The effects of ankaferd blood stopper and microporous polysaccharide hemospheres on epidural fibrosis in rat laminectomy model

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ABSTRACT

PURPOSE: To investigate whether topically administered hemostatic agents ankaferd blood stopper and microporous polysaccharide hemospheres can decrease epidural fibrosis after laminectomy in rats.

METHODS: Eighteen adult male Sprague-Dawley rats were equally and randomly divided into three groups. In the treatment groups, ankaferd blood stopper and microporous polysaccharide hemospheres topically administrated upon duramater surface after laminectomy. Fibroblast count, epidural fibrosis and arachnoideal involvement were evaluated and graded histopathologically.

RESULTS: Our data revealed that the count of fibroblasts, the grading of epidural fibrosis and arachnoideal involvement in the rats treated with microporous polysaccharide hemospheres were significantly less than the control group. Although the arachnoideal involvement in ankaferd blood stopper group were significantly less than the control group, there were no statistical differences when comparing the grading of epidural fibrosis and the fibroblasts count between the treatment groups and the control group.

CONCLUSION: The ankaferd blood stopper and microporous polysaccharide hemospheres reduced epidural fibrosis and arachnoideal involvement after laminectomy in rats.

Key words: Fibrosis. Laminectomy. Polysaccharides. Hemostasis. Rats.
Introduction

Surgery for lumbar disc herniations are performed over than one million patients all over the world in every year1-2. Unsatisfactory result rates according to failed back syndrome are 8 to 25% of the patients3-4. The causes of failed back syndrome are disectomy for wrong level, recurrent or persistent disc herniation, iatrogenic instability, central or lateral stenosis, arachnoiditis, and spinal epidural fibrosis3-5. Spinal epidural fibrosis (EF) is one of the major causes (10–24%) of failed back syndrome. The average incidence of EF in the MRI series is 16.1%, whereas the incidence (83.3%) is definitely higher in the epiduroscopy series6.

Epidural fibrosis cause nerve root traction, restricting of nerve root movements, extradural compression, and decreases the arterial supply of the nerve root. Impaired axoplasmic transport and excretion of inflammatory mediators following the decreasing of arterial supply lead to intractable radicular pain1,3,5,7. Both back and radicular pain associated with the EF are resistant to physical, medical and surgical treatments1-8. Epidural fibrosis is also associated with an increased complication rate in revision spine surgery9,10.

Epidural fibrosis is a part of the physiological tissue response after laminectomy. Histologic studies have demonstrated that destruction of epidural fat, epidural hematoma accumulation and muscle invasion of the laminectomy site are the main responsible factors for the formation of dense EF. The destruction of epidural fat results that duramater is exposed to neighboring structures and create a cavity where blood refill as hematoma. If the hemATOMA is more extensive, this may also cause more dense and thicker scar adhesions throughout inducing the invasion of fibrous tissue elements by the erector spine muscles6,8,10. Regarding to hypothesis, the inadequate control of epidural bleeding during surgical interventions is strongly associated with epidural fibrosis3,5,10.

The aim of this study is to investigate topically administered hemostatic agents, Ankaferd Blood Stopper (ABS) and Microporous Polysaccharide Hemospheres (MPH) can prevent epidural fibrosis and reduce arachnoideal involvement in a rat laminectomy model.

Methods

All experimental procedures were approved by the Animal Research Ethical Committee of Gazi University and the study was carried out at the Animal Breeding and Experimental Research Laboratory Center of Gazi University.

Surgical procedure and sample preparation

The surgical procedures were performed under general anesthesia. Rats were sedated with 5 mg/kg of Xylazine hydrochloride (Rompun/Bayer/Turkey) intraperitoneally (ip) and anesthetized with 45 mg/kg ketamine hydrochloride ip (Ketalar / Eczacıbaşı/Turkey). Rats were stabilized on the operation table in a prone position after deep anesthesia. The lower back of each rat, which is the surgical site, was sterilized with povidone. Following sterile isolation, long posterior midline surgical incisions were performed between the L4 and L6 levels. The lumbar fascia was opened approximately 2–2.5 cm over the spinous processes. Bilaterally, the paravertebral muscles were subperiostally dissected to expose the L3-4-6 laminae. A total L5 laminectomy and flavectomy were performed, and epidural fat tissues were removed, leaving the duramater clean and fully exposed. The rats were then randomly allocated into three groups with six rats per group. After treatment, the wounds were primarily sutured in anatomical layers (Prolen polypropylene sutures, Ethicon, Ethicon Endo-Surgery, Inc., Cincinnati, OH, