

Research Article

The effect of adding carboxymethylcellulose and alginate to hyaluronic acid on reducing epidural fibrosis in a lumbar laminectomized rat model

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ABSTRACT

Objective: This study aimed to compare the anti-epidural fibrosis and anti-inflammation effects of hyaluronic acid (HA)-carboxymethylcellulose (CMC)-alginate hydrogel, pure HA, and normal saline using a lumbar laminectomized rat model.

Methods: Thirty lumbar laminectomized adult rats were randomly assigned to three groups. The control group received normal saline, the HCA group received HA-CMC-alginate gel, and the HA group received pure HA gel soaked over the dura of the laminectomized area before closing the surgical wound. All rats were housed for eight weeks, then epidural fibrosis (EF) was histologically graded. In addition, the fibroblast and inflammatory cell density were computerized for evaluation.

Results: The mean fibroblast densities were $32.03 \times 10^2 \pm 488$, $13.22 \times 10^2 \pm 200$, and $14.52 \times 10^2 \pm 368$ cell/mm² in the control, HCA, and HA groups, respectively. The mean inflammatory cell density was $30.74 \times 10^2 \pm 459$, $5.90 \times 10^2 \pm 129$, and $11.08 \times 10^2 \pm 282$ cell/mm² in the control, HCA, and HA groups, respectively. The mean fibroblast and inflammatory cell densities in the HCA and HA groups were significantly lower than in the control group ($P < 0.05$). The HCA group had a significantly lower inflammatory cell density than the HA group ($P < 0.05$). The fibrous adherence grading of HCA and HA was significantly lower than the control ($P < 0.05$).

Conclusion: HA-CMC-alginate gel and HA hydrogels seem to have a better preventative effect on EF than no treatment (control). HA-CMC-alginate can exhibit a better anti-inflammatory effect than HA. HA-CMC-alginate can be effective in reducing EF and inflammation after lumbar laminectomy.

Introduction

Laminectomy for the treatment of neural compression is one of the most common spinal surgical procedures. Many authors have reported excellent results from the procedure.¹ Nevertheless, a substantial number of patients continue to have recurrent back pain, radicular pain, and neurological deterioration after decompressive laminectomy. This relapse is called failed back surgery syndrome (FBSS) or postlaminectomy syndrome.² Many FBSS patients undergo repeat spinal surgery to alleviate the recurrent symptoms and release the affected neural tissues, but the failure rate is high.³ Moreover, repeated spinal surgery after a failed laminectomy increases the surgical risk to the dura and spinal nerve roots because of difficult scar dissection, which in turn can increase the operative time, intraoperative blood loss, associated anesthesia risk, and healing time, which is associated with greater fibrotic scarring.

Postoperative epidural fibrosis is an inevitable sequela of spinal surgery and is part of the postoperative healing process. To prevent or reduce fibrosis, several surgical techniques, synthetic materials, and bioabsorbable materials have been developed and tested.⁴

The principles for preventing epidural fibrosis include minimizing the inflammatory process, reducing hematoma, and using a barrier between the dura and surrounding muscle.

Hyaluronic acid (HA) is a natural substance that inhibits fibroblast proliferation and collagen deposition.⁵ Hyaluronic acid has had a positive effect on epidural fibrosis prevention in animal models with high biocompatibility and no biotoxicity.⁶⁻⁹ Various forms of HA have been developed in the last decade to prolong its activity, such as high molecular weight liquid form barrier sheet.⁷⁻⁹ Moreover, HA has also been combined with other substances—such as carboxymethylcellulose (CMC)—to delay its absorption and prolong the duration of its anti-adhesion effect.¹⁰ Alginate acts as an inhibitor of the complement cascade and inflammatory cytokines,¹¹ resulting in an anti-inflammatory effect. Anti-inflammatory substances can reduce epidural fibrosis.² We hypothesize that adding CMC and alginate to HA will have a synergistic effect that improves the anti-inflammatory and antifibrotic activity of HA. The purpose of this experiment was to compare the anti-epidural fibrosis and anti-inflammation effects among HA-CMC-alginate, pure HA, and normal saline using a lumbar laminectomized rat model.

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Materials and Methods

The study protocol was reviewed and approved by the Institutional Animal Research Ethics Committee before the study began (ACUC-KKU-65/60). All animal housing, care, and procedures were conducted at our institute's Animal Laboratory Center, which maintains a level one animal biosafety standard.

Study samples

Thirty Sprague–Dawley rats aged 8 weeks were included. Each rat was anesthetized using vaporized isoflurane. A single dose of prophylactic antibiotic (150 mg of cefazolin) was injected intraperitoneally. Once the rat's pedal reflex diminished, the back hair along the lumbosacral spine was shaved off and scrubbed using a betadine scrub solution. The rat was placed prone on the operating table. The skin was prepared with betadine solution, and the surgical field was draped.

A 4.5-cm posterior longitudinal incision was made midline between the L5 and L6 spinous processes. The back fascia was incised to expose the spinous processes of the L5-L6 vertebrae. The paraspinal muscles were subperiosteally detached using the periosteal elevator. A self-retaining retractor was used to retract the muscles bilaterally. The spinous process, ligamentum flavum, and lamina of the L5-L6 vertebrae were meticulously resected using a small bone cutter to avoid damaging the dural sac. Any rats with complications from the laminectomy (viz., dural tear, cerebrospinal fluid leakage, or death) were classified as ineligible and excluded from the study. However, to ensure good anesthetic, operative, and housing techniques, we piloted these techniques with 2 rats. The first rat had a dural tear and cerebrospinal fluid leakage, so we repaired the dura with a dural patch and routinely closed the wound. The second pilot rat had no operative complications. Both rats were housed for an additional 8 weeks without any complications. Good wound healing was found, and the sutures were removed on day 7. These 2 rats were not included in the study. For all the included rats ($n=30$), no complications were associated with the anesthetic, operative, or housing techniques. After dural exposure, the rats were randomly allocated using permuted block randomization (with stratification for a 1 : 1 male : female ratio) into 1 of 3 groups. The control group received normal saline, the hyaluronan–carboxymethylcellulose–alginate (HCA) group received sodium HA-CMC and alginate gel (Protescal; LG Life Sciences, Seoul, Korea), and the HA group received pure HA gel (Synvisc; Genzyme, Naarden, Netherlands) soaked over the dura of the laminectomized area before surgical wound closure. Each number code was sealed in an opaque envelope. The surgical wound was then closed layer by layer using absorbable Vicryl 4-0 for

the myofascial layer and Nylon 3-0 for the skin. The skin suture was removed on day 7 after the operation.

Pathological assessments

All rats were housed for 8 weeks after the operation and then euthanized using a lethal dose of carbon dioxide. The L5-L6 vertebrae—together with the surrounding soft tissue—were cut using en bloc resection. All specimens were transversely and serially cut into 5- μ m slices (10 per rat) and then stained with hematoxylin and eosin for histopathologic assessment, including fibroblast density, inflammatory cell density, and fibrous adherence grading. The fibroblast density and inflammatory cell density were counted and calculated in units of cells/ mm^2 using ImageJ (version 1.45s National Institutes of Health, Bethesda, Md, USA). In addition, the fibrous adherence classification ranging from 0 (no adhesion) to 3 (severe adhesion)¹² was evaluated by a pathologist blinded to the goals of the study.

Statistical analysis

The level of statistical significance was set at 0.05, and the type II error of the study was set at 0.2. The sample size determination was 10 rats per treatment group.

