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| Original Article |

Effects of Low and High Molecular Weight Hyaluronic Acids on Peridural Fibrosis and Inflammation in Lumbar Laminectomized Rats

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Background:

Postlaminectomy peridural fibrosis is inevitable. Some studies have compared and identified the effects of high molecular weight hyaluronic acids (HMWHA) and low molecular weight hyaluronic acids (LMWHA) on peridural fibrosis in postlaminectomy animal models. However, no studies have been found that compare pain behaviors between hyaluronic acids or among hyaluronic acids and other solid materials. The purpose of this study was to examine the correlation between pain-related behaviors and histopathologic changes in laminectomized rats using various peridurally administered materials.

Methods:

Forty male Sprague-Dawley rats, laminectomized at the L5 and L6 levels, were divided into four groups: group C, laminectomy only; group L, laminectomy and LMWHA application; group H, laminectomy and HMWHA application; group F, laminectomy and fat interposition. Pain behaviors were checked before, 3 days, 1 week, and 3 weeks after surgery. Histopathological changes were checked at the L5 level 3 weeks after the surgery.

Results:

The 50% withdrawal thresholds in groups L and H were higher than that in groups C and F three days after laminectomy ($P < 0.05$). The paw withdrawal time did not change among the groups and in each group during the study period. Peridural fibrosis in group F was significantly lower than in the other groups ($P < 0.05$).

Conclusions:

Hyaluronic acids significantly reduced mechanical allodynia but not thermal hyperalgesia. Peridural fibrosis did not show any correlation with pain behaviors. There have been limited studies on the correlation between peridural fibrosis and pain behavioral change, which should be verified by further studies. (Korean J Pain 2011; 24: 191-198)

Key Words:

allodynia, failed back surgery syndrome, hyaluronic acid, laminectomy.

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INTRODUCTION

Laminectomy has been used to decompress spinal nerves but postoperative peridural fibrosis may follow [1]. The excess formation of peridural fibrosis causes leg and back pains by tethering of the spinal nerve roots and dura, and poses significant surgical hazards in subsequent operations [2,3].

There have been some experimental methods developed to prevent peridural fibrosis [4−15]. Non-biological methods to prevent such fibrosis have been performed using gelatin foam, microfibrillar collagen, barrier gel, or collagen gel [4−7]. Biological methods have been tried using fat, ligamentum nuchae, carboxymethyl cellulose, polylactide absorbable film, gelatin sheet, bioelastic polymer, polylactic acid containing elastase, tissue plasminogen activator gel, or hyaluronic acid (HA) [8−15]. Generally, the results have been inconsistent because of the variety of materials used.

It has been shown that peridural fibrosis after laminectomy is formed by fibrous connective tissue ingrowths into the surgical hematoma from the posterior spinal muscle [8] and the majority of the materials preventing ingrowths of fibrous tissues were developed with the hypothesis that limiting the contact between the dura and hematoma by the materials could prevent peridural fibrosis.

HA is a high molecular weight polysaccharide composed of N-acetyl glucosamine and glucuronic acid [16]. HA serves as important function in lubricating and protecting articular cartilage and soft tissue surface during joint function [17,18]. Many studies have reported that high molecular weight hyaluronic acid (HMWHA) showed more effective results than those of low molecular weight hyaluronic acid (LMWHA) to improve joint functions and to prevent inflammation [19−22]. In a postlaminectomy animal model, HMWHA can prevent peridural fibrosis in dogs [23].

There are some problems in the previous studies about the prevention of peridural fibrosis by HA. First, even though it has been proven that HA prevents peridural fibrosis in some animal studies in proportion to the molecular weight [13,23], those studies did not evaluate the relation between the degree of fibrosis and the behavioral changes, such as motor function, sensory function, or pain assessment. Those studies also did not evaluate the behavioral changes in proportion to the molecular weight of HA. Second, in previous studies, the correlation between peridural fibrosis and LMWHA, with a molecular weight of less than 1,000,000 Daltons, has not been studied [23,24]. However, LMWHA has been used clinically in many countries including Korea. The effects of LMWHA on the prevention of peridural fibrosis and pain behavior should therefore be evaluated. Third, there have been no studies comparing the effects of HMWHA and LMWHA on the prevention of peridural fibrosis. One study performed in a laminectomized dog model showed HMWHA decreased peridural fibrosis [23], but in their study, only HMWHAs at 4,000,000 and 3,000,000 Daltons were used.

