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ORIGINAL ARTICLE

Strong adhesiveness of a new biodegradable hydrogel glue, LYDEX, for use on articular cartilage

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ABSTRACT

***Aim*: Until recently, only fibrin glue has been available for clinical usage to repair articular cartilage, although its adhesiveness is not strong enough for use with articular cartilage, and it is derived from human blood and thus carries the risk of contamination. Recently, LYDEX, a new biodegradable hydrogel glue, has come onto the market. The purpose of this study was to evaluate the adhesive strength and cytotoxicity of LYDEX when used on articular cartilage.**

***Materials and Methods*: The differing adhesive strengths of collagen membrane and articular cartilage with LYDEX versus with fibrin glue were measured using a tensile tester. In addition, the cytotoxity of LYDEX in vitro was evaluated. The cytotoxity of LYDEX for the articular cartilage of rats was evaluated histopathologically.**

***Results*: The adhesive strength of LYDEX was significantly stronger than that of fibrin glue, giving values about 3.8 times higher. LYDEX has no discernible effect on normal articular cartilage.**

***Conclusions*: Our study is the first to assess the usefulness and safety of LYDEX for use on articular cartilage.**

Key words: Articular cartilage, Adhesive strength, Biodegradable hydrogel glue, Collagen membrane, Cytotoxity

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INTRODUCTION

Several studies have reported on the use of fibrin glue for articular cartilage repair, such as for the reattachment of chondral or osteochondral fragments, and as a seal for sutured collagen or for periosteum membrane in autologous chondrocyte implantation (ACI) (1, 2). However, fibrin glue is derived from blood and thus carries the risk of contamination. As one of several available adhesives, fibrin glue has been available for clinical use; however, its adhesiveness is not strong enough for use with articular cartilage.

Numerous studies have reported on the development of adhesives which are able to bond soft tissue. Cyanoacrylate (3), aldehyde-based (4) and fibrin glue (5) have all been used in clinical applications. However, several studies have indicated that cyanoacrylate and aldehyde-based glues can cause chronic inflammation or that they can delay wound healing (6, 7). Fibrin glue has been reported to have low bonding strength and also to carry the risk of viral infections because it is made from blood. In 2007, Nakajima et al (8) reported on the development of LYDEX, a self-degrading bioadhesive. LYDEX was created by introducing aldehyde groups into an α-glucan (e.g., dextran (polysaccharide) and starch) through oxidation, followed by a reaction with polylysine, consisting of linearly linked lysines (an essential amino acid). The reaction between the aldehyde groups of the aldehyde dextran and the amino groups of the polylysine forms Schiff bonds. Polylysine is widely used as a safe food additive. The key characteristic of LYDEX is that it is created solely from medical and food additive sources.

In contrast to a fibrin glue, LYDEX holds great potential for the development of an effective cartilage repair treatment.

In the search for methods of treating osteoarthritic knees with wide cartilage defects in middle-aged patients, many strategies for cartilage repair have been developed (9-11). In clinical usage, such cases may be treated by drilling (12), microfracture (13), abrasion arthroplasty or destruction arthroplasty (14). However, these treatments are not always satisfactory. More efficient treatment is therefore needed for wide cartilage defects in chondral defective or osteoarthritic patients. It seems very likely that the focus for cartilage repair treatment is shifting from fibrin glue to LYDEX. Therefore, in our study we deemed it essential to first of all investigate the adhesive strength

