IN SITU FORMING OXIDISED HYALURONIC ACID/ADIPIC ACID DIHYDRAZIDE HYDROGEL FOR PREVENTION OF EPIDURAL FIBROSIS AFTER LAMINECTOMY

M.H. Hu[1,2], K.C. Yang[3], Y.H. Sun[2], Y.C. Chen[4], S.H. Yang[2,*]and F.H. Lin[1,5]

[1] Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
[2] Department of Orthopaedics, National Taiwan University College of Medicine and National Taiwan University Hospital, No.1, Sec. 1, JenAi Road, Taipei 100, Taiwan
[3] School of Dental Technology, College of Oral Medicine, Taipei Medical University, 250 Wuxing Street, Taipei 110, Taiwan
[4] Department of Orthopaedics, Far Eastern Memorial Hospital, No.21, Sec. 2, Nanya S. Road, Banciao District, New Taipei City 220, Taiwan
[5] Institute of Biomed Engineering and Nanomedicine, National Health Research Institutes, 35 Keyan Road, Zhunan, Miaoli County 350, Taiwan

Abstract

Post-operative epidural fibrosis is a biological response after laminectomy that may lead to clinical symptoms, such as radicular pain. An ideal material for prevention of epidural fibrosis should be able to inhibit fibroblast adhesions and reduce formation of scar tissue. An injectable hydrogel would be the material of choice for this purpose, since it could fill an irregular surgical defect completely, gelate *in situ* and be delivered in a minimally-invasive manner. The objective of this study was to evaluate, *in vitro* and *in vivo*, the cytocompatibility and anti-adhesive effect of an oxidised hyaluronic acid/adipic acid dihydrazide (oxi-HA/ADH) hydrogel. Different cell types present in the spine were used to test the cytocompatibility of the hydrogel. The hydrogel extraction medium had no deleterious effects on neural cells (PC-12), but reduced fibroblasts viability (NIH/3T3). Although the hydrogel did not change the release of lactate dehydrogenase from myoblasts (C2C12) and Schwann cells (RSC96), the extraction medium concentration slightly affected the mitochondrial activity of these two cell types. qPCR showed that the hydrogel down-regulated *S100a* and *P4hb* expression in NIH/3T3 cells, supporting the hypothesis that the hydrogel might inhibit fibroblast activity. The animal study showed a reduction of scar tissue formation as well as severity of adhesion between scar tissue and the dura mater in a rat laminectomy model. Superficially, the peel-off test showed significantly decreased tenacity. In conclusion, the oxi-HA/ADH hydrogel is a promising injectable and thermosensitive material for prevention of post-operative epidural fibrosis.

Keywords: Injectable hydrogel, thermosensitive, hyaluronic acid, epidural fibrosis, laminectomy.

*Address for correspondence: Dr Shu-Hua Yang, Department of Orthopaedics, National Taiwan University College of Medicine and National Taiwan University Hospital, No.1, Sec. 1, JenAi Road, Taipei 100, Taiwan. Telephone: +886 23123456   Fax: +886 23224112   E-mail: shuhuayang@ntu.edu.tw

Introduction

Laminectomy is used to explore the spinal canal and decompress the neural elements during spinal surgery. Post-operative epidural fibrosis is a normal biological response after laminectomy, which leads to post-laminectomy dura mater adhesion (Semra *et al.*, 2015). Fibrosis is the replacement of epidural fat with fibrotic tissue, which binds the dura mater and the nerve roots to the anterior and posterior structures (de Tribolet *et al.*, 1998; Robertson, 1996). In addition, it can cause symptomatic problems, such as radicular pain. Ross *et al.* (1996) show a close relationship between epidural adhesions and recurrent radicular pain or lower back pain after a successful lumbar discectomy. In addition, epidural adhesion leads to difficulties during a revision surgery. The rate of complications, such as iatrogenic nerve root injury or dura mater tear, remains unpredictably high in revision surgeries (Burton, 1991). Therefore, prevention of epidural fibrosis and adhesion is an imperative for a successful laminectomy.

The growth of fibrous connective tissue into the surgical haematoma of the paraspinal muscle results in fibrosis after laminectomy (Yong-Hing *et al.*, 1980; Jacobs *et al.*, 1980). To avoid haematoma infiltration, anti-adhesive materials are proposed to act as an occupying spacer preventing blood from entering

the epidural space after laminectomy (Jacobs *et al.*, 1980). A variety of biological, pharmacological and synthetic materials have been tested *in vitro* or used *in vivo* as space-occupying agents. Free or pedicle fat graft (Barberá *et al.*, 1978), Silastic (Alkalay *et al.*, 2003), methylene blue (Farrokhi *et al.*, 2011), ADCON-L (Einhaus *et al.*, 1997), Vicryl mesh (Nussbaum *et al.*, 1990), methacrylate (Lawson *et al.*, 1991), carboxymethyl cellulose (Rodgers *et al.*, 2003), polyethylene oxide (Rodgers *et al.*, 2003), haemostatic agents (Doğulu *et al.*, 2009; Songer *et al.*, 1995), mitomycin C (Lee *et al.*, 2004; Lee *et al.*, 2006), pimecrolimus (Cemil *et al.*, 2009), absorbable cement (Mehdi *et al.*, 2014), topical high-molecular-weight hyaluronan (Kato *et al.*, 2005; Massie *et al.*, 2005; Songer *et al.*, 1990; Chen *et al.*, 2014; Semra *et al.*, 2015) and anti-inflammatory medications (Ross *et al.*, 1996; Cekinmez *et al.*, 2010) have been used with inconsistent results. Therefore, development of a new anti-adhesive material to prevent epidural fibrosis shall benefit spine laminectomy surgery.

Hyaluronic acid (HA), a heteropolysaccharide formed by binding repetitive disaccharide units of D-glucuronic acid and N-acetyl-glucosamine through b-4 bindings, is highly biocompatible and does not cause a foreign-body reaction (Semra *et al.*, 2015). In addition, exogenous HA inhibits foetal fibroblast proliferation and has effective roles in reducing fibrosis and scar formation during the early stages of tissue healing (He *et al.*, 1995). However, HA is water soluble at room temperature and it can only stay in place for a short time. For this reason, it is not suitable in most clinical situations. Compared with HA solution, cross-linked high-molecular-weight HA is effective in retarding hydrolytic degradation and resides in the tissue for up to 7 d (Cencetti *et al.*, 2011). In a few animal studies, it reduces epidural fibrosis formation (Chen *et al.*, 2014; Semra *et al.*, 2015). During the proliferative phase of the wound healing process, recruited fibroblasts and newly-produced extracellular matrix (including collagen and proteoglycan) accumulate at the lesion site and form the granulation tissues before the 3rd week after wound formation. This process, at its highest activity period of four weeks, is critical for the formation of scar tissue that adheres to the apposed tissues (Baum and Arpey, 2005; Lin *et al.*, 2015). Currently available HA derivatives are still far from ideal agents for epidural fibrosis inhibition.

