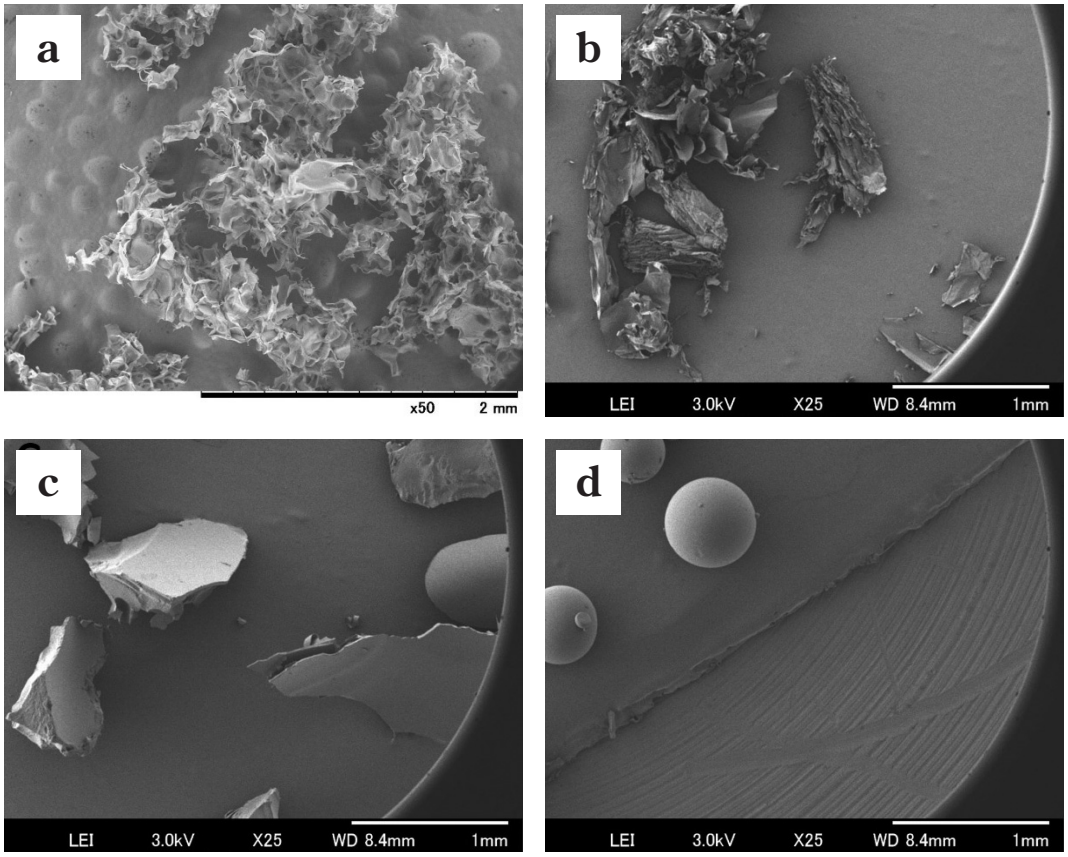

Figure 4. The microscopical forms of the powdered gelatins

a: Flakeballs. b: Flakes. c: Polyhedra. d: Spheres.

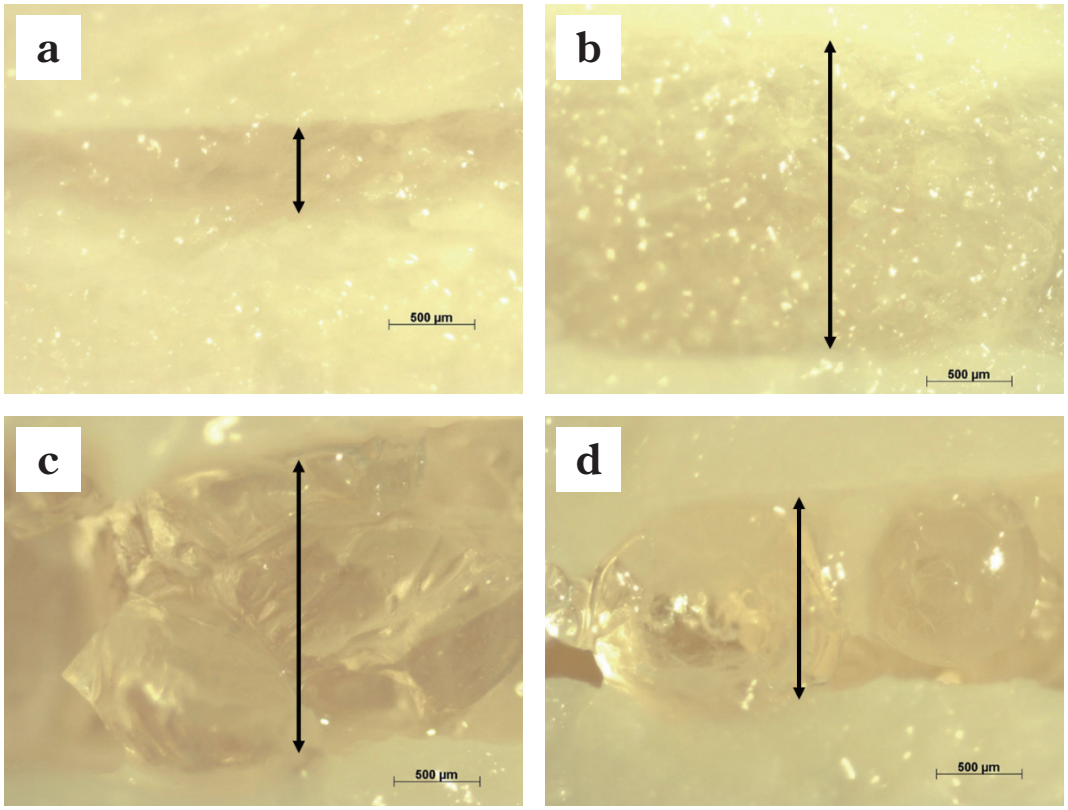

Figure 5. The appearances of the gels

a:Flakeballs. b: Flakes. c: Polyhedra. d: Spheres.

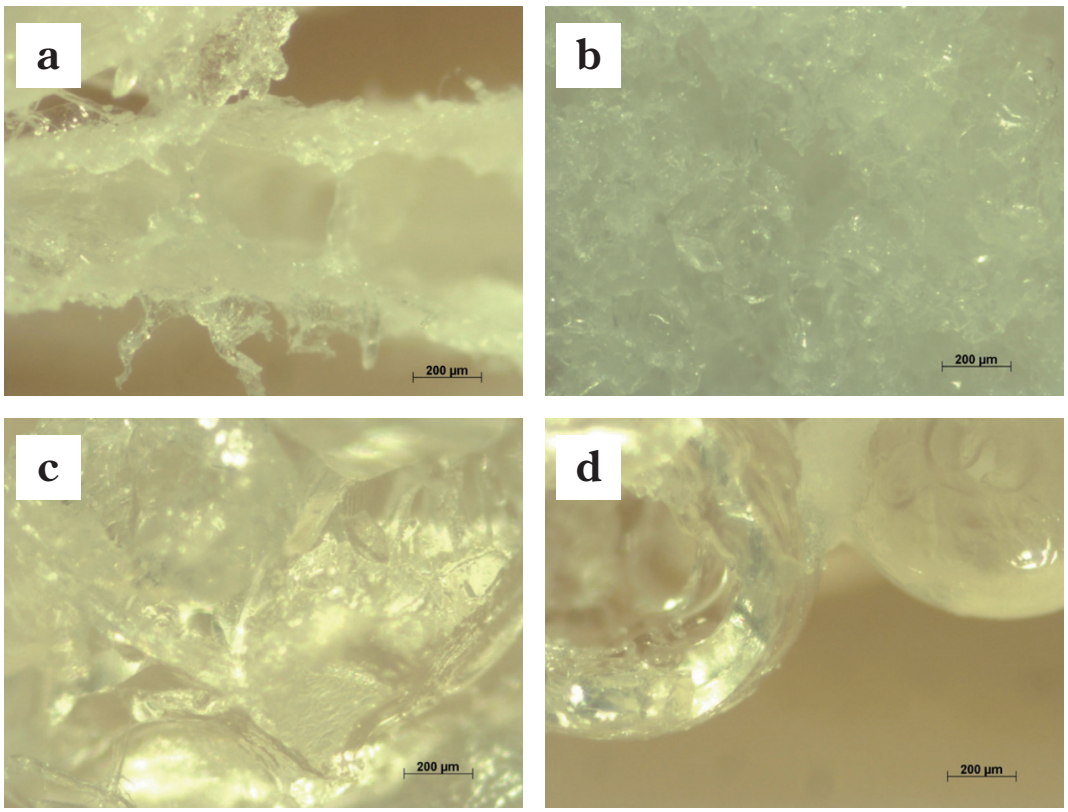

Figure 6. The inner structures of the gels

a: Flakeballs. b: Flakes. c: Polyhedra. d: Spheres.

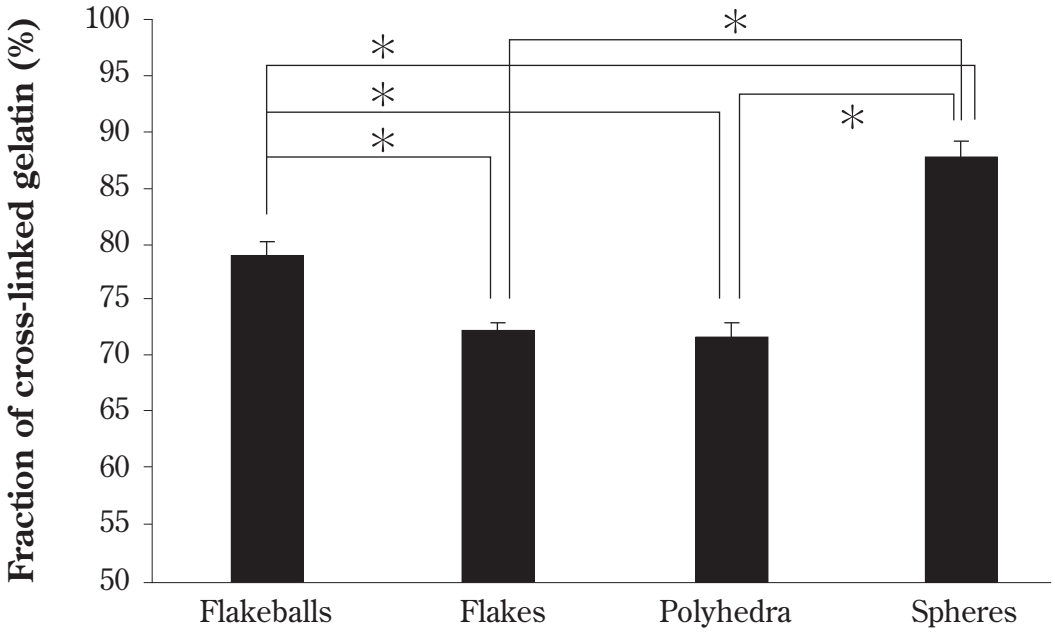

Figure 7. The fraction of cross-linked gelatin

The significant differences are indicated by * (p<0.001).

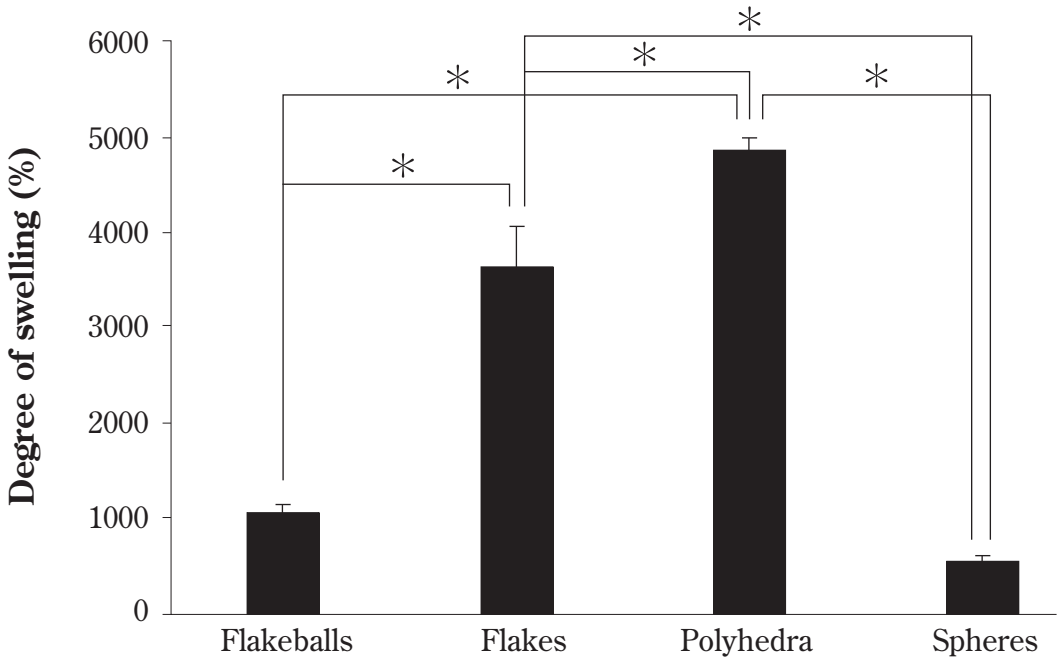

Figure 8. The degree of swelling

The significant differences are indicated by * (p<0.001).

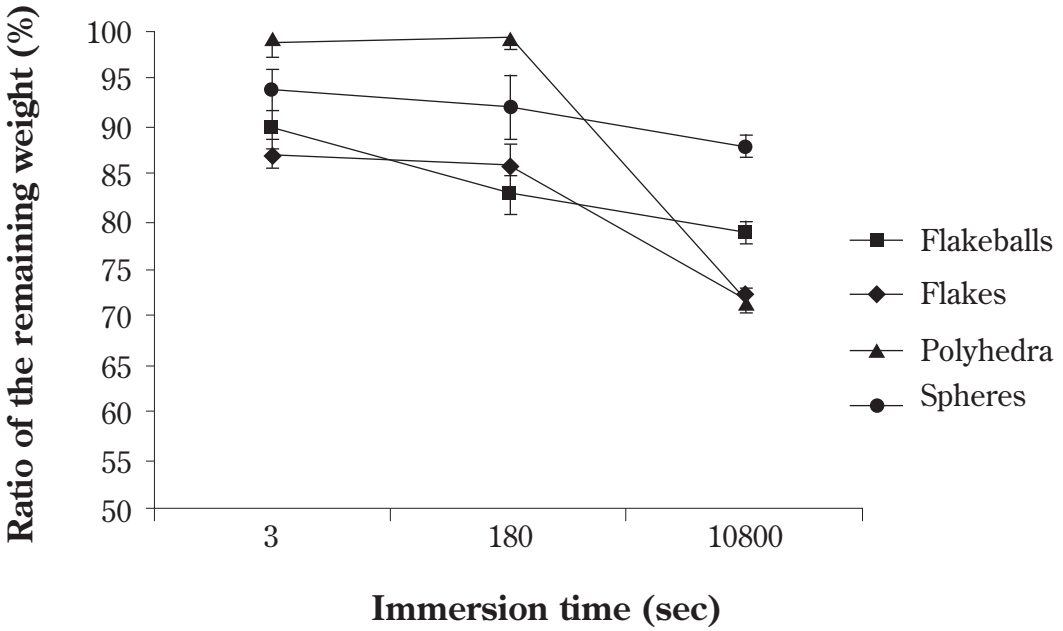

Figure 9. The speed of dissolving

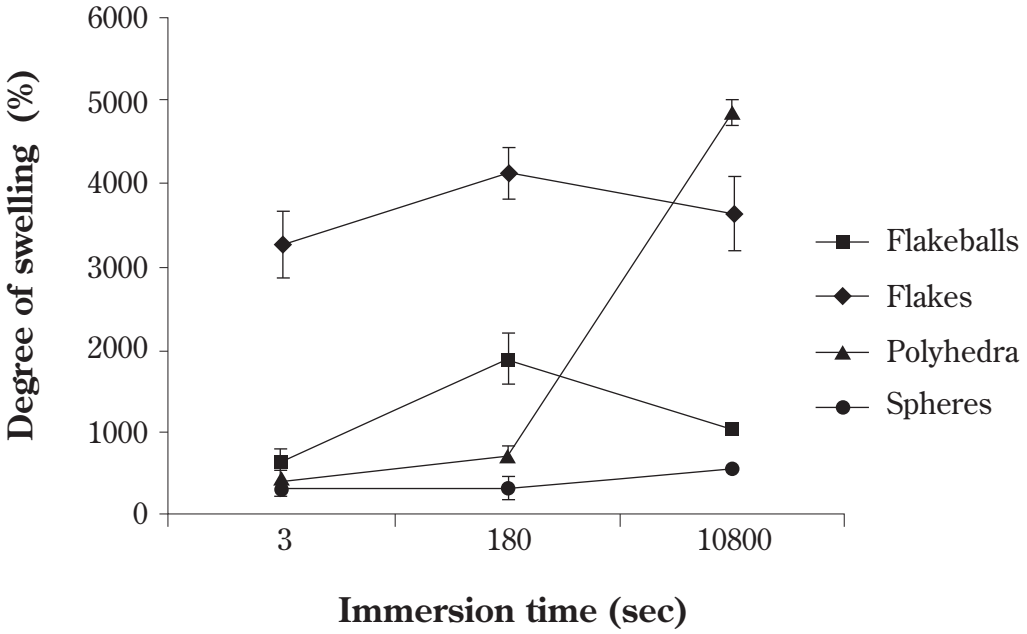

Figure 10. The speed of swelling

STUDY III-2

Fundamental Properties of Gelatin Flakes as a New Anti-adhesive Material for Laparoscopic Surgery — A Preliminary Study —

TABLE OF CONTENTS (STUDY III-2)

ABSTRACT

Background:

Postsurgical adhesions often cause serious complications, such as chronic abdominal pain, functional intestinal obstruction, and female infertility. Therefore, postsurgical adhesions are clinical problems. At present, sodium hyaluronate and carboxymethyl-cellulose (HA/CMC) film (Seprafilm®) is commonly used as an anti-adhesive material. However, cellulose film has some disadvantages, including its poor degradability and poor usability in surgery. In order to develop a new anti-adhesive material for thoracoscopic and laparoscopic surgery, we focused on flake-form gelatin. In this study, our aim was to make gelatin flakes with various particle sizes and various degrees of thermal cross-linking and to evaluate the fundamental properties of gelatin flakes *in vitro*.

Materials and Methods:

In this study, we made gelatin flakes with various particle sizes (below 500, 500-1000 and 1000-2000 μm) and cross-linked for 0, 3, 5, 8 and 14 h. About these samples, scanning electron microscope (SEM) observations and measurements of water solubility, the degree of swelling, degradability in collagenase solution and water absorption times were performed.

Results:

Gelatin flakes had many folds on their surface and a large surface area. As is shown in the results of the water solubility test, the water-insoluble portion of the gelatin flakes was formed by thermal cross-linking. So the water-insoluble portion was cross-linked gelatin and turned into a soft gel when it absorbed water, thereby acting as a mechanical barrier. The fraction of cross-linked gelatin increased as the thermal cross-linking time increased, up to 8 h, while the degree of swelling decreased with prolongation of the thermal cross-linking time, up to 8 h, above which the values became almost constant. The rate of degradation in collagenase solution decreased as the thermal cross-linking time increased. The water absorption speed increased by the introduction of thermal cross-linking.

Conclusion:

Gelatin flakes exhibit high water absorbability and turn into a soft gel, thereby acting as a mechanical barrier on tissue. In addition, the biodegradation time can be controlled by changing the thermal cross-linking time. Because of these benefits, thermal cross-linked gelatin flakes possess superior fundamental properties as a new anti-adhesive material.

ゼラチンフレークの腹腔鏡手術用新規癒着防止剤としての基本特性
―予備的検討―

要約

背景：

術後癒着は慢性腹痛、腸閉塞、不妊などの重篤な合併症の要因となり得る。術後癒着を軽減するべく、現在ではヒアルロン酸ナトリウム・カルボキシメチルセルロース（HA/CMC）フィルム（Seprafilm®）が広く使用されている。しかし、Seprafilm®は生体内での分解性および、術中での操作性などいくつかの課題がある。胸腔鏡および腹腔鏡など内視鏡手術における新たな癒着防止材としてゼラチンフレークを開発した。本研究では、様々な粒子サイズと熱架橋度において特性を評価した。

材料および方法：

種々の粒子サイズ（500未満、500-1000、1000-2000μm）および熱架橋時間（0、3、5、8、14時間）のゼラチンフレークを作成し、これらについてSEMによる形態観察、水溶性、膨潤性、コラゲナーゼによる被分解性、吸水時間を検討した。

結果：

ゼラチンフレークは多くの襞壁を表面に持ち、比表面積は大である。水溶性試験結果に示すように、水溶性を示す部位は熱架橋により生じる。水溶性部分は、水分を吸収すると、ソフトゲルに変化し、よれゆえ物理的障壁として作用する熱架橋時間が８時間に達するまで熱架橋時間の増加に伴い、。熱架橋ゼラチンの割合は増加し、その後は一定となる。コラゲナーゼ分解性は、その逆に、熱架橋時間の増加に伴い、低下する。水分吸収速度は、熱架橋の導入により増加する。

結語：

ゼラチンフレークは高い水分吸収性を持ち、ソフトゲル化する。それゆえ組織表面の物理的障壁として作用する。それに加えて、熱架橋時間を変えることにより生体内分解性の調節が可能である。これらの利点により、ゼラチンフレークは新規癒着防止材としての基本的特性に優れると考えられる。

INTRODUCTION

Postsurgical adhesions that arise after abdominal surgery often cause serious complications, such as chronic abdominal pain, (1) functional intestinal obstruction (2) and female infertility. (3) (4) Therefore, postsurgical adhesions are clinical problems.

To prevent intra-abdominal adhesions and related morbidities, numerous anti-adhesive materials have been developed, some of which have been reported to be effective in animal models and clinical practices. (5)

At present, sodium hyaluronate and carboxymethyl-cellulose (HA/CMC) film (Seprafilm®, Genzyme Corporation, Cambridge, MA) is commonly used as an anti-adhesive material to prevent damaged serosal surfaces from coming into contact with each other for a specific period of time in surgery. (6-8) Seprafilm® has been reported to turn into a gel within 24 to 48 h after being placed on intraperitoneal tissue and remains on the site for up to 7 days to reduce adhesion-formation by acting as a mechanical barrier that separates the adjacent traumatized serosa during the critical early stages of wound repair. (9, 10) However, Seprafilm® has some disadvantages. One is its poor degradability. Because carboxymethyl-cellulose is composed of polysaccharides, Seprafilm® is a non-mammal polysaccharide-based material and not degraded

enzymatically in mammals. Therefore, it has difficulties in being degraded *in vivo* and remains as foreign body. As a result, it may prevent tissue regeneration and cause infection. (11) The other disadvantage of Seprafilm® is its poor usability in surgery. Seprafilm® is a brittle film-type material and tears easily. In thoracoscopic and laparoscopic surgery, in particular, there is the problem that cellulose film is often broken when it is introduced into the body through the trocar. (12, 13)

In our previous studies, we developed gelatin film and two-layered gelatin sheet composed of film-form and sponge-form materials as anti-adhesive materials and reported superior anti-adhesion effects with excellent wound healing. (14-16) Gelatin is denatured collagen, which is the most abundant extracellular matrix protein in mammals, so it has been used for medical applications due to its safety and good biodegradability in the human body. In general, the biodegradation time *in vivo* can be readily controlled by changing the degree of intermolecular cross-linking of gelatin by ultraviolet irradiation, using chemical agents or thermal treatment. (17-21) Especially sponge-form gelatin anti-adhesive material exhibits high attachment stability onto tissue and superior anti-adhesion effects, even in the presence of blood. But sponge-form material is difficult to introduce through the trocar during thoracoscopic and laparoscopic surgery.

Therefore, in this study, in order to develop a new anti-adhesive

material for thoracoscopic and laparoscopic surgery utilizing the advantages of the sponge-form, we focused on flake-form gelatin made by pulverizing gelatin sponges into powder. Flakes are useful in thoracoscopic and laparoscopic surgery because they can be blown into a desired location in the body by spraying in compressed air through the trocar. (13, 22)

In this study, our aim was to make gelatin flakes with various particle sizes and various degrees of thermal cross-linking and to evaluate the fundamental properties of gelatin flakes, such as biodegradability and water absorption, *in vitro*.

MATERIALS AND METHODS

1. Preparation of gelatin flakes (GFL)

Medi grade gelatin (type I collagen, "Medi gelatin", Nippi Co. Ltd., Shizuoka, Japan) with an isoelectric point of 5 was dissolved in distilled water (Otsuka distilled water, Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan) to a concentration of 4.8wt% aqueous gelatin solution. The solution was frothed with a homogenizer (Excel Auto, Nihonseiki Kaisha Ltd., Tokyo, Japan) at 8000 rpm for about 3 minutes, then flowed into an aluminum tray (160 mm × 120 mm). The solution was frozen in a freezer (ULTRA LOW, SANYO Electric Co.

Ltd, Osaka, Japan) at -80℃ for about 30 minutes and freeze-dried for about 24 h with a freeze dryer (TF 10-80 A TA, Takara, Tokyo, Japan), then gelatin sponges were obtained. Thermal cross-linking was introduced by a vacuum oven (AVO-250N, As One Co. Ltd., Osaka, Japan) at 140℃ for 3, 5, 8, and 14 h. Next, cross-linked gelatin sponges were pulverized with a coffee mill (KHN22-5889, Kohnan Syouji Co. Ltd., Osaka, Japan) into flaky powder, gelatin flakes. The gelatin flakes were passed through three meshes (TESTING SIEVE (sieve size 150 × 60, aperture 0.5, 1, 2 mm), TOKYO SCREEN CO.LTD., Tokyo, Japan) in the order of decreasing aperture. Flakes remaining on the 1 mm aperture were defined as 1000-2000 μm in size, particles remaining on the 0.5 mm aperture were defined as 500-1000 μm in size and particles passing through the 0.5 mm aperture were defined as below 500 μm in size.

Finally, we obtained thermally cross-linked gelatin flakes (GFL) with various particle sizes and cross-linked for various times. In this paper, gelatin flakes cross-linked for 0, 3, 5, 8 and 14 h are referred to as GFL-0, GFL-3, GFL-5, GFL-8 and GFL-14, respectively.

2. *In Vitro* Examination

2.1. Scanning electron microscope (SEM) observation

Scanning electron microscope (SEM) observation of GFL-0 with a particle size of 500-1000 μm was carried out using a Miniscope TM-

1000 (Hitachi-High-Technologies Co., Tokyo, Japan).

2.2. Water solubility test

Water solubility tests of gelatin flakes thermally cross-linked for 0, 3, 5, 8 and 14 h with a particle size of 500-1000 μm were performed. The ratio of the remaining weight of gelatin flakes after being immersed in water was evaluated.

Gelatin flakes dried under vacuum at room temperature overnight in advance were weighed about 40 mg (W*s*).

The gelatin flakes were immersed in distilled water at 37℃ for 1, 2 and 3 h. After being immersed, the gelatin flakes were dried under vacuum at 60℃ overnight using a vacuum drier VACUUM OVEN AVO-250N (As One Co. Ltd., Osaka, Japan). The dry weight of gelatin flakes (W*d*) was measured.

The ratio of the remaining weight of gelatin flakes was calculated from the equation (A). (23)

$$\text{The ratio of the remaining weight (\%)} = (W_d / W_s) \times 100 \quad \text{(A)}$$

where W*s* is the initial weight of gelatin flakes before immersion, and W*d* is the dry weight of gelatin flakes after immersion.

When the ratio of the remaining weight was constant, even after extending the immersion time, the value indicated “the fraction of cross-linked gelatin”.

2.3. Measurement of the degree of swelling

The degree of swelling of gelatin flakes thermally cross-linked for 3, 5, 8 and 14 h with particle sizes of below 500, 500-1000 and 1000-2000 μm was measured.

Gelatin flakes dried under vacuum at room temperature overnight in advance were weighed about 40 mg.

The gelatin flakes were immersed in distilled water at 37℃ for 3 h. After being immersed for 3 h, the flakes were taken out from the distilled water and the excess water on the container was removed. Then, the weight of the gelatin flakes containing water (W*w*) was measured. After that, the gelatin flakes were dried under vacuum at 60℃ overnight using a vacuum drier VACUUM OVEN AVO-250N (As One Co. Ltd., Osaka, Japan). After vacuum drying, the dry weight (W*d*) was measured. The degree of swelling was calculated from the equation (B).

The degree of swelling (%) = [(W*w* – W*d*) / W*d*] × 100……(B)

where W*w* is the weight of the wet gelatin flakes and W*d* is the weight of gelatin flakes after complete drying at 60°C. (24)

2.4. Measurement of degradability in collagenase solution

The degradability in collagenase solution of gelatin flakes thermally cross-linked for 3, 5, 8 and 14 h with a particle size of 500-1000 μm was measured. The ratio of the remaining weight of gelatin in

collagenase solution was evaluated.

Gelatin flakes dried under vacuum at room temperature overnight in advance were weighed about 20 mg (W*s*). The gelatin flakes were immersed in collagenase solution (0.5 unit/mL, 50 mM Tris-HCl Buffer, 10 mM CaCl2, pH 7.4) at 37℃ for 0.5, 1 and 2 h. After being immersed, the gelatin flakes were taken out from collagenase solution and immersed in distilled water for a few minutes. Then, the flakes were taken out and immersed in new distilled water for a few minutes in the same manner. This immersion in distilled water was conducted three times, and the collagenase solution inside the gelatin flakes was completely replaced with distilled water. Subsequently, the gelatin flakes were dried under vacuum at 60℃ overnight using a vacuum drier VACUUM OVEN AVO-250N (As One Co. Ltd., Osaka, Japan). The weight of the flakes after enzymatic degradation (W*a*) was measured. The ratio of the remaining weight of gelatin was calculated from the equation (C).

The ratio of the remaining weight of gelatin (%) =

$$(W_a / W_s) \times 100 \quad \text{(C)}$$

where W*s* is the weight of the flakes before enzymatic degradation and W*a* is the weight of the flakes after enzymatic degradation.

2.5. Measurement of the water absorption time

Gelatin flakes were weighed about 20 mg and put in a cell strainer

(40 μm Nylon) (BD Falcon, Tokyo, Japan). Gelatin sponges (about 20 mg, about 4 mm thick) were put in a cell strainer in the same manner. Patent blue (about 6.5 mg) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to distilled water, and the concentration of the patent blue solution was set to about 0.3 g/L. About 10 μL of the patent blue solution was dropwised onto the surface of the samples from the height of about 1.5 cm. Then, the "absorption time", the time from when the solution dropped until the superior surface of the droplet was completely absorbed by the sample when viewed from the side, was measured.

RESULTS

1. Scanning electron microscope (SEM) observation

The scanning electron micrograph of GFL-0 with a particle size of 500-1000 μm was shown in Figure 1. The gelatin flakes exhibited flake-ball structures with many folds on the surface and were not mere globular particles.

2. Water solubility test

Changes in the immersion time and the ratio of the remaining weight of GFL-3, GFL-5, GFL-8 and GFL-14 with a particle size of 500-

1000 μm are shown in Figure 2. Until an immersion time of 2 h, the ratios of the remaining weight of all gelatin flakes decreased. After an immersion time of 2 h, the ratios of the remaining weight maintained almost constant values. On the other hand, GFL-0 was completely dissolved as soon as it was immersed in distilled water. As the thermal cross-linking time of gelatin flakes increased, the ratio of the remaining weight tended to increase.

In Figure 2, the value at an immersion time of 3 h means the ratio of cross-linked gelatin in gelatin flakes because uncross-linked gelatin is soluble in water and thermally cross-linked gelatin is not. The relationship between the fraction of cross-linked gelatin and the thermal cross-linking time is shown in Figure 3.

As the thermal cross-linking time increased, the fraction of cross-linked gelatin tended to increase.

In order to evaluate the difference in the relationship between the fraction of cross-linked gelatin and the thermal cross-linking time, the fractions of cross-linked gelatin of various particle sizes of gelatin flakes were measured, as shown in Figure 4. The fraction of cross-linked gelatin increased as the thermal cross-linking time increased in all cases of various particle sizes (below 500, 500-1000 and 1000-2000 μm). The fraction of cross-linked gelatin seemed not to be different between the various particle sizes.

3. Measurement of the degree of swelling

The degree of swelling of gelatin flakes with various particle sizes (below 500, 500-1000 and 1000-2000 μm) and cross-linked for different thermal cross-linking times (3, 5, 8 and 14 h) is shown in Figure 5. The degree of swelling of gelatin flakes was greater than 1000% and showed high values in all gelatin flakes. The degree of swelling decreased as the thermal cross-linking time increased. There were no differences in the degree of swelling between GFL-8 and GFL-14. There were almost no differences in the degree of swelling between particle sizes (below 500, 500-1000 and 1000-2000 μm).

4. Measurement of degradability in collagenase solution

The ratios of the remaining weight of GFL-3, GFL-5, GFL-8 and GFL-14 in collagenase solution are shown in Figure 6. In all gelatin flakes, the ratio of the remaining weight decreased as the immersion time in collagenase solution increased, and the gelatin flakes almost disappeared after being immersed for 2 h. In addition, the ratio of the remaining weight of gelatin flakes had a tendency to decrease slowly as the thermal cross-linking time increased.

5. Measurement of the water absorption time

The water absorption times of gelatin sponges and flakes cross-linked for various times were measured, as shown in Figure 7 and

Table 1.

When making a comparison between the gelatin sponges and gelatin flakes, the water absorption time of the flakes was found to have a tendency to be shorter than that of the sponges regardless of the thermal cross-linking time. In both cases of gelatin flakes and gelatin sponges, the water absorption times of the thermally cross-linked samples were shorter than those of the uncross-linked samples. However, the differences in water absorption time between the thermally cross-linked samples for 3, 5, 8 and 14 h were unclear in both cases of gelatin flakes and gelatin sponges.

In case of the flakes, the droplet was absorbed and moved downward quickly. In contrast, in case of the sponges, the droplet spread around the dropping site.

DISCUSSION

As shown in Figure 1, gelatin flakes had flake-ball structure with many folds on the surface. Gelatin flakes had a large surface area, resulting in good water solubility and high attachment stability onto tissue in the presence of blood, similar to gelatin sponge. In the same way as gelatin sponges shown in previous studies, (15, 16, 25) unique properties that globular particles and liquid type anti-adhesive

materials do not have, such as high attachment stability onto tissue even in the presence of blood, can be expected.

As observed in the result of the solubility test shown in Figure 2, it was found that the thermally cross-linked gelatin flakes were composed of water-soluble portion and water-insoluble one. In general, it is known that intermolecular cross-linking between polymer chains makes gelatin insoluble in water. Therefore, the insoluble portion of gelatin flakes is cross-linked gelatin, and the soluble portion is not cross-linked gelatin. (19)

As shown in Figures 3 and 4, as the thermal cross-linking time increased, the cross-linking points in gelatin flakes increased, resulting in increases in the fraction of cross-linked gelatin. As seen in Figure 3, the fraction of cross-linked gelatin increased with prolongation of the thermal cross-linking time up to 8 h, above which increase in the fraction became moderate. As shown in Figure 4, the fraction of cross-linked gelatin increased as the thermal cross-linking time increased, and there was a tendency to maintain an almost constant fraction of cross-linked gelatin when thermally cross-linked for more than 8 h. This is because the cross-linking points may be almost fully produced by thermally cross-linking for 8 h.

According to these results, gelatin flakes with various degrees of cross-linking can be made by thermal cross-linking.

With regard to gelatin flakes with various particle sizes and

degrees of cross-linking, measurements of the degree of swelling, degradability in collagenase solution and water absorption time were performed.

As observed in the result of the degree of swelling shown in Figure 5, the degree of swelling decreased with prolongation of the thermal cross-linking time up to 8 h, above which the values became almost constant. In case of gelatin film, it is known that the amount of water that gelatin film can retain decreases due to intermolecular cross-linking between the polymer chains of gelatin. (19) It is considered that the same is true for gelatin flakes.

In order to investigate the effects of the thermal cross-linking time on degradability, the degradability of gelatin flakes in collagenase solution was measured, as shown in Figure 6. The residual rate of gelatin was smaller than 20% for GFL-3 and GFL-5, while the residual rate of gelatin was over 20% for GFL-8 and GFL-14. It is considered that thermal cross-linking slows the degradation rate. Gelatin flakes are degraded enzymatically in the body, so similarly in the body, the biodegradation rate decreases as thermal cross-linking time increases. The biodegradation time in the body can be controlled by changing the thermal cross-linking time.

For anti-adhesive materials, a short absorption time is important because flakes quickly absorb the water, such as body fluids and blood, on the tissue surface when they are placed on tissue.

Therefore, gelatin flakes are expected to exhibit high attachment stability. As is shown in the result of the measurement of the water absorption time in Figure 7, the water absorption time decreased by the introduction of thermal cross-linking. When the particles of the uncross-linked gelatin flakes make contact with water, the particles dissolve quickly and turn immediately into a gel. As a result, it is difficult for water to pass through the dissolved portion, and the water absorption speed decreases. On the other hand, in case of thermal cross-linking, the cross-linked portion does not dissolve in water. So, spaces among the flakes can remain, and water can pass through them easily. In this way, the water absorption speed of cross-linked gelatin flakes is fast. The differences in the water absorption speeds among the gelatin flakes cross-linked for various cross-linking times were unclear in this method of the measurement.

In this study, gelatin flakes with various degrees of cross-linking and various particle sizes were made, and the fraction of cross-linked gelatin, the degree of swelling, the degradability and the water absorption speed were evaluated.

Gelatin is hydrophilic, and the flake-form has a very large surface area. In addition, there are a lot of spaces among the flakes, so the capillary force is strong. Therefore, gelatin flakes exhibit good water absorbability and attachment stability as an anti-adhesive material. Furthermore, because the insoluble portion is formed by thermal

cross-linking, the form is remained and acts as a mechanical barrier after the flakes are placed on tissue and the insoluble cross-linked portion turns into a soft gel when it absorbs water. So the gel fits the surface of the tissue and shows great attachment. And the gel does not injure the surrounding tissue. Because of these benefits, thermal cross-linked gelatin flakes possess outstanding fundamental properties as a new anti-adhesive material.

Among them, the fraction of cross-linked gelatin and degradability are considered to be especially important properties. With respect to the fraction of cross-linked gelatin, the longer cross-linking time is better because the more mechanical barrier on the wound site is better. On the other hand, with respect to degradability, gelatin flakes should remain on the wound site for about 7 days and be degraded without remaining as a foreign body for 14 days. Therefore, considering the fraction of cross-linked gelatin and degradability, gelatin flakes have a proper thermal cross-linking time for an anti-adhesive material. In this study, differences in the fraction of cross-linked gelatin and degradability among particle sizes were not clear.

In the future, further evaluations of the properties of gelatin flakes, such as attachment properties on tissue, safety and anti-adhesive effects in the body and usability in laparoscopic and thoracoscopic surgery, are required.

Gelatin flakes exhibit high water absorbability and turn into a gel,

thereby acting as a mechanical barrier on tissue. In addition, the biodegradation time can be controlled by changing the thermal cross-linking time. Because of these benefits, thermal cross-linked gelatin flakes possess great fundamental properties as a new anti-adhesive material.

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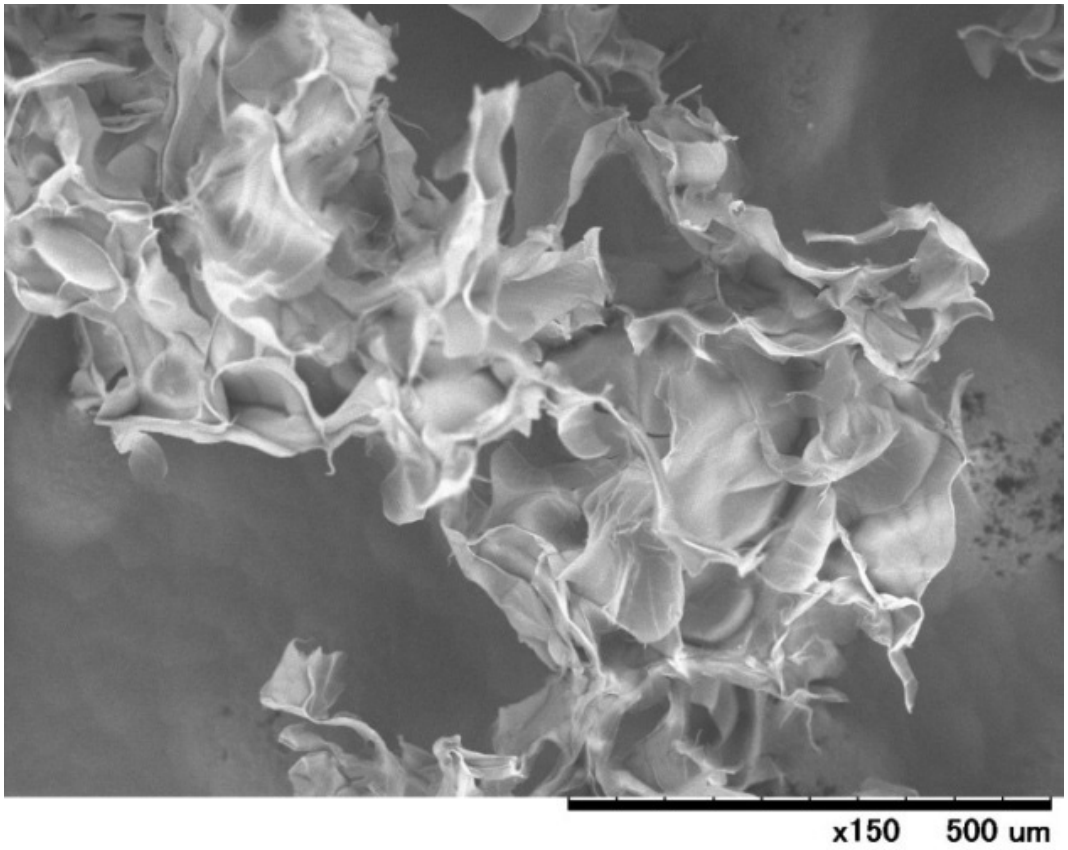

Figure 1. SEM image of GFL-0 (500-1000 μm) (×150)

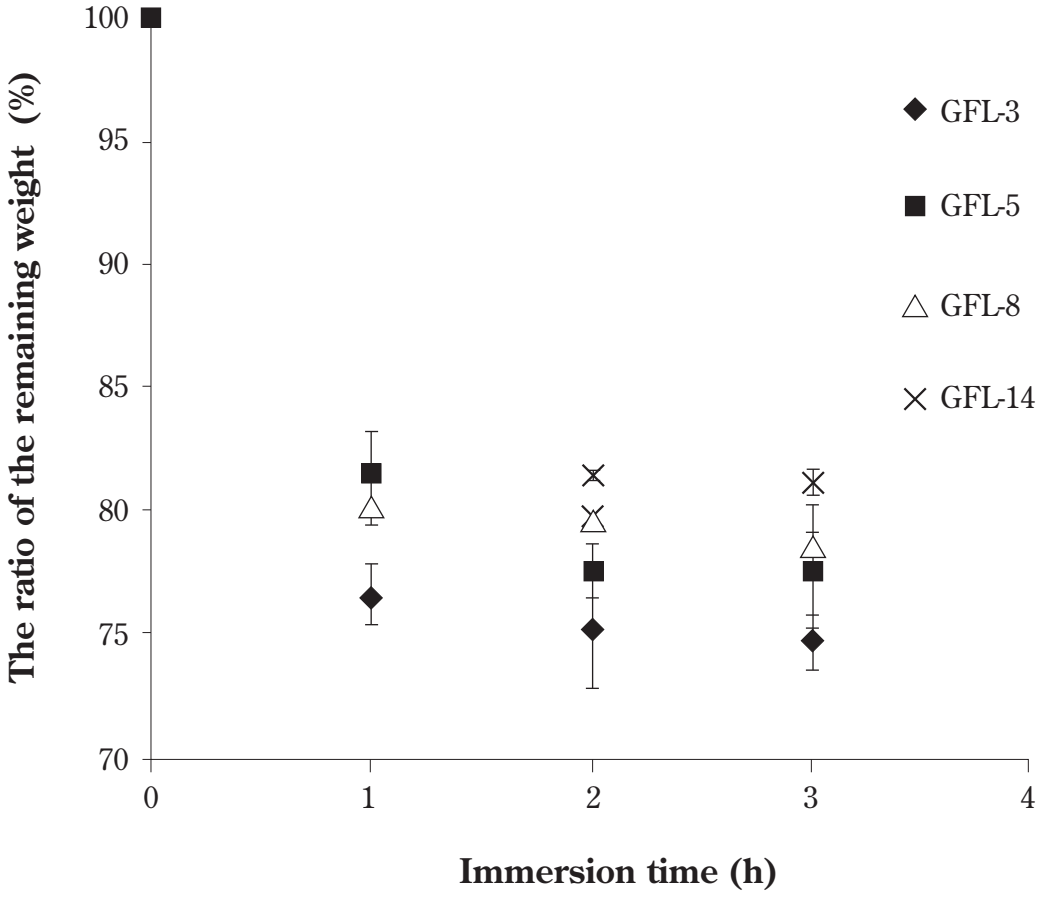

Figure 2. Changes in the immersion time and the ratio of the remaining weight

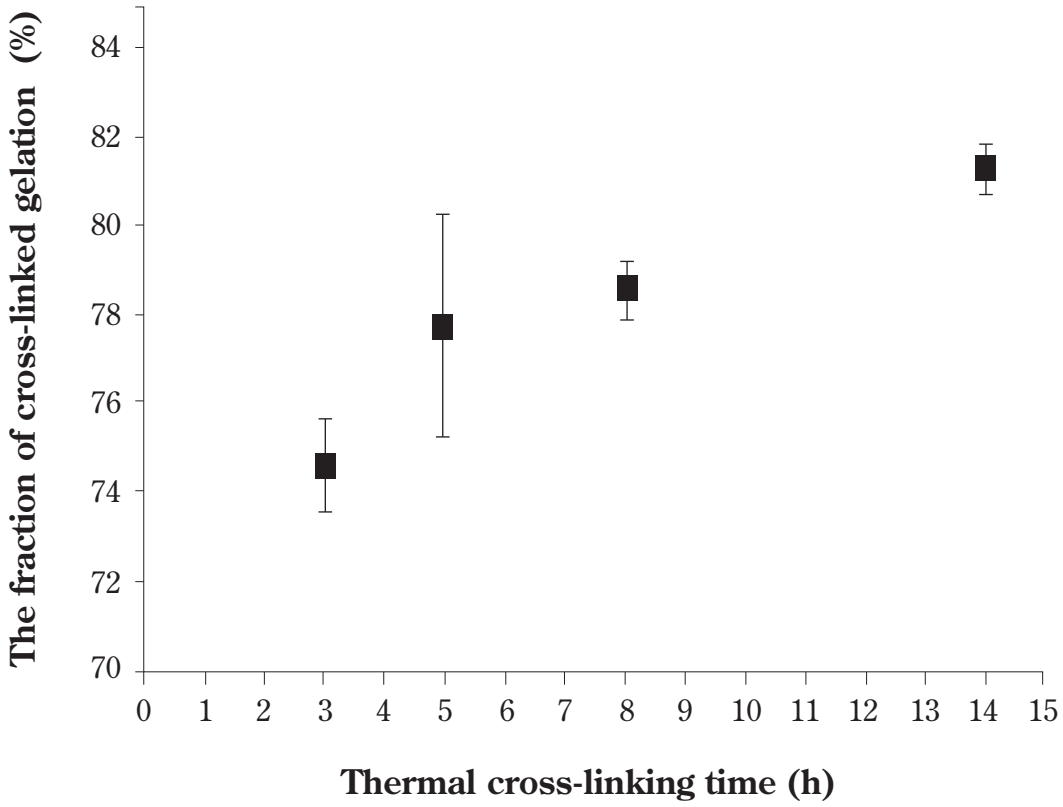

Figure 3. The relationship between the fraction of cross-linked gelatin and the thermally cross-linking time

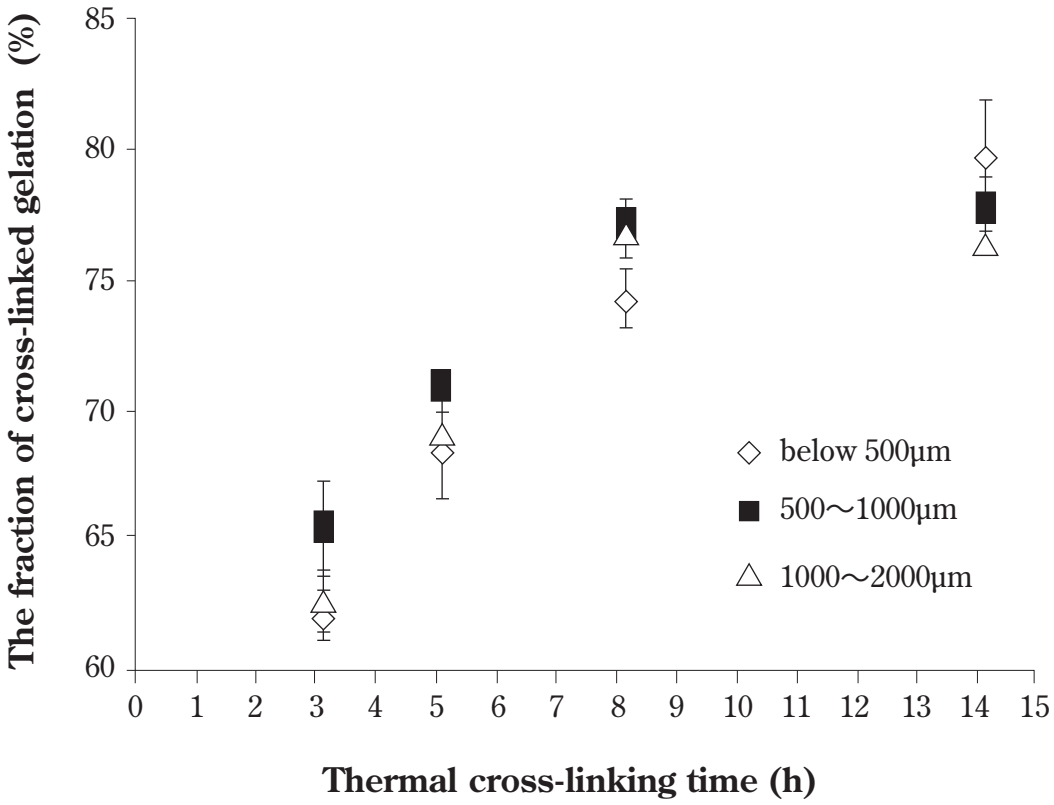

Figure 4. The fractions of cross-linked gelatin of various particle sizes of gelatin flakes (GFL)

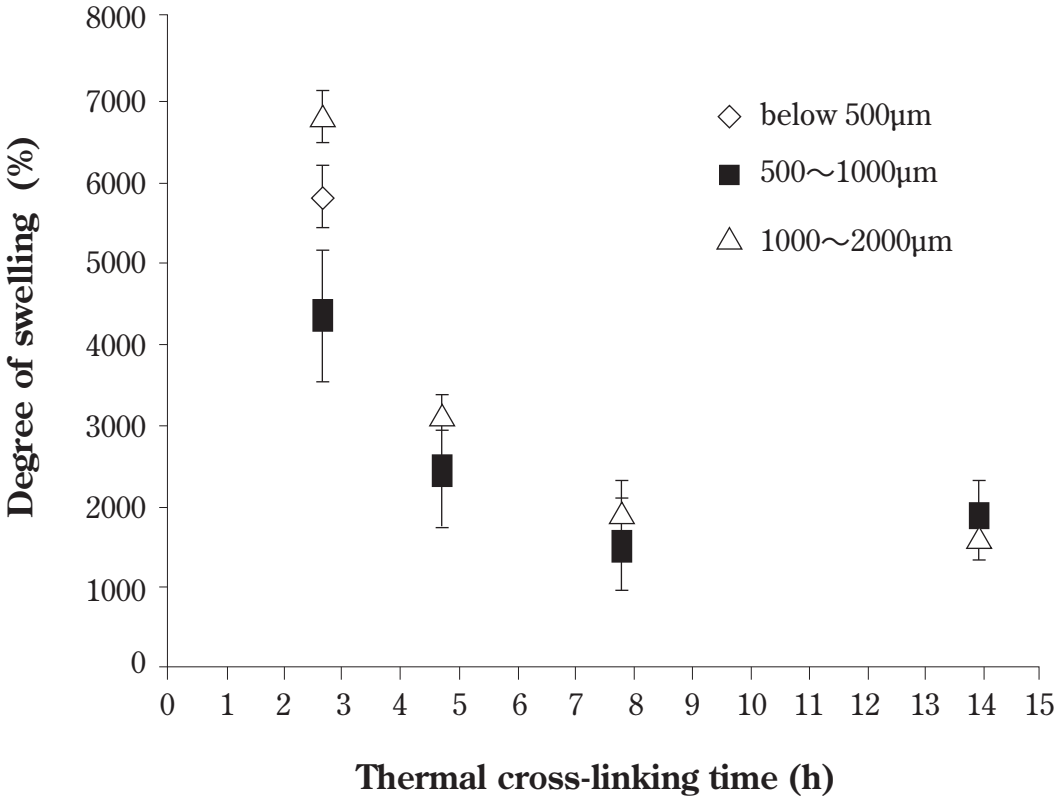

Figure 5. The result of the measurement of the degree of swelling

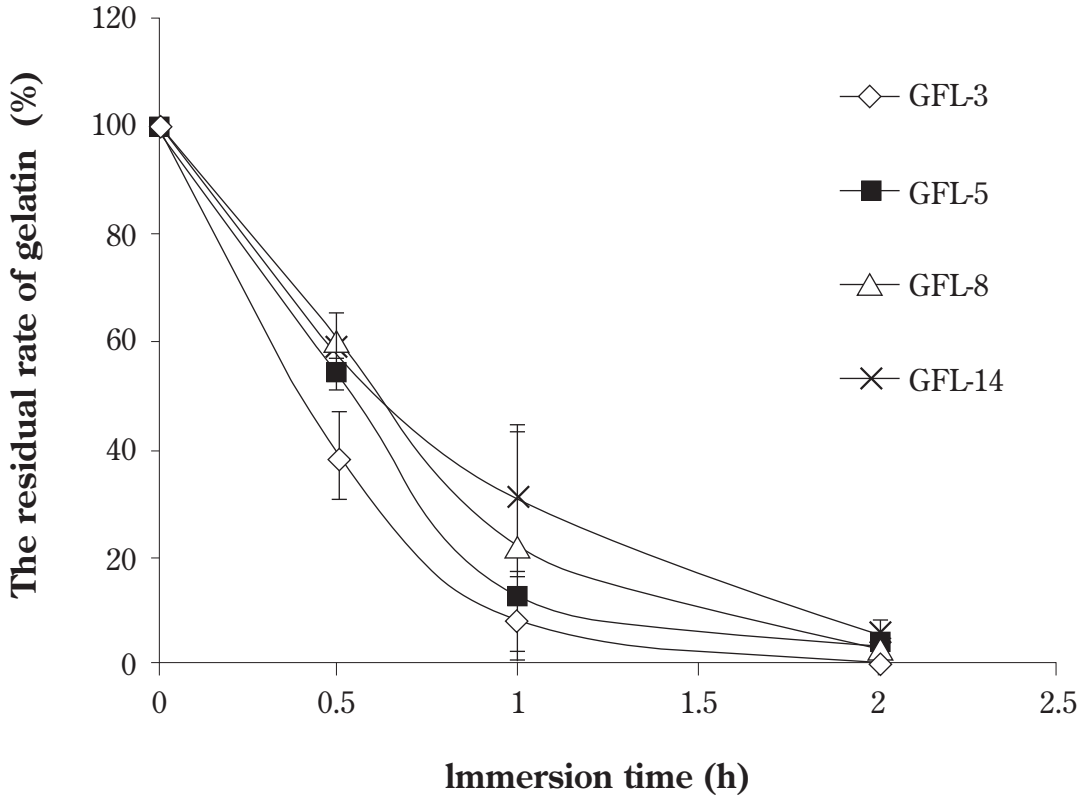

Figure 6. The result of the measurement of degradability in collagenase solution

Table 1. Water absorption time

	Average of the water absorption time (s)	
Thermal crosslinking time (h)	flakes	sponge
0	19.8	57.6
3	5.1	14.0
5	4.0	9.4
8	2.8	12.8
14	4.5	7.2

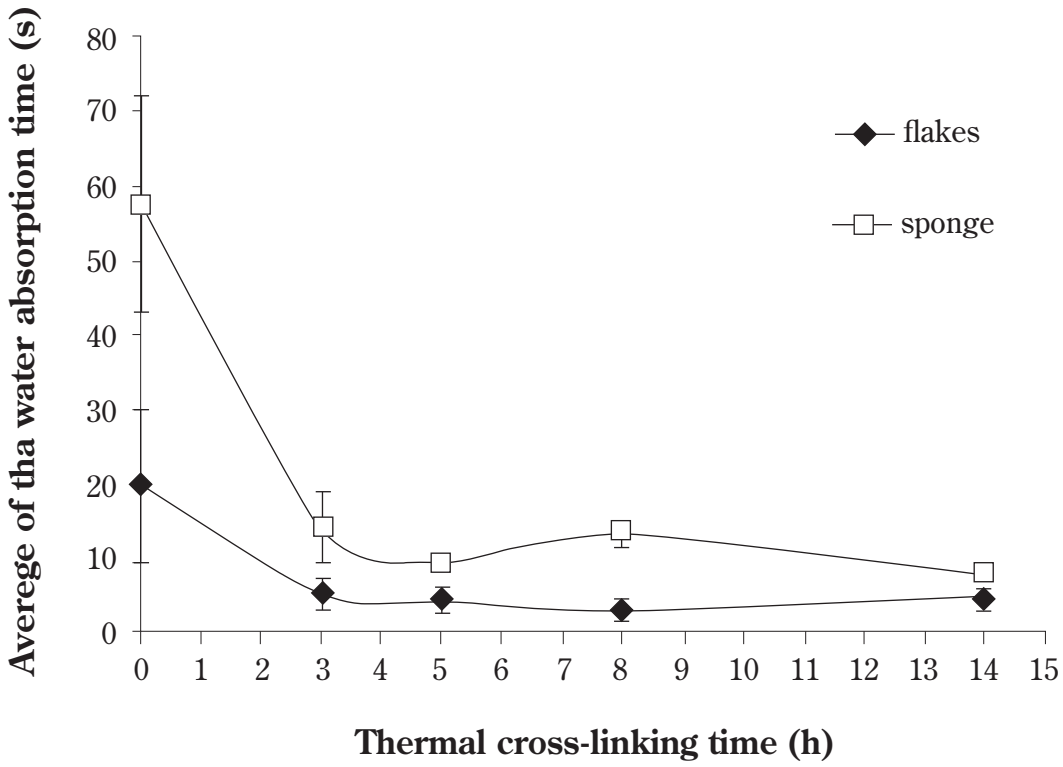

Figure 7. Water absorption time

STUDY III-3

The Anti-adhesive Effects and Biodegradability of Gelatin Flakes in Rats

TABLE OF CONTENTS (STUDY III-3)

ABSTRACT

Background:

Currently, film- or sheet-type anti-adhesive materials, such as a gelatin film® comprising a combination of hyaluronic acid and carboxymethyl cellulose, are commonly used in open surgery. However, these film- or sheet-type anti-adhesive materials are generally thin and fragile and therefore easily break during surgery. In particular, such materials are quite difficult to handle when applied in thoracoscopic and laparoscopic surgery. To address these problems, we developed a new type of anti-adhesive material, "gelatin flakes" (GFC). In this study, we present the findings of *in vivo* examinations of gelatin flakes using a rat adhesion model, paying particular attention to anti-adhesive effects and biodegradability.

Materials and Methods:

We made gelatin flakes which were thermally cross-linked for 3 different periods of time: 0, 5, 14 hours (GFL-0, GFL-5 and GFL-14, respectively). In this study, we used a rat cecal abrasion model designed to create adhesion between the cecum and the abdominal wall, and applied gelatin flakes to the abraded cecum under various conditions (i.e., various treatment doses of flakes or additional thermal cross-linking). In the model, we macroscopically observed

the status of the applied gelatin flakes over time and evaluated the anti-adhesive effects of the flakes by scoring the degree of adhesion according to the adhesion grading scales.

Results:

In the pilot study, gelatin flake groups showed significantly lower scores in both categories of the extent and the severity in comparison to the non-treated group. In the time-course observation, the flakes without cross-linking macroscopically turned into gel and disappeared very soon after applied. However, the flakes with thermal cross-linking remained in a gel state at the applied sites beyond one week with excellent anti-adhesive effects. In the study to determine the optimal treatment dose of gelatin flakes, the adhesion scores tended to decrease in association with increasing treatment doses.

Conclusion:

These results suggest that gelatin flakes have satisfying anti-adhesive effect and favorable biodegradability, which were affected by the treatment dose and thermal cross-linking. Although further examinations are needed to optimize, the gelatin flakes are a potentially useful anti-adhesive material.

ラットの盲腸擦過モデルにおけるゼラチンフレークの癒着防止効果および生体内の分解性の検討

要約

背景：

現在、フィルムやシートタイプの癒着防止材が外科手術では広く使用されている。これらのフィルムやシートタイプの製材は通常開腹手術で使用されるものである。しかしながらこれらは、一般に薄く脆弱で、それゆえ手術中に容易に破損する。特に胸・腹腔鏡手術時の取り扱いは困難である。この問題点に対し、新規の癒着防止材「ゼラチンフレーク」を開発した。この研究報告では、ラットの癒着モデルを用い、癒着防止効果と生体内分解性について報告する。

材料および方法：

３時間架橋ゼラチンを架橋時間０、５、14時間の３種類の異なる熱架橋熱架橋を行いこれらを粉砕して、３種類の熱架橋ゼラチンフレーク（GFL-０、GFL-５、GFL-14）を作成した。ラット盲腸擦過モデル（盲腸と腹壁の間の癒着形成）を用い、架橋時間の違いと投与量の違いの条件下で癒着防止材を使用し、検討した。癒着防止効果は、長短期間の効果を肉眼的評価スコアにより評価した。

結果：

パイロットデストでは、５と14のいずれの架橋時間でも未架橋フレークに比較して有意に優れた癒着防止効果を示した。経時的検討では、未架橋フレークは、非常に早期にゲル化し視認出来なくなった。他方架橋フレークは、１週間以上にわたりゲル状で肉眼的に患部表面に視認され、優れた癒着防止効果を示した。投与量の検討を行った試験では、投与量の増加に伴い癒着防止効果も向上した。

結語：

上記の結果から、熱架橋ゼラチンフレークは癒着防止効果と生体内分解性を十分に有し、これらは熱架橋時間と投与量により可変であることがわかった。今後さらなる検討を行えば、ゼラチンフレークは有用な癒着防止材となる可能性を持つと考えられる。

INTRODUCTION

Abdominal adhesions occur in more than 90 % of the patients who have undergone abdominal surgery. (1) Postsurgical adhesions often result in serious complications, such as small bowel obstruction, chronic abdominal pain and female infertility, and increase the technical difficulty of performing reoperative surgery. (2-4) Therefore, to prevent abdominal adhesions, numerous anti-adhesive materials have been developed, some of which have been used clinically.

Currently, Seprafilm® comprising a combination of hyaluronic acid and carboxymethyl cellulose film is an anti-adhesive material commonly used in open surgery. When placed into injured tissue, Seprafilm® rapidly turns in to gel, absorbing the surrounding moisture, and it thereafter remains at the site for approximately seven days as a barrier to prevent adhesion to adjacent organs. (5-7) In fact, several studies have reported that Seprafilm® decreases the incidence or severity of adhesions, both experimentally and clinically. (8-12)

However, Seprafilm® did not decrease the incidence of bowel obstruction in clinical trials, although it did decrease the incidence of severe cases that required reoperation. In addition, Seprafilm® induces a higher incidence of intestinal anastomotic leakage,

especially when used to directly wrap suture or staple lines of fresh bowels. (9, 10, 12)

To generate more effective and safe anti-adhesive materials, we previously developed a thermally cross-linked gelatin film (GFM). We reported that the film exhibited superior anti-adhesive effects with excellent peritoneal regeneration and could be used safely on intestinal anastomoses, in comparison to Seprafilm®. (13) In addition, we recently developed a two-layered gelatin sheet that combined the gelatin film with a gelatin sponge. (14) This two-layered gelatin sheet had not only excellent anti-adhesive effects, but also a superior ability to attach to injured tissue by absorbing both moisture and blood. However, these film- or sheet-type anti-adhesive materials, including Seprafilm®, gelatin film and two-layered gelatin sheet, are generally thin and fragile and therefore easily break during surgery. In particular, it is quite difficult to handle these materials when they are applied in thoracoscopic and laparoscopic surgery. (5, 15, 16)

To address the problems of these film- or sheet-type anti-adhesive materials, we developed a new type of anti-adhesive material, "gelatin flakes", made of pulverized gelatin sponges. This flake-shaped material can be applied more easily through thoracoscopic and laparoscopic instruments while maintaining a superior ability to attach to injured tissue and absorb both moisture and blood. (17) In this study, we present our findings of *in vivo* examinations of gelatin

flakes using a rat adhesion model, and while paying particular attention to their anti-adhesive effects and biodegradability.

MATERIALS AND METHODS

1. Preparation of gelatin flakes

Medical grade gelatin (type-I collagen, Medigelatin®) extracted from porcine skin, with an isoelectric point of 5 was supplied by Nippi Co, Ltd. (Shizuoka, Japan). The gelatin was dissolved in distilled water (Otsuka Distilled Water, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) to a final concentration of 4.8 wt%. The solution was frothed with a homogenizer (Nissei Excel Auto Homogenizer DX-4, Nihonseiki Kaisha Ltd., Nigata, Japan) at 8,000 rpm for three minutes, frozen at -80℃ with a deep freezer (ULTRA LOW, SANYO Electric Co., Ltd, Osaka, Japan) for approximately 30 minutes and freeze-dried for 24 hours with a freeze dryer (TF10-80ATA, Takara, Tokyo, Japan) until a sponge like appearance was attained.

Additional dehydrothermal cross-linking was introduced by a vacuum oven (Vacuum Oven AVO-250N, As One Co. Ltd., Tokyo, Japan) at 140℃ for three different time periods: 0 (no heating), 5 or 14 hours (GFL-0, GFL-5 and GFL-14, respectively). Then, the gelatin sponges were pulverized into small pieces (gelatin flakes) with a

coffee mill (Coffee Mill KHN 22-5889, Kohnan Shoji Co. Ltd., Osaka, Japan).

Then, the gelatin flakes were fractionized by two sieves with different mesh size (mesh size: 500 μm, 1000 μm, Test sieves JIS Z 8801, Tokyo Screen Co., Ltd, Tokyo, Japan) and only the flakes with particles measuring 500 - 1000 μm were collected for further experiments (Figures 1 and 2). Finally, the gelatin flakes with or without thermal cross-linking were sterilized with ethylene-oxide gas in preparation for the animal experiments.