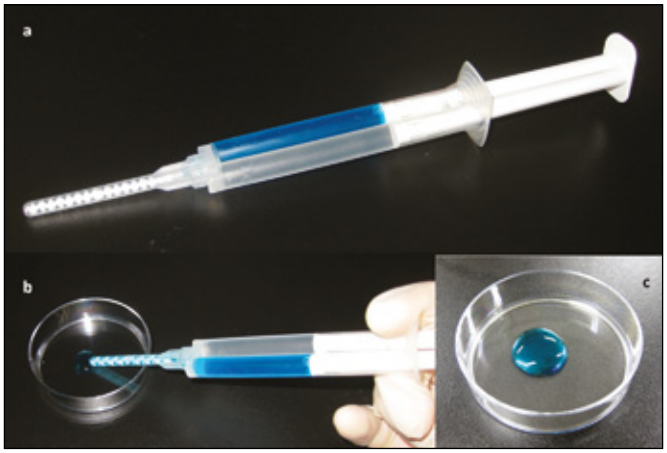

Fig. 1 - *a)* *LYDEX was prepared in a syringe-like container with 2 cylinders: The colorless liquid in the syringe is 20 w/w% dextran aldehyde (molecular weight 70 kDa, aldehyde introduction = 0.46/sugar unit); the blue-colored liquid (brilliant Blue FCF, food additive, 50 ppm) in the syringe is 10 w/w% of ε-poly(L-lysine) containing 3.0 w/w% acetic anhydride.* ***b)*** *The container has a special mixing tip which can mix the 2 liquids together in equal volumes as they pass through it when the plunger is depressed, allowing it to be applied directly as a glue.* ***c)*** *After 13 seconds, the mixed liquids have gelled.*

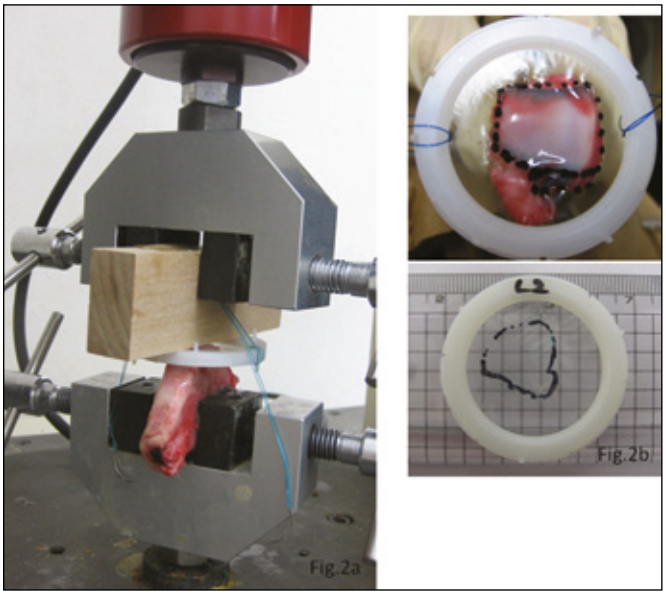

Fig. 2 - *a)* *Bonding strength was measured using a tensile tester. The articular cartilage was placed on the tensile tester, and 0.5 mL of glue was applied to the femoral or tibial joint cartilage, then the atelocollagen membrane was placed on top.* ***b)*** *The adhesive area was checked and marked on the atelocollagen membrane. The adhesive area was measured using Image J software.*

and safety of LYDEX for application on articular cartilage. The purpose of our study was to evaluate the adhesive strength and cytotoxicity of LYDEX when used on articular cartilage of the knee joint.

MATERIALS AND METHODS

Preparation of adhesive

Liquid LYDEX was provided by the Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan (Fig. 1). LYDEX is a hydrogel adhesive prepared by mixing 2 kinds of liquid – polysaccharide aldehydes and ε-poly(L-lysine) – which form a Schiff base. The colorless liquid shown in the syringe is 20 w/w% aldehyde dextran (molecular weight [MW] = 70 kDa, aldehyde introduction = 0.46/sugar unit). The blue-colored (brilliant Blue FCF, food additive, 50 ppm) liquid in the syringe is 10 w/w% ε-poly(L-lysine) (MW = 4 kDa) containing 3.0 w/w% acetic anhydride. Both liquids were sterilized with a syringe filter (0.2-μm pore size) (Fig. 1a). The container has a special mixing tip which can mix equal volumes of the 2 liquids together as they pass through it when the plunger is depressed, allowing the mixture to be applied directly as a glue (Fig. 1b). Once mixed, the glue has a gelation time of about 13 seconds at 37°C (Fig. 1c).

Fibrin glue (Bolheal™) was obtained from Astellas Pharma Inc, Tokyo, Japan, for comparison.

Preparation of animals

Five normal mini-pigs from the Hiroshima animal laboratory were used for evaluation of adhesive strength. The pigs were aged approximately 13 months, with a mean weight of 25 kg. Six normal Sprague Dawley (SD) rats from CLEA Japan Inc. were used for evaluation of cytotoxity. The rats were between 11 and 13 weeks old, and their mean weight was 400 g. All procedures were performed according to the *Guide for Animal Experimentation,* Hiroshima University, and were approved by the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Measurement of adhesive strength using a tensile tester

We used normal cartilage from the femur or tibia of a mini-pig to evaluate the bonding strength of the glue. The distal femora and the proximal tibiae of the pigs were resected. The articular cartilages of the femur or tibia were bisected sagittally and placed on the tensile tester (Model-1840nt; Aikoh Engineering Co., Osaka, Japan) (Fig. 2a). The cartilage was wiped with a dry towel and kept dry while 0.5 mL of glue was applied to the femoral or tibial joint cartilage. The glue used was LYDEX in group L and Bolheal™ in group F. Next, an atelocollagen membrane (AteloCell®, Koken Co., Tokyo, Japan) was placed on the adhesive. Immediately, the adhesive area was examined, and the atelo collagen membrane was marked (Fig. 2b).

The collagen membrane was fixed under standardized conditions which maintained an even tension on the thread used to fix the collagen membrane, with the distance of the thread between the end of the collagen membrane and the tensile tester set at 3 cm. After loading 100 g of force for 5 minutes at room temperature (25°C), the bonding strength was measured using a tensile tester with a shearing speed of 10 mm/min.

The adhesive area was measured using Image J software. The tensile strength per square centimeter was then determined by dividing the initial tension, measured with the tensile tester, by the adhesive area.

Cytotoxicity test in vitro

The cytotoxicity of the new glue was evaluated by a V79 fibroblasts colony assay, according to the national standard guidelines of cytotoxicity tests for biomaterials (15, 16). A V79 Chinese hamster-established fibroblast cell line was obtained from the Japanese Health Science Foundation, Tokyo, Japan. Eagle's Minimum Essential Medium (MEM) (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10 v/v% of fetal calf serum (FCS) was used for the general cell culture. MEM Earle's (Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 5 v/v% of FCS was used for the cytotoxicity assay. Monolayer V79 cells were recovered with 0.05 v/v% trypsin containing 0.02 v/v% of ethylenediaminetetraacetic acid (EDTA) and were then resuspended in MEM Earle's at a concentration of 100 cells/mL, after which 0.5 mL of the cell suspension was poured into a 24-well culture plate. After 6-hour incubation at 37°C in 5% CO_2, the culture medium was sucked out and MEM Earle's containing different concentrations of test substances was added, followed by 6 days of incubation at 37°C. After this incubation period, the cells were fixed with 10% formaldehyde and stained with 0.1% methylene blue to count the colony number (each colony consists of >50 cells).

Aldehyde dextran (MW = 70 kDa, -CHO = 0.46 / sugar unit, 10 w/w%) and ε-poly(L-lysine) (MW = 4 kDa, 10 w/w% containing 3.0 w/w% acetic anhydride) were separately diluted with MEM Earle's to provide them with different concentrations (0-5 mg/mL).