The demographic data were reported as the mean $\pm$ standard deviation (SD) except for sex, which was reported as the number of rats. The fibroblast density and inflammatory cell density were continuous data, and a one-way analysis of variance (ANOVA) was consequently used to analyze the differences in the means of fibroblast and inflammatory cell densities among the treatment groups. The independent *t*-test with Bonferroni correction was used to compare the differences in the means between each pair of treatment groups. Fibrous adherence grading is categorical data, and the Kruskal–Wallis test was consequently used to detect differences in the median fibrous adherence grading among the treatment groups. A pairwise comparison was used to compare the differences in fibrous adherence grading between each pair of treatment groups. All outcomes were analyzed using International Business Machines Statistical Package for the Social Science Statistics (version 20, IBM Corporation). The post hoc power analysis of the study was calculated using G*Power (version 3.0.10).¹³

Results

A total of 30 rats were included in the study. The male to female ratio was 1 : 1. The average preoperative and preeuthanized weights of the rats are shown in Table 1. Serious surgical or postsurgical complications (i.e., dural tears, cerebrospinal fluid leakage, massive bleeding, nerve root injury, or infection) did not occur. Ten rats, including 5 males and 5 females, were allocated to each intervention group. Sex did not significantly affect fibroblast density ($F(1, 28)=0.01$, $P\text{-value}=0.92$), inflammatory cell density ($F(1, 28)=0.01$, $P\text{-value}=0.94$), or fibrous adherence grading ($F(1, 28)=0.43$, $P\text{-value}=0.52$). The respective

HIGHLIGHTS

- Postoperative epidural fibrosis is a sequela of spinal surgery and is part of the physiological healing process. To prevent or reduce fibrosis, many different techniques and materials have been developed and tested. This study aimed to investigate the synergistic effect of adding carboxymethylcellulose (CMC) and alginate to hyaluronic acid (HA) on the anti-inflammatory and antifibrotic activity of HA using a rat model.
- The results showed that addition of CMC and alginate (HCA group) did not have any effect on fibroblast density compared to HA alone. However, the mean inflammatory cell density of HCA group was significantly lower than that of HA and control groups. Additionally, Fibrous adherence grading of HCA and HA groups was significantly lower compared to the control group.
- The results from this study may be extrapolated that HCA and HA may provide protection against epidural fibrosis compared to saline, and that HCA may have better anti-inflammatory properties compared to HA alone.

Table 1. Baseline characteristics

	Control group n = 10	HCA group n = 10	HA group n = 10
Pre-operative weight (g)			
Male	354.4 $\pm$ 13.7	350.0 $\pm$ 15.6	342.1 $\pm$ 14.3
Female	224.7 $\pm$ 1.7	225.9 $\pm$ 5.5	235.3 $\pm$ 10.1
Pre-euthanize weight (g)			
Male	479.9 $\pm$ 7.9	477.4 $\pm$ 13.1	473.7 $\pm$ 13.1
Female	345.4 $\pm$ 2.1	337.2 $\pm$ 7.6	338.3 $\pm$ 9.3

HCA, hyaluronic acid-carboxymethylcellulose and alginate gel; HA, hyaluronic acid; Values represent respective mean $\pm$ standard deviation. The male and female ratio was 1:1.

Table 2. Mean fibroblast cell density and inflammatory cell density

Group	Fibroblast cell		Inflammatory cell	
	Density (cell/mm ²)	95% CI	Density (cell/mm ²)	95% CI
Control	3203 ± 488	2854-3552	3074 ± 459	2745-3402
HCA	1322 ± 200	1179-1465	590 ± 129	598-683
HA	1452 ± 368	1188-1714	1108 ± 282	906-1310

Values within the table represent respective mean ± standard deviation.
HCA, hyaluronic acid-carboxymethylcellulose-alginate gel; HA, hyaluronic acid.

means ± SD of the fibroblast cell densities of the control, HCA, and HA groups were 3203 ± 488, 1322 ± 200, and 1452 ± 368 cell/mm², whereas the respective means ± SD of inflammatory cell densities were 3074 ± 459, 590 ± 129, and 1108 ± 282 cell/mm² (Table 2, Figures 1 and 2). The respective median fibrous adherence grades were 3, 0.5, and 1 (Table 3).

