Development of tough hydrogel for intervertebral disc nucleus pulposus replacement to treat degenerative disc disease

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Development of tough hydrogel for intervertebral disc nucleus pulposus replacement to treat degenerative disc disease

Directed by Professor Seong-Hwan Moon

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Science

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December 2023

This certifies that the Doctoral Dissertation of
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December 2023

ACKNOWLEDGEMENTS

To begin, I would like to express my deepest respect and gratitude to my mentor and supervisor, Professor Seong-Hwan Moon, for his invaluable advice and guidance throughout this research. Additionally, I am profoundly thankful for the insightful advice and contributions from the members of my thesis committee, Professor Si-Young Park, Professor Hwan-Mo Lee, Professor Keung-Nyun Kim, and Professor Ho-Joong Kim.

I am also thankful to Senior Researcher Young Mi Kang for her dedicated assistance and support in laboratory experiments. Thanks should also go to all the researchers and individuals at GS Medical, Seoul National University Department of Materials Science & Engineering, and Yonsei Laboratory Animal Research Center. This endeavor would not have been possible without their work and support.

Lastly, I wish to acknowledge my family, especially my wife, children, parents, and parents-in-law. Their belief in me, love, and unwavering support have made it possible for me to successfully complete this study.

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ABSTRACT

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(Directed by Professor Seong-Hwan Moon)

Degenerative disc disease is a condition where lower back pain occurs due to degenerative changes in the intervertebral discs. If conservative treatments do not show improvement, surgical interventions such as spinal fusion may be necessary. Tissue engineering, cell therapy, and disc transplantation have been attempted as new treatments for degenerative disc disease, but no successful results have been demonstrated to date. Hydrogel, with its excellent biocompatibility and properties similar to the intervertebral disc nucleus pulposus, has recently gained attention as a potential material for nucleus pulposus replacement. In this study, we aimed to develop a tough hydrogel with enhanced mechanical properties and explore its potential for clinical application as a nucleus pulposus replacement. The tough hydrogel was fabricated by incorporating silk fibers treated with 2-hydroxyethyl methacrylate (2-HEMA), which contains hydrophilic group, into the core of a polyvinyl alcohol (PVA) hydrogel. In vitro experiments were conducted, including cytotoxicity tests, cell adhesion and cell proliferation tests. Cell viability in the extract was assessed, and cell adhesion and proliferation were evaluated through absorbance measurements. Mechanical tests, including swelling, static compression and tensile test, and dynamic mechanical analysis, were performed. Swelling ratio was measured by comparing the weights in dry

and wet states, and the elastic modulus and tan δ were measured using an electromechanical testing machine. In vivo experiments were carried out by implanting the tough hydrogel into the intervertebral disc of pigs. Under general anesthesia, surgical incisions were made to remove the pig's lumbar disc nucleus pulposus, and the tough hydrogel was implanted. After 3 weeks, the pigs were sacrificed, and X-ray, micro-CT, and histological analyses were conducted. The cell toxicity test showed that the cell viability in the extract of the tough hydrogel was over 80% of the control group, indicating no cytotoxicity. In cell adhesion test, it exhibited 205% higher absorbance compared to the control group, and cell proliferation tests showed an increasing absorbance difference from the control group over time. Through in vitro experiments, the tough hydrogel demonstrated suitability for cell adhesion and proliferation. The swelling test showed a swelling ratio of 202%, and the compression and tensile elastic modulus were 3.75 kPa and 16.50 MPa, respectively. Tan δ gradually decreased with increasing frequency, converging to 0.09. Mechanical tests confirmed that the tough hydrogel exhibited properties similar to the intervertebral disc nucleus pulposus in terms of elasticity and viscoelasticity. In the pig intervertebral disc implantation surgery, no complications related to the surgery occurred. Histological analysis and imaging results confirmed that the tough hydrogel was located within the intervertebral disc nucleus pulposus, and it integrated well without any adverse reactions to the disc tissue. Through this study, we confirmed that the tough hydrogel possesses excellent biocompatibility and properties, making it a promising candidate for nucleus pulposus replacement in the treatment of degenerative disc disease. For future clinical applications of the tough hydrogel, further research is required to develop minimally invasive and effective implantation methods into the intervertebral disc.

Key words : tough hydrogel, degenerative disc disease, nucleus pulposus, PVA, silk

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I. INTRODUCTION

Low back pain is a leading cause of disability in both developed and developing countries[1,2]. It has been estimated that 70-85% of all people have low back pain at some time in life[3]. In 2015, the global point prevalence of activity-limiting low back pain was 7.3%[4]. In Korea, the prevalence of the patients presenting low back pain was 8.5% among adults in 2007[5]. The economic and public health burden related to low back pain are enormous[6]. Since the number of people affected is increasing world-wide, the burden is expected to increase[1].

A well understood pathological cause such as vertebral fracture, malignancy, or infection is identified in only a small portion of people with low back pain. The veritable cause of low back pain still remains unclear in most cases[7]. However, a correlation with intervertebral disc degeneration and low back pain has been documented in numerous literatures[8-13]. When degenerative changes in the intervertebral disc are confirmed and considered to be the cause of chronic back pain, it can be diagnosed as a degenerative disc

disease.

The intervertebral disc is a fibrous cartilaginous complex which connects upper and lower vertebral bodies and makes stability of vertebral column. It consists of a central nucleus pulposus and an outer annulus fibrosus. The nucleus pulposus is composed of type II collagen and proteoglycans which enable the tissue to retain high levels of water and make it suited for resisting compressive loads[14,15]. The annulus fibrosus is a structure attached to the vertebral body and cartilage endplate. It surrounds the nucleus pulposus and resists tensile forces.

Degeneration of the intervertebral discs is a progressive cascade of cellular, compositional, and structural changes that is closely linked with aging[15,16]. In the degeneration process, the nucleus pulposus loses its capacity to bind water[17]. The pressure in the nucleus pulposus decreases, and thus, disc height is lost. The annulus fibrosus shows bulging, de-lamination, and radial fissures[18]. Due to an increase in microscopic and macroscopic damage to the endplate, the subchondral bone and the endplate shows sclerotic change[19].

Whether the disc degeneration is a typical aging process or a pathological condition, remains a subject of debate[20,21]. However, severe and persistent low back pain without a clear cause, accompanied by evident signs of disc degeneration, may lead to a diagnosis of degenerative disc disease. Conservative treatment approaches for degenerative disc disease typically involve medications, injections, physical therapy, and exercise. However, if conservative treatments fail to improve the condition, surgical treatment such as spinal fusion may be necessary. The efficacy of spinal fusion in eliminating the pain is well proved by various studies[22]. Nevertheless, reduced spine flexibility and the possibility of adjacent segmental degeneration are the disadvantages[23]. Total disc replacement with

artificial discs has been developed, however, there is still controversy regarding the long-term clinical outcomes[24].

The ultimate goal to treat degenerative disc disease would be regeneration of the intervertebral disc and restoration of its mechanical function. Several new technologies have been tried to restore and augment the biomechanical function of nucleus pulposus. Researches on tissue engineering, cell therapy, intervertebral disc transplantation, and biofabrication using 3D printing have been attempted, however, they have not yet demonstrated successful result with long-term efficacy[25]. Hydrogel is one the biomaterials which has been considered suitable as a substitute for nucleus pulposus because of its mechanically and biologically competent properties[26]. Hydrogels from natural origin, such as alginate, hyaluronan, chitosan, collagen, and gellan gum, offer a wide range of biological advantages, however, the physical properties are not satisfactory. On the other hand, synthetic hydrogels, such as polyethylene glycol, polyvinyl alcohol, and polyvinyl-pyrrolidone, provide good physical properties and low degradation rate[27]. In addition, hydrogels combined with various components including hyaluronic acid, collagen II, and carboxymethylcellulose have been evaluated in vitro for nucleus pulposus regeneration[28-30].

In this study, we developed a novel tough hydrogel matrix reinforced with silk fibers. Our objective was to assess the biologic and mechanical compatibility of the tough hydrogel through in vitro experiments, mechanical tests, in vivo implantation in a porcine model, and ex vivo evaluation. Through these validations, we explored the potential of utilizing the tough hydrogel as a replacement for the intervertebral disc nucleus in the treatment of degenerative disc disease.

II. MATERIALS AND METHODS

1. Tough hydrogel synthesis

Hydrogel matrix was synthesized based on 13 wt% polyvinyl alcohol (PVA) polymer. To increase elastic modulus and strength, hydrogel matrix was reinforced by 14.8 vol% silk fiber with a diameter of 650 μm in the core. The surface of the silk fiber was treated with 2-hydroxyethyl methacrylate (HEMA), which contains a hydrophilic group (-OH), in order to prevent sliding and increase the interfacial energy between the silk fiber and the hydrogel. The surface-treated silk fiber was positioned in the center, and then PVA solution was injected into the inner part of the outer mold. The synthesis of the tough hydrogel was completed by repeating the freezing-thawing cycle of 6 hours of freezing and 3 hours of thawing for a total of 9 cycles. The tough hydrogel can be fabricated in various forms, and for this study, it was prepared in the form of fiber with a diameter of 2 mm to be implanted into porcine lumbar discs. The finally produced tough hydrogels are shown in Figure 1.

2. In vitro cell cytotoxicity test

The tough hydrogel was sterilized and exposed to Dulbecco's modified Eagle's medium (DMEM) for 24 hours at 37 °C to make extract fluid. Positive and negative control were latex and polyethylene film, respectively, and titanium was also compared as a medically approved material. The same extracting procedure was performed for each material. The proportion of each material and the medium was 0.1 g per 1 ml of the medium. The extract fluid of each material as well as 2-fold diluted extract of the tough hydrogel was prepared. Wistar Institute–38 (WI–38) cells, which are human fibroblast-like fetal lung cells, and human intervertebral disc cells were separately seeded and incubated at 37 °C to a density of over 80% of the area of each culture well. The culture media was replaced with the

extract fluid, and then the cells were incubated for further 24 hours. After incubation, each well was washed with phosphate-buffered saline (PBS) and incubated with water-soluble tetrazolium salts (WST) assay reagent for 1 hour. Each well plate was placed in the absorbance microplate reader and absorption was detected at 450nm. The viability of the cells exposed to each material was calculated. The test was repeated 3 times.

Figure 1. Photograph of the tough hydrogel. The tough hydrogel has a diameter of 2 mm and contains a silk fiber with a diameter of 650 μm in the center.

3. In vitro cell adhesion and proliferation test

The tough hydrogel was sterilized and prepared in a 12–well plate with a diameter of 2.1 cm and a surface area of 3.9 cm^2. A blank 12–well plate was prepared for control. WI–38 cells were seeded with a density of 2×10^4 cells/cm^2 and incubated with DMEM for 4 hours, 1 day, 3 days, and 7 days. DMEM is replaced with PBS and WST assay reagent for 1 hour. Each well plate was placed in the absorbance microplate reader and absorption was detected at 450nm. Serial difference of optical density between tough hydrogel and control was obtained to evaluate cell proliferation. The test was repeated 3 times.

4. Swelling test

A tough hydrogel sample was prepared with a diameter of 3mm and a length of 13cm. The sample was subjected to lyophilization for 24 hours, and its weight in the dried state was subsequently measured. Following this, the sample was immersed in PBS at 37 ℃ for 24 hours, and its weight in the swollen state was measured. Experiments were repeated 3 times. Swelling ratio was calculated using following equation.

$$\textit{Swelling ratio}\ (\%) = 100 \times (W_s - W_d)/W_d$$

$$W_s = \textit{Weight of the swollen state}$$

$$W_d = \textit{Weight of the dried state}$$

5. Static compression test

A cylindrical tough hydrogel sample, which had been immersed in PBS for 1 hour, was placed in Acumen® 3 electrodynamic test machine (MTS Systems Co, Eden Prairie, MN, USA) and its diameter was measured. The gauge length was set to 5 mm, and a compressive

force was applied to the tough hydrogel sample using flat jigs at a speed of 2 mm/min (Figure 2). The stiffness and elastic modulus were determined by calculating the slope of the linear region of the load–displacement curve and stress–strain curve. The test was repeated 6 times.

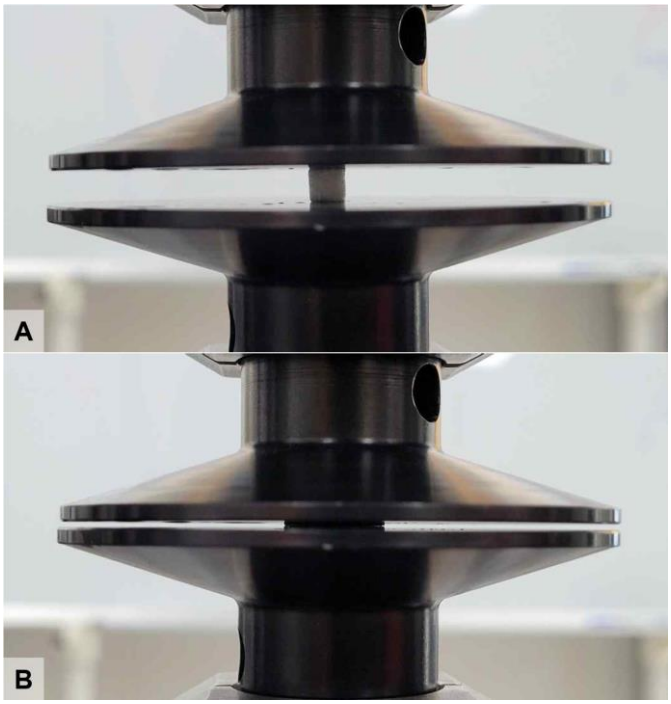

Figure 2. Static compression test. (A) Before and (B) after applying a compressive force. Flat jigs were used, and a compressive force was applied at a speed of 2mm/min.

6. Static tension test

A cylindrical tough hydrogel sample, which had been immersed in PBS for 1 hour, was placed in Acumen® 3 electrodynamic test machine (MTS Systems Co, Eden Prairie, MN, USA) and its diameter was measured. The gauge length was set to 5 mm, and a tensile force was applied to the tough hydrogel sample using clamp-shaped jigs at a speed of 5 mm/min (Figure 3). Elastic modulus, ultimate strength, and elongation were measured. The test was repeated 6 times.

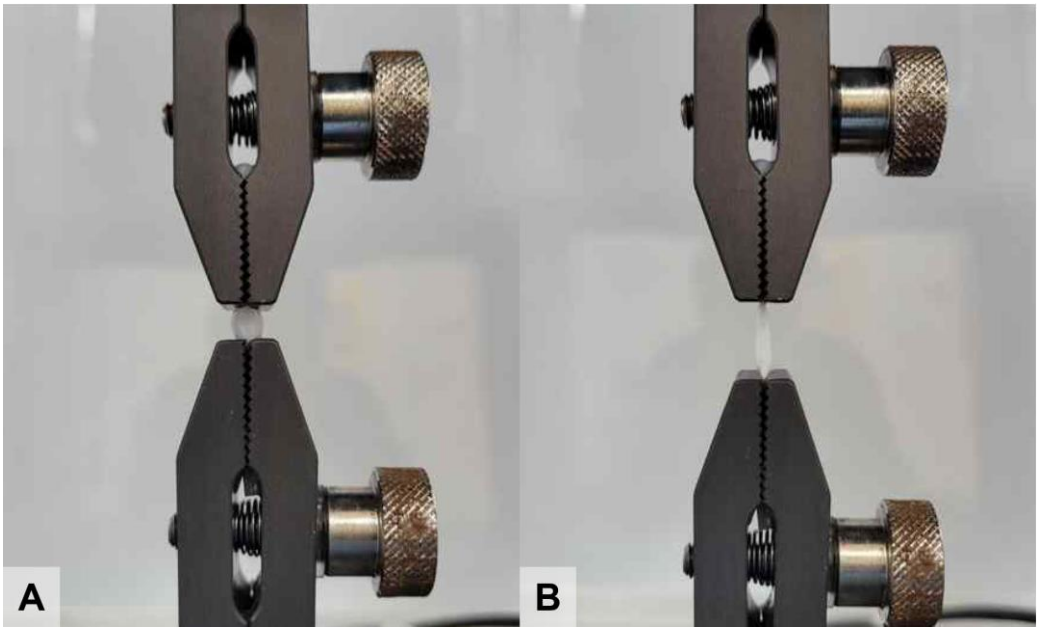

Figure 3. Static tension test. (A) Before and (B) after applying a tensile force. Clamp-shaped jigs were used, and a tensile force was applied at a speed of 5mm/min.

7. Dynamic mechanical analysis

A cylindrical tough hydrogel sample, which had been immersed in PBS for 1 hour, was placed in Acumen® 3 electrodynamic test machine (MTS Systems Co, Eden Prairie, MN, USA) and its diameter was measured. For dynamic mechanical analysis (DMA), a load-controlled cyclic tensile load was applied. The common elastic range of all tested specimens, derived from the static tension test, was set as the repeated load range of 10 to 30 N using a sine wave. The frequency range was set from 1 to 15 Hz, and the analysis was performed by sequentially increasing the frequency by 1 Hz increments. The test was repeated 3 times. The viscoelasticity (tangent of delta, tan δ) is the ratio of the loss stiffness to the storage stiffness and was calculated using the following equation.

$$K^* = K' + iK'' \ (\textit{complex stiffness})$$

$$\delta = phase\ lag$$

$$K' = \frac{\sigma_0}{\varepsilon_0}\cos\delta \ (\textit{storage stiffness})$$

$$K'' = \frac{\sigma_0}{\varepsilon_0}\sin\delta \ (\textit{loss stiffness})$$

$$\tan\delta = K''/K' \ (\textit{viscoelasticity})$$

8. In vivo implantation in a porcine model

With Institutional Animal Care and Use Committee (IACUC) approval, we utilized three 5-month-old pigs weighing 50 kg for in vivo implantation of the tough hydrogel. Pigs were anesthetized via intramuscular injection of alfaxan and were intubated and maintained on an isoflurane-oxygen mixture during the surgical procedure. We placed the pig in semi-lateral position with left side up. The draping was performed aseptically in the ordinary orthopedic manner. The intervertebral discs between lumbar vertebrae 3, 4, and 5 were accessed through an anterior approach, similar to the anterior lumbar approach commonly used in human surgeries. Confirmation of the level was performed by C-arm fluoroscopy. Oblique skin incision was made on the lateral side of the abdomen. We incised subcutaneous fat, divided abdominal muscles, and dissected through the plane between retroperitoneal fat and psoas fascia. Peritoneal cavity was retracted medially, and psoas muscle was retracted laterally. Vertebral bodies and intervertebral discs were identified. We made a small incision on annulus fibrosus and removed nucleus pulposus. Then, the tough hydrogel was implanted at the core of the intervertebral disc with a coil shape. For one pig, we utilized a titanium fiber with a thickness of 0.2 mm embedded within the tough hydrogel material to assess the positioning of the implanted tough hydrogel within the disc.

The surgical wound was closed layer by layer.

Postoperatively, the pigs were managed by veterinary staff and were administered intravenous ketorolac (1 mg/kg) and subcutaneous meloxicam (0.2 mg/kg) for pain control. For antimicrobial prophylaxis, intramuscular cefazolin (30 mg/kg) was administered preoperatively, and oral amoxicillin clavulanate (0.14 mg/kg) was administered postoperatively for seven days.

9. Ex vivo evaluation

On postoperative day 21, the pigs were euthanized by overdose intravenous potassium chloride injection under general anesthesia by intramuscular injection of alfaxan (1 mg/kg), xylazine (2 mg/kg), and azaperone (2 mg/kg). The lumbar 3-4-5 spine segment was harvested and fixed in neutral buffered formalin. The sample was decalcified and sectioned at 10 μm. Sections were stained with hematoxylin and eosin and Masson's trichrome. The segments with titanium fiber underwent X-ray and micro-computed tomography (CT) imaging to visualize the distribution of the tough hydrogel in the intervertebral disc.

10. Statistical analysis

Data were expressed as mean ± standard deviation (SD) from the results of independent experiments. A two-tailed Student's t-test was used to compare the results of the two groups. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using R (version 4.3.0).

A schematic diagram of the study methods is shown in Figure 4.

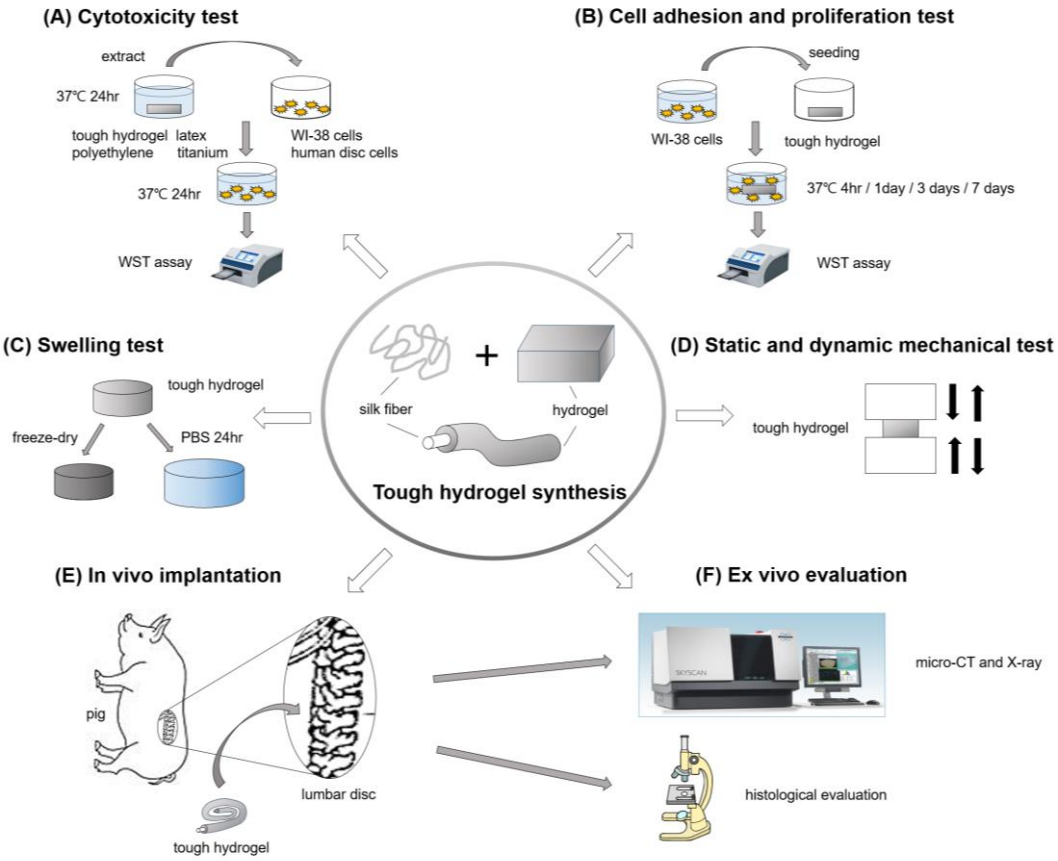

Figure 4. Schematic diagram of the study methods. Tough hydrogel was synthesized and evaluated in the following experiments: (A) Cytotoxicity test, (B) Cell adhesion and proliferation test, (C) Swelling test, (D) Static and dynamic mechanical test, (E) In vivo implantation, and (F) Ex vivo evaluation.
WST, water-soluble tetrazolium salts; PBS, phosphate-buffered saline; CT, computed tomography

III. RESULTS

1. Cell cytotoxicity test

The mean cell viability of WI-38 cells was 84.82 ± 6.60% and 96.49 ± 6.77% for 100% and 50% tough hydrogel extract fluid, respectively. For disc cells, the mean cell viability was 86.82 ± 7.99% and 92.83 ± 9.87% for 100% and 50% tough hydrogel extract fluid, respectively (Figure 5). The cell viability of the 100% tough hydrogel extract exceeded 80%, indicating that the tough hydrogel material is non-cytotoxic and exhibits high biosafety. The cell viability of both 100% and 50% tough hydrogel was significantly higher than that of latex, the positive control ($P < 0.001$).

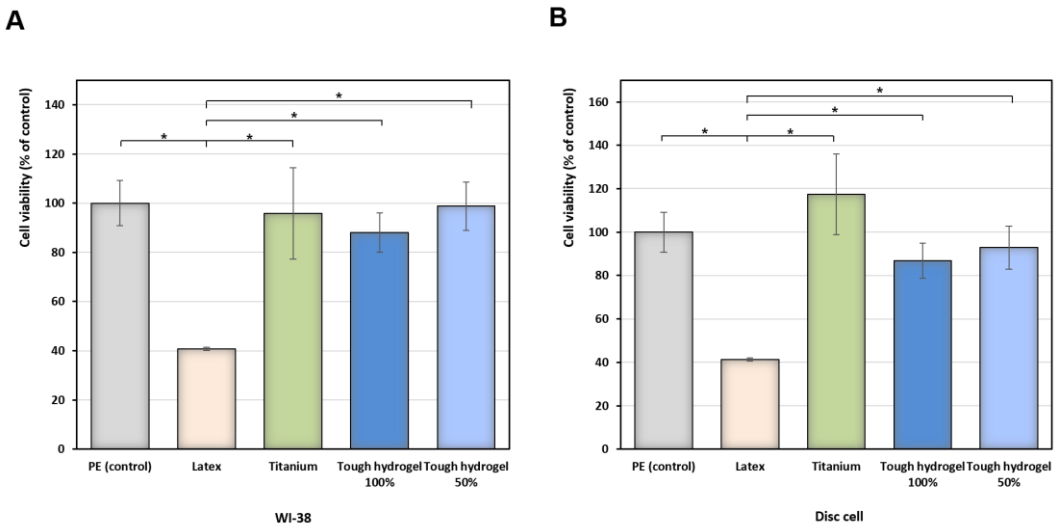

Figure 5. Cell viability results of (A) WI-38 and (B) disc cell for each material extract fluid. The tough hydrogel showed similar results to the control material, indicating its non-toxicity and biosafety. Data were shown mean ± standard deviation from 3 independent experiments.
*Statistically significant difference with $P < 0.05$.
PE, polyethylene; WI, Wistar Institute

2. Cell adhesion and proliferation test

After a 4-hour incubation period, the mean optical density (OD) at 450 nm of the tough hydrogel was 0.21 ± 0.07, which was 205% higher than that of the control ($P = 0.002$). This result demonstrates the excellent cell adhesion capability of the tough hydrogel. The difference of optical density between the tough hydrogel and control increased over time, indicating that the tough hydrogel is conducive to cell proliferation (Figure 6).

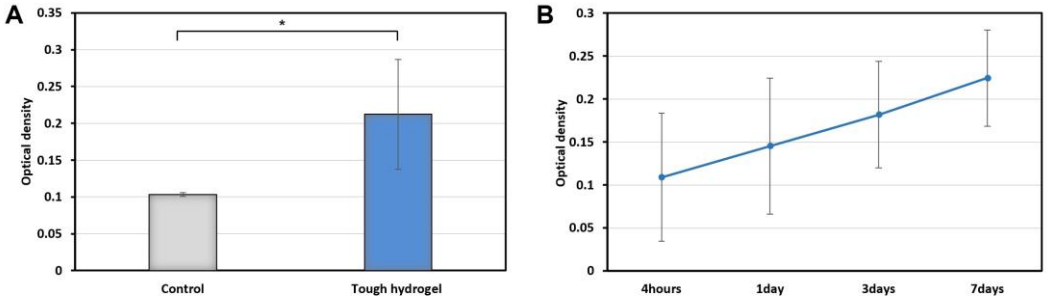

Figure 6. Results of in vitro cell adhesion and proliferation test. (A) The mean optical density at 450 nm of the tough hydrogel was 205% higher than that of the control. (B) The difference of optical density between the tough hydrogel and the control showed increasing pattern over time. Data were shown mean ± standard deviation from 3 independent experiments.
*Statistically significant difference with $P < 0.05$.

3. Swelling test

The mean weight of the tough hydrogel in the swollen state was 1.38 ± 0.13 g, which was significantly higher compared to the dried state weight of 0.46 ± 0.05 g ($P < 0.001$) (Figure 7). The swelling ratio of the tough hydrogel was 202%.

4. Static compression test

The mean stiffness of the tough hydrogel sample was 1.46 ± 0.33 N/mm, and the mean elastic modulus was 3.75 ± 0.86 kPa (Table 1).

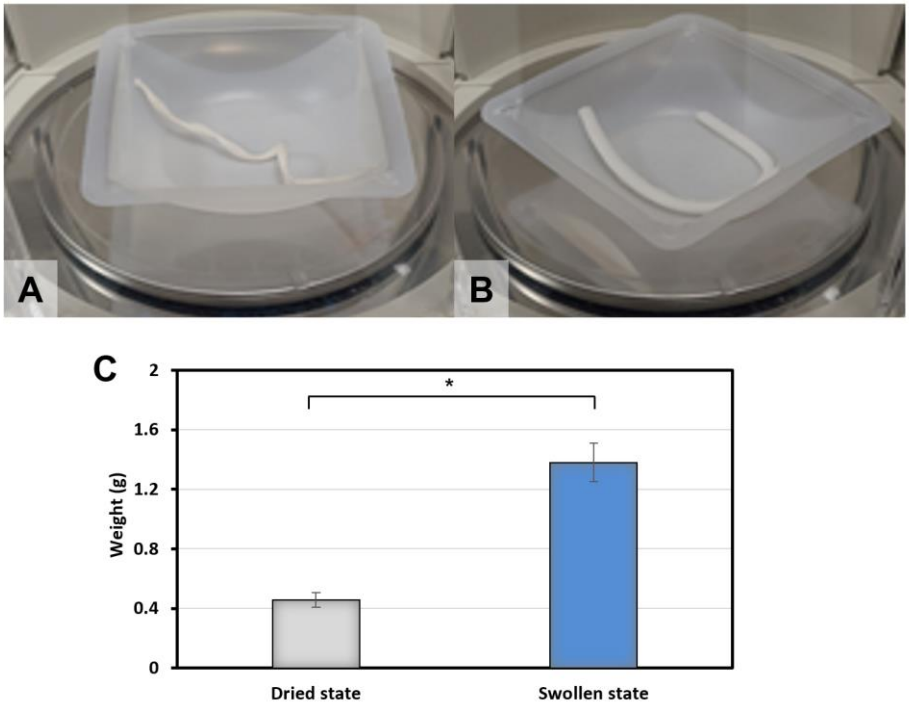

Figure 7. The tough hydrogel in (A) dried state and (B) swollen state. (C) The mean weight of the tough hydrogel in the swollen state was higher compared to the dried state. Data were shown mean ± standard deviation from 3 independent experiments.
*Statistically significant difference with $P < 0.05$.

Table 1. Results of the static compression test

	Stiffness (N/mm)	Elastic modulus (kPa)
#1	1.73	4.46
#2	1.08	2.75
#3	1.24	3.16
#4	1.69	4.31
#5	1.18	3.03
#6	1.85	4.78
Mean ± SD	1.46 ± 0.33	3.75 ± 0.86

SD, standard deviation

5. Static tension test

The 6 stress–strain curves were presented in Figure 8. The mean elastic modulus of the tough hydrogel was 14.11 ± 0.81 MPa. The mean ultimate strength was 12.54 ± 0.78 MPa, and the mean elongation was 103.07 ± 13.85% (Table 2).

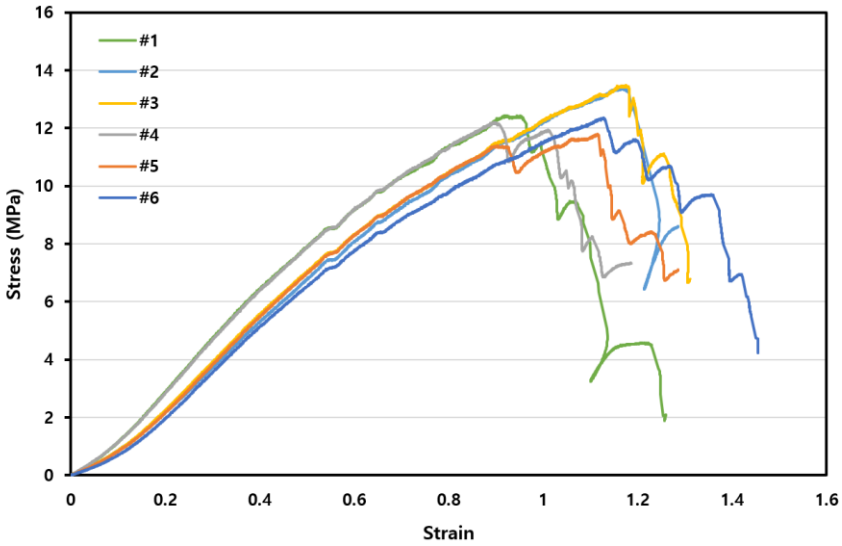

Figure 8. The stress-strain curves of the tough hydrogel in 6 independent static tension tests. The elastic modulus was determined by calculating the slope of the linear region.

6. Dynamic mechanical analysis

Measured complex stiffness ranged from 106 to 136 N/mm and showed gradual increase as the frequency increased (Figure 9). The storage stiffness curve was similar to the complex stiffness curve (Figure 10). The loss stiffness showed a gradual decrease and plateau pattern (Figure 11). The tan δ of the tough hydrogel ranged from 0.09 to 0.15. As the frequency increased, the tan δ decreased and reached a plateau around 0.09 (Figure 12).

Table 2. Results of the static tension test

	Elastic modulus (MPa)	Ultimate Strength (MPa)	Elongation (%)
#1	17.86	12.44	91.82
#2	14.82	13.37	116.77
#3	16.21	13.49	117.46
#4	17.98	12.20	89.51
#5	16.41	11.40	90.28
#6	15.73	12.35	112.60
Mean ± SD	16.50 ± 1.23	12.54 ± 0.78	103.07 ± 13.85

SD, standard deviation

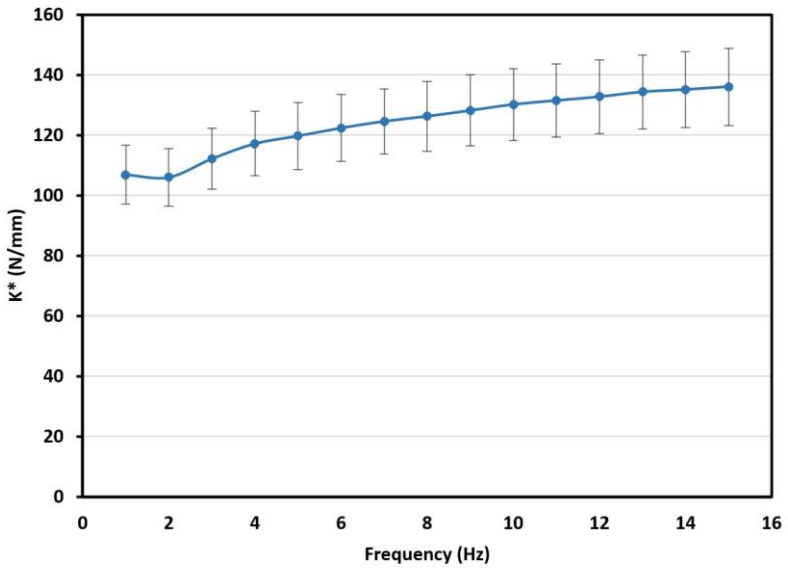

Figure 9. Complex stiffness (K*) of the tough hydrogel. The complex stiffness exhibited a gradual increase as the frequency increased. Data were shown mean ± standard deviation from 3 independent experiments.

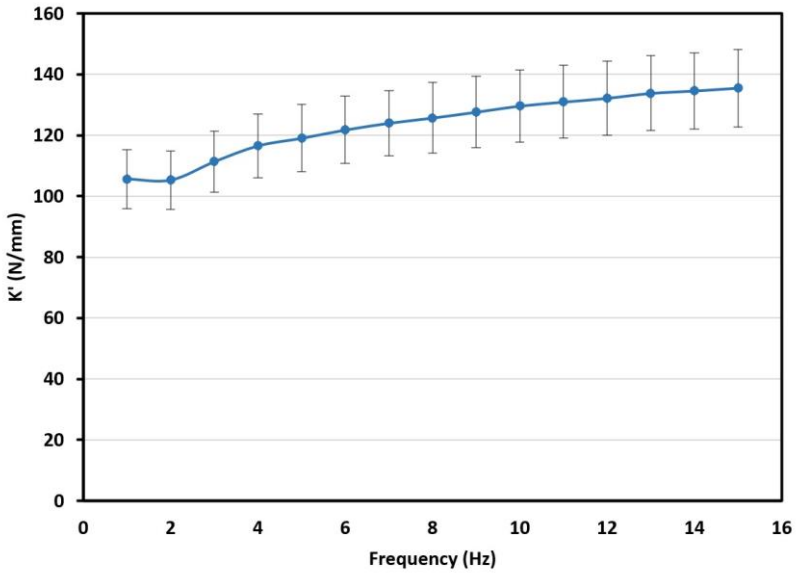

Figure 10. Storage stiffness (K') of the tough hydrogel. The storage stiffness displayed similar results to the complex stiffness, showing a gradual increase as the frequency increased. Data were shown mean ± standard deviation from 3 independent experiments.

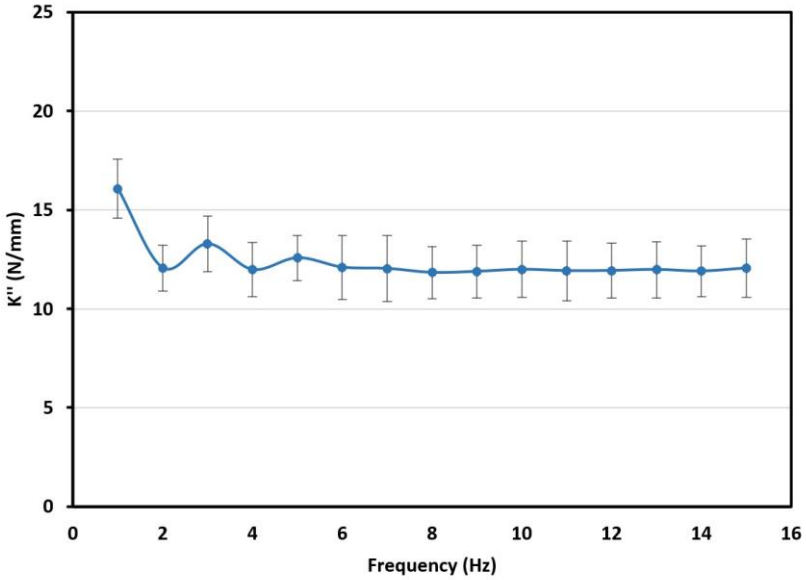

Figure 11. Loss stiffness (K'') of the tough hydrogel. The loss stiffness gradually decreased and reached a plateau of approximately 12 N/mm as the frequency increased. Data were shown mean ± standard deviation from 3 independent experiments.

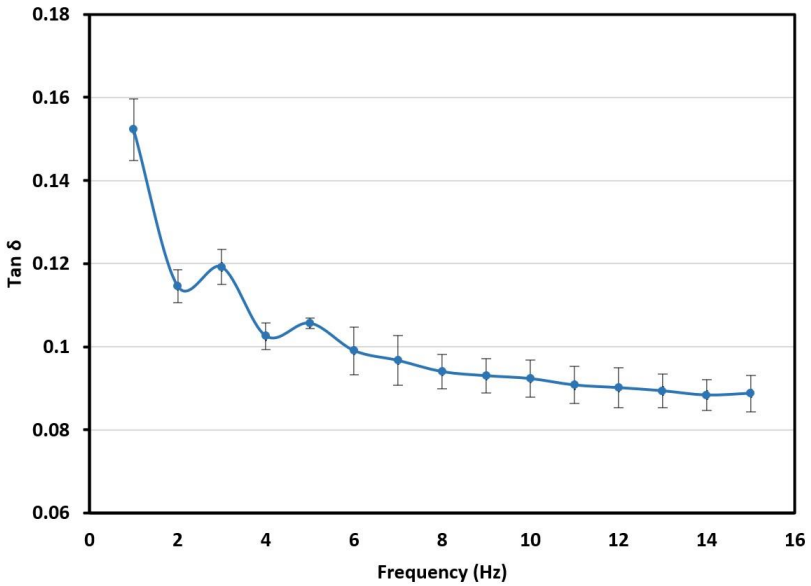

Figure 12. Tan delta of the tough hydrogel. Tan delta, which represents the viscoelasticity, gradually decreased and reached a plateau of approximately 0.09 as the frequency increased. Data were shown mean ± standard deviation from 3 independent experiments.

7. In vivo implantation in a porcine model

All the pigs tolerated well during the surgery and there were no complications such as paraplegia due to nerve injury. There was minimal bleeding observed during the procedure. The surgical wound healed well, and the stitches were removed 2 weeks after the surgery. Surgical photos were shown in Figure 13.

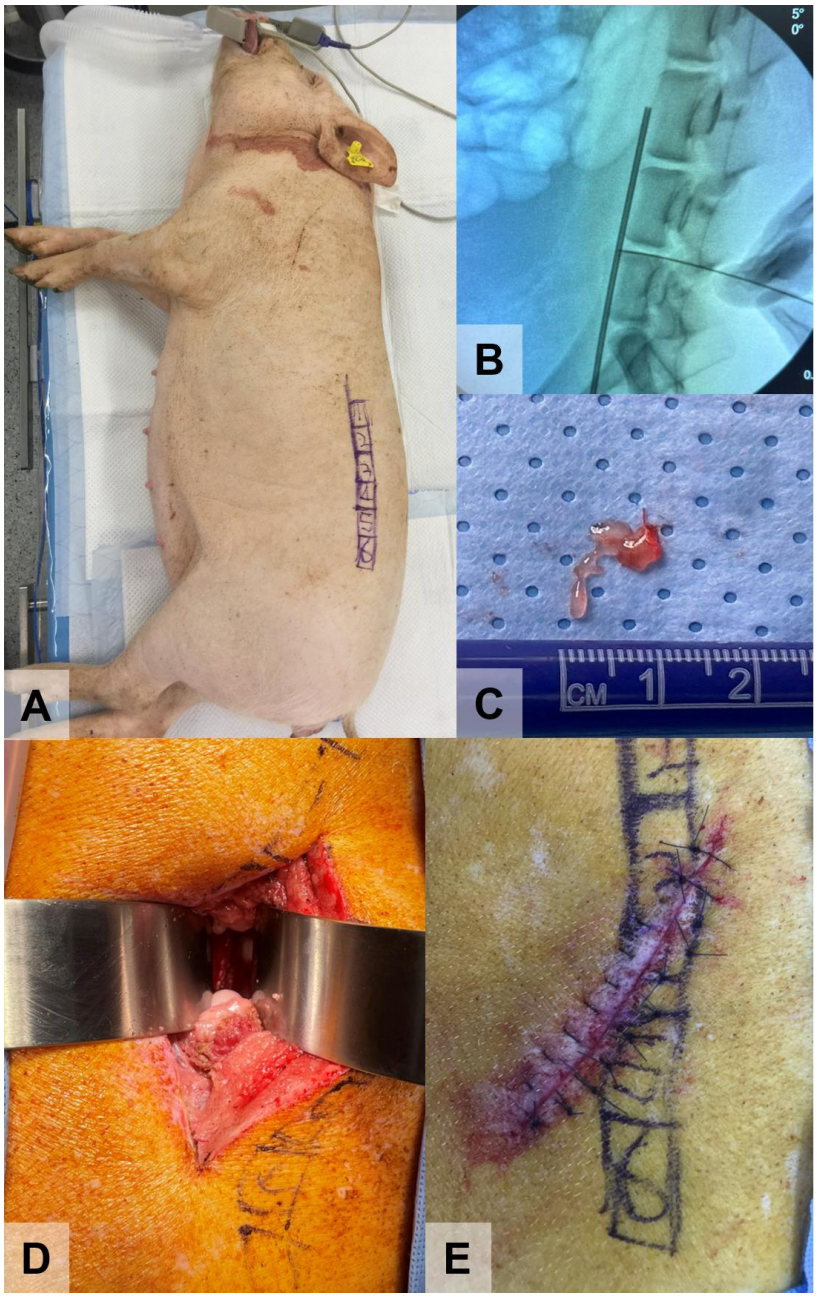

Figure 13. In vivo implantation of the tough hydrogel in a porcine model. (A) A pig with lateral position on the operating table. (B) Confirmation of the surgical level using C-arm fluoroscopy. (C) Resected clear, jelly-like normal nucleus pulposus of a porcine disc. (D) Deaver retractors were used to obtain a surgical field of view and approach to the disc. (E) Sutured skin with nylon.

8. Ex vivo evaluation

The X-ray and micro-CT scans confirmed that the titanium fiber embedded within the tough hydrogel was located in the central region of the intervertebral disc. (Figure 14). In the histological examination, it was confirmed that the tough hydrogel was situated within the nucleus pulposus, with intact adjacent vertebral endplates and annulus fibrosus. The implanted tough hydrogel was enveloped by a thin fibrous capsule, without any abnormal immune reaction observed. It exhibited close contact with the highly collagenous remnant nucleus pulposus (Figure 15).

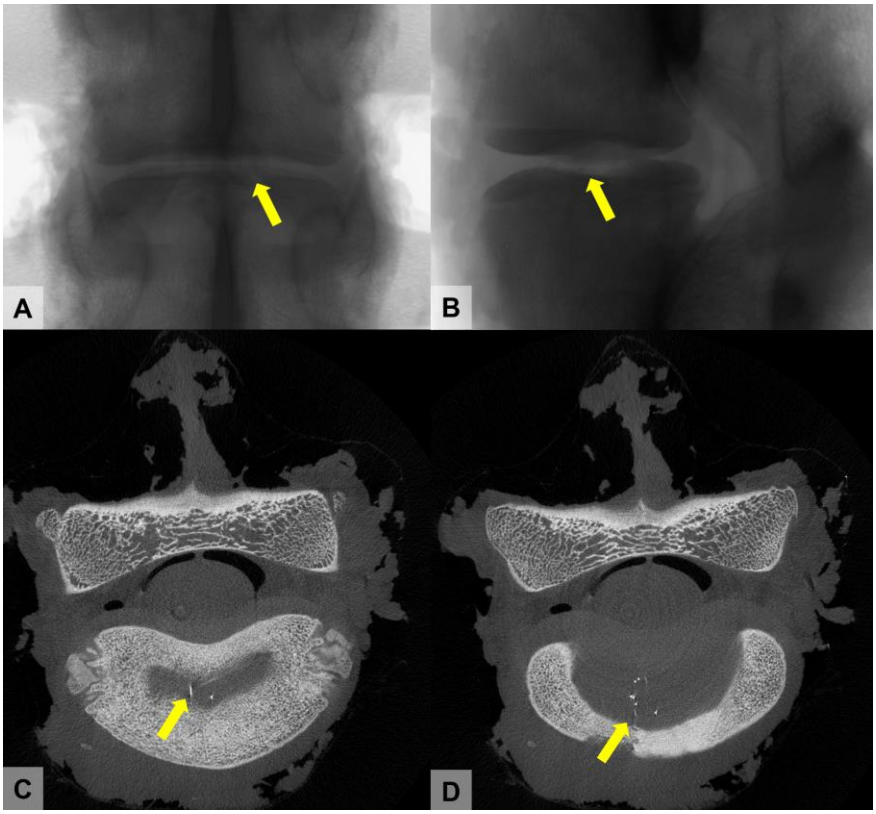

Figure 14. X-ray and micro-CT evaluation of the implanted tough hydrogel. A titanium fiber with a thickness of 0.2 mm was detected in the central portion of the porcine intervertebral disc using X-ray (A and B) and micro-CT (C and D) imaging. The fiber was embedded within the tough hydrogel material.

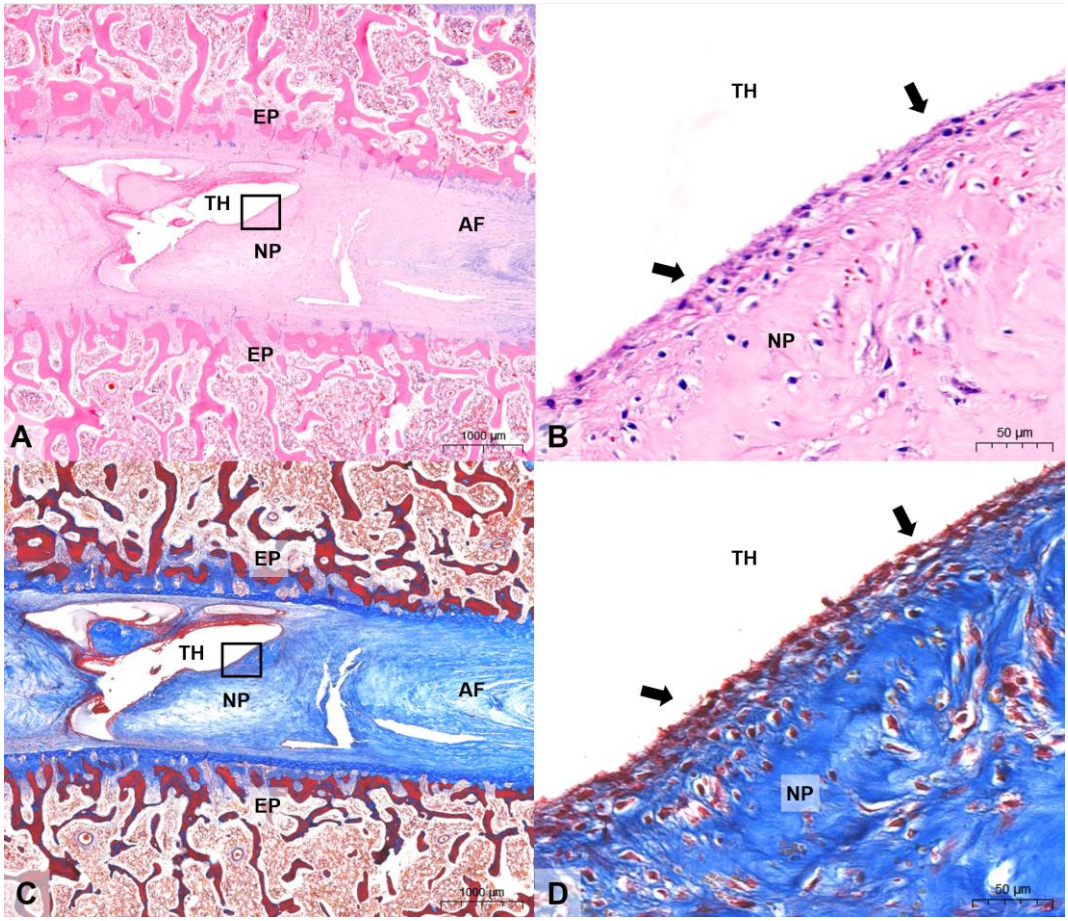

Figure 15. Histological evaluation of the tough hydrogel at 3 weeks after implantation in a porcine model. Hematoxylin and eosin stain for (A) and (B), and Masson's trichrome stain for (C) and (D). The boxed areas in (A) and (C) (×10) are depicted at higher magnification in (B) and (D) (×170). Implanted tough hydrogel was surrounded by a thin fibrous capsule (black arrow) without abnormal immune reaction, showing close contact with highly collagenous remnant nucleus pulposus (D). EP, endplate; NP, nucleus pulposus; TH, tough hydrogel; AF, annulus fibrosus.

IV. DISCUSSION

In this study, we conducted in vitro experiments, mechanical tests, in vivo implantation, and ex vivo evaluations to investigate the potential use of the reinforced tough hydrogel as a nucleus pulposus substitute. A mechanically reinforced tough hydrogel was created using PVA hydrogel and silk fibers, and in vitro experiments confirmed its non-cytotoxic nature and observed favorable cell adhesion and proliferation. Through mechanical testing, the tough hydrogel demonstrated excellent swelling properties, elastic modulus, strength, and viscoelasticity. In the large animal experiments conducted using pigs, it was confirmed that the tough hydrogel remained within the disc for an extended period without causing any abnormal immune or inflammatory response.

Hydrogels are widely recognized for their excellent biocompatibility, and they are currently used in various products such as contact lenses, wound dressings, drug delivery, and hygiene products[31]. PVA hydrogel has been found to have minimal toxicity and is widely used in biomedical engineering research, particularly in applications such as vascular stents and cartilage, due to its highly strong and ultrapure characteristics[32,33]. Silk also demonstrates high biocompatibility and is utilized as a medical material in various applications, including sutures[34]. In this study, a cytotoxicity test was conducted on the final product of the tough hydrogel to evaluate the potential risks associated with residual monomers or degradation by-products during the fabrication process. Even in the 100% tough hydrogel extract, the cell viability showed over 80%, meeting the standards set by the International Organization for Standardization (ISO).

PVA/silk composite hydrogels have gained significant attention in the field of tissue engineering due to their excellent cell adhesion and proliferation properties[35,36]. In this study, silk fibers treated with 2-HEMA were utilized, and we observed outstanding

results in terms of both cell adhesion and proliferation. These findings indicate the high biocompatibility of the tough hydrogel and its potential for applications in tissue engineering, where the attachment of growth factors or bioactive molecules can be used to regulate the proliferation and differentiation of surrounding cells[37].

Hydrogels are composed of hydrophilic polymers capable of retaining a large amount of water, resulting in their characteristic swelling behavior. The swelling property of hydrogels allows for easier minimally invasive surgeries, as the hydrogel can be inserted into the body with fewer incisions when it is in a dry and compacted state. There are literature reports documenting successful replacement after nucleotomy using dried-state hydrogels in porcine and canine model lumbar discs, utilizing these characteristics[38,39]. The swelling ratio of the tough hydrogel was 202%, which was similar to the results mentioned in the aforementioned literatures, and it was considered satisfactory at a desirable level.

Intervertebral disc nucleus replacement materials need to withstand high loads, requiring excellent mechanical properties. According to a cadaveric study[40], the compressive elastic modulus of the nucleus pulposus was 5.8 kPa, and it increased with the progression of degenerative changes. In another cadaveric study by Cloyd et al.[41], the mean elastic modulus of the nucleus pulposus was 5.39 ± 2.56 kPa. The tough hydrogel in our study had an elastic modulus of 3.75 kPa, which was similar to that of the cadaveric nucleus pulposus mentioned above. While the elastic modulus of the tough hydrogel we studied was slightly lower than that of the cadaveric nucleus pulposus, it exhibited over tenfold greater stiffness compared to typical PVA hydrogel[42]. It is possible that the slightly lower elastic modulus of the tough hydrogel compared to the elastic modulus of cadaveric nucleus pulposus may be due to conducting the measurement without the presence of surrounding annulus fibrosus, which could have prevented hoop strain. Further research is needed to

investigate this aspect in more detail.

In contrast to the static compression test, the static tension test revealed a higher elastic modulus for the tough hydrogel. By incorporating silk fibers with hydrophilic surface treatment using 2-HEMA into the core of the PVA hydrogel, it was possible to achieve higher tensile strength and elastic modulus. There have been several studies focused on creating composite PVA hydrogels to enhance their mechanical properties. Xiang et al. fabricated PVA/hydroxyapatite/tannic acid hydrogels and reported a tensile strength of 0.43 MPa[43]. Hou et al. reported PVA/silk fibroin composite hydrogels with an increased elastic modulus of 161 kPa[35]. In this study, the tough hydrogel exhibited a tensile ultimate strength of 12.54 MPa and a tensile elastic modulus of 16.50 MPa. The use of coarse silk fibers in their unmodified state, in contrast to previous studies, is considered the primary factor contributing to the significant enhancement of mechanical properties observed in our study.

It is widely known that the nucleus pulposus exhibits viscoelastic properties[44-47]. In a study conducted by Gadd et al.[48], which involved 12 ovine lumbar discs, it was observed that storage stiffness increased with frequency, while loss stiffness remained relatively constant across the frequency range. In this study, the tough hydrogel also exhibited an increase in storage stiffness with increasing frequency, while the loss stiffness remained relatively constant above 8 Hz. The tan δ, often referred to as the loss factor, measures the relationship between the loss and storage stiffness and can be considered as a representative value of a material's viscosity. A tan δ value greater than one signifies a more liquid-like behavior, while a tan δ value closer to zero suggests more solid-like characteristics. In this study, the tan δ of the tough hydrogel ranged from 0.09 to 0.15, and as the frequency increased, the tan δ reached a plateau around 0.09. This finding closely

aligns with the tan delta values observed in the study by Gadd et al.[48], indicating that the tough hydrogel exhibits a viscoelastic behavior similar to that of the nucleus pulposus.

Based on the experimental results mentioned above, we performed large animal experiments to assess the feasibility of clinical application for the tough hydrogel. Pigs are extensively utilized in various experiments and also for training purposes in the field of spinal surgery, primarily due to their anatomical resemblance to humans in terms of spinal structures[49]. We performed surgery using a porcine model to remove the lumbar disc nucleus pulposus and insert the tough hydrogel, and no significant complications were observed during the procedure. This result holds significant clinical significance since previous studies have demonstrated that although hydrogels used for nucleus pulposus augmentation can replicate native disc mechanical properties during ex vivo testing, they frequently exhibit failure when implanted in large animal models in vivo[50-52]. Indeed, in our preliminary study, we initially performed disc replacement surgery in pigs using a posterior approach. However, we encountered complications such as infection, lower limb paralysis due to nerve injury, and fatalities due to excessive bleeding. As a result, we changed the approach to an anterolateral approach for disc access, and we were able to achieve favorable outcomes. While the use of an anterolateral approach for disc access is not yet common in humans, we believe that with the development of minimally invasive spine surgery tools, it has the potential to become more widely adopted in the future. Additionally, in humans, unlike pigs, surgical facilities are equipped to facilitate bleeding control and nerve root manipulation, making the posterior approach a viable option.

In our ex vivo evaluation, we confirmed that the tough hydrogel was well positioned in the nucleus pulposus region of the disc and did not elicit any inflammatory response. The biocompatibility of PVA hydrogel and silk has already been established, and

there are similar research findings to ours[53-55]. However, our study holds significance as we used pigs as the animal model and performed open surgery involving the removal of the nucleus pulposus and insertion of the tough hydrogel, rather than a simple injection of hydrogel.

There are several limitations in this study. The tough hydrogel was designed in a fiber form to facilitate insertion into the disc, but in the porcine model, the insertion could not be achieved ideally. It should be noted that porcine discs are considerably smaller than human discs, and when applying this technique to human discs, appropriate insertion instruments need to be developed. Second, during the insertion process of the tough hydrogel, the intact annulus fibrosus may become damaged. Even in degenerated discs with annular fissures, it is important to minimize the artificial injury inflicted. Therefore, it is necessary to enable the insertion of the tough hydrogel with minimal annular incisions. Further research utilizing the swelling characteristics of the hydrogel and other approaches may be required to address this issue. Lastly, the longevity of the tough hydrogel inside the disc tissue was not evaluated in this study. For the clinical application of the tough hydrogel, additional research on the in vivo longevity of the tough hydrogel is necessary.

V. CONCLUSION

The tough hydrogel, created by combining PVA hydrogel and silk fibers, underwent rigorous in vitro experiments, mechanical testing, in vivo implantation, and ex vivo evaluations. The in vitro experiments confirmed its biocompatibility, as there were no observed cytotoxic effects, and it promoted cell adhesion and proliferation. Mechanical testing showcased its outstanding properties, including excellent swelling behavior, elastic modulus, strength, and viscoelasticity. In large animal experiments using pigs, the tough hydrogel demonstrated long-term presence within the disc without triggering any adverse immune or inflammatory reactions. Based on the overall findings of this study, the tough hydrogel appears to be a suitable material for use as a nucleus pulposus substitute. With further research, it may be possible to explore its clinical application and attempt its implementation in practice for the treatment of degenerative disc disease.

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ABSTRACT(IN KOREAN)

퇴행성 추간판 질환 치료를 위한
추간판 수핵 대체용 터프 하이드로젤 개발

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이 수 빈

퇴행성 추간판 질환은 추간판의 퇴행성 변화로 인해 요통이 발생하는 질환으로 보존적 치료에 호전이 없을 경우 척추 유합술 등의 수술적 치료가 필요할 수 있다. 퇴행성 추간판 질환의 새로운 치료법으로 조직 공학, 세포 치료, 추간판 이식 등이 시도되었으나 성공적으로 밝혀진 것은 현재까지 없다. 하이드로젤은 생체 적합성이 우수하며 추간판 수핵과 유사한 물성을 보여 추간판 수핵을 대체할 수 있는 소재로 최근 주목받고 있다. 본 연구에서는 물성을 강화한 터프 하이드로젤을 개발하여 추간판 수핵 대체제로서 임상 적용의 가능성을 확인하고자 하였다. 터프 하이드로젤은 실크 섬유를 친수성기를 가진 2-하이드록시에틸메타크릴레이트 (2-HEMA)로 처리한 후 폴리비닐알콜 (PVA) 하이드로젤의 중심부에 배치하여 제작하였다. 시험관내 (in vitro) 실험으로 세포독성시험 및 세포 부착, 세포 증식 시험을 진행하였다. 용출액에서의 세포 생존율을 확인하였고 세포 부착 및 증식 정도를 흡광도를

통해 확인하였다. 물성 시험으로는 팽윤 시험, 정적 압축 및 인장 시험, 동적 기계 분석을 시행하였다. 건조 및 침윤 상태의 무게를 비교하여 팽윤도를 측정하였고 전기역학 시험기를 이용하여 탄성계수, tan δ를 측정하였다. 생체내 (in vivo) 시험으로 돼지의 추간판 내 터프 하이드로젤 주입을 시행하였다. 전신 마취 하에 수술적 절개를 통해 돼지 요추 추간판 수핵을 제거하였고 터프 하이드로젤을 삽입하였다. 3주 뒤 돼지를 희생하여 X-ray, micro-CT, 조직학적 분석을 시행하였다. 세포독성시험 결과 터프 하이드로젤 용출액의 세포 생존율은 대조군의 80% 이상으로 세포 독성이 없는 것을 확인하였다. 세포 부착 시험에서는 대조군 보다 205% 높은 흡광도를 보였고, 세포 증식 시험에서는 시간이 경과함에 따라 대조군과의 흡광도 차이가 증가하였다. In vitro 실험을 통해 터프 하이드로젤이 세포 부착 및 증식에 적합하다는 것을 확인하였다. 팽윤 시험에서 터프 하이드로젤은 202%의 팽윤도를 보였고 압축 및 인장 탄성계수는 각각 3.75 kPa, 16.50 MPa이었다. tan δ는 진동수가 증가함에 따라 점차 감소하여 0.09에 수렴하는 것을 확인하였다. 물성 시험을 통해 터프 하이드로젤이 추간판 수핵과 유사한 탄성 및 점탄성을 가진다는 것을 확인하였다. 돼지 추간판 내 터프 하이드로젤 주입 수술 결과 수술과 관련된 합병증은 발생하지 않았다. 조직학적 분석 및 영상 검사 결과 터프 하이드로젤은 추간판 수핵에 위치하고 있었으며 추간판 조직과 이상 반응 없이 생착한 것을 확인하였다. 본 연구를 통해 터프 하이드로젤은 우수한 생체 적합성과 물성을 가지고 있음을 알 수 있었고, 퇴행성 추간판 질환 치료를 위한 수핵 대체제로서의 가능성이 충분함을 확인하였다. 앞으로 터프

하이드로젤의 임상 적용을 위해서는 최소침습적이고 효과적인 추간판 내 주입 방법에 대한 추가적인 연구가 필요하다.

핵심되는 말 : 터프 하이드로젤, 퇴행성 추간판 질환, 수핵, PVA, 실크