In the case of gelling materials, the extraction method was as follows: The same volume of aldehyde dextran solution (20 w/w%) and ε-poly(L-lysine) (10 w/w%) was mechanically mixed to prepare gel formation. After 2 minutes at 25°C, 29.15 mL of MEM Earle's to 1 g of the hydrogel was added for the extraction. In this step, 5 mg of aldehyde dextran with ε-poly(L-lysine) was extracted with 1 mL of water and MEM Earle's. This extraction (5 mg/mL) was diluted with MEM Earle's and used for a cytotoxicity test after 24 hours of extraction at 37°C. In this experiment, GRF glue (MicroVal, Saint-Just Malmont, France), one of the commercially available tissue adhesives, was selected as a reference material. The same extraction (5 mg/mL) was prepared with MEM Earle's.

Cytotoxicity test in vivo

Surgical procedure for adhesion of collagen membrane

The surgical procedures in rats were carried out under general anesthesia induced by an intraperitoneal injection of 1 ml/kg sodium pentobarbital. The patella was everted through the medial approach, then the articular cartilage of the distal femur's patellar groove was exposed. The cartilage was wiped with a dry towel and kept dry while an atelocollagen membrane (AteloCell) was glued onto the joint cartilage of the patellar groove with LYDEX. The arthrotomy was closed with interrupted 5-0 nylon sutures. This was group L. Sham groups were operated on in the same way and were categorized as group S.

One week after surgery, the rats in groups L and S were killed by intraperitoneal injection of a lethal dose of pentobarbital sodium. The whole knee joints were resected en bloc and fixed in 4% paraformaldehyde for 24 hours. They were then decalcified in a 0.5 M of EDTA solution. Next, the specimens were embedded in paraffin and cut into 5-μm serial sections along the sagittal plane. For histological evaluation, the sections were stained with hematoxylin and eosin (HE), and safranin-O/fast green.

Immunohistochemistry

Sections washed in phosphate-buffered saline (PBS) were treated for 20 minutes at 90°C with a retrieval solution (Dako Cytomation; Dako Japan Co, Tokyo, Japan). After blocking the sections for 30 minutes with a blocking reagent (Block Ace; DS Pharma Biomedical Co, Osaka, Japan), they were incubated with a primary antibody at appropriate dilutions for 1 hour at room temperature. For immunohistological evaluation, the primary antibodies used were as follows: rabbit anti-rat collagen type II polyclonal antibody (Millipore, Billerica, MA, USA), rat anti-goat TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat anti-goat IL-6 (Santa Cruz Biotechnology). The secondary antibodies used were as follows: peroxidase-labeled polymer-horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG for collagen type II and Alexa Fluor 488–conjugated rabbit anti-goat IgG (Molecular Probes, Eugene, OR, USA) for TNF-α and IL-6. DAPI (4,6-diamidino-2-phenylindole) solution (Dojindo Laboratories, Kumamoto, Japan) was used for 10 minutes as a nuclear counterstain. Diaminobenzidine (DAB) was used as the chromogen for collagen type II. Negative controls were prepared in the same manner, but without the primary antibody.

TABLE I - HISTOPATHOLOGICAL SCALE FOR GRADING THE SEVERITY OF KNEE ARTHRITIS

Tissue	Finding	Criteria for evaluation of each finding		
		Score	Grade	Scale for grading
Synovial Membrane	• Edema • Inflammatory cell infiltration • Proliferation of synovial cells • Granulation tissue formation • Fibrosis • Exudation in the joint cavity	0	No changes	No difference from normal
		1	Minimal	There are minimal changes or lesions within the limits of ¼ area of the whole
Trochlear sulcus of the femur Patella	• Pannus formation • Destruction (degeneration, fibrosis) of the cartilage • Destruction (resorption) of the bone	2	Slight	There are obvious changes or lesions within the limits of 1/4-1/2 areas of the whole
		3	Moderate	There are relatively severe changes or lesions within the limits of 1/2-3/4 areas of the whole
		4	Severe	There are very severe changes or lesions within the limits of 3/4-4/4 areas of the whole

Histological evaluation

The histopathological scale for grading the severity of knee arthritis was used for evaluation. The synovial membrane, trochlear sulcus of the femur and sagittal surface of the patella-from the knee joints of the rats were examined. Changes were classified into 5 stages according to the items and criteria for arthritis shown in Table I (17) as follows: no change (score 0), minimal change (score 1), slight change (score 2), moderate change (score 3) and severe change (score 4). Synovial tissues were assessed by scoring the following items: edema, inflammatory cell infiltration, proliferation of synovial cells, granulation tissue formation, fibrosis and exudation into the joint cavity. The trochlear sulcus of the femur and patella were assessed by scoring the following items: pannus formation, destruction of the cartilage and destruction of the bone. The maximum score was 48 points.

Statistical analysis

The bonding strength in each group and the histopathological scales for grading the severity of knee arthritis in the 2 groups were calculated as means ± standard deviation. The Student's unpaired *t*-test was used to compare the different treatments. A P value of <0.05 was considered to be statistically significant. All statistical analyses were performed on a personal computer using the statistical package Excel-Toukei 2010 (Social Survey Research Information Co.).

RESULTS

Adhesive strength test using a tensile tester

In strength tests, the adhesive strengths of the adhesive in group L were a minimum of 0.97 N/cm^2 and a maximum of 1.95 N/cm^2; those in group F were a minimum of 0.25 N/cm^2 and a maximum of 0.65 N/cm^2 (Tab. II). The mean adhesive strength of group L was 1.5 ± 0.4 N/cm^2, and that of group F was 0.4 ± 0.2 N/cm^2. The adhesive strength of LYDEX was therefore significantly stronger than that of fibrin glue, giving values about 3.8 times higher (*t*-test; $P<0.05$) (Fig. 3). In all cases, neither the thread fixed to the collagen membrane nor the collagen membrane itself was ruptured.

TABLE II - RESULTS OF ADHESIVE STRENGTH TESTING

Group L			Group F		
LYDEX (N)	Adhesion area (mm^2)	Adhesive strenght (N/cm^2)	Fibrin glue (N)	Adhesion area (mm^2)	Adhesive strength (N/cm^2)
3.5	305.69	1.15	1.9	292.40	0.65
2.6	269.54	0.97	1.1	302.02	0.36
3.0	154.25	1.95	2.6	467.40	0.56
5.3	282.64	1.88	1.2	354.47	0.34
2.6	193.75	1.34	0.5	203.00	0.25

Group L= LYDEX Group, Group F=Fibrin glue Group.

Low cytotoxity of LYDEX in vitro

Aldehyde dextran and ε-poly(L-lysine) were separately diluted with MEM Earle's, and colony formation of V79 cells in their presence was evaluated. The results are shown in Figure 4. The colony formation was suppressed with the increase of aldehyde dextran and ε-poly(L-lysine). In contrast, almost no suppression or cytotoxicity was observed after gel formation, as shown in Figure 5. values (mg/mL), at which the colony formation was suppressed to 50%, was calculated, and the results are summarized in

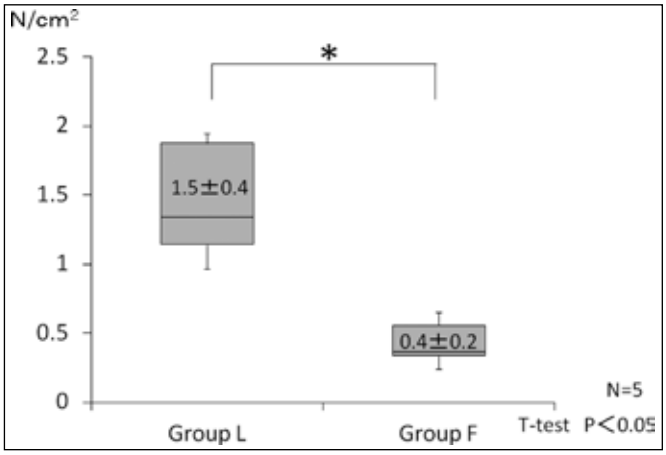

Fig. 3 - *Results of adhesive strength tests. The strength of LYDEX was 3.8 times greater than that of fibrin glue; *P<0.05.*