One-way ANOVA showed a significant difference in the mean fibroblast density and inflammatory cell density (P -values=.0001). The post hoc pairwise comparisons showed that the HCA group had a lower mean fibroblast cell density than the control group but not the HA group (P -value=.0001 and P -value=1.0, respectively) (Table 4). The mean inflammatory cell density of the HCA group was significantly lower than those of both the HA and control groups (P -value=.0001 and P -value=.004) (Table 4). The fibrous adherence grading of the HCA and HA groups was significantly lower than that of the control group (Figure 3). However, the fibrous adherence grading did not significantly differ between the HCA and HA groups (P -value=.408). As shown in Figure 4, the histopathological image demonstrated thin, nonadhering fibrosis to the dura in the HCA and HA groups, whereas fibrosis was thick and adhered to the dura in the control group. The post hoc power analysis of the study was 98.37%.

Discussion

The results demonstrate that the HCA and HA groups exhibited a superior preventative effect on epidural fibrosis compared to the control. We compared fibroblast cell density and inflammatory cell density between the treatment groups. The HCA and HA groups showed

significantly lower fibroblast cell densities, inflammatory cell densities, and fibrous adherence grading than the control group. Based on the prevention of epidural fibrosis, hyaluronan in both the HCA and HA groups was the main active ingredient, as evidenced by a lower degree of fibroblast density than in the control group. Hyaluronan prevents fibrosis by inhibiting fibroblast proliferation and collagen deposition. The HCA group showed a significantly lower degree of inflammatory cell density than the HA and the control groups. We propose that the primary anti-inflammatory mechanism is mediated by CMC and alginate, which inhibit proinflammatory cytokines. The lower degree of inflammation may result in a lesser degree of postoperative pain. In a study by Zhang et al.¹⁴ postoperative pain control after lumbar spine surgery with nonsteroidal anti-inflammatory drugs—used to minimize the inflammatory process—was effective after various types of surgery. The anti-inflammatory or pain issues should be further studied. In the current study, HCA was no better at preventing fibrosis than pure HA, which may be due to the inadequate dosage of CMC and alginate within the HCA group. As shown in a previous study by Sae-Jung and Jirattananaphochai,² a lower dosage of the anti-inflammatory drug (2 mg parecoxib) did not prevent epidural fibrosis, whereas a higher dosage (6 mg parecoxib) effectively controlled fibrosis.

Hyaluronan combined with CMC (HC) has been reported to prevent fibrosis adhesions after intraperitoneal, uterine horn, pericardial, and sinus surgeries.¹⁵⁻¹⁸ These cited studies reported that HC reduced fibrosis, and 1 study reported no additive effect on the anti-inflammatory activity of HC compared to a sham operation.¹⁶ In the current study, the HCA and HA groups showed a significant reduction in epidural fibrosis compared to the control group. The HCA group showed a greater anti-inflammatory effect than the other groups. This difference might be due to the additive effect of alginate, which is an important anti-inflammatory ingredient (that is not found in CMC).

We found that the fibroblast and inflammatory cell densities correlated well with fibrous adherence grading (Pearson's correlation coefficient ranged between 0.92 and 0.98, P -value=.0001). More

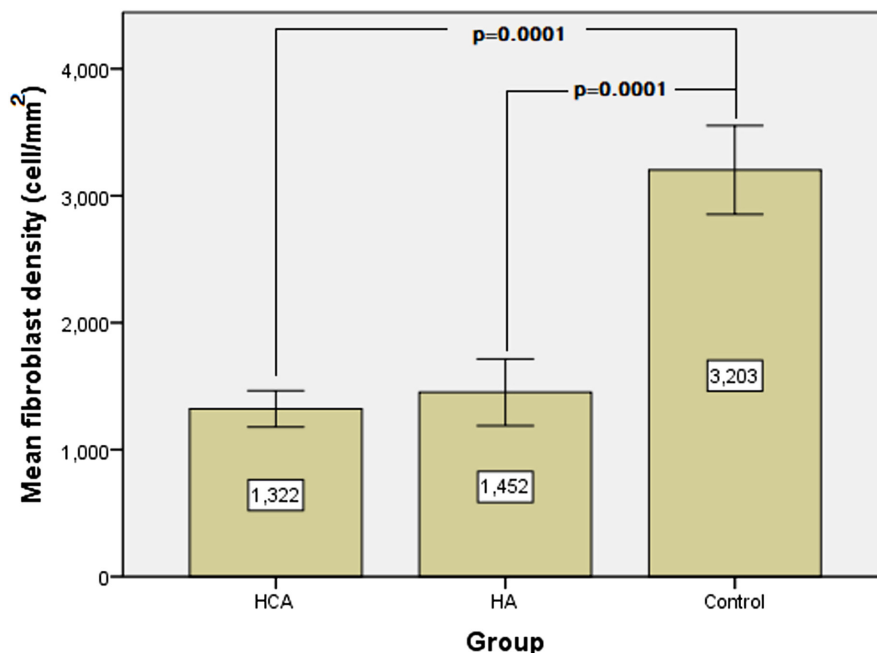


Figure 1. The bar graph demonstrates the mean fibroblast density (cells/mm²) and the 95% CI for all interventions. The error bar indicates the 95% CI.

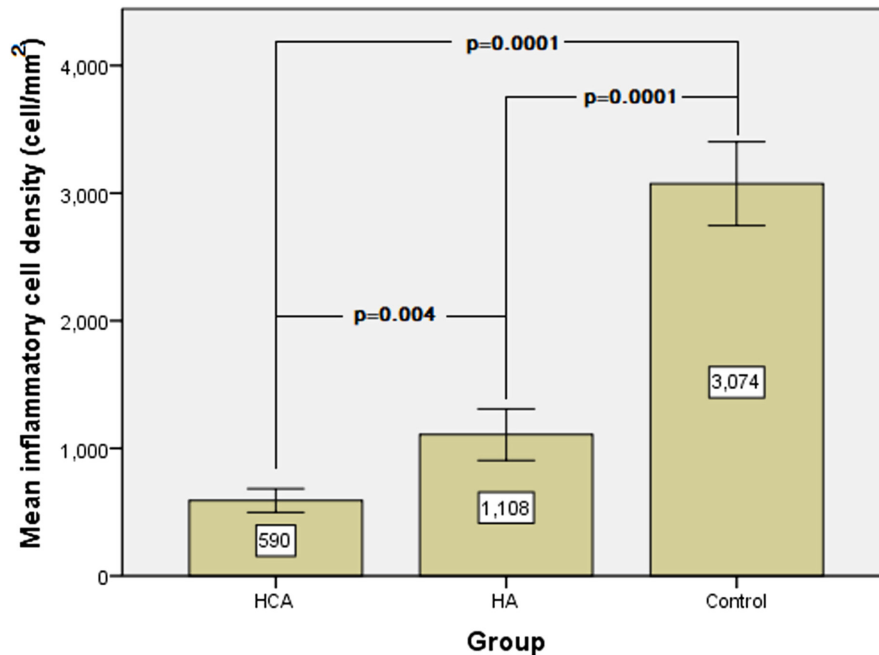


Figure 2. The bar graph demonstrates the mean inflammatory cell density (cells/mm²) of each treatment group. The error bar represents the 95% CI. HCA, hyaluronan-carboxymethylcellulose-alginate; HA, hyaluronic acid; control, normal saline.

fibroblast cells and inflammatory cells were counted, resulting in more fibrous adhesions to the dura. Conversely, the fibroblast density and fibrous adherence grading were significantly lower in the HCA and HA groups than in the control group. The fibrous adherence grading may reflect clinical complications after surgery. Such severe adhesions indicate a high possibility of postoperative fibrosis that adheres to the dura or nerve root.

Few articles have provided rationales for their selection of the sex of the rats used. According to Cross et al.¹⁹ the thinner skin found in female rats has a faster rate of wound healing and a higher rate of wound contraction. By contrast, wounds in male rats are more likely to heal by epithelialization. Additionally, the US Food and Drug Administration now requires the use of females in human clinical studies.²⁰ Therefore, female rats were included in the current study. For the follow-up period, a study of epidural fibrosis described that epidural fibrosis could occur 6 weeks after the operation.²¹ To ensure

epidural fibrosis formation, we sacrificed the rat's spine and evaluated epidural fibrosis 8 weeks after lumbar laminectomy, as in our earlier study.²

Biases/limitations

First, a surgical learning curve bias may be possible; specifically, increased soft-tissue dissection may have increased the potential for fibrous tissue proliferation and adhesion. We addressed this bias by using the pilot surgery, using the same surgical team but randomized

Table 3. Mean and median fibrous adherence grading in each treatment group

Group	Mean	SD	Median
Control	2.8	0.4	3
HCA	0.5	0.5	0.5
HA	1.1	0.6	1

Control, normal saline; HCA, hyaluronan-carboxymethylcellulose-alginate gel; HA, hyaluronic acid; SD, standard deviation.