The purpose of this study was to compare the roles of HMWHA (3,000,000 Daltons) and LMWHA (800,000 Daltons) in autologous fat as fibrotic inhibitors and anti-inflammatory agents in a laminectomized rat model and to evaluate the pain-related behaviors and histopathological changes between the two different molecular weight HAs as well as the autologous fat graft.

MATERIALS AND METHODS

1. Materials and surgery

The institute's animal Care and Use Committee (IACUC) approved all experimental protocols used in this study (No. 13-2006-006-6). Forty male Sprague-Dawley rats (150−250 g) were equally divided into four groups.

Animals were anesthetized with sevoflurane in oxygen and laminectomies were performed at the L5 and L6 levels. The L5 and L6 spinous processes were removed with rongeur under microscopy and the remaining thin layer of the lamina was carefully chipped away with blunt microforceps and a rongeur.

Any rats showing neuropathic pain behavior before laminectomy or evidence of cerebrospinal fluid leakage during surgical manipulations were excluded from the study.

In group C (n = 10), only laminectomy was done. In group L (n = 10), laminectomy was done and 0.5 ml of LMWHA (Hyruan®, LG Pharm Co., Seoul, Korea; 800,000 Daltons) was applied to the laminectomy defect. In group H (n = 10), the same procedure as in group L was applied except HMWHA (Hyruan Plus®, LG Pharm Co., Seoul, Korea; 3,000,000 Daltons) was applied. In group F (n = 10), laminectomy was done and fat pads were interposed between the exposed surface of dura and the posterior spinal muscles.

After the end of procedure, the muscles and skin were closed around the wound.

2. Pain-related behavior testing

All animals were evaluated for pain behavior before surgery and 3 days, 1 week, and 3 weeks after surgery using two tests: mechanical allodynia and thermal hyperalgesia tests. To assess the nociceptive response to thermal stimuli, we assessed the time (s) of withdrawal response to heat applied to the plantar surface of the hind paw using a Plantar Test (Hargreaves Apparatus) analgesiameter (Ugo Basile, Comerio, Italy) [25]. Rats were placed in a clear plastic chamber with a plastic floor and allowed to stay there for 15 min before testing. After the acclimation period, a radiant heat source was placed under the floor beneath the plantar surface of the hind paw. The withdrawal latency to 0.1 s was determined using an electric clock circuit and microcomputer.

Mechanical allodynia test assessed the withdrawal responses to the filaments applied to the plantar surface of the hind paw using von-Frey filaments (Stoeling Co., Chicago, IL, USA) and the 50% paw withdrawal threshold with the up-down method [26]. The test was started using a 2.0 g hair. Six responses were checked to calculate the withdrawal threshold in the range of 0.4–15.1 g. In cases where continuous positive and negative responses were observed to the exhaustion of the stimulus set, values of 0.4 g and 15.1 g were assigned, respectively. The resulting pattern of positive and negative responses was tabulated using the conversion. The 50% response threshold was interpolated using the following formula: 50% withdrawal threshold = $(10^{[Xf + \kappa\delta]})/10{,}000$, where Xf is the value (in log units) of the final von-Frey hair used; κ is the tabular value for the pattern of positive/negative responses; and δ is the mean difference (in log units) between stimuli. In this test, $\delta = 0.244$ [23].