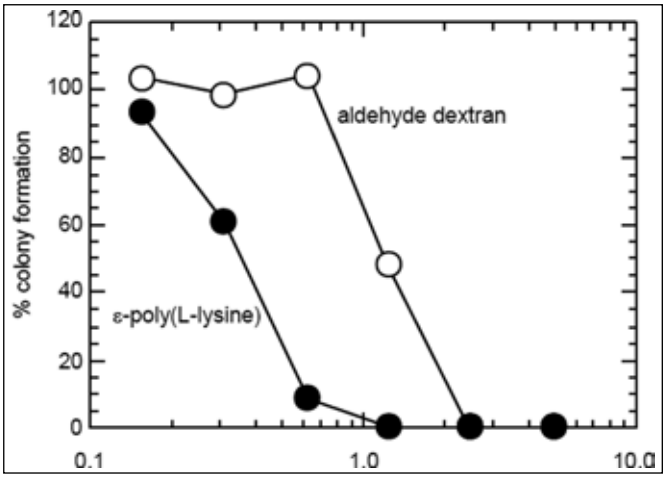

Fig. 4 - *Colony formation of V79 cells in the presence of aldehyde dextran and ε-poly(L-lysine). It was suppressed by the increase of aldehyde dextran and ε-poly(L-lysine).*

Table III. The IC50 of gelling of the glue (aldehyde dextran + ε-poly(L-lysine)) was higher than 5 mg/mL. On the other hand, IC50 for the GRF extraction was 0.11 mg/mL. High cytotoxicity of GRF-cured glue was due to the remaining low aldehyde molecules, such as glutaraldehyde and formaldehyde, contained in GRF. These findings suggest that no cytotoxic materials remained in the new glue after gel formation, and no cytotoxicity to surrounding tissue in clinical application would be expected.

Low cytotoxity of LYDEX for articular cartilage in vivo

Macroscopic examination found no signs of infection, swelling or redness of the joint in either of the groups of rats. The adhesive area was evaluated after staining with HE and safranin-O / fast green from serial sections for microscopic evaluation (Fig. 6). There were no significant differences between group L and group S with regard to

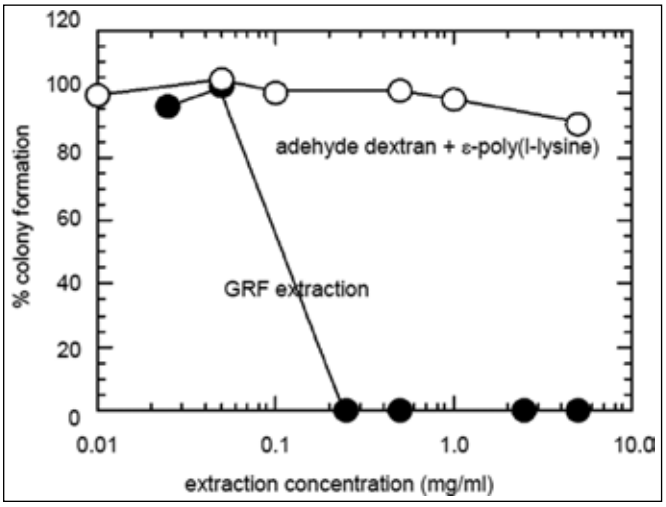

Fig. 5 - *Colony formation of V79 cells in the presence of the extraction of aldehyde dextran + ε-poly(L-lysine) and GRF glue. Almost no suppression or cytotoxicity was observed after gel formation, as shown.*

TABLE III - CYTOTOXICITY OF ALDEHYDE DEXTRAN, ε-POLY (L-LYSINE) AND THEIR GELLING SPECIMEN

Testing substance	IC50 (mg/ml)
Aldehyde dextran	1.2
ε-poly(L-lysine)	0.3
Aldehyde dextran + ε-poly(L-lysine)	>5
(gelling specimen, extraction)	
GRF, extraction	0.11

GFR = commercially available tissue adhesive; IC50 = half maximal inhibitory concentration.

inflammatory reactions. The synovial membrane, trochlear sulcus of the femur and patella were evaluated according to the histopathological scale for grading the severity of knee arthritis (Tab. I). These results showed no significant difference between the 2 groups (Fig. 7).