Table 4. Mean respective difference of fibroblast and inflammatory cell densities between treatment groups

Group 1 vs. group 2	Fibroblast		Inflammatory cell	
	MD (95% CI)	P	MD (95% CI)	P
HCA vs. control	-1881 (-2305 to -1475)	0.0001	-2483 (-2849 to -2118)	0.0001
HA vs. control	-1751 (-2175 to -1327)	0.0001	-1966 (-2331 to -1601)	0.0001
HCA vs. HA	-130 (-554 to 294)	1.0	-517 (-883 to -152)	0.004

Control, normal saline; HCA, hyaluronan-carboxymethylcellulose-alginate gel; HA, hyaluronic acid; MD, mean difference; - indicates group 1 had less fibroblast density than group 2.

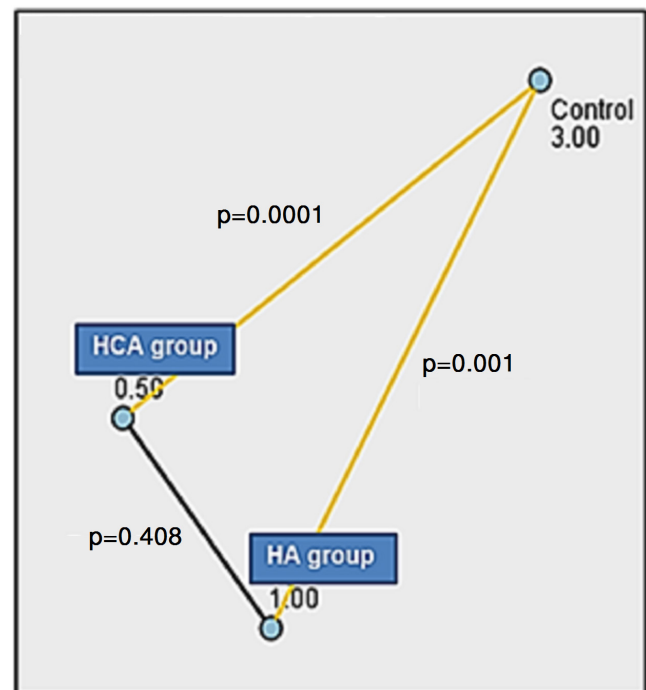


Figure 3. Multiple pairwise comparisons showed that HCA and HA had a significantly lower degree of fibrous adherence grading than the control. HCA, hyaluronan-carboxymethylcellulose-alginate; HA, hyaluronic acid.