2. Animal experiments

2.1. Animals

Female Wistar/ST rats, 8 weeks of age weighing approximately 200 g were purchased from Shimizu Animal Laboratory (Kyoto, Japan). During the experimental period, these rats were maintained under standard specific pathogen-free (SPF) conditions (a light-dark cycle of 12:12 hours, a mean temperature of 23°C, and mean humidity of 50%). Standard laboratory rodent chow and water were freely available. Before the study, the rats were maintained in the laboratory for one week. On the experimental day, all rats were checked for their overall health condition. All animal care protocols and the following animal experiments performed in this study were approved by the Animal Experimentation Committee of Doshisha University. All

surgeries were performed under sterile conditions and all procedures were performed by one surgeon.

2.2. Surgical procedures used to create a rat adhesion model

To examine the anti-adhesive effects of the gelatin flakes under conditions or to observe the status of gelatin flakes applied to experimental animals, we used a rat cecal abrasion model designed to create adhesions between the rat's cecum and abdominal wall. (17) The common procedures of the experiments were as follows:

The rats were anesthetized using isoflurane (Escain®, Mylan, Inc., Osaka, Japan) inhalation. Then, sodium pentobarbital (Sommnopentyl®, KyoritsuSeiyaku, Tokyo, Japan) was administered intraperitoneally at a dose of 35 mg/kg of body weight with a tuberculin syringe and a 23-G injection needle. Under the above-described general anesthesia, the rats were fixed in the dorsal position. A 4-cm-long midline incision was made on the abdominal wall. The serosal surface of the cecum was abraded 10 mm in diameter with dental sanding paper (Dental grinding material, Sharp Mini (size: microfine), Ohki Chemical Industry Co., Ltd.) until small blood drops appeared (Figure 3-a). Another 10-mm-diameter abrasion was made on the right lateral internal abdominal wall directly opposite the abraded cecum.

Next, the gelatin flakes in the conditions described laterwere

placed onto thesites of the abraded cecum (Figure 3-b). The two abraded surfaces were approximated with 7-0 prolene sutures (monofilament; Asflex, Kono Seisakusho Co., Ltd., Chiba) to induce adhesion since the cecum floated freely in the abdominal cavity. The laparotomy incision was closed with 4-0 nylon sutures (Natsume Co, Tokyo, Japan). In the non-treated group, no gelatin flakes were placed, and the laparotomy incision was closed with 4-0 prolene sutures after the two abrasions were approximated.

The rats were sacrificed by a lethal dose of pentobarbital at the predetermined period following surgery. The abdomen was opened again, and the adhesions were scored macroscopically according to an adhesion grading scale (Adhesion scores, Table 1). (18) The evaluation of the adhesion score was performed by a researcher who was blinded to the animal assignments. Statistical comparisons were made by the Dunn test following the Kruskal-Wallis test (Experiment 1) and by the Shirley-Williams test (Experiment 4). A value of less than 0.05 was considered to be significant.

2.3. Evaluation of the anti-adhesive effects of the gelatin flakes

To confirm whether the gelatin flakes has anti-adhesive effects and to optimize the degree of thermal cross-linking or the amount of gelatin flakes, we examined the anti-adhesive effects of the gelatin flakes under various conditions, as described below.

Experiment 1: A pilot study to confirm the anti-adhesive effects of the gelatin flakes

To confirm whether the gelatin flakes has any anti-adhesive effects, we designed a pilot study using the following conditions. 21 rats were randomly assigned to three groups: the GFL-0 group (n=9), the GFL-14 group (n=8), and the non-treated group (n=5). Each treatment dose of gelatin flakes was 20 mg. The evaluation date was two weeks after surgery.

Experiment 2: A preliminary study of macroscopic observation of the gelatin flakes and the anti-adhesive effects over time

To observe the status and anti-adhesive effects of the gelatin flakes over time after applied to the experimental animals, we designed an experiment using the following conditions. 32 rats were randomly assigned into four groups consisting of eight rats each: the GFL-0 group, the GFL-5 group, the GFL-14 group, and the non-treated group. Each treatment dose of gelatin flakes was 20 mg. At 20 minutes, four, seven and 14 days after surgery, we observed the macroscopic findings of the applied gelatin flakes and evaluated the anti-adhesive effects in two animals in each group.

Experiment 3: A preliminary study to examine the anti-adhesive effects in lower doses of gelatin flakes

To examinethe anti-adhesive effects in lower doses of gelatin flakes (less than 20 mg), 21 rats were randomly assigned to seven groups consisting of three rats treated with various types of flakes and treatment doses: the GFL-0, 5mg group, the GFL-0, 10mg group, the GFL-5, 5mg group, the GFL-5, 10mg group, the GFL-14, 10mg group, the GFL-14, 10mg group, and the non-treated group. The evaluation date was three weeks after the surgery.

Experiment 4: A larger-scale study to determine the optimal treatment dose of gelatin flakes

Based on the results of Experiments 1 and 3, we designed a larger-scale study to determine the optimal treatment dose ofgelatin flakes. 30 rats were randomly assigned to fivegroups of six rats each: the GFL-5, 5mg group, the GFL-5, 10mg group, the GFL-5, 15mg group, the GFL-5, 20mg group and the non-treated group. The evaluation date was three weeks after the surgery.

RESULTS

Experiment 1: A pilot study to confirm the anti-adhesive effects of the gelatin flakes

The adhesion scores obtained in the pilot study are shown in Figure 4 and Table 2. The GFL-0 and GFL-14 groups exhibited significantly lower scores in both categoriesof extent and severityin comparison to that observed in the non-treated group. However, there were no significant differences between the two flake groups.

Experiment 2: A preliminary study for macroscopic observation of the gelatin flakes and the anti-adhesive effects over time

The macroscopic status of the gelatin flakes:

When gelatin flakes were applied into the abraded sites of the cecum in the rat adhesion model, the flakes rapidly turned into a gel absorbing the surrounding moisture and blood, and remained at the sites without flowing out, irrespective of the types of flake. However, 20 minutes after implantation, the gel in the GFL-0 groups disappeared macroscopically. In contrast, the gels in the GFL-5 and GFL-14 groups remained at 20 minutes after implantation. These gels were found between the abraded cecum and abdominal wall at four and seven days after surgery. At 14 days after surgery, we could not find any flakes or gel macroscopically inany of the flake groups.

The macroscopic status of adhesion and the adhesion scores:

We evaluated the status of adhesion and the adhesion scores four, seven and 14 days after surgery. The adhesion scores are shown in Figure 5 and Table 3. On day 4, adhesions were observed between the cecum and the abdominal wall in all groups. However, the adhesions in the flake groups were weak and delicate and fell apart easily by hand, exhibiting lower scores of adhesion, especially in the category of severity. On day 7, the adhesions became stronger than those observed on day 4, while remaining gels were observed in the GFL-5 and -14 groups. On day 14, the non-treated group showed higher adhesion scores in both categories, and the flake groups, especially the GFL-5 group, exhibited lower adhesion scores.

Experiment 3: A preliminary study to examine the anti-adhesive effects in lower doses of gelatin flakes

The adhesion scores are shown in Figure 6 and Table 4. The adhesion scores were generally high (more than 2 points on average). There seemed to be no differences among the evaluated groups, irrespective of the treatment dose (5 mg or 10 mg) or the type of gelatin flake (GFL-0, -5 or -14), thus indicating no significant anti-adhesive effects in these groups with such lower treatment doses.

Experiment 4: A larger-scale study to determine the optimal treatment dose of gelatin flakes

The adhesion scores are shown in Figure 7 and Table 5. Among the flake groups, only the 20 mg group showed significantly lower adhesion scores in both categories of the extent and severityin comparison to the non-treated group. The 10 and 15 mg groups showed significantly lower adhesion scores in the category of severity. The adhesion scores in the flake groups tended to be lower in both categories of extent and severity depending on the treatment dose.

DISCUSSIONS

As described in the introduction, we newly developed a flake-shaped anti-adhesive material made from gelatin that can be applied easily in thoracoscopic and laparoscopic surgery. However, as an optimal anti-adhesive material, gelatin flakes should possess the following properties: (1) satisfactory anti-adhesive effects achieved by covering the injured sites until the site is no longer susceptible to adhesion, (2) excellent biodegradability evidenced by disappearing quickly after the critical period of reperitonization to avoid foreign body reactions to the material, which can cause other sites of

adhesion and (3) sufficient mechanical properties required for ease of handling in thoracoscopic and laparoscopic surgery. (5, 15)

Under these prerequisites, we firstly attempted to confirm whether gelatin flakes exhibit anti-adhesive effects in a pilot study (Experiment 1). As shown in Table 2, the gelatin flakes clearly exhibited significant anti-adhesive effects in comparison to that observed in the non-treated group. In our previous study examining the anti-adhesive effectsof thermally cross-linked gelatin film, the non-thermally cross-linked gelatin film showed little anti-adhesive effect. (19) However, in the pilot study, the non-thermally cross-linked gelatin flakes (GFL-0) demonstrated significantly superior anti-adhesive effects to that observed in thenon-treated group.

As a next step, to further evaluate the properties of the gelatin flakes, we observed the status and anti-adhesive effects of the gelatin flakes over time after applied to the experimental animals (Experiment2). The flakes without cross-linking (GFL-0) macroscopically turned into gel, but disappeared very soon after being applied. The flakes with thermal cross-linking (GFL-5 and GFL-14) remained in a gel form between the injured cecum and the abdominal wall for at least seven days. This means that the period of thermal cross-linking affects the biodegradability of the gelatin flakes. However, while the GFL-0 and GFL-14 flakes exhibited consecutively higher adhesion scores, the GFL-5 flakes ultimately exhibited lower

adhesion scores on day 14. Therefore, longer or shorter thermal cross-linking of the gelatin flakes may result in reduced anti-adhesion efficacy. Hence, further experiments are required to optimize the period of thermal cross-linking for generating gelatin flakes with favorable biodegradability and satisfying anti-adhesive effects.

Then, in Experiment 3, we examined the anti-adhesive effects in lower doses of the gelatin flakes (less than 20 mg). However, significant anti-adhesive effects were observed in lower treatment doses such as 5 or 10 mg, irrespective of thermal cross-linking. In the first pilot study (Experiment1) using the higher treatment dose of 20 mg, significant anti-adhesive effects were observed in the flake groups. The difference in results between the two experiments suggests that the treatment dose affects the anti-adhesive efficacy.

Based on these results, we designed a larger-scale study to determine the optimal dose of gelatin flakes using various treatment doses. As shown in Figure 7, the adhesion scores in the flake groups tended to be lower depending on the treatment dose. In particular, the groups treated with the dose of 20 mg exhibited significant anti-adhesive effects in both categories of extent and severity. Therefore, in this rat adhesion model, the treatment dose of gelatin flakes should be greater than 20 mg.

In this study, we found that gelatin flakes have satisfying anti-adhesive effects and favorable biodegradability, which were affected

by the treatment dose and thermal cross-linking. Although further examinations are needed to optimize, the gelatin flakes are a potentially useful anti-adhesive material.

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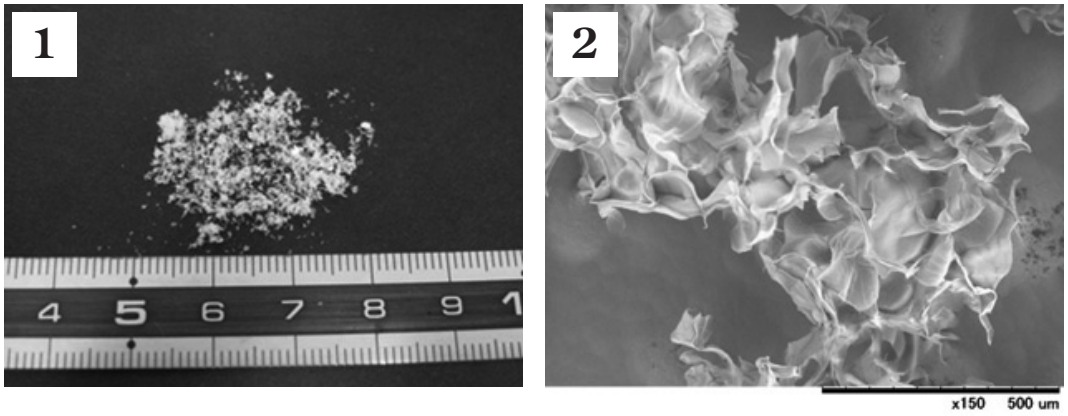

Figures 1 and 2.

1: Macroscopic view of the GFL.

2: Electron microscopic view of the GFL (the black bar indicates 500 µm).

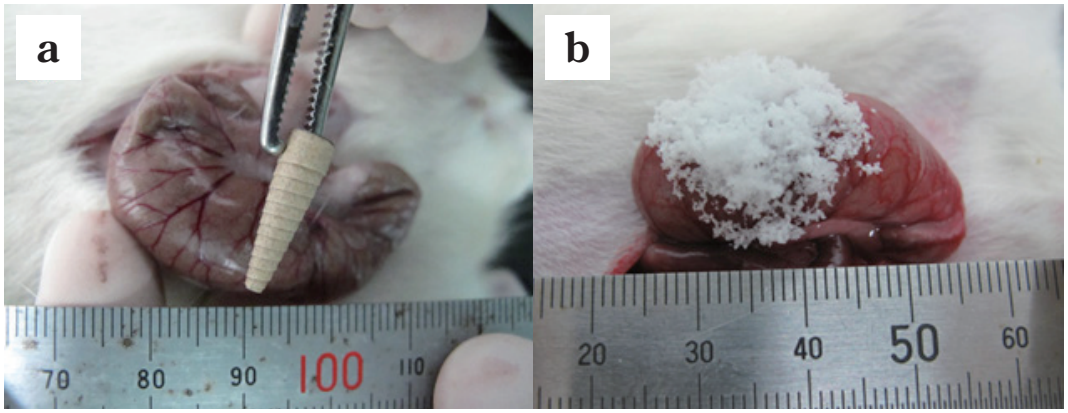

Figure 3. Procedure used to develop the rat adhesion model

a: An abrasion was made on the cecum using a dental sanding tip.

b: Flakes placed onto the abraded cecum.

Table 1. Adhesion scores

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 adhesions 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

Table 2. Adhesion scores obtained in the pilot study (Experiment 1)

		Scores (Extent)						Scores (Severity)					
		0	1	2	3	4	Mean±SD	0	1	2	3	4	Mean±SD
Non-treated group	n=5	0	0	0	1	4	3.80±0.45[a, b]	0	0	0	1	4	3.80±0.45[c, d]
GF-0	n=9	4	3	2	0	0	0.78±0.83[a]	4	2	3	0	0	0.78±0.83[c]
GF-14	n=8	2	5	1	0	0	0.86±0.64[b]	2	3	3	0	0	1.13±0.83[d]

a, b, c: $p<0.01$.
d: $p<0.05$.

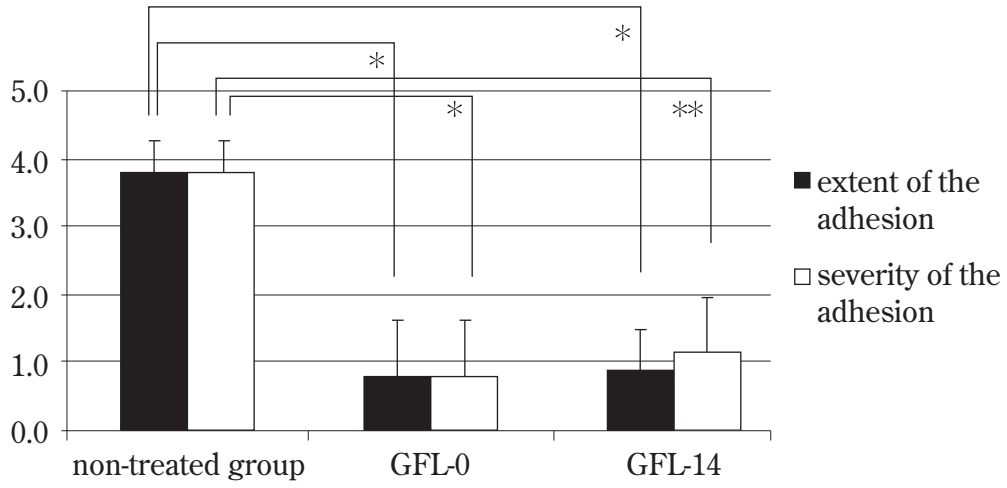

Figure 4. Adhesion scores obtained in the pilot study (Experiment 1)

Significant differences are indicated by * (p<0.01) and ** (p<0.05).