Immunohistochemistry showed the same level of staining for collagen type II in the cartilage underneath the adherent collagen membrane in both groups. The expression of TNFα and IL-6 in articular cartilage was not up-regulated in either of the 2 groups. The expression of TNFα in synovium was also not up-regulated in either group (Fig. 8).

DISCUSSION

Nakajima et al (8) have reported several favorable properties of their new glue as a tissue adhesive for medical applications. LYDEX carries no risk of transmitting infectious materials of human or animal origin, because only medical and food additive sources are

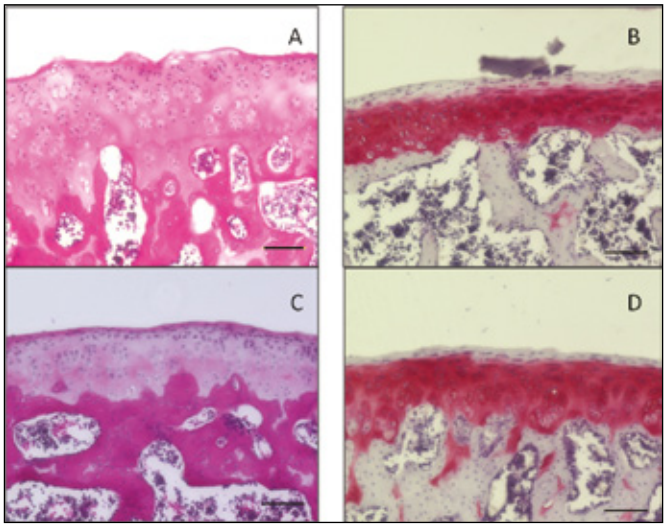

Fig. 6 - *Histological findings.* ***A)*** *Hematoxylin and eosin (HE) staining of group L;* ***B)*** *safranin-O staining of group L;* ***C)*** *HE staining of group S;* ***D)*** *safranin-O staining of group S. There were no significant differences between group L and group S with regard to inflammatory reactions (magnification ×20, bar 100 μm).*

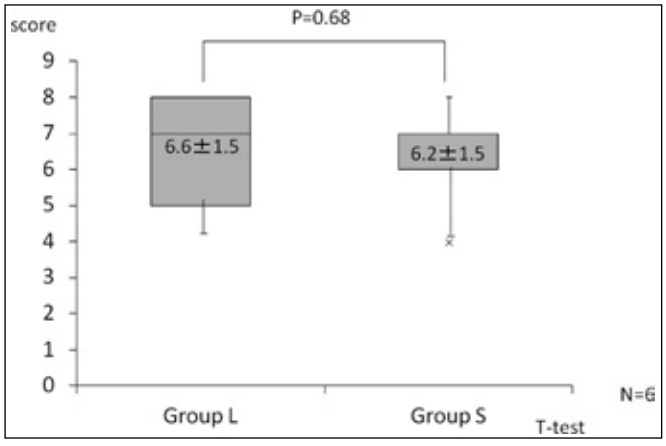

Fig. 7 - *Histopathological grading of knee arthritis. There was no significant difference between the 2 groups.*

selected as the starting materials, instead of human plasma and animal-derived components such as are used for other adhesives. Furthermore, it has a high degree of flexibility and a bonding strength higher than that of fibrin glue. After the first report in 2007, LYDEX was tested in various fields. In thoracic surgery, Araki et al (18) reported that LYDEX had sufficient sealing properties to prevent air leakage from large pleuroparenchymal defects and was significantly superior to fibrin glue as a sealant in their beagle model. In this study, normal lung structure was restored without fibrosis by 6 months. In ophthalmology, Takaoka et al (19) reported a safe and simple technique for sutureless amniotic membrane transplantation using LYDEX, which promoted a secure and rapid adhesion onto the sclera in vivo without the need for