The paw withdrawal time and 50% withdrawal threshold of the left and right hind paws were averaged. The 50% withdrawal threshold (g) and paw withdrawal time (s) were

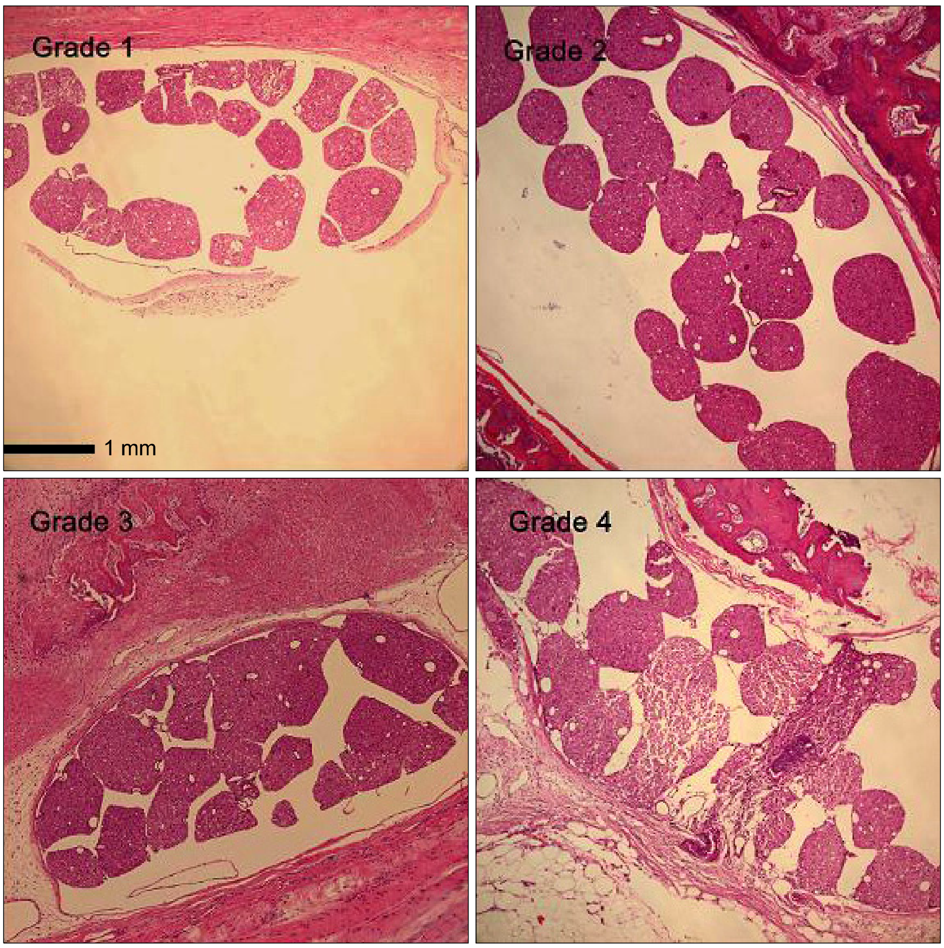

Fig. 1. The grade of cauda equina clumping on the light microscopic findings at L5 or L6 level at the 21st day after laminectomy in ×10 scale (hematoxylin and eosin staining). In grade 1, most rootlets are distributed separately in the subarachnoid space. In grade 2, more than half of the rootlets are grouped to one another. In grade 3, all nerve rootlets are grouped or adhered to form a lump. In grade 4, severe peridural adhesion can be seen.

compared over the testing period using a repeated measured ANOVA with post hoc Bonferroni test. Measures were compared among the groups using a Kruskal-Wallis test with a post hoc Mann-Whitney test.

3. Histopathology

At the 3rd week postoperatively, all rats were perfused intracardially with saline, followed by 4% buffered formaldehyde perfusion, and the lumbar 5^{th} and 6^{th} spinal columns were cropped. Vertebrae were decalcified by immersing the spinal columns in 10% w/v ethylenediaminetetraacetic acid for 24 h. The lumbar 5^{th} or 6^{th} vertebra was cropped and embedded in paraffin. Cross sections (4 μm) were stained with hematoxylin and eosin staining.

Cauda equina adhesion was classified based on the number/severity of adhesive changes (Fig. 1): grade 1: most rootlets are distributed separately in the subarachnoid space; grade 2: more than half of the rootlets are grouped to one another; grade 3: all nerve rootlets are grouped or adhered to form a lump; and group 4: lumped adhesion of all nerve rootles are adhered to the dura and the extradural scar tissue [27].