Many researchers and companies have focused on the development of injectable hydrogels for different kinds of tissues (Barbucci *et al.*, 2002; Sakai *et al.*, 2015; Varma *et al.*, 2014; Su *et al.*, 2010). Injectable hydrogels can be maintained in the liquid state before injection and harden *in situ* after transplantation *in vivo*. The sol-gel transformation property allows for irregular surgical defects to be completely filled, reducing the risk of material migration. Testing is known of different hydrogels that show effective anti-adhesive behaviour on other tissues, for the prevention of epidural fibrosis. Examples of this are zwitterionic

hydrogels on subcutaneous implantation (Zhang *et al.*, 2013) and chitosan dextran gel on abdominal peritoneum (Rajiv *et al.*, 2017). In addition, efforts are made to develop hydrogels with more *in situ* durability, either by using new materials, such as silk-polyethylene glycol hydrogel (Wang *et al.*, 2015) and poloxamer-based gel (Shin *et al.*, 2016), or by modulating the crosslinking process (Lin *et al.*, 2015). Due to their tuneable physical properties, hydrogels have become versatile tools for drug delivery and combinations of pharmacologic agents and hydrogels are used in epidural adhesion prevention (Lin *et al.*, 2016). In a previous study, we show development of an injectable oxidised hyaluronic acid/adipic acid dihydrazide (oxi-HA/ADH) hydrogel (Su *et al.*, 2010). Since the gelation time of the oxi-HA/ADH hydrogel at 37 °C is between 143 and 175 s, the change from liquid state into a gel-like matrix occurs within 3 min after injection into the human body. The hydrogel can maintain the gel-like state for at least 5 weeks, with a percentage degradation *in vitro* of 40 % under static incubation in phosphate buffered saline (PBS) at 37 °C and 5 % carbon dioxide (Su *et al.*, 2010). These characteristics make the oxi-HA/ADH hydrogel a good candidate for limiting fibroblast ingrowth at the laminectomy site, from surrounding paraspinal muscles, during the critical period of scar tissue adhesion. The purpose of this study was to test the biocompatibility of this hydrogel for use in spine surgery and to examine the ability of the oxi-HA/ADH hydrogel to reduce epidural fibrosis, using the rat laminectomy model. Fig. 1 shows the scheme of the experimental setup.

Materials and Methods

Materials and reagents
All materials and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Hyaluronic acid (MW = 180 kDa) was purchased from Q. P. Corporation (Tokyo, Japan).

Preparation of oxidised hyaluronic acid
1 % (w/v) HA was dissolved in double-distilled water at room temperature and then 2.67 % sodium periodate ($NaIO_4$) was gently added under stirring. The molar ratio of $NaIO_4$ to HA was 1 : 1. The oxidation reaction proceeded in a dark environment for 2 h at room temperature. The reaction was stopped by the addition of 0.5 mL of ethylene glycol. In order to obtain a purified oxidised HA, a Cellu-Sep T-series® dialysis membrane (8030-32; Orange Scientific, Braine-l'Alleud, Belgium) was used to separate the by-product and the oxidised HA. Double-distilled water was used as a dialysis buffer solution and the water was changed three times per day. 1 % silver nitrate ($AgNO_3$) was used to check the amount of periodate in the outer dialysis buffer, with water changes required until there was no more precipitate

visible. The final oxidised HA product was obtained by freeze-drying (FDU-1200; EYELA, Tokyo, Japan). The average yield of oxidised HA was about 87 %.

Preparation of hyaluronic acid-ADH hydrogel

For topical usage at laminectomy sites, PBS solution (pH 7.4) was selected as an optimal solvent source. 6 % (w/v) oxidised HA was dissolved in PBS overnight at 4 °C and gently mixed with 8 % (w/v) adipic acid dihydrazide (ADH) to form the oxi-HA/ADH hydrogel.

Cell cultivation

Fibroblasts (NIH/3T3; 60008), myoblasts (C2C12; 60083), Schwann cells (RSC96; 60507) and neural cells (PC-12; 60048) were chosen for the cytocompatibility studies of the oxi-HA/ADH hydrogel. RSC96 and PC-12 cells were used to evaluate possible deleterious effects of the hydrogel on spinal cord, dura sac and nerve root; C2C12 cells to test whether wound muscle healing would be affected; NIH/3T3 cells to measure the epidural fibrosis reduction. All cell lines were purchased from the Bioresource Collection and Research Centre (BCRC, Hsinchu, Taiwan). NIH/3T3, C2C12 and RSC96 were cultured in 90 % high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 4 mM L-glutamine, 10 % foetal bovine serum (FBS, 26140-079; Gibco, Life Technologies, Carlsbad, CA, USA) and 1 % antibiotic (15240-062; Gibco), at 37 °C in a humid atmosphere containing 5 % CO_2. PC-12 were cultured in 85 % Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 2 mM L-glutamine, 10 % heat-inactivated horse serum, 5 % FBS and 1 % antibiotic.

Cytocompatibility of the oxi-HA/ADH hydrogel

Cytocompatibility of the oxi-HA/ADH hydrogel was evaluated by testing the extraction medium with four different cell lines, according to ISO standards (International Standardisation Organisation, 1992). The extraction medium was prepared by incubating the oxi-HA/ADH hydrogel for 72 h at 37 °C with standard culture medium (as described above) at a 0.75 cm² mL⁻¹ extraction ratio. 5×10^3 cells (PC-12 and NIH/3T3) and 2.5×10^3 cells (C2C12 and RSC96) were seeded into a 96-well polystyrene cell culture plate (Advangene Consumables, Lake Bluff, IL, USA) and cultured overnight in standard medium. Then, the culture medium was changed to extraction medium and the cells where cultured for additional 72 h. In order to minimise the effect of the serum, FBS was added into both standard and extraction medium prior to cell seeding. In addition, a serum-free extraction medium group was added. Groups in the study included negative control (standard culture medium), positive control (medium containing 0.1 % Triton X-100), extraction medium, diluted extraction medium (1 : 2 dilution) and serum-free extraction medium. Furthermore, fluorescence-based live/dead staining (Molecular Probes, Waltham, OR, USA) was performed on cells cultured in different media/extract to identify cell survival under different conditions.

After been cultured in extraction or standard medium for 72 h, cell viability was evaluated. First, test media were collected and preserved for testing the cytotoxicity. Then, cells were washed twice with PBS and subsequently 0.1 mL water-soluble tetrazolium-8 (WST-8; Enzo Life Sciences, Farmingdale, NY, USA) working solution was added into each well. After 2 h incubation, the WST-
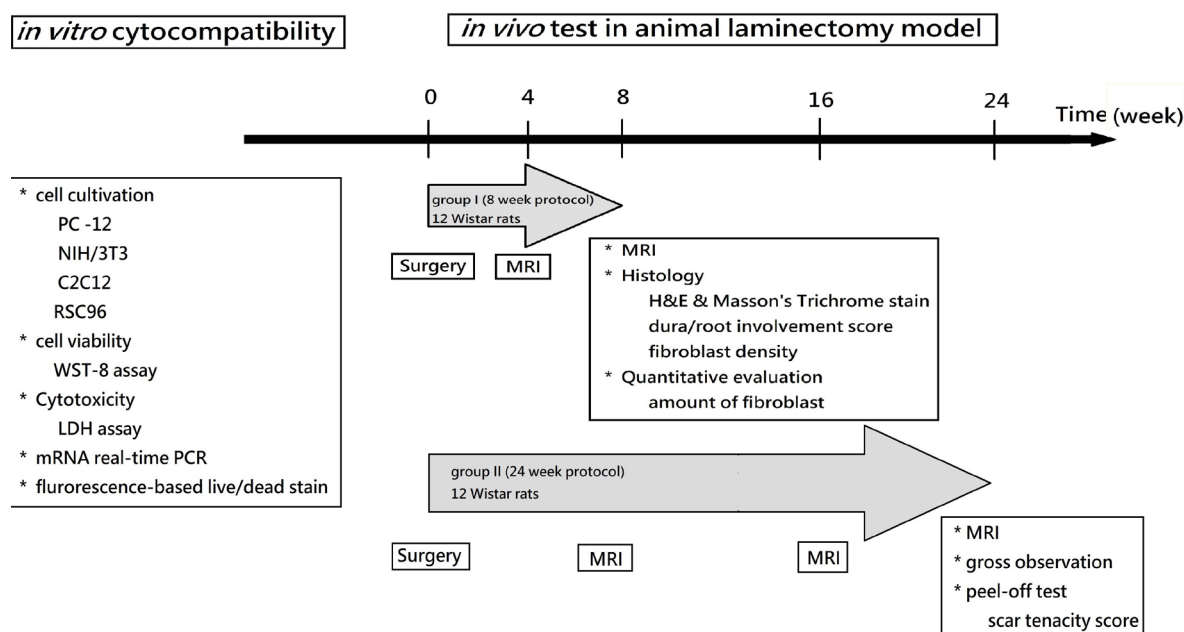

Fig. 1. Scheme of the experimental setup: *in vitro* cytocompatibility test of the hydrogel was performed first, followed by examination of the effect of the oxi-HA/ADH hydrogel on the reduction of the epidural fibrosis using a rat laminectomy model.