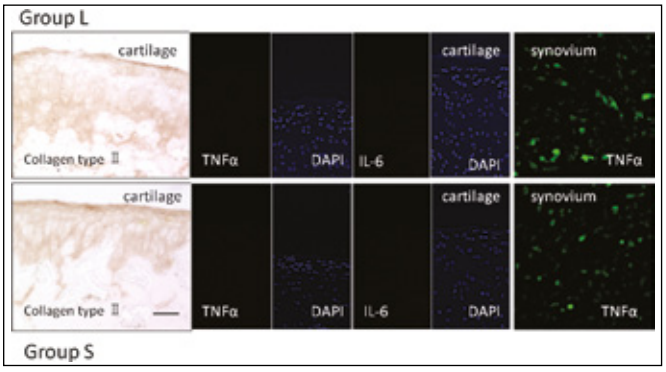

Fig. 8 - *Immunohistochemistry showed the same level of staining for collagen type II in the cartilage underneath the adherent collagen membrane in both groups. The expression of TNFα and IL-6 in articular cartilage was not different between the 2 groups. The expression of TNFα in the synovium was also not different between the groups. (Magnification ×20, bar 100 μm.)*

suturing. They found no significant differences between the sutured and nonsutured models with regard to inflammatory reactions. In orthopedics, Yamamoto et al (20) reported that LYDEX combined with hydroxyapatite granules was useful for repairing rabbit bone defects. Our study is the first to assess LYDEX for the repair of articular cartilage of the knee joint, and it has proved that the combination of LYDEX and collagen membrane is safe to use for articular cartilage.

Nakajima et al (8) have developed and reported LYDEX as a self-degradable bioadhesive. In their report, commercial fibrin glue was used as a reference. In 2007, they reported that LYDEX gave 4.0 times greater strength than fibrin glue when tested on cow skin. Concurring with this, we found that LYDEX gave 3.8 times more strength than fibrin glue when tested on articular cartilage.

We have demonstrated that LYDEX has no discernible effect on normal articular cartilage when examined histopathologically. We needed to examine whether LYDEX application had any effects on articular cartilage. The results of our study will markedly advance the treatment of cartilage injury.

Recently, Wegener et al (21) reported the use of bone marrow mesenchymal stem cells implanted into the area of cartilage injury using implants with added fibrin glue. Jung et al (22) reported that chondrogenic-differentiated mesenchymal stem cells derived from human adipose tissues combined with fibrin glue were able to proliferate and form new cartilage. Therefore, studies of the treatment for articular cartilage combined with fibrin glue are ongoing and are progressing. However, fibrin glue is derived from blood and thus carries the risk of contamination. The development of a treatment using LYDEX instead of a fibrin glue for cartilage repair has great potential.

This study is limited by the following: (i) articular cartilage is round and not flat, thus precluding the uniform application of LYDEX containing the ingredients used

in this study; (ii) in this study, LYDEX was applied to a dry surface, although in clinical practice, it is difficult to achieve a dry surface. The above problems can be solved by altering the qualities of LYDEX to increase its viscosity.

In addition, LYDEX is notably characterized by the fact that polylysine is a food additive widely used as an antibacterial agent and has potent antibacterial effects. LYDEX is potentially effective against infection, which is sometimes a complication of surgery. LYDEX should therefore be further investigated for application in various therapies. This is the first study to demonstrate that LYDEX provides greater strength adhesion than that of fibrin glue and has low cytotoxity for articular cartilage, which will allow its use as a novel material for injuries in the joint.

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Conflict of interest: None.

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