Table 3. Adhesion scores over time (Experiment 2)

			Scores (Extent)						Scores (Severity)					
			0	1	2	3	4	Mean±SD	0	1	2	3	4	Mean±SD
Day 4	Non-treated group	(n=1)	0	0	0	0	1	4.00±0.00	0	0	0	0	1	4.00±0.00
	GFL-0	(n=2)	0	0	0	1	1	3.50±0.71	0	2	0	0	0	1.00±0.00
	GFL-5	(n=2)	1	0	0	0	1	2.00±2.83	1	1	0	0	0	0.50±0.71
	GFL-14	(n=2)	0	0	0	1	1	3.50±0.71	0	2	0	0	0	1.00±0.00
Day 7	Non-treated group	(n=2)	0	0	0	0	2	4.00±0.00	0	0	0	0	2	4.00±0.00
	GFL-0	(n=2)	0	0	0	0	2	4.00±0.00	0	0	2	0	0	2.00±0.00
	GFL-5	(n=2)	0	0	1	0	1	3.00±1.41	0	1	0	0	1	2.50±2.12
	GFL-14	(n=2)	0	0	1	0	1	3.00±1.41	0	1	0	0	1	2.50±2.12
Day 14	Non-treated group	(n=2)	0	0	0	0	2	4.00±0.00	0	0	0	1	1	3.50±0.71
	GFL-0	(n=2)	0	0	1	0	1	3.00±1.41	0	0	2	0	0	2.00±0.00
	GFL-5	(n=2)	1	0	1	0	0	1.00±1.41	1	0	1	0	0	1.00±1.41
	GFL-14	(n=2)	0	0	1	1	0	2.50±1.41	0	0	1	1	0	2.50±0.71

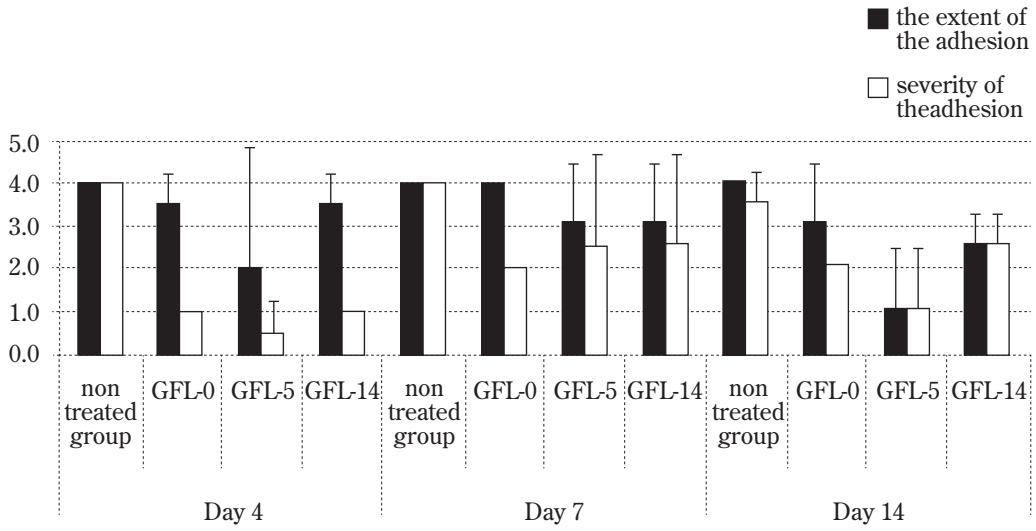

Figure 5. Adhesion scores over time (Experiment 2)

Table 4. Adhesion scores in lower doses of gelatin flakes (Experiment 3)

			Scores (Extent)						Scores (Severity)					
			0	1	2	3	4	Mean±SD	0	1	2	3	4	Mean±SD
Non-treated group		(n=3)	0	0	0	0	3	4.00±0.00	0	0	0	1	2	3.67±0.58
5 mg	GFL-0	(n=3)	0	0	2	1	0	2.33±0.58	0	0	0	3	0	3.00±0.00
	GFL-5	(n=3)	0	0	1	1	1	3.00±1.00	0	0	1	1	1	3.00±1.00
	GFL-14	(n=3)	0	0	0	2	1	3.33±0.58	0	0	1	2	0	2.67±0.58
10 mg	GFL-0	(n=3)	0	0	0	0	3	4.00±0.00	0	0	0	2	1	3.33±0.58
	GFL-5	(n=3)	0	0	1	0	2	3.33±1.15	0	0	1	1	1	3.00±1.00
	GFL-14	(n=3)	1	0	0	1	1	2.33±2.08	1	0	0	1	1	2.33±2.08

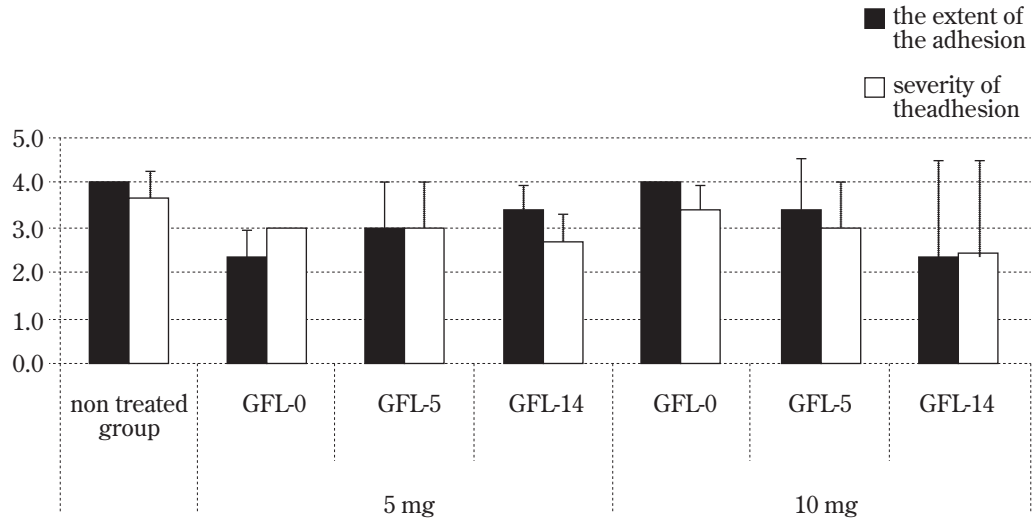

Figure 6. Adhesion scores in lower doses of gelatin flakes (Experiment 3)

Table 5. Adhesion scores obtained in the larger-scale study conducted to determine the optimal dose of gelatin flakes (Experiment 4)

			Scores (Extent)						Scores (Severity)					
			0	1	2	3	4	Mean±SD	0	1	2	3	4	Mean±SD
Non-treated group		(n=6)	0	0	1	0	5	3.67±0.82[a]	0	0	1	0	5	3.67±0.82[b, c, d]
GFL-5	5 mg	(n=6)	0	0	0	0	6	4.00±0.00	0	0	0	0	6	4.00±0.00
	10 mg	(n=6)	0	1	0	3	2	3.00±1.10	0	0	1	4	1	3.00±0.63[b]
	15 mg	(n=6)	0	0	2	2	2	3.00±0.89	0	0	4	1	1	2.50±0.84[c]
	20 mg	(n=6)	0	1	2	1	2	2.67±1.21[a]	0	0	4	2	0	2.33±0.52[d]

a, b, c, d: $p<0.05$.

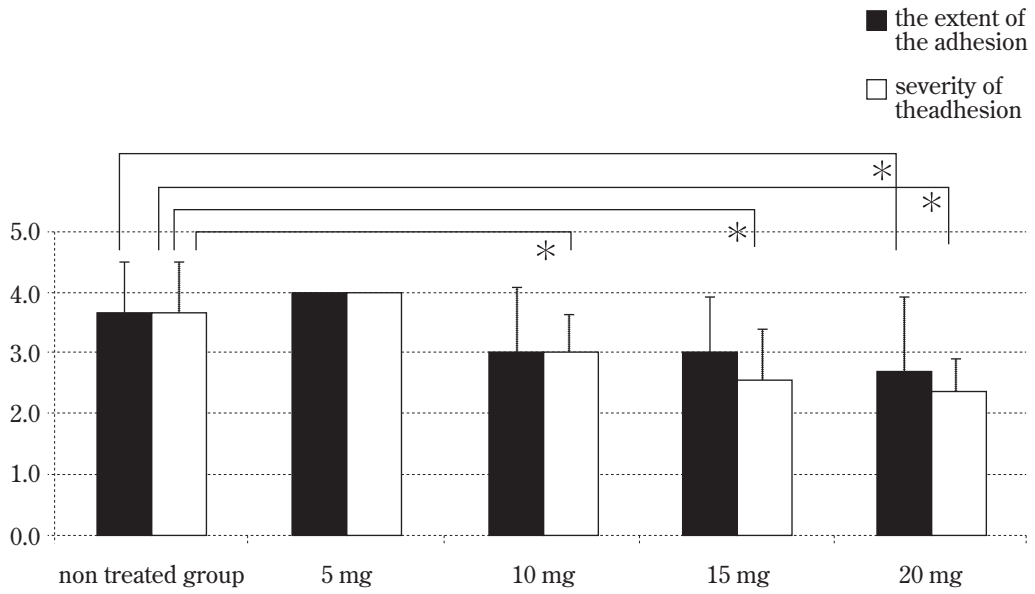

Figure 7. Adhesion scores obtained in the larger-scale study conducted to determine the optimal dose of gelatin flakes (Experiment 4)

Significant differences are indicated by * (p<0.05).

Conclusion and discussion

In gynecologic surgery to preserve fertility, prevention of postoperative adhesion is an important challenge. In one study, laparoscopy revealed the presence of adhesion in 55-95% of patients with a history of surgery. (1) The number of institutions in which laparoscopic surgery is performed for benign diseases, such as uterine myoma and ovarian tumor, has increased recently. Additionally, another study showed that laparoscopic surgery reduces postoperative adhesion compared with abdominal operation. Thus, the use of anti-adhesive materials is essential.

Conventional anti-adhesive materials, such as Seprafilm® and INTERCEED®, which are widely used, are positioned between the lesion and surrounding tissues as a physical barrier to provide anti-adhesive effects. There are several problems in conventional anti-adhesive materials, as described previously by other research groups. (2-4) To overcome these problems, we developed a new anti-adhesive material containing gelatin.

Gelatin was the selected material for the following reasons: (1) it is safe; (2) it can be resolved and absorbed in vivo; (3) it does not inhibit wound healing; (4) its nature can be controlled by changing the crosslinking time; and (5) there are few hurdles to clinical application of gelatin.

Gelatin is made from resolved trimers of collagen; its antigenicity is low, and it can be used safely because it is not associated with virus, endotoxin, or prion infectivity. In addition, gelatin is degraded by proteolytic enzymes, indicating that it can be resolved and absorbed in vivo. In contrast, our study showed that Seprafilm® and INTERCEED® (conventional materials) are still present several weeks after surgery on histological evaluation and engulfed as foreign bodies by phagocytes. In a cell growth experiment in this study, conventional materials showed almost no growth of cells, while gelatin material showed cell growth, suggesting that gelatin material did not inhibit wound healing.

Because simple thermal crosslinking can control the features of gelatin and has already been used for medical products, such as soft capsule and suppositories, the number of processes needed to initiate clinical applications of newly developed anti-adhesive materials containing gelatin may be small.

The conclusions from Studies I-III are as follows:

STUDY I: Development of Gelatin Film

STUDY I-1: Biological Properties and Extent of Thermal Crosslinking in Gelatin Film Used as an Anti-adhesive Material

In this study, we found that the water content, solubility, and enzymatic degradation by collagenase of thermally crosslinked

gelatin film (GFM) were closely related to the duration of thermal crosslinking, indicating that this property also reflected the extent of thermal crosslinking.

The initial 5-7 days are thought to be important for reperitonization. In particular, separating the site of injury from the adjacent tissue during this period is quite critical with respect to the efficacy of anti-adhesive barriers in preventing adhesion. (5) In this study, various GFMs with different crosslinking times were used to examine residual GFM and its anti-adhesive effects in rats in vivo. GFM-3, GFM-8, and GFM-14 showed significant anti-adhesive effects compared with those in the untreated group. These results could be explained by the presence of the materials in vivo for 5-7 days, during the critical period.

In a cell culture study, the cells in GFM-3, GFM-8, and GFM-14 decreased as compared with those in the control group. These results could be explained by differences in the solubility of the material after crosslinking. The effects of these films on fibroblast growth may play a role in preventing adhesion in addition to acting as a mechanical barrier. However, all the films showed cell growth, indicating that they may not inhibit wound healing completely.

This study suggested that thermally crosslinked gelatin films could control the resolving time according to the thermal crosslink time (1) and showed excellent anti-adhesive effects as compared with the

untreated group (2).

STUDY I-2: The Effects of Thermally Crosslinked Gelatin Film on Adhesion and Intestinal Anastomosis in Canine Models

In this study, the gelatin films showed significant anti-adhesive effects in terms of both extent and severity as compared with those in the untreated group, whereas Seprafilm® as a conventional material showed no significant differences as compared with the untreated group. These results may be explained by the histological findings; indeed, in an assessment at 3 weeks after surgery, the GFM group showed lower inflammatory cell scores and proliferation of the peritoneum than the Seprafilm® group. Mesothelial cell injury and subsequent unveiling of submesothelial tissues result in adhesion formation under pathological conditions, such as abdominal surgery, trauma, and inflammation. In addition, prolonged inflammation also induces adhesion through the suppression of fibrinolysis in injured peritoneal tissues. (5) Therefore, rapid reperitonization and reduced inflammation are thought to be advantageous for anti-adhesion. These results are thought to be associated with the observation that Seprafilm® was engulfed as a foreign body by phagocytes in vivo, whereas gelatin was resolved and absorbed in vivo without inhibiting cell growth.

In an experiment in an anastomosis model, GFM did not decrease the burst pressure of the anastomosis at 3 or 7 days after surgery. In

contrast, Seprafilm® significantly decreased the burst pressures of the intestinal anastomosis, particularly at 3 days after the surgery. According to previous animal experiments and our present data, the burst pressures of the anastomosis 3 days after surgery were obviously lower than those at 7 days after surgery. (6, 7) Even in histological analysis, GFM showed findings similar to those in the untreated group and did not appear to inhibit wound healing.

From the above findings, GFM is thought to have excellent anti-adhesive material effects and be able to be used safely for intestinal anastomotic sites, which are typically contraindicated for conventional materials.

STUDY II: Development of Two-layered Gelatin Sheets

STUDY II-1: Hemostatic Effects of Two-layered Gelatin Sheets

In this experiment, two-layered gelatin sheets were found to be significantly superior to conventional hemostatic materials (TachoSil), possibly owing to the absorbency and permeability of the materials. The absorbency of the two-layered gelatin sheet was significantly higher than that of TachoSil. Both the two-layered gelatin sheet and TachoSil had low permeability, indicating that the two-layered gelatin sheets had high absorbency and low permeability. The sponge layer absorbs blood quickly with two-layered gelatin sheets; thus, blood clotting is initiated immediately. Additionally, the sponge layer shows

superior adhesiveness due to the occurrence of gelation. Furthermore, the gelatin film layer inhibits effusion of blood from bleeding sites contralaterally, thereby reducing adhesion to contralateral tissues and gauze. Accordingly, the gauze can be removed easily, suggesting that gelatin material shows superior functionality.

Based on these data, two-layered gelatin sheets were shown to be superior as hemostatic materials and to show excellent adhesiveness to the tissues and improved functionality.

STUDY II-2: Anti-adhesive Effects of the Newly Developed Two-layered Gelatin Sheet in Dogs

In this experiment, the two-layered gelatin sheet group showed significantly improved anti-adhesive effects as compared with the untreated group, whereas there were no significant differences among the untreated, Seprafilm®, and INTERCEED® groups. However, the anti-adhesive effects of Seprafilm or INTERCEED® for applications in pelvic surgeries are still controversial. (8-10).

Histologically, matured single-layered mesothelium, suggesting the regeneration of the peritoneum in two-layered gelatin sheets, was observed at 3 weeks after surgery similar to that in Study II. No mesothelial cells were found at 3 weeks after surgery in the Seprafilm® group. Immature mesothelial cells were observed at 3 weeks after surgery in the INTERCEED® group. In the Seprafilm®

and INTERCEED® groups, the results showed that the materials were engulfed as foreign bodies by macrophages. Because the peritoneum plays a role in the prevention of fibrin adhesion, which is the first adhesion stage, rapid regeneration of the peritoneum was thought to contribute to the prevention of adhesion. (5, 11) This is why the two-layered gelatin sheets showed excellent anti-adhesive effects. Furthermore, the reason for the early regeneration of the peritoneum appeared to be related to the results of the cell growth experiment. In the three types of cell growth experiments, cell growth was inhibited in all the cells in the Seprafilm® and INTERCEED® groups, but was promoted on both the sponge and film surfaces on the two-layered gelatin sheets. Thus, the two-layered gelatin sheets were not thought to inhibit the regeneration of the tissues, indicating that they did not inhibit wound healing.

The above results suggested that the two-layered gelatin sheet had excellent anti-adhesive effects and did not inhibit wound healing.

STUDY III: Development of Gelatin Flakes

STUDY III-1: Anti-adhesive Effects of Gelatin Powders with Different Particle Forms

In the assessment of anti-adhesive effects, flakeballs and flakes showed adhesion scores similar those of Seprafilm®. Flakeballs and flakes are soft and have large surface areas; therefore, they absorb

water immediately and gelate. Both of these materials also aligned closely after they gelated. In contrast, polyhedras and spheres aligned with space. Taking account of their roles as physical barriers to prevent fibrin deposition, flakeballs and flakes, which aligned closely, seemed to be more useful.

These above results suggested that flakeballs and flakes gelated immediately, showed high adhesion to tissues, and were equivalent to Seprafilm® as conventional materials in their anti-adhesive effects. These materials may be useful in laparoscopic surgery.

STUDY III-2: Fundamental Properties of Gelatin Flakes as a New Anti-adhesive Material for Laparoscopic Surgery

Based on the results of the solubility analysis in this study, gelatin that had not been crosslinked was found to be soluble in water but may form insoluble gelatin by thermally crosslinking (crosslinked structures were formed between gelatin molecules). The fraction of crosslinked gelatin and the degree of swelling were measured using three particle diameters (less than 500, 500-1000, and 1000-2000 μm). There were no differences related to changes in particle diameter. However, the degree of swelling decreased when the fraction of crosslinked gelatin increased. In other words, these data indicated that the preservable fluid volume was decreased.

From the data showing the degradability of gelatin flakes in collagenase solution, the residual rate of gelatin was higher as the

thermal crosslinking time increased (GFL became difficult to resolve); thus, we assumed that the biodegradation time in the body could be controlled by changing the thermal crosslinking time. In the results of measurement of the water absorption time, the water absorption time decreased following the introduction of thermal crosslinking. Thus, the gelatin appeared to dissolve when non-crosslinked gelatin absorbed water, inhibiting the penetration of water. In contrast, the thermally crosslinked gelatin absorbed water rapidly because the amount of gelatin resolved by crosslinking reduced the inhibition of water penetration. In addition, the water supply time of GFL tended to be shorter than that of the gelatin sponge, regardless of the thermal crosslinking time. This could be explained by the many gaps in GFL, resulting in rapid absorption of water.

These results demonstrated that GFL was powdery, allowing it to easily be used for laparoscopy. Additionally, GFL was superior in hydrophilia/retentiveness of moisture and was able to control the nature of gelatin according to the thermal crosslinking time.

STUDY III-3: Anti-adhesive Effects and Biodegradability of Gelatin Flakes in Rats

In experiment 1, adhesion scores were significantly reduced for GFL-0 and GFL-14 compared with those in the untreated group. These data suggested that GFL had sufficient anti-adhesive effects. In

experiment 2, in which we analyzed GFL-0, GFL-5, and GFL-14, we observed the status and anti-adhesive effects of the gelatin flakes over time after application to the experimental animals. To prevent adhesion, the materials should act as a physical barrier for about 7 days. GFL-5 and GFL-14 remained in the gel form between the injured cecum and the abdominal wall for at least 7 days. The adhesion scores tended to be lower with GFL-5 and higher with GFL-0 and GFL-14. Therefore, these data suggested that excessively long or short thermal crosslinking times may reduce the anti-adhesive effects of the material. In experiments 3 and 4, the optimal dose of GFL was examined. As compared with the untreated group, in addition to the extent and severity, anti-adhesion scores were significantly lower at 20 mg. At 20 mg or less, the adhesion score tended to decrease as the dose increased. Thus, the anti-adhesive effects of GFL appeared to depended on the dose, and the minimum dose was found to be 20 mg. However, further studies are needed to determine the optimal dose.

From the above results, we found that gelatin flakes had sufficient anti-adhesive effects and that additional studies are needed to determine the dose providing the maximum anti-adhesive effects.

Generally, gelatin material showed the following features: (1) a simple and easy thermal crosslinking procedure could be used to control its nature and form, and therefore, its nature and form could be altered depending on the environment in which the material would

be used; (2) gelatin could provide adequate anti-adhesive effects without inhibiting wound healing, like conventional materials; (3) gelatin could be safely used for intestinal anastomotic or breeding sites, which are contraindicated for conventional materials; and (4) flakes of gelatin material could be changed into a form available for laparoscopic surgery, which is not feasible with conventional materials.

According to these findings, gelatin appeared to be a highly useful anti-adhesive material.

One of the future challenges in the application of gelatin material is its use in laparoscopic surgery. The optimal dose of gelatin flakes in laparoscopic surgery should be examined. In addition, a device to disperse the flakes during laparoscopic surgery must be developed. Gelatin is particularly beneficial because its flakes absorb water quickly and gelate. However, it may absorb water and gelate in a device before being administered to the lesion in a wet environment, such as the abdominal cavity. Then, the formed gel may occlude the lumen of the device, making it unusable. Accordingly, it will be necessary to develop a device that can be used for appropriate administration to lesion by preventing the flakes from absorbing water in the device before administration.

In addition, further studies are needed to assess the potential clinical applications of this material and to examine the anti-adhesive

effects of this material in humans. In Japan, for example, Juntendo University performed laparoscopic surgery (second look) for examination at specific times after surgery using anti-adhesive materials and evaluated the adheision. However, there are various problems associated with human studies, including high cost, patient consent, and shortage of staff; thus, such studies are not easy.

Overcoming these challenges will allow us to prepare materials with better anti-adhesive effects, e.g., by adding growth factors, such as vascular endothelial growth factor, to gelatin material, mixing other materials, or using other processes.

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Appendix

A Preliminary Study of the Physical Properties of a New Anti-Adhesive Material Made of Thermally Cross-Linked Gelatin Film

TABLE OF CONTENTS (Appendix)

ABSTRACT

Background:

To generate a more effective and safer anti-adhesive material, we developed a new thermally cross-linked gelatin film. We previously reported that this film had superior anti-adhesive effects compared to cellulose film and could be used safely on the intestinal anastomosis in canine models. To evaluate the handling of the gelatin film during surgery, we investigated the physical properties of the gelatin film and compared it with cellulose film.

Materials and Methods:

We performed tensile and shear tests to evaluate the maximum loads, the elastic modulus and the fracture strains of the gelatin film, paying special attention to the relationship between the time required for the thermal cross-linking and those physical properties.

Results and Conclusions:

The maximum tensile and shear loads of each thermally cross-linked gelatin film were significantly higher than those of cellulose film. The fracture strains of each gelatin film were also significantly higher than those of cellulose film. However, there were no significant differences in the elastic modulus between the gelatin films and the

cellulose film in terms of both the tensile and shear tests. There were no significant differences in these physical properties among the gelatin films allowed to thermally cross-link for different lengths of time.

In conclusion, thermally cross-linked gelatin film has a higher physical strength and ductility than cellulose film, regardless of the time allowed for thermal cross-linking. These physical properties of the gelatin films are considered to be advantageous for their handling during surgery.

新規癒着防止材熱架橋ゼラチンフィルムの物性試験

要約

背景：

より有効で安全な癒着防止材の作成のために、筆者らは新規の熱架橋ゼラチンフィルムを開発してきた。このフィルムはイヌの実験モデルにおいてセルロースフィルムに比較してより優れた癒着防止効果を持ち、腸管吻合部上でも安全に使用できることを、以前に我々は報告している。本研究では、このゼラチンフィルムの手術中の操作性を評価するべく、物理的性質を評価しセルロースフィルムと比較した。

材料および方法：

ゼラチンフィルムについて引張試験、せん断試験を行い、限界強度、弾性、破壊ひずみを、特に熱架橋時間との相関に注目して検討した。

結果および結語：

引張試験とせん断試験の限界強度について各ゼラチンフィルムはセルロースフィルムより有意に高く、また破壊ひずみも上記と同じであった。しかし引張試験とせん断試験における弾性は、ゼラチンフィルムとセルロースフィルム間で有意な差異は無かった。また上

記の物理的性質は、ゼラチンフィルムの熱架橋時間の違いで検討しても差異がなかった。上記の結果から、熱架橋ゼラチンフィルムは強さと柔軟性において、熱架橋時間に関わらずセルロースフィルムより優れること、またこれらの性質は、ゼラチンフィルムが手術中の操作性に優れると、と結論される。

INTRODUCTION

Postoperative adhesion, where injured sites become attached to the surrounding peritoneum or organs, is a serious problem after abdominal and gynecological surgery, (1) because it often leads to severe complications such as intestinal obstruction, (2) female sterility and chronic abdominal pain. (3, 4) To prevent such adhesion, various kinds of anti-adhesive materials have been developed and been used experimentally and clinically. (5)

Currently cellulose film, which is composed of hyaluronate sodium and carboxymethyl-cellulose, is widely used as an anti-adhesive material in the clinical setting. When placed on the injured sites during an operation, it rapidly turns to a gel form absorbing the surrounding moisture and remains in place for about a week, thereby preventing direct contact between the injury and other tissues as a physical barrier. (6)

However, the cellulose film has several drawbacks. First, it is quite difficult to handle the cellulose film due to its fragility. In addition, the cellulose film has been contraindicated for wrapping directly the stapled and sutured lines of gastro-intestinal anastomoses because several studies reported that the film induced a high frequency of leakage. (7)

To solve the problems of the cellulose film, we have developed a

new anti-adhesive material made of thermally cross-linked gelatin film. We previously reported that the gelatin film had superior anti-adhesive effects compared to the cellulose film with excellent peritoneal regeneration and that it could be used safely on the intestinal anastomosis in canine models. (8)

To evaluate the handling of the gelatin film, we investigated its physical properties and compared them with those of cellulose film. We performed tensile and shear tests to evaluate the maximum loads, the elastic modulus and the fracture strains of the gelatin film, paying special attention to the relationship between the time allowed for the thermal cross-linking and the physical properties.

MATERIALS AND METHODS

1. Materials

Medical grade gelatin extracted from porcine skin (type-I collagen, Medigelatin®) with an isoelectric point of 5 was supplied by Nippi Co. (Shizuoka, Japan). To prepare the gelatin film, the gelatin was dissolved in distilled water to a final concentration of 4.8%. Next, the solution was cast in plastic plates (Kanto Chemical Co., Tokyo, Japan) and allowed to dry in a clean bench with a consistent air flow at room temperature for two days, yielding a film of approximately 30 μm in

thickness. (9) After the films were taken off from the plates, thermal cross-linking was induced by a vacuum oven (AVO-250N, As One, Osaka, Japan) at 140 oC for 0 h (no heating), 1 h, 3 h, 8 h or 14 h.

As a control material, we used a commercial cellulose film (Seprafilm®, Genzyme Co., Cambridge, MA, USA), which is a commonly used anti-adhesive material for abdominal surgery. The cellulose film has an approximately 50 μm thickness. Finally, each film was cut into an oblong-shaped piece of 10 × 50 mm in size, and was kept in a dry state in a desiccator until the following physical analyses were performed.

2. Evaluation of the physical properties of the films

2.1. Tensile test

Each oblong piece of film was set on two folders of the testing apparatus (CPU gauge: MODEL-RX10, TESTSTAND: MODEL-1356R, Aikoh Engineering, Osaka, Japan), by grasping both film ends at a distance of three centimeters, as shown in Figure 1. Next, the film was drawn automatically into opposite directions at the fixed speed of 5 mm/minute. To analyze the maximum tensile load, the fracture strain and the Young's modulus, six oblong pieces of each type of film were used and the stress-strain ($\sigma_{nom} = f(\varepsilon_{nom})$) diagrams were recorded until the films were broken. Six pieces of each gelatin and cellulose film were examined. The value was calculated using the

equation.

$\sigma_{nom} = F/A_0$ and $\varepsilon_{nom} = \delta / L_0$

where, F was applied force; A_0, the initial cross-section; δ, the change in gauge length; L_0, the initial gauge length.

2.2. Shear test

Each oblong piece of film was set on two specific folders of the testing apparatus (CPU gauge: MODEL-RX10, TESTSTAND: MODEL-1356R, Aikoh Engineering) by grasping both film ends and the film center to give two distances of one centimeter each, as shown in Figure 2. Next, the film was drawn automatically into opposing directions at the fixed speed of 5 mm/minute. To analyze the maximum shear load, the fracture strain and the shear modulus, six oblong pieces of each film were used and the stress-strain diagrams ($\tau_{nom} = f(\gamma_{nom})$) were recorded until the films were broken. Six pieces of each gelatin and cellulose film were examined. The value was calculated using the equation.

$\tau_{nom} = F/2A_0$ and $\gamma_{nom} = \delta / L_0$

where, F was the applied force; A_0, the initial cross-section; δ, the change in gauge length; L_0, the distance between folders (1 cm) (Figure. 2).

The measured values were shown as the means ± standard deviation (SD). After processing by a one-way layout analysis of

variance, the Tukey test was used as a post-hoc test. A p value <0.05 was considered to be significant for the test.

RESULTS

1. Tensile test

The maximum tensile loads of the gelatin films thermally cross-linked for 0, 1, 3, 8 and 14 h were 30.9 ± 7.5N, 32.6 ± 1.7N, 33.4 ± 4.8N, 34.0 ± 5.9N and 30.0 ± 8.8N (mean ± SD) respectively. The tensile load of the cellulose film was 19.4 ± 2.5N (mean ± SD) (Figure. 3). The maximum tensile load of each of the gelatin films was significantly higher than that of the cellulose film (p<0.05). However, there were no significant differences among the gelatin films.

The fracture strain of the gelatin films cross-linked thermally for 0, 1, 3, 8 and 14 h were 8.98 ± 2.84%, 11.02 ± 1.93%, 8.34 ± 1.85%, 9.03 ± 1.37% and 7.75 ± 2.19%, (mean ± SD) respectively. The fracture strain of the cellulose film was 3.42 ± 1.15% (mean ± SD) (Figure. 4). The fracture strain of each of the gelatin films was significantly higher than that of the cellulose film (p<0.05). However, there were no significant differences among the gelatin films.

The data regarding the Young's modulus are shown in Figure. 5. There were no significant differences between the gelatin films and

the cellulose film.

2. Shear test

The maximum shear loads of the gelatin films thermally cross-linked for 0, 1, 3, 8 and 14 h were 15.3 ± 3.5N, 17.4 ± 2.3N, 17.2 ± 3.2N, 16.8 ± 1.5N and 18.0 ± 2.0N (mean ± SD) respectively. The maximum shear load of the cellulose film was 7.6 ± 1.0N (mean ± SD). The maximum shear load of each gelatin film was significantly higher than that of the cellulose film ($p<0.05$) (Figure. 7). However, there were no significant differences among the gelatin films.

The fracture strain of the gelatin films thermally cross-linked for 0, 1, 3, 8 and 14 h were 12.56 ± 4.13%, 17.39 ± 4.28%, 14.82 ± 3.65%, 14.53 ± 1.83% and 22.93 ± 3.69%, (mean ± SD) respectively. The fracture strain of the cellulose film was 5.01 ± 0.79% (mean ± SD) (Figure. 8). The fracture strain of each of the gelatin films was significantly higher than that of cellulose film ($p<0.05$). However, there were no significant differences among the gelatin films.

The data regarding the shear modulus are shown in Figure. 9. There were no significant differences between the shear modulus of each of the gelatin films and that of the cellulose film.

DISCUSSIONS

As needed physical conditions of ideal anti-adhesive films, it should be hardly broken and be handled easily during surgery. (10) In addition, the film should be able to be crumpled and folded without tearing when it is used in laparoscopic surgery. However, cellulose film does not satisfy these conditions due to its fragility, although it has been used clinically. In this study, to examine whether the thermally cross-linked gelatin film met these requirements, we measured the physical strength of the film, and compared it with the cellulose film.

In both the tensile and shear tests of this study, there were no significant differences in the elastic modulus between the thermally cross-linked gelatin films and the cellulose film. This means that both films have similar elasticity. In contrast, the maximum tensile and shear loads of the gelatin films were significantly higher than those of cellulose film, even though the thicknesses of the thermally cross-linked gelatin films are thinner than that of the cellulose film. These results indicate that the thermally cross-linked gelatin films have higher physical strength than the cellulose film.

In terms of the fracture strain, there were also significant differences between the gelatin films and the cellulose film. Therefore, the gelatin films were considered to have higher ductility

than the cellulose film. These results suggest that the gelatin films with or without thermal cross-linking may have better handling properties than the cellulose film.

In this study, we also examined the relationship between the physical properties of the gelatin films and the length of time allowed for thermal cross-linking. However, there were no significant differences in the physical properties, such as the maximum load, the fracture strain and the elastic modulus among the gelatin films allowed to cross-link for different lengths of time. This result indicates that the gelatin film may have stable physical properties, regardless of the thermal cross-linking. However, a previous report showed that chemical cross-linking with genipin significantly decreased the fracture strain of the gelatin films, although it did not affect the maximum stress. (11) Therefore, the modality used for cross-linking may affect the physical properties of the gelatin films, rather than the degree of the cross-linking.

CONCLUSION

Thermally cross-linked gelatin film has higher physical strength and ductility than cellulose film, even though the thickness of the gelatin film is thinner than that of the cellulose film. These properties

of the gelatin films are considered to be advantageous for its handling during surgery.

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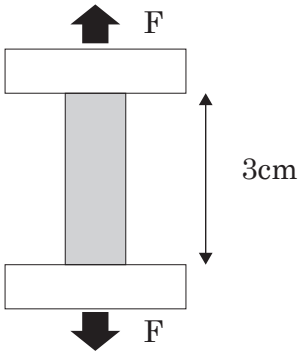

Figure 1. A schema of the tensile test.

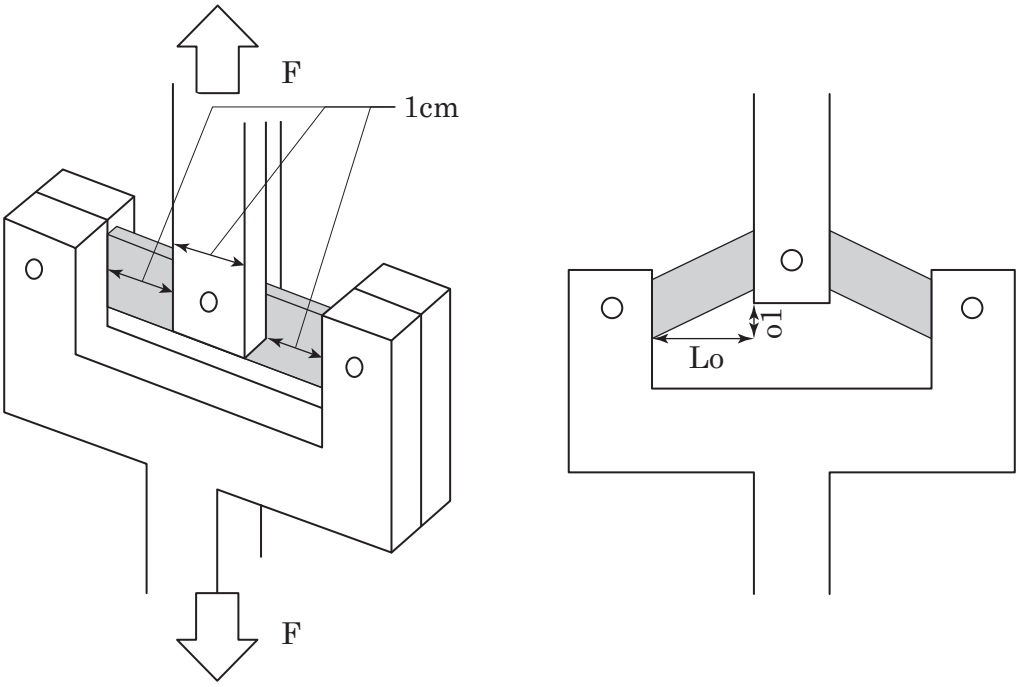

Figure 2. A Schema of the shear test (A) and a front view (B).

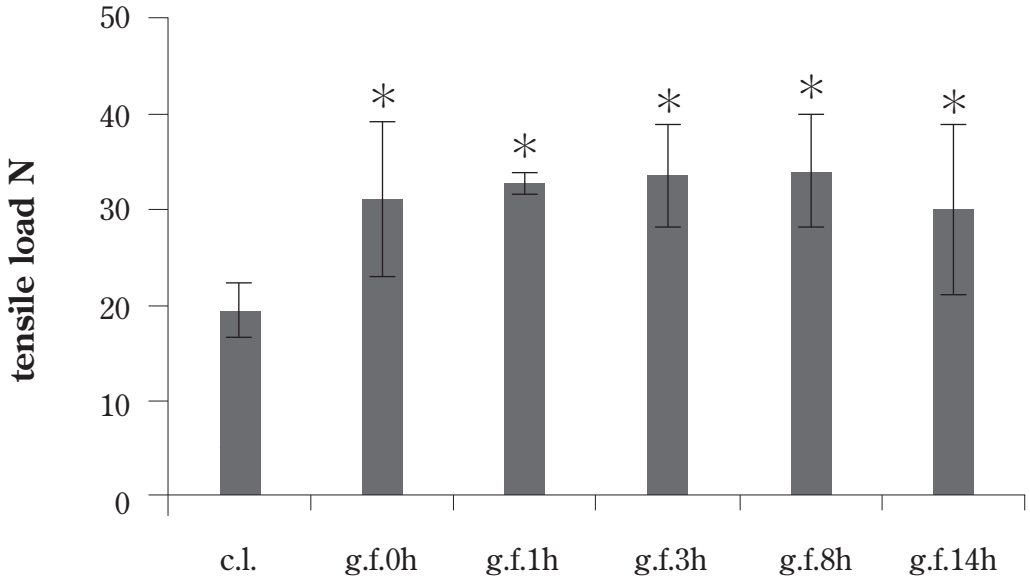

Figure 3. The maximum tensile load in the tensile test (n = 6).

*: $p<0.05$ relative to cellulose film

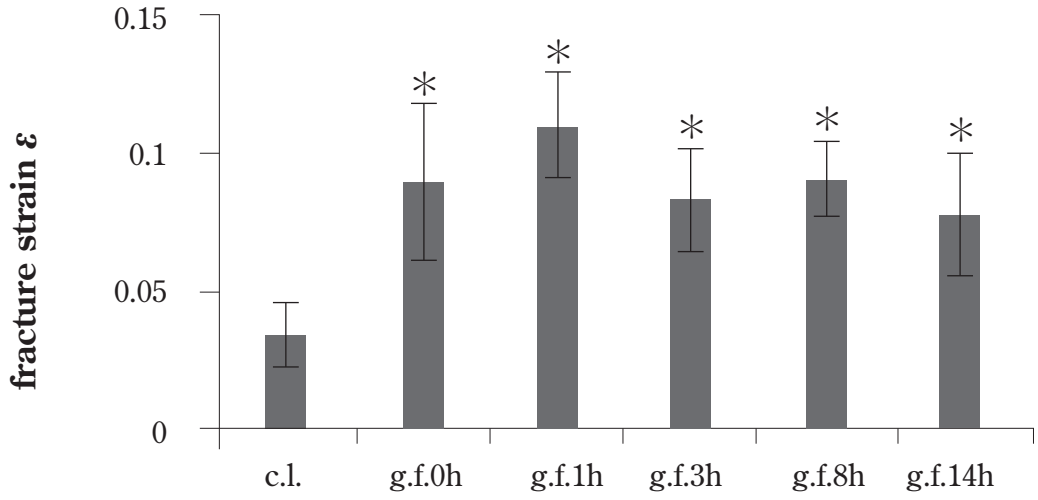

Figure 4. The fracture strain in the tensile test.

*: $p<0.05$ relative to the cellulose film

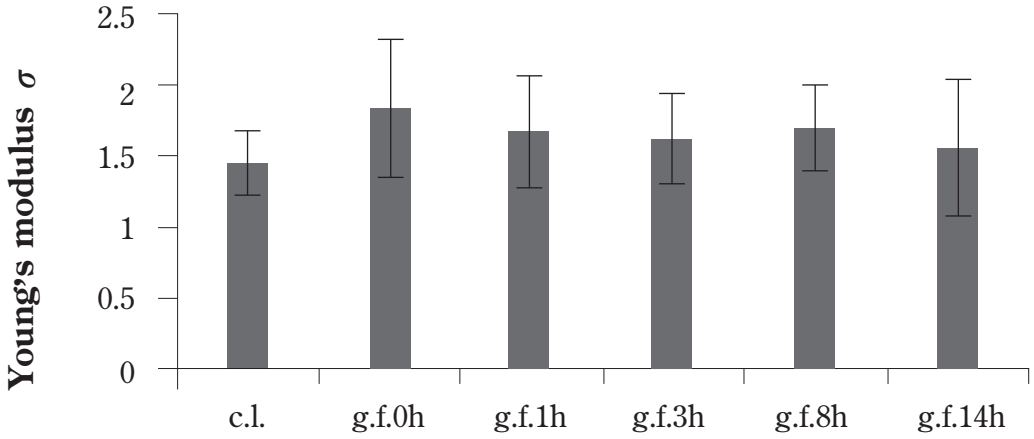

Figure 5. Young's modulus in the tensile test.

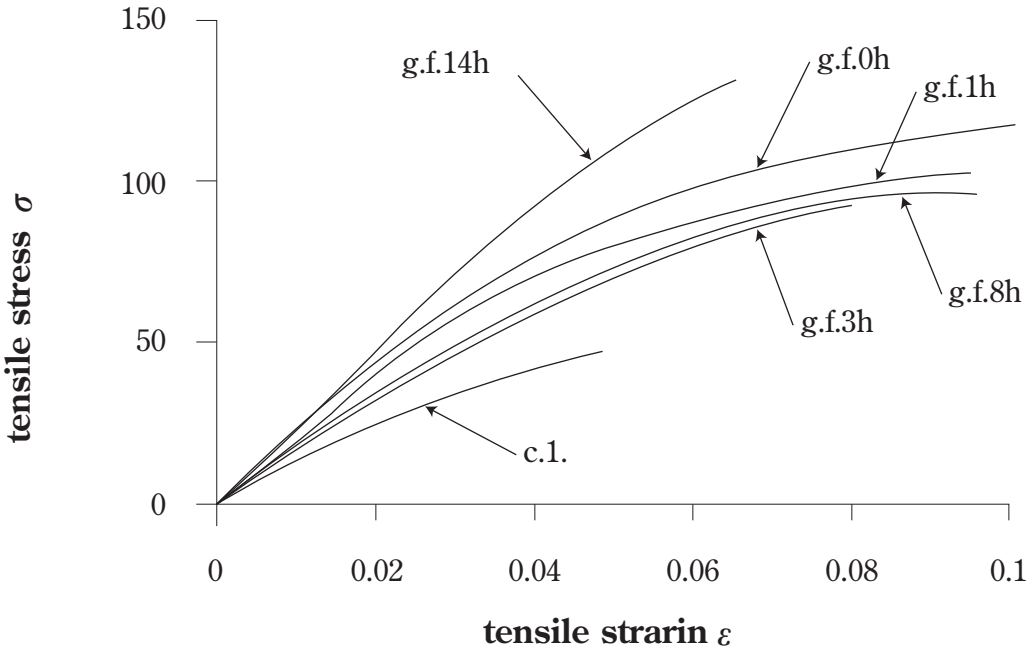

Figure 6. Representative stress-strain diagrams in the tensile test.

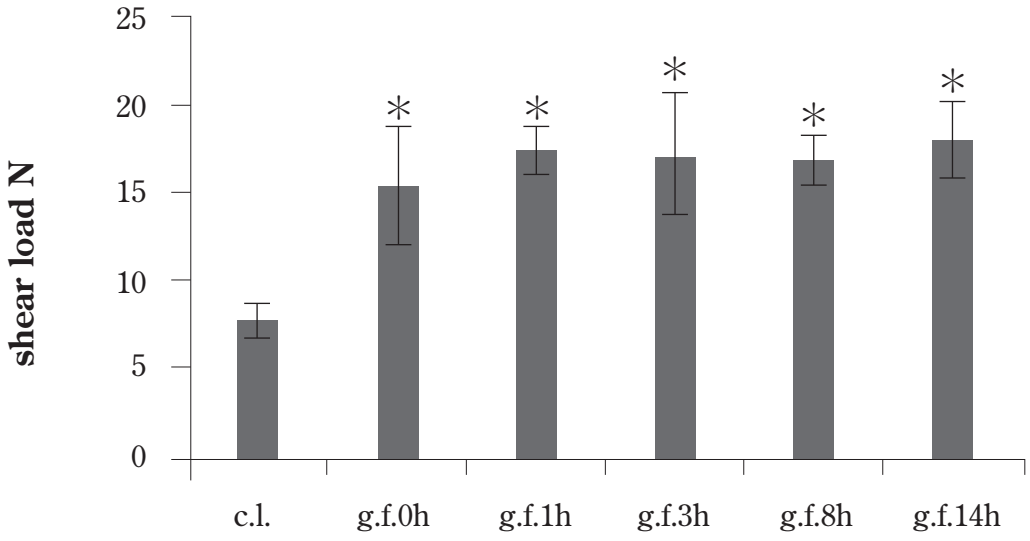

Figure 7. The maximum shear load in the shear test.

n=6, *: $p<0.05$ relative to the cellulose film

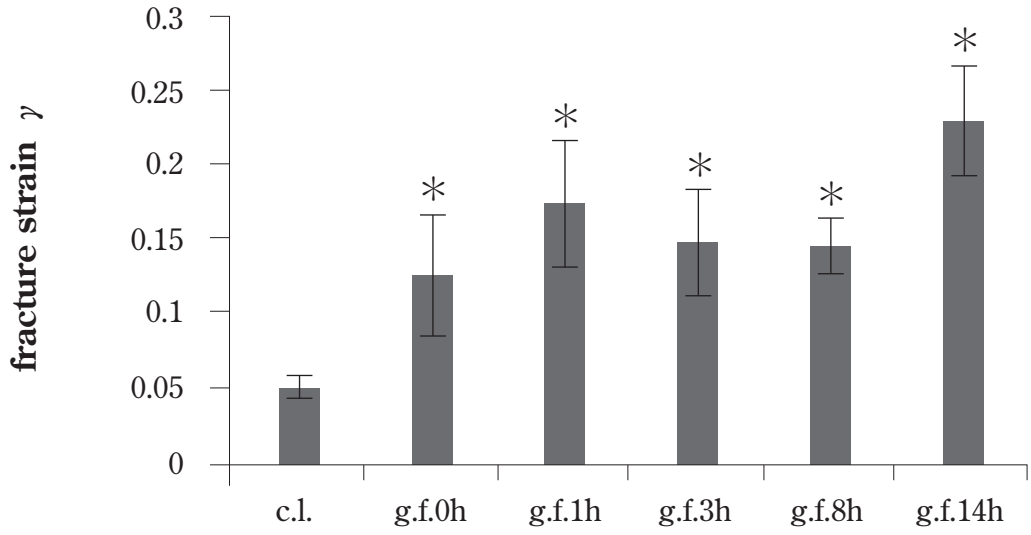

Figure 8. The fracture strain in the shear test.

n=6, *: p<0.05 relative to the cellulose film

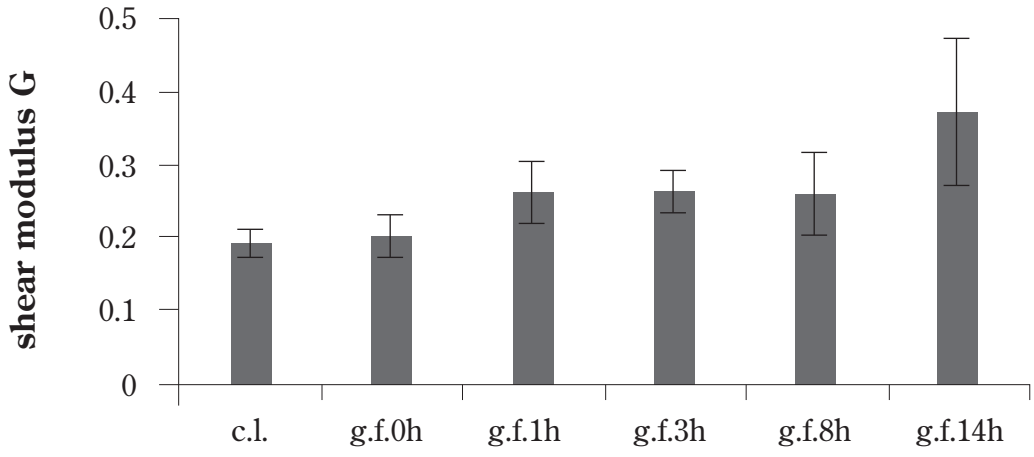

Figure 9. The shear modulus in the shear test.

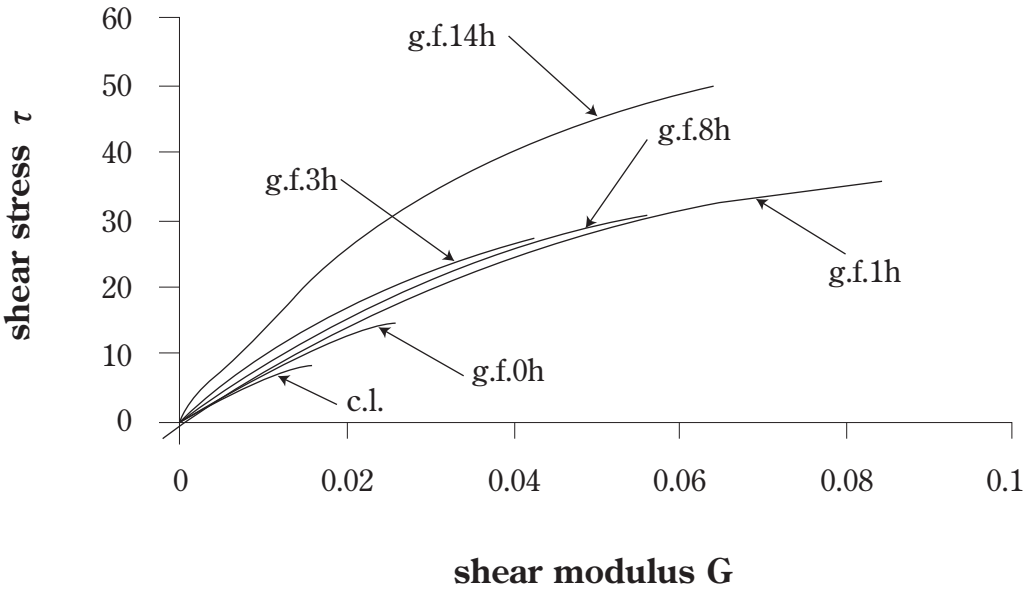

Figure 10. Representative stress-strain diagrams in the shear test.

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