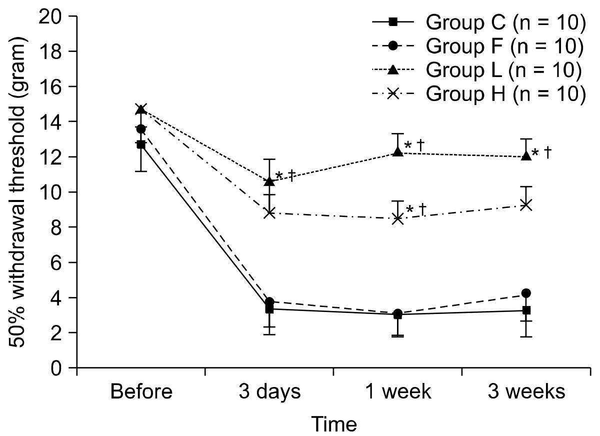

Fig. 2. The changes of 50% withdrawal threshold before laminectomy and 3 days, 1 week, and 3 weeks after laminectomy. Values are mean ± SE. Group C: laminectomy only, group L: laminectomy and low molecular weight hyaluronic acid application, group H: laminectomy and high molecular weight hyaluronic acid application, and group F: laminectomy and fat interposition. In groups C and F, 50% withdrawal thresholds are decreased significantly 3 days after laminectomy and maintained thereafter. Overall the mechanical allodynia in groups C and F are more significant than that of other groups from 3 days after laminectomy. $*P < 0.05$ vs. group C and $^{\dagger}P < 0.05$ vs. group F.

The three most inflamed sites were selected on ×40 scale and the mean number of inflammatory cells, such as neutrophil, lymphocyte, and macrophage in ×400 scale was measured on each slide.

The thickness of fibrosis near the laminectomy site was measured with a ruler in ×10 scale and classified based on the thickness of the extradural scar tissue: grade 0: less than 1 mm; grade 1: 1-2 mm; grade 2: 2-3 mm; grade 3: 3-4 mm; and grade 4: more than 4 mm [28].

Measures were compared among the groups using the Kruskal-Wallis test and between two groups with the Mann-Whitney test. Correlation between histopathology and paw withdrawal time or 50% withdrawal threshold at 3 weeks postoperatively was evaluated using a Pearson's correlation.

RESULTS

1. Pain-related behavior testing

Two rats that showed neuropathic pain behaviors were excluded from this study. No rats showed leakage of cerebrospinal fluid during the surgery. The 50% withdrawal thresholds to the mechanical stimulation in groups L and H were higher than that in groups C and F seven days after the laminectomy ($P < 0.05$), but there were no significant

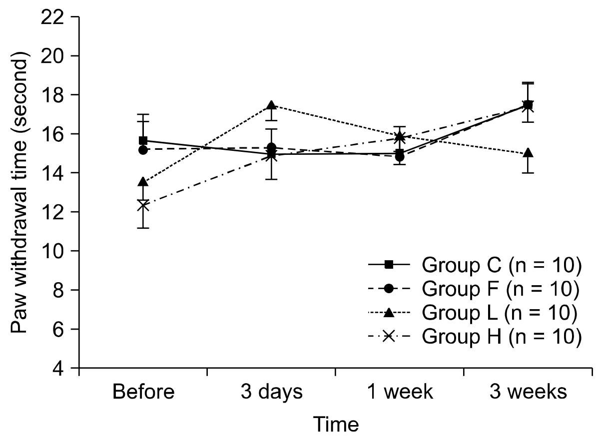

Fig. 3. The changes of paw withdrawal time before laminectomy and 3 days, 1 week, and 3 weeks after laminectomy. Values are mean ± SE. Group C: laminectomy only, group L: laminectomy and low molecular weight hyaluronic acid application, group H: laminectomy and high molecular weight hyaluronic acid application, and group F: laminectomy and fat interposition. There was no significant difference in thermal hyperalgesia among groups.

Table 1. Microscopic Evaluation of Peridural Fibrosis at Laminectomy Site

Grade of fibrosis	Groups			
	C	L	H	F*
0	0	0	0	0
1	0	0	0	4
2	4	3	2	4
3	2	5	6	0
4	3	1	2	1

Values are numbers of rats. Group C: laminectomy only, Group L: laminectomy and low molecular weight hyaluronic acid application, Group H: laminectomy and and high molecular weight hyaluronic acid application, Group F: laminectomy and fat interposition. The grade of thickness of peridural fibrosis: Grade 0: less than 1 mm, Grade 1: 1–2 mm, Grade 2: 2–3 mm, Grade 3: 3–4 mm, and Grade 4: more than 4 mm. $^*P < 0.05$ vs. corresponding data of other groups.

Table 2. Microscopic Evaluation of Cauda Equina Adhesion

Grade of adhesion	Groups			
	C	L	H	F
1	0	0	0	0
2	8	6	6	3
3	1	4	3	5
4	0	0	1	1

Values are numbers of rats. Group C: laminectomy only, group L: laminectomy and low molecular weight hyaluronic acidapplication, group H: laminectomy and and high molecular weight hyaluronic acid application, and group F: laminectomy and fat interposition. The grade of cauda equina adhesion: grade 1: most rootlets are distributed separately in the subarachnoid space, grade 2: more than half of the rootlets are grouped to one another, grade 3: all nerve rootlets are grouped or adhered to form a lump, and group 4: lumped adhesion of all nerve rootles are adhered to the dura and the extraduralscar tissue.

differences between group L and group H (Fig. 2). It was shown that the 50% withdrawal thresholds to mechanical stimulation dropped significantly 3 days after the laminectomy in groups C and F ($P < 0.05$) but not in groups L and H.

Paw withdrawal time to heat stimulation did not drop during the 3 weeks after the laminectomy in all groups and there were no significant differences among the groups in the paw withdrawal time (Fig. 3).

2. Histopathology

Fibrosis in group F was significantly lower than that in the other groups ($P < 0.05$, Table 1). There were no significant differences among the groups in cauda equina clumping (Table 2) and inflammation.

The numbers of inflammatory cells at ×400 scales were as follows: group C: 32.1 ± 25.9, group L: 24.4 ± 27.4, group H: 12.6 ± 6.9, and group F: 28.6 ± 17.5 ($P > 0.05$).

We assumed that the greater the fibrotic change, the inflammatory cell count, or the clumping of the cauda equina, the lower the 50% withdrawal threshold or paw withdrawal times were. However, there were no significant correlations between pain behaviors and histopathological findings (Figs. 4, 5).

Neutrophils were not found but lymphocytes, macrophages, and plasma cells were observed in all slides, which imply that the inflammation change 3 weeks after the laminectomy was chronic.

DISCUSSION

In this study, fat interposition reduced peridural fibrosis significantly, whereas the HA-applied groups and laminectomy only group did not. Another important result of this study was that the reduction of mechanical allodynia was observed only in the HA-applied groups. From these results, it is believed that perineural fibrosis around the neural foramen may have a more close correlation to pain behavioral changes than the degree of peridural fibrosis at the laminectomy site, and HA, itself may have an analgesic effect. Unfortunately, we did not evaluate the degree of perineural fibrosis around the neural foramen or nerve. Further studies will be required on the correlation between neural foraminal fibrosis and changes in the pain behaviors to verify such an assumption. One study showed that HA with a molecular weight of greater than 40,000 Daltons produced a high and long-lasting analgesia [29], which supports our assumption. In their study, such effect of HA appeared to be caused by the interaction between HA and its receptors.

Regardless of the molecular weight, HAs were effective in preventing mechanical allodynia but not thermal hyperalgesia in this study. Even though the mechanism of the difference between mechanical allodynia and thermal hyperalgesia is unclear, some studies showed the differ-