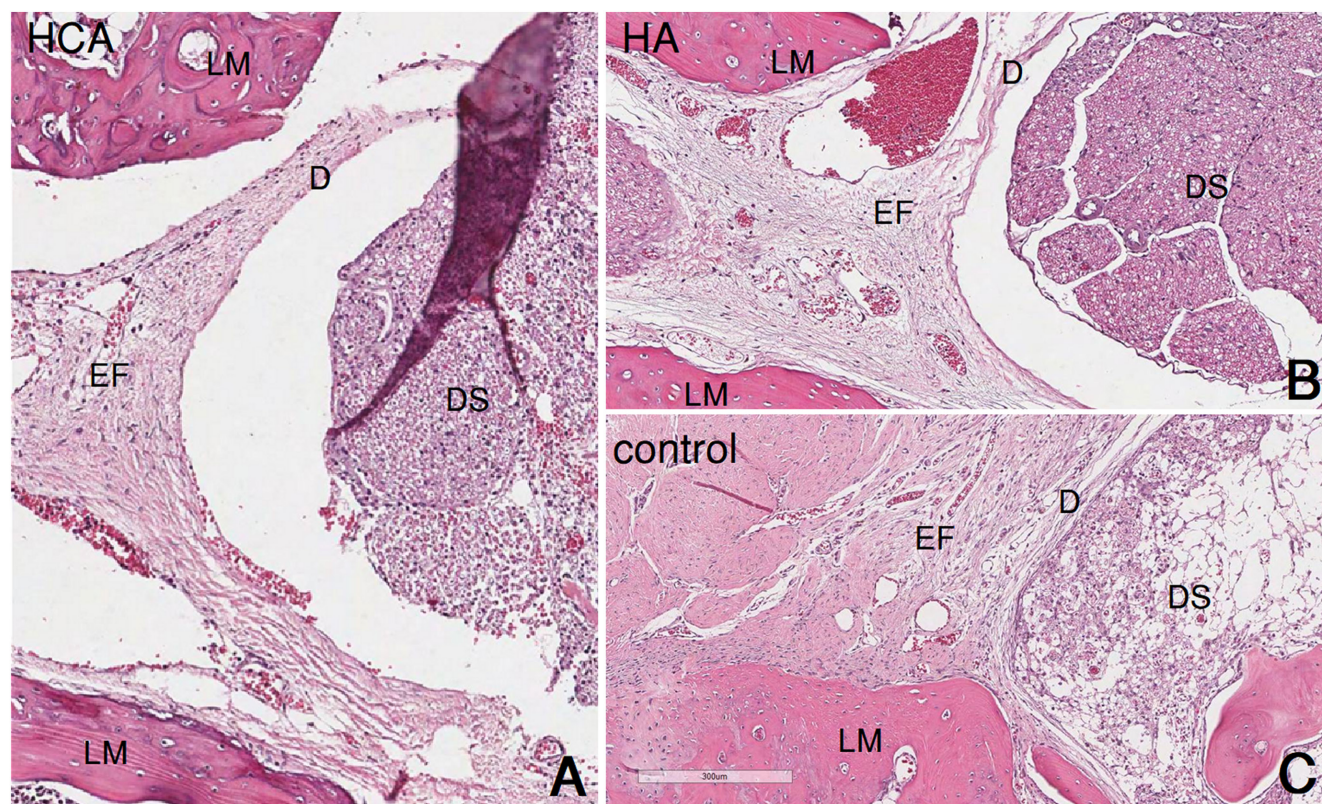


Figure 4. A-C. A comparison of epidural fibrosis between the treatment groups; (A) HCA group, thick fibrosis, completely adhered to the dura along the laminectomy site, (B) HA, and (C) control group: thin fibrosis did not adhere to the dura along the laminectomy site. EF, epidural fibrosis; LM, laminectomy area; DS, dural sac; D, dura; HCA, hyaluronan-carboxymethylcellulose-alginate; HA, hyaluronic acid; control, normal saline.

by allocation. Randomization resolved the bias by equally distributing the known and unknown confounders among the treatment groups. The second bias was an outcome assessment bias that might have occurred at each step of outcome measurement. This bias was prevented by having the pathologist blinded to all processes of the study. The histopathological evaluation was performed and calculated using the ImageJ software. The third limitation was the lack of postoperative pain assessment; therefore, we cannot claim that HCA has a benefit over HA vis-à-vis postoperative pain management, although the HCA group showed significantly less inflammatory cell density than the HA group. A postoperative pain study is warranted for a further clinical trial. Postoperatively, none of the rats had neurological deficits, so these substances can be safely placed adjacent to the rat's neural tissue.

In conclusion, HCA and HA were better able to prevent epidural fibrosis compared with the control (normal saline). Hyaluronan-carboxymethylcellulose-alginate has a better anti-inflammatory effect than HA. Hyaluronan-carboxymethylcellulose-alginate should be recommended to prevent epidural fibrosis and possibly minimize the pain from an inflammatory process associated with spine surgery.

Ethics committee approval: The study protocol has been reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (ACUC-KKU-65/60).

Informed Consent: N/A.

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Declaration of Interests: The authors have no conflicts of interest to declare.

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