8 working solution should show a colour change due to cleavage of the tetrazolium salt by cellular mitochondrial dehydrogenase and formation of formazan. Cell viability was quantitatively assessed by spectrophotometer readout at 450 nm, with a reference wavelength at 650 nm.

For cytotoxicity evaluation, 50 µL of the incubation medium were transferred into a 96-well ELISA plate, mixed with 50 µL of substrate mix and incubated for 30 min in the dark. The tetrazolium salt in the substrate mix could react with the lactate dehydrogenase (LDH) to give a red formazan product (CytoTox 96® Non-Radioactive Cytotoxicity Assay, G1780; Promega, Madison, WI, USA). LDH released was quantitatively assessed by spectrophotometer readout at 490 nm. The extraction media were prepared from three batches of hydrogels and used separately. The extractions were diluted with serum-free medium (volume ratio 1 : 1). The toxicity of undiluted and diluted extraction media were tested in four cell types (n = 4 for each cell line).

Quantitative real-time polymerase chain reaction
Cells ($2 × 10^5$ cells for C2C12 and RSC96 and $4 × 10^5$ cells for NIH/3T3 and PC12) were suspended in 200 µL of the oxi-HA/ADH hydrogel and placed in an incubator for 10 min for gelation. Then, the cell/gel constructs were transferred into a 24-well culture plate and 1.5 mL culture medium was added. For comparison, the same quantity of cells was cultured in monolayer in a 24-well culture plate. After one week of culture, two samples of each culture type were pooled together for analysis of gene expression. Quantitative real-time polymerase chain reaction (qPCR) analysis was performed on pooled samples of both monolayer cultured cells and cells encapsulated within the oxi-HA/ADH hydrogel. Total RNA was extracted using the PureLink™ Mini RNA kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). During extraction, RNA was treated with RNase-free DNase (Qiagen, Chatsworth, CA, USA) to eliminate any residual DNA. An aliquot of RNA was used to quantify RNA yield by recording the absorbance at 260 nm with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The reverse transcription (RT) reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 10 µL of RNA were added to 10 µL 2×RT master mix, containing RT buffer, deoxynucleotides (dNTPs) mix, RT random primers and MultiScribe reverse transcriptase. The RT reaction mixture was incubated at 25 °C for 10 min, then at 37 °C for 120 min and finally at 85 °C for 5 min to inactivate the enzyme. Complementary DNA, synthesised from 20 ng of RNA, was subjected to PCR amplification using the TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) (n = 3 for each cell line). The following

expression profiles were examined: *S100b* (a small EF-hand calcium and zinc binding protein, highly expressed in the adult vertebrate nervous system) in PC12 and RSC96 cells; *Pax7*, *Myf6* and *Myod1* (transcription factors playing a role in myogenesis and muscle differentiation) in C2C12; *S100a4* (a member of the S100 family) and *P4hb* (encoding the beta subunit of the prolyl 4-hydroxylase and identified in fibroblast-cell-derived exosomes) in NIH3T3. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control for gene expression. mRNA expression for each of the target genes was normalised to the housekeeping gene *GAPDH*. The ΔCT was calculated by subtracting the cycle threshold (CT) of *GAPDH* from the CT of each target gene. The ΔCT for each treated group was further normalised to the monolayer group to obtain the ΔΔCT. Relative expression was calculated using the $2^{-ΔΔCT}$ method.

Surgical procedures
24 female Wistar rats (3 months old and weighing 350-400 g; purchased from BioLASCO Taiwan Co., Ltd; fed at the Laboratory Animal Centre, National Taiwan University College of Medicine, Taipei, Taiwan) were used in this study. Animals were maintained in accordance with the guidelines for the care and use of laboratory animals. Experimental protocols and surgical procedures were approved by the National Taiwan University Hospital College of Medicine Institutional Animal Care and Use Committee (IACUC). Rats were fed with Purina laboratory chow (PMI Nutrition International Certified LabDiet, St. Louis, MO, USA), had access to tap water *ad libitum* and were housed in a temperature-, humidity- and light-controlled environment. Animals were marked to aid the individual identification and acclimatised in their cages for 28 d before the start of the study.

Under anaesthesia by intra-abdominal injection of 30 mg/kg sodium pentobarbital, rats were fixed in a prone position, with skin being disinfected and draped. In each rat, two midline skin incision, approximately 3 cm in length, were made at the thoracic and lumbar area, separately. The spinous processes were exposed and the paraspinous muscles were dissected bilaterally. Total laminectomy with a longitudinal length of 10 mm and transverse width of 5 mm at the L4-L5 level and mid-thoracic level were performed by the same spine surgeon using an operative microscope.

The two laminectomy areas of each rat were assigned one to treatment group and the other to control group, respectively. Treatment and control group between the two spine levels were randomly assigned, considering the anatomical differences between thoracic and lumbar spine. In the treatment group, the dura mater was exposed after the laminectomy and the topical hydrogel (0.1 mL) was applied at the laminectomy site to cover the entire exposed dura theca and root. In contrast, in the control group, laminectomy was followed by

no additional procedure (Fig. 2). The wounds were closed layer by layer after attaining haemostatic control.

The 24 Wistar rats were divided into two groups subjected to different protocols.

Preparation of specimens

4 and 8 weeks after surgery of group I animals (8 weeks protocol), a 7T-magnetic resonance imaging (MRI) (Bruker BioSpec 70/30 MRI; Core Lab of the Neurobiology and Cognitive Science Centre, National Taiwan University, Taipei, Taiwan) was used for morphological evaluation of the fibrotic tissue overlying the exposed dura sac.

8 weeks after surgery, group I animals were euthanised by intraperitoneal administration of a high dose (75-100 mg/kg) of sodium pentobarbital. The whole thoraco-lumbar spinal column was removed. The specimens were fixed for 2 d in 10 % buffered formalin solution. Next, they were immersed for 3 d in a decalcification solution (0.5 M aluminium chloride, 8.5 % HCl and 5 % formic acid). Subsequently, 2 mm-thick specimens were cut at the laminectomy site, transverse to the spinal canal. These tissues were processed according to routine tissue-processing techniques. Serial sections of 4 μm were cut from paraffin-embedded blocks using a microtome (Leica, Wetzlar, Germany) and stained with haematoxylin and eosin (H&E) and Masson's trichrome kits (Polysciences, Warrington, PA, USA).

Histopathological examination

All tissue samples were evaluated by a blinded observer. Using a light microscope, each section was evaluated and scored for the extent of fibrosis, dura adhesion and density of fibroblasts. The dura adhesion parameters were evaluated according to the criteria proposed by He and colleagues, already proved to have good inter-rater reliability (He *et al.*, 1995; Sae-Jung *et al.*, 2015). Fibroblast cell density was calculated in each field at 40× magnification, following the classification system proposed by

Hinton *et al.* (2015), according to which the definition of grade 1 was fewer than 100 fibroblasts in each field, grade 2 100-150 fibroblasts in each field and grade 3 more than 150 fibroblasts in each field. The number of fibroblasts was counted and the average recorded for all 12 rats (2 laminectomy level in each rat, with one being treatment group and the other control group), repeating the procedure for three fields per sample (one from the middle of the laminectomy area and two from the margins, one on each side). The cell counting resulting for each group was expressed in cell number per mm^2 (Tao and Fan, 2009).

Superficial observation and peel-off testing

8, 16 and 24 weeks after surgery of group II animals (24 weeks protocol), 7T-MRI images were acquired. 24 weeks after surgery, the rats were sacrificed, as described above. Using forceps, the scar tissue was peeled off manually. The tenacity of adhesion between the scar tissue and dura mater was evaluated and the difficulty of separating the scar from the dura mater was also taken into consideration. A six-level scoring system (grades 0-5) was used to indicate the separation difficulty. Grade 0 indicated no adhesion between the dura and scar tissue; grade 1, very slight adhesion to the dura mater and tissue easily detachable without applying manual force; grade 2, some adhesion to the dura and the tissue easily detachable by moderate traction; grade 3, less than 50 % of the operated area had adhesion and could be detached by strong traction; grade 4, more than 50 % of the operated area had adhesion and could be bluntly detached by strong traction; grade 5, severe adhesion not detachable without disruption of the dura mater, unless sharp dissection was used (Einhaus *et al.*, 1997). The result of the scoring was defined as the dura/root involvement score.

MRI

Spinal coil T2-weighted MRI images were acquired in transverse plane (TR = 3000 ms, TE = 26.7 ms, slice thickness = 1 mm) with a 7.0T sigma MRI instruments
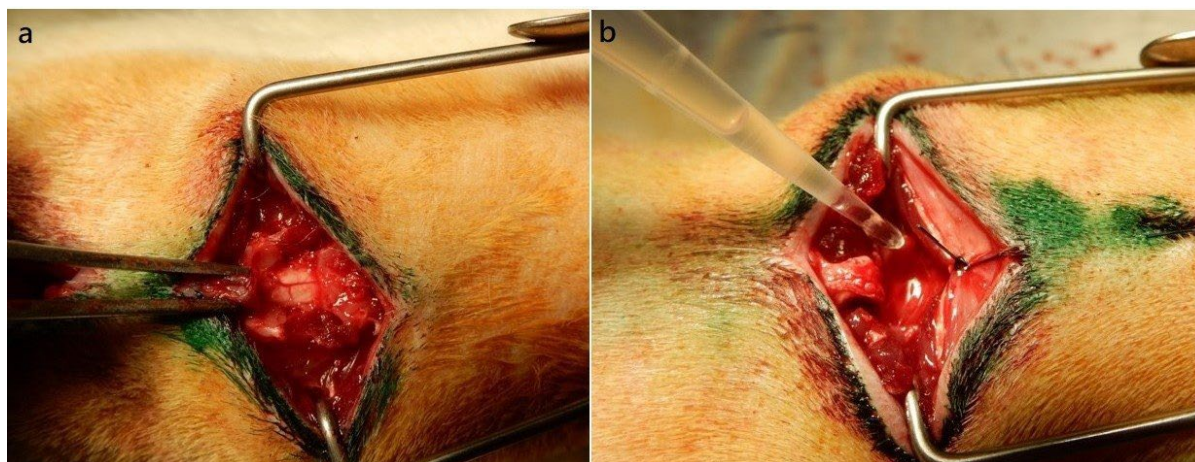

Fig. 2. Images of surgery. (**a**) In control group, only laminectomy was performed without any additional procedures; (**b**) in treatment group, after laminectomy, the topical hydrogel was applied at the laminectomy site to cover the entire exposed dural theca and root.

(Bruker BioSpec 70/30 MRI) using a standard phase array. The MR images were used for measuring the area of scar adhesion.

Statistical analysis
All data were represented as means ± standard error of the mean (SEM). Wilcoxon matched-pairs signed ranks test was used for comparison of control *vs.* treatment group. Statistical significance was considered at $p < 0.05$.

<div align="center">

Results

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Cytocompatibility of the oxi-HA/ADH hydrogel
3 d after cultivation of the 4 different cell lines with the test media, cell viability and cytotoxicity were evaluated by WST-8 and LDH assays, respectively. As shown in Fig. 3**a**, the WST-8 optical density (OD) at 450 nm for PC-12 cells cultivated in extraction medium, diluted extraction medium and standard medium was 0.148 ± 0.017, 0.196 ± 0.027 and 0.196 ± 0.052, respectively. For NIH/3T3 cells, extraction medium, diluted extraction medium and control medium ODs were 0.477 ± 0.011, 0.547 ± 0.007 and 0.569 ± 0.01, respectively. When compared to cells cultured in standard medium, extraction medium caused a relatively lower viability ($p < 0.05$) in PC-12 and NIH/3T3 cells. Although NIH/3T3 cells in diluted extraction medium had a lower viability ($p < 0.05$) compared to standard medium, there was no significant difference between these two groups and PC-12 cells. For C2C12 cells, extraction medium, diluted extraction medium and standard medium ODs were 0.254 ± 0.083, 1.203 ± 0.059 and 1.487 ± 0.101, respectively and for RSC96, they were 1.028 ± 0.067, 1.669 ± 0.124 and 2.166 ± 0.148, respectively. In the four different cell lines, extraction-medium-cultivated cell had lower absorbance values

compared to cell cultivated in standard medium and diluted extraction medium. Moreover, although the cell activity in extraction medium was lower than that in standard medium, the cell viability was still higher compared to cell cultivated in positive control medium and serum-free extraction medium.

Additionally, the LDH OD at 490 nm showed no significant differences among cells cultivated in extraction medium, diluted extraction medium and standard medium. A significantly higher LDH OD at 490 nm ($p < 0.05$) was observed in positive control compared to the other 3 groups (extraction medium, diluted extraction medium and standard medium) for PC12, NIH/3T3 and RSC96 (Fig. 3**b**).

In a fluorescence-based live/dead staining, similar cell numbers and survival rates were found for PC12, NIH/3T3 and RSC96 cells cultivated in extraction medium, diluted extraction medium and standard medium (Fig. 4). However, for C2C12 cells, lower cell density and more dead cells were found in the extraction medium (Fig. 4**j**) compared to diluted extraction medium (Fig. 4**k**) and standard medium (Fig. 4**l**).

mRNA gene expression
To evaluate the specific gene expression of the four different cell lines cultured as monolayer or encapsulated within the oxi-HA/ADH hydrogel, a qPCR analysis was performed following 7 d of cultivation. *S100b* mRNA level (0.967 ± 0.62) in PC-12 cells cultivated in the oxi-HA/ADH hydrogel was equal to that of the monolayer culture. However, *S100b* expression (3.865 ± 0.23, $p < 0.05$) was significantly up-regulated in RSC96 cells cultivated in the oxi-HA/ADH hydrogel, compared to the monolayer culture (Fig 5**a**). *S100a4* (0.079 ± 0.005, $p < 0.05$) and *P4hb* (0.087 ± 0.006, $p < 0.05$) gene expressions were significantly down-regulated when NIH/3T3 cells were cultured in the oxi-HA/
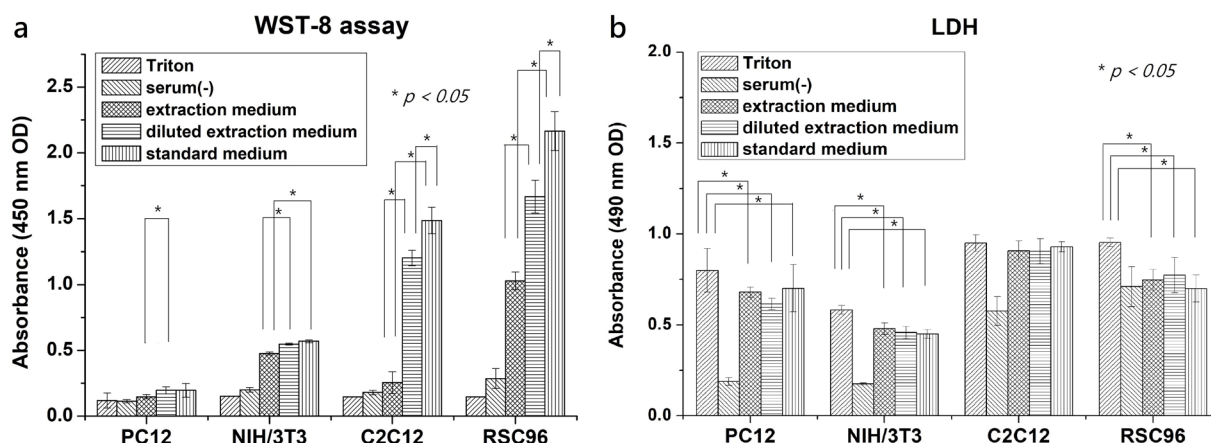

Fig. 3. Cytocompatibility of the oxi-HA/ADH hydrogel. Diluted extraction medium (1 : 2) and serum-free extraction medium were tested, whereas 0.1 % Triton-X-100-containing medium was used as a positive control. (**a**) When compared with the cells cultured in the standard medium, the extraction medium caused a relative lower viability ($p < 0.05$) in PC-12 and NIH/3T3 cells. In the 4 different cell lines, extraction medium had lower absorbance levels when compared to standard medium and diluted extraction medium. (**b**) LDH OD at 490 nm showed no significant difference between extraction medium, diluted extraction medium and standard medium.

ADH hydrogel. Similarly, down-regulations of *Pax7* (0.159 ± 0.003, $p < 0.05$), *Myf6* (0.166 ± 0.037, $p < 0.05$) and *Myod1* (0.088 ± 0.084, $p < 0.05$) mRNA levels were observed when C2C12 cells were encapsulated within the oxi-HA/ADH hydrogel (Fig 5**b**).

Surgical outcome

All rats included in this study could walk after surgery, without any obvious neurological problems. Skin, fascia and muscle around the surgical sites healed well in both group I and II and no infection, local swelling or discharge from the wound was noted at sacrifice.

Histological analysis

Significant differences ($p < 0.05$) were observed between group I and II regarding epidural fibrosis, dura adhesion and fibroblast cell density (Fig. 6**a**). The mean of the dura/root involvement score for the extent of scar adhesion was significantly lower in hydrogel group (1.17. ± 1.17), compared to control group (2.33 ± 0.52) ($p < 0.05$) (Fig. 6**a**). Furthermore, variable grades of epidural fibrosis were observed in the rats of both groups. However, microscopical examination showed that the tissue treated with hydrogel presented more discontinuous areas of

hypointense signals between the dura mater and the surrounding scar tissue and a smaller number of inflammatory cells in the scar tissue at both thoracic and lumbar laminectomy site (Fig. 7). The thickness of the epidural scar tissue was relatively wider in control group, which was also shown in the MRI sequence, whereas the MRI showed a relatively loose epidural tissue in hydrogel group, 24 weeks after the surgery (Fig. 8). In addition, in both groups, a time-dependent correlation of scar tissue thickness was found, with the scar being thicker at 24 weeks compared to 8 weeks after surgery.

The density of fibroblast cell score was significantly less in the hydrogel group with 1.33 ± 0.52, whereas the mean was 2.33 ± 0.52 in the control group ($p = 0.004$) (Fig. 6**a**).

Superficial observation and peel-off test

Superficially, no residual hydrogel could be seen at sacrifice in all rats of the treatment group. Three rats were scored as grade 1, with no adhesion between the dura and scar tissue, which were easily separable (Fig. 6**b**). The exposed dura after revision surgery were surprisingly smooth. Oppositely, in the control group, all rats scored 3 or more, with disruption of the dura mater in one animal.
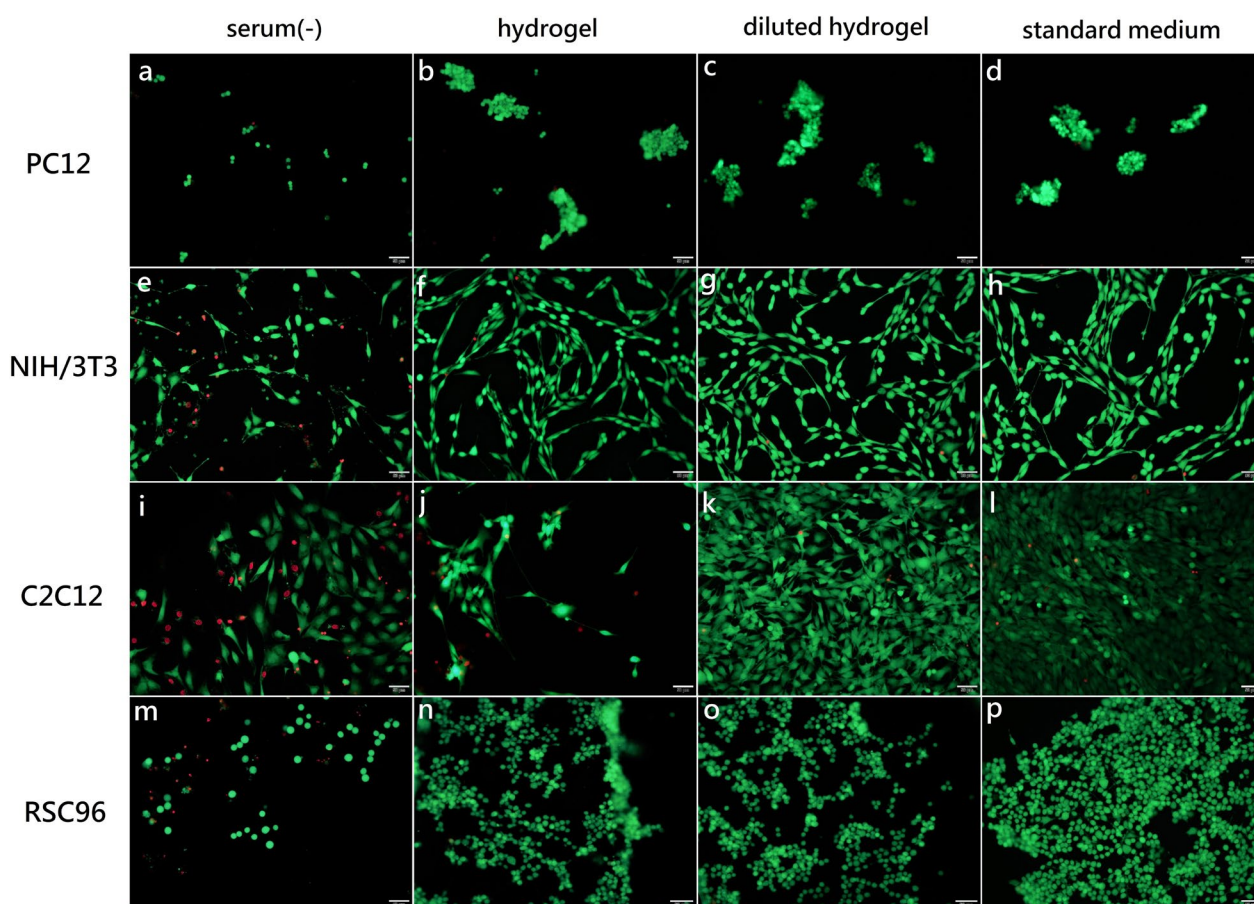

Fig. 4. Fluorescence-based live/dead staining for (**a-d**) PC12, (**e-h**) NIH/3T3, (**i-l**) C2C12 and (**m-p**) RSC96. Similar cell numbers and survival rates were observed for PC12, NIH/3T3 and RSC96 cells cultivated in extraction medium, diluted extraction medium and standard medium. For C2C12 cells, a relative smaller cell number and lower survival rate were observed when cells were cultured in (**j**) extraction medium compared to (**k**) diluted extraction medium and (**l**) standard medium. Scale bar = 50 μm.

In manual peel-off testing, the mean grade were 4 ± 0.63 for the control group and 1.67 ± 0.82 for the treatment group (Fig. 7). The tenacity of the adhesion between the scar tissue and dura was significantly different in the two groups ($p < 0.0001$).

Quantitative histology evaluation: number of fibroblasts

8 weeks postoperatively, the number of fibroblasts was significantly larger in the control group compared to treatment group. The average fibroblast number in the control group was $1,916 \pm 546$ per mm^2 and the average fibroblast number in the treatment group was $1,106 \pm 176$ per mm^2 ($p = 0.026$) (Fig. 9).

Discussion

Post-operative epidural fibrosis is a normal biological response after laminectomy, which will result in post-laminectomy dura adhesion. The origins of postoperative fibrosis are multifactorial and the effects of the unavoidable fibrotic tissue formation

on postoperative pain are not fully understood (He *et al.*, 1995). In fact, fibrosis and adhesion are the results of an inflammatory reaction, an inevitable process during tissue healing, and are caused by the organisation of the fibrin matrix (Chen *et al.*, 2014). Several mechanisms have been proposed to explain the presence of post-laminectomy dura adhesion. LaRocca and colleagues have concluded that fibrosis originates from the posterior invasion of fibroblasts, extending from the *erector spinae* muscle to the dura and then growing into the haematoma (LaRocca and MacNab, 1974). Accordingly, Holtz suggests an approach that could possibly decrease the fibrosis formation, including separation of fibrin by a physical barrier and inhibition of fibroblast proliferation (Holtz, 1980). Based on this theory, a variety of biological, pharmacological and synthetic materials have been tested since LaRocca and MacNab first designated the laminectomy membrane in 1974 (LaRocca and MacNab, 1974).

HA, a highly biocompatible heteropolysaccharide, is widely used in the biomedical field. HA reduces foetal fibroblast proliferation and effectively inhibits fibrosis and scar formation during the early stages
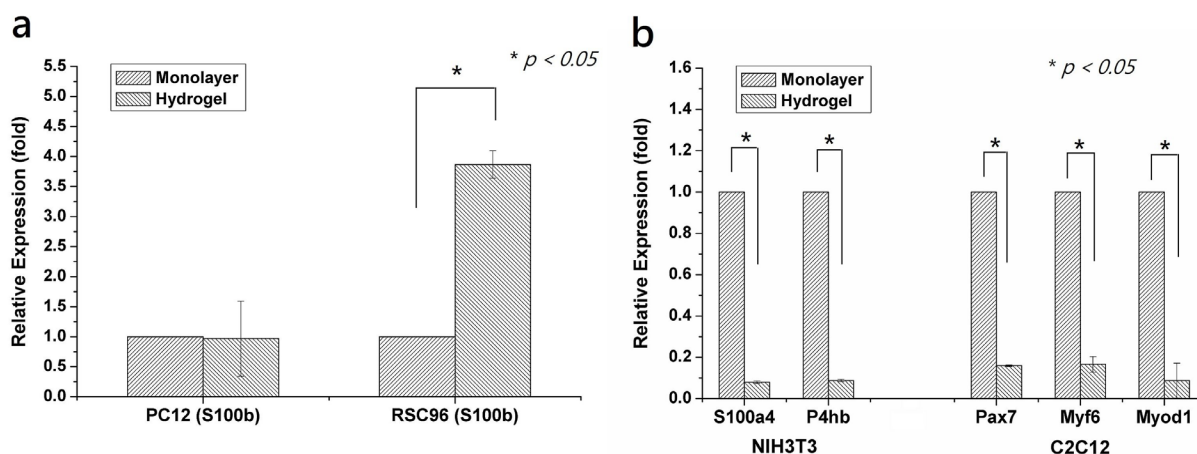

Fig. 5. (**a**,**b**) Gene expression in fibroblast cell (NIH/3T3), myoblast cell (C2C12), Schwann cell (RSC96) and neural cell (PC 12) cultivated as monolayer and encapsulated within the oxi-HA/ADH hydrogel, including *S100b* for PC12 and RSC96, *S100a4* and *P4hb* for NIH/3T3 and *Pax7*, *Myf6* and *Myod1* for C2C12.
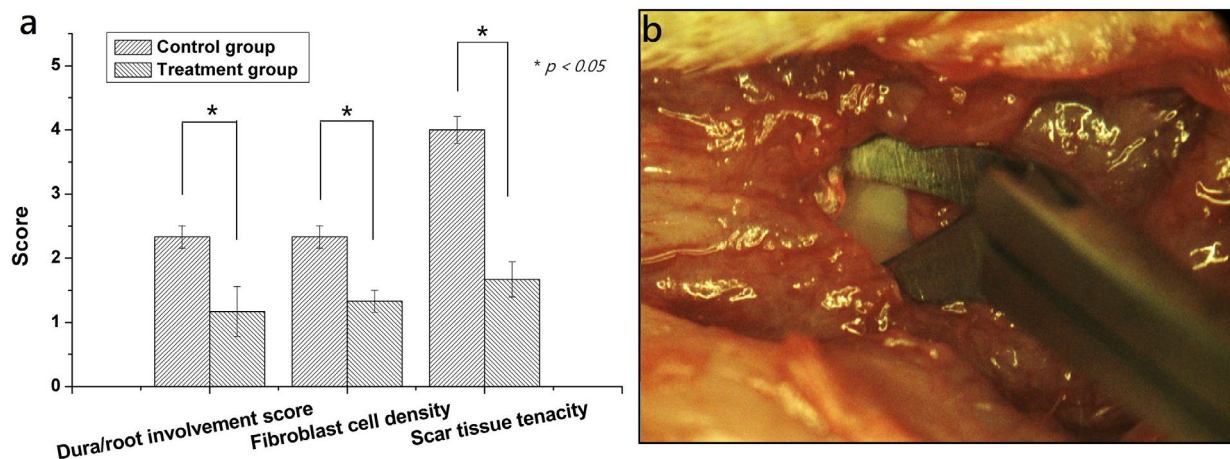

Fig. 6. (**a**) Epidural fibrosis, dura adhesion, fibroblast cell density and peel-off test were significantly different between control and treatment groups ($n = 12$). (**b**) Superficially, no residual hydrogel at sacrifice was observed in all rats of the treatment group. Dura and scar tissue were easily separated at previous laminectomy site, which was covered with the oxi-HA/ADH hydrogel.

of tissue healing (He *et al.*, 1995; Semra *et al.*, 2015). Recently, it has been used as an anti-adhesive material in other surgical fields, such as different kinds of intra-abdominal surgeries (Yeo *et al.*, 2007; Huberlant *et al.*, 2015), ophthalmology surgeries (Yaacobi *et al.*, 1992) and post-surgery tendon adhesions (Liu *et al.*, 2008; Kaux *et al.*, 2016). Recently, HA, including HA solutions and cross-linked high-molecular-weight HA gels, have drawn researchers' attention for the prevention of post-laminectomy epidural fibrosis (Kato *et al.*, 2005; Chen *et al.*, 2014; Semra *et al.*, 2015; Shih *et al.*, 2004). Semra *et al.* (2015) show that cross-linked high-molecular-weight HA (HA gel) has positive effects on the prevention of epidural fibrosis and reduction of fibrotic tissue density. However, no statistically significant differences are detected between HA and HA gel in either the histopathological evaluation or the biochemical analysis (Semra *et al.*, 2015). Compared to HA, HA gel effectively retards hydrolytic degradation and remains longer in the tissues. However, it is still resorbed through HA's pathways within 7 d, which could be too short to overcome active healing process (Cencetti *et al.*, 2011). Likewise, Chen *et al.* (2014) conclude that HA-based gelatine may be effective in preventing post-laminectomy dura adhesion in a rabbit animal study. This HA-based gelatine shows positive effects on the prevention of dura adhesion.

However, to date, there is still no solid conclusion regarding an ideal material to be clinically used for the prevention of epidural adhesion.

Due to the complicated procedure of a spine surgery, two major issues need to be solved. First, the implanted barrier with an anti-adhesion purpose may migrate somewhere else. Kato *et al.* (2005) find that, compared to an HA solution, an HA sheet forms a more solid interpositional barrier and exhibits better anti-inflammatory effects. Moreover, an HA sheet can easily cover the whole exposed nerve structure, and, unlike liquid solutions, it can remain *in situ* instead of migrating. However, the sheet is not an optimal form during surgery. For surgeon and patient, it could be time consuming to cut the surgical sheet to match the shape of an irregular laminectomy site for a full dura coverage during operation. Second, the biodegradation/bioresorption time of the anti-adhesive material in surgical site must be considered. A cross-linked high-molecular-weight HA resides in the tissue for about 7 d (Cencetti *et al.*, 2011). By using different cross-linking agents, Hahn *et al.* (2007) report that the degradation time of a HA hydrogel can be extended up to 29 d. However, the degradation time of these HA-based materials is far from ideal for epidural fibrosis inhibition. In general, the highest activity of fibrous connective tissue ingrowth will last for 4-6 weeks.
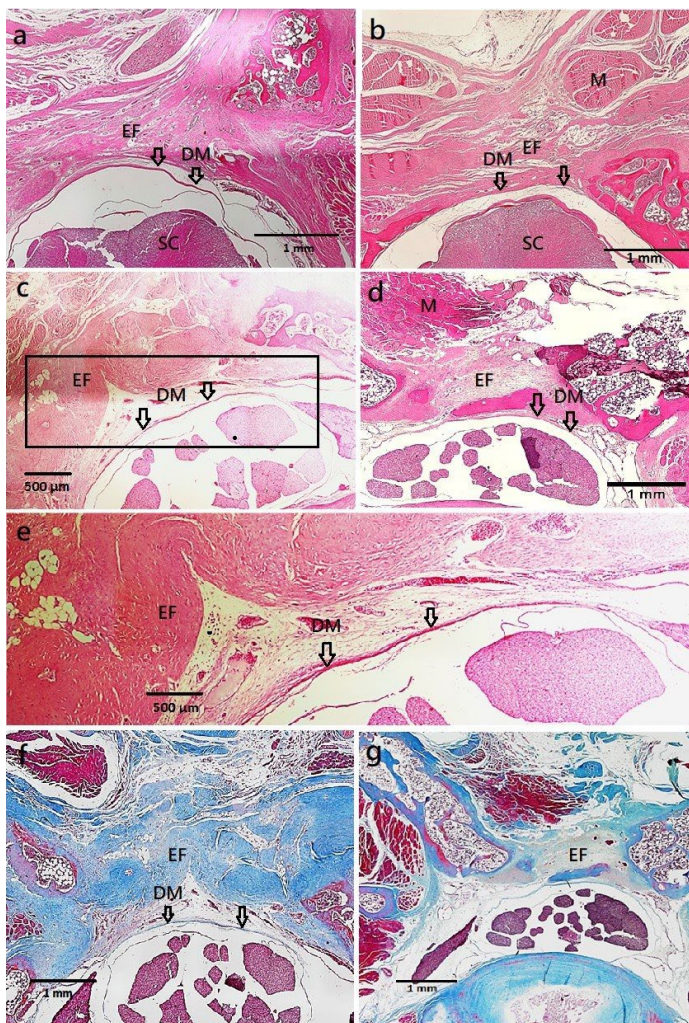

Fig. 7. Histology results 8 weeks post-operatively. (**a**) 100× magnification image of tissue treated with hydrogel and stained with H&E at thoracic spine. Only thin fibrosis bands between the epidural fibrotic scar tissues and the dura mater, compatible with grade 1 adhesions, were observed. (**b**) 100× magnification image of control group tissue stained with H&E at thoracic spine. Scar adhesion was large and involved more than two-thirds of the laminectomy defect, compatible with grade 3 adhesions. In addition, reduction of dura mater with spinal cord compression was found. 100× magnification images of tissue treated with hydrogel and stained with (**c**) H&E and (**f**) Masson's trichrome showed grade 0 adhesions, with the dura mater free from scar tissue. The rectangle in **c** indicated the area represented in **e**, in which fibroblast density was low and a larger distance from the dura to scar tissues was found. Also, normal muscular structure with some post-surgical fibroblast infiltration of retracted muscle was observed. It showed good muscle and soft tissue healing at the surgical site. (**d,g**) Control group at lumbar spine showed dominant fibroblast formation (grade 3), with severe adhesion between dura and epidural fibrosis [EF: epidural fibrosis; DM (arrow): dura matter; SC: spinal cord; M: paraspinal muscle].

An injectable hydrogel could solve these problems. The hydrogels with a sol-gel transformation property could completely fill the desired region, reduce the risk of migration and lessen the infection rate at the wound site. In the present study, an *in situ* cross-linked oxi-HA/ADH hydrogel was used and it could be kept in the liquid state for up to 8 min and transformed into a gel-like matrix within 3 min. This handling property allows surgeons to have sufficient time to prepare and apply this hydrogel during surgery. Since the gelation time is short and the viscosity of the hydrogel is high, the risk of ectopic leakage is supposedly low. From the results of our *in vitro/in vivo* studies, as well as other groups' reports, the hydrogel should be biocompatible. However, it was noticed that the concentration of extraction medium from the hydrogel may affect the mitochondrial activity of C2C12 and RSC96 cells. The PCR results showed that three specific gene markers were decreased in C2C12 cells encapsulated within the oxi-HA/ADH hydrogel, while the viability was also influenced when the cells were cultured in the hydrogel extraction medium. On the other hand, according to the findings of live/dead staining, similar cell number and survival were found when RSC96 where cultured in extraction medium, diluted extraction medium and standard medium. Instead, a smaller cell number was found for C2C12 cultured in hydrogel extraction medium. Therefore, the possible effect of hydrogel on muscle cells should be further addressed. Although *in vivo* toxicity testing might provide solid evidence to identify whether the material does harm normal tissue or not, *in vitro*

tests can also reveal possible toxicity at the cellular level. In this study, there was no clinical sign of toxicity, *i.e.* abnormal animal body weight and food consumption, in the rats treated with the hydrogels. All of the rats showed normal vitality and no rat died during dosing or observational periods. Furthermore, skin, fascia and muscle around the surgical sites healed well. In addition, no infection, local swelling or discharge from the wound was noted at sacrifice. The histology results also demonstrated healthy and well-healed paraspinal muscular structure.

The present study showed that the oxi-HA/ADH hydrogel was an effective and safe anti-adhesion material. It was associated with a reduction of scar tissue, as well as, the degree of adhesion between scar tissue and dura mater. In addition, superficial observation and peel-off test, mimicking the revision surgery that most clinical surgeons are concerned about, showed significantly decreased tenacity at sacrifice, 24 weeks post-operatively. In addition, preventing migration of fibroblasts at the surgical level is a theoretically applicable measure to reduce epidural fibrosis, since fibroblasts are important for the formation of peridural fibrosis originating from the para-spinal muscles. In the present study, we found significantly smaller fibroblast number in the hydrogel group compared to control group. It was hypothesised that our oxi-HA/ADH hydrogel was a biocompatible material, which might induce a reduced inflammatory response.

In clinical practice, an appropriate sterilisation of the materials is another concern. Among the various sterilisation methods, the simplest one is the passage
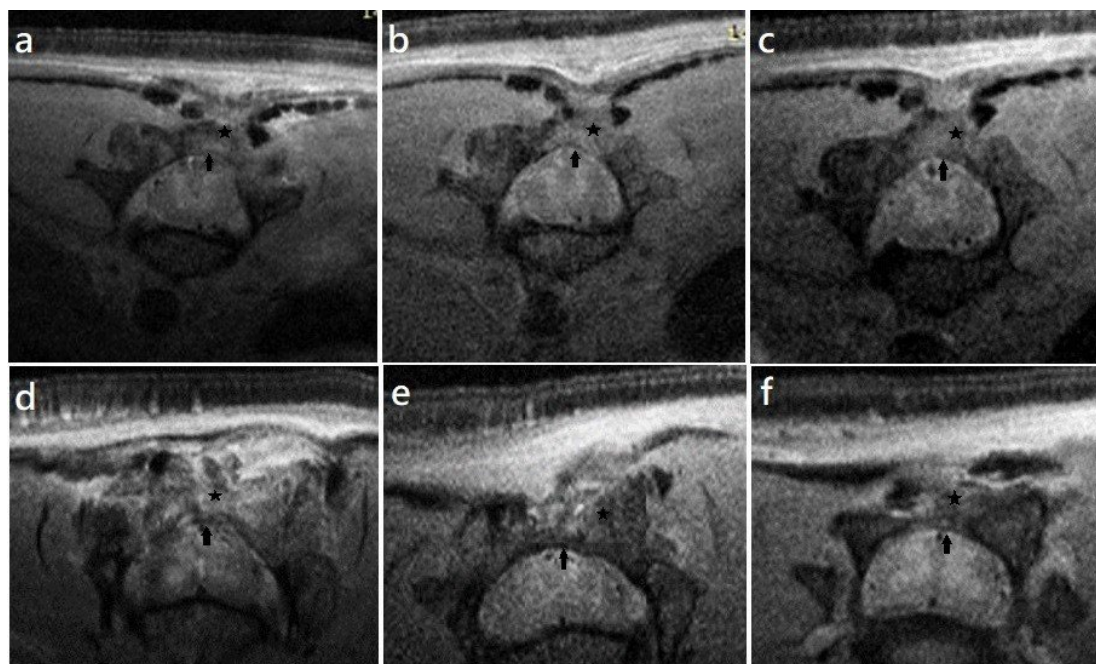

Fig. 8. T2-weighted MRI. (**a**-**c**) Control group: 8, 16 and 24 weeks, respectively. A thick layer of epidural fibrotic tissue with dura connection was found. (**d**-**f**) Hydrogel group: 8, 16, 24 weeks, respectively. The epidural fibrosis was relatively loose and thin and limited dura contact was found. Moreover, in both groups, a time-dependent correlation of scar tissue thickness was found, with the scar being thicker 24 weeks compared to 8 weeks after surgery (star: epidural fibrosis; black arrow: dura).
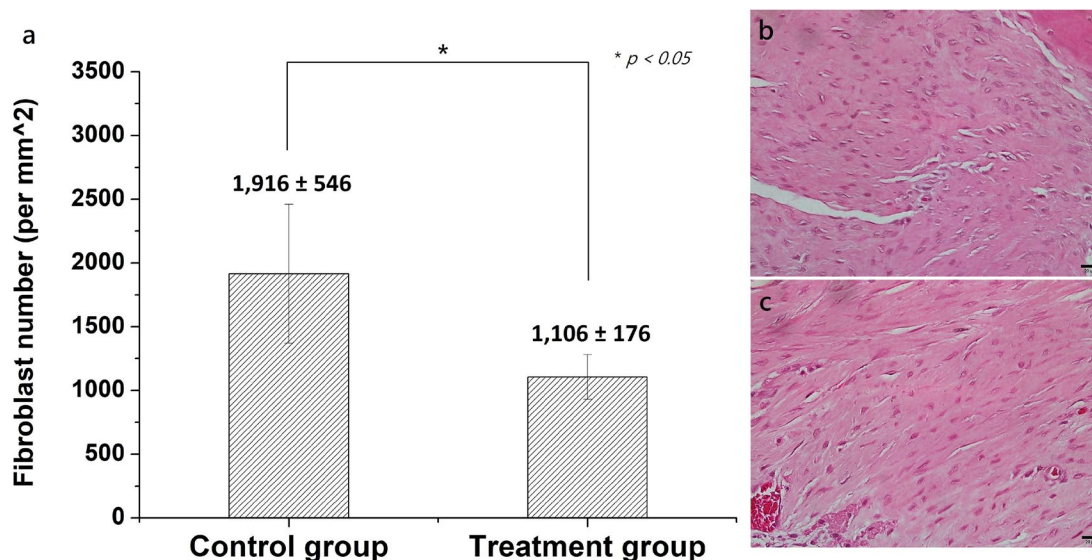
Fig. 9. (**a**) Fibroblasts number per mm^2 of scar tissue , 8 weeks postoperatively (n = 12, mean ± SD). (**b**) The control group showed significant larger number of fibroblasts in epidural scar tissue in comparison with (**c**) treatment group. The average fibroblast number of control group was 1,916 ± 546 per mm^2 and the average fibroblast number of treatment group was 1,106 ± 176 per mm^2 (p value indicated control group *versus* treatment group; * $p < 0.05$). Scale bar in b,c = 20 μm.

of the solution through a 0.22 μm filter. It is not easy for native HA to pass through a 0.22 μm filter due to its high viscosity, but the viscosity significantly decreases after the oxidation process. For our oxi-HA/ADH hydrogel, we were able to sterilise the oxi-HA and ADH solutions separately by passaging through a 0.22 μm filter (Barbucci *et al.*, 2002). Another advantage of the oxi-HA/ADH hydrogel was that we could mix therapeutic agents with the liquid state HA/ADH solution and inject it at the surgical site. Since reduction of the initial inflammatory reaction and exudation could be a possible way for reducing fibrosis (Kato *et al.*, 2005), developing a drug-eluting hydrogel mixed with some anti-inflammatory or fibrolytic agents may be a potential future work.

Conclusion

In order to prevent post-operative epidural fibrosis after laminectomy, the use of the injectable oxi-HA/ADH hydrogel as an anti-fibrotic adhesion barrier was suggested. Based on the experimental findings, the hydrogel had reasonable biocompatibility for neural cells, Schwann cells, fibroblasts and myoblasts. In a rat study, it showed a good sol-gel transformation property, so that it could be handled composedly and easily in order to cover the exposed neural structure in the laminectomy site. Also, it showed good anti-adhesive characteristics based on MRI, histology and peel-off tenacity test. In conclusion, the oxi-HA/ADH hydrogel was an effective and promising material in the prevention of epidural fibrosis after spine surgery. Nevertheless, the efficacy of this agent should be further explored in additional experimental and clinical studies.

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Discussion with Reviewers

Marianna Peroglio: What could be the challenges in translating the use of the hydrogel from rat to human laminectomy for the prevention of epidural fibrosis?
Authors: Our concern would be how to determine the safety and assess the efficacy of this hydrogel as an anti-adhesion agent after discectomy or laminectomy. Close monitoring and collection of safety data is very important before the routine use of a new device and it should not be undervalued. Such further studies should meet the essential requirements of the Medical Devices Directive. Unlike the animal study, direct evidences of dura or root adhesion can only be collected from gross evaluation in limited patients who receive reoperation. Indirect evidences from patient-reported outcome assessment are sometimes debatable, whereas postoperative back or leg pain should be multifactorial. An objective clinical tool, such as myelography, may be helpful.

Marianna Peroglio: For which other applications could this hydrogel potentially be used for, besides the prevention of epidural fibrosis?
Authors: Besides the prevention of epidural fibrosis, there are some potential clinical applications for which this hydrogel could be used. First, it is a potential vitreous substitute after vitrectomy (Su *et al.*, 2011; additional reference). Second, it plays a probable role in preventing other post-operative adhesions, such as intra-abdomen adhesions after open or endoscopic abdomen surgery, intrauterine adhesions after operative hysteroscopy and tendon adhesions. Third, this injectable hydrogel is a good candidate for cell or drug carrier. For example, this hydrogel could be a suitable cell carrier for nucleus pulposus (NP) cells in the treatment of NP degeneration. In addition, it should be a promising drug compound (*e.g.* epidural morphine-hydrogel compound for postoperative pain, anti-inflammatory drug-eluting hydrogel for facilitating epidural fibrosis prevention), whose cytocompatibility in the spine environment was proved in the current study.

Zhen Li: Are there already similar products available on the market? What are the main advantages of the current hydrogel compared with those?
Authors: The only FDA-approved adhesion barrier gel for spine surgery is carboxymethycellulose

(CMC)/polyethylene oxide (PEO) gel (Oxiplex®/SP Adhesion Barrier Gel, manufactured by FzioMed, San Luis Obispo, CA, USA and distributed under the trade names of Oxiplex®/SP Adhesion Barrier Gel, DePuy International, Leeds, UK and Medishield™ Adhesion Barrier Gel, Medtronic International Trading SARL, Tolochenaz, Switzerland). It is a flowing, viscoelastic gel with a degradation time of about 4 weeks, which is stored at room temperature and supplied sterile in a 3 mL syringe with a flexible applicator.

The possible advantages of our thermosensitive oxi-HA/ADH hydrogel is its longer degradation time and its sol-gel transformation property, which lessens the risk of ectopic leakage, especially in early upright activity after surgeries.

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