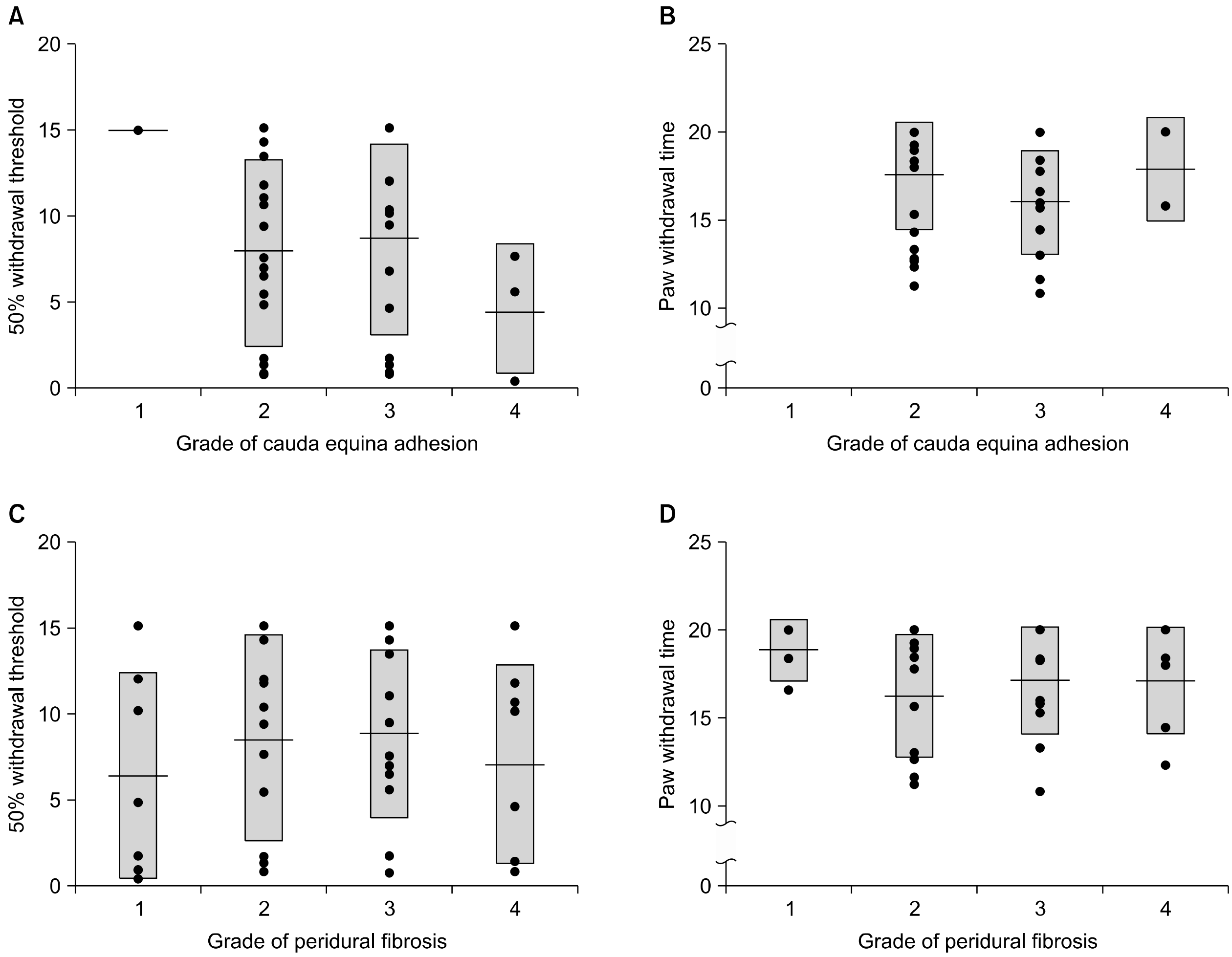

Fig. 4. The correlations between pain behaviors and histopathologic findings. (A) Correlations between 50% withdrawal threshold and grade of cauda equina adhesion. (B) Correlations between paw withdrawal time and grade of cauda equina adhesion. (C) Correlations between 50% withdrawal threshold and grade of eridural fibrosis. (D) Correlations between paw withdrawal time and grade of peridural fibrosis. Boxes are mean ± SD and center lines are means. It is supposed that the more the fibrotic change or cauda equina adhesion, the less the 50% withdrawal threshold or paw withdrawal time. However, there are no significant correlations between pain behaviors and histopathological findings.

ence in different animal neuropathic pain models [30,31]. One study suggested the mechanism for the difference was caused by the difference in the regeneration and maturity of injured nerves between A fibers and C fibers, which transmit tactile and thermal sensations, respectively [32].

There are not many studies on the effects of HA in reducing peridural fibrosis in animal and clinical laminectomized studies, and no studies have evaluated the anti-fibrotic effect in terms of the molecular weight of the HAs. Songer et al. [13] showed that HMWHA with a molecular weight of 4,000,000 Daltons reduced the formation of fibrotic tissue more than LMWHA with a molecular weight of 3,000,000 Daltons. We evaluated the effectiveness of LMWHA in reducing peridural fibrosis as well as HMWHA. Both the 3,000,000 and 800,000 Dalton HAs did not reduce peridural fibrosis. No difference was observed in the antifibrotic action between them possibly because HAs less than 3,000,000 Daltons were used in this study. First, even though there are some studies that show HA reduces fibrosis in animal studies, HAs could act as foreign bodies whereas autologous fat pads cannot. Second, HAs less than 3,000,000 Daltons could disappear from the laminectomy site before peridural fibrosis occurs, but as Songer et al. [13], HAs with a molecular weight of 4,000,000

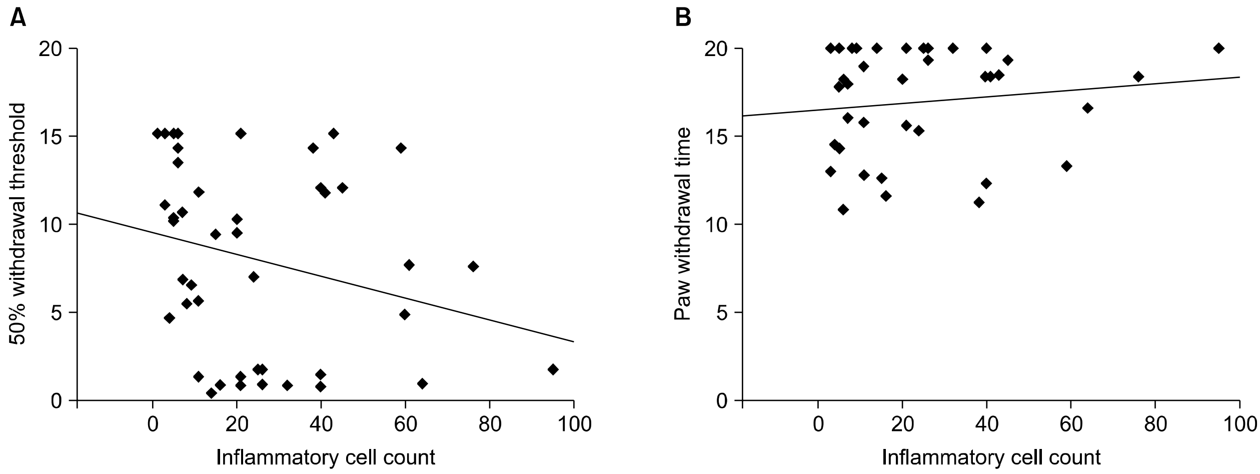

Fig. 5. The correlations between pain behaviors and inflammatory cells count. It is supposed that the more the inflammatory change, the less the 50% withdrawal threshold or paw withdrawal time. However, there are no significant correlations between pain behaviors and inflammatory cell count. In graph (A) y0 = 9.5 and slope = −0.062 and in graph (B) y0 = 16.5 and slope = 0.019. y0 means y-intersect where x value is 0.

showed decreased fibrosis. Further study might be needed with HA using a molecular weight of 4,000,000 or continuous injection of HA to the laminectomized site through epidural catheter.

HA plays an important role in the extracellular regulation of the migration of inflammatory cells [33,34]. HA inhibits the migration of lymphocytes, macrophages, and granulocyte in *in vitro* studies [35]. HA has been shown to have an inhibitory effect on cytokine formation, which explains the reduced inflammatory reactions [36]. However, we could not identify any anti-inflammatory effects of HAs in this study.

In conclusion, HAs significantly reduced mechanical allodynia. This finding suggests that peridural application of HAs regardless of the molecular weight before surgical closure in patients who received laminectomy could reduce agonizing mechanical allodynia, which could occur in laminectomized patients. Peridural fibrosis did not show any correlation with pain behaviors. There have been limited studies on the correlation between peridural fibrosis and pain behavioral changes, which should be verified through further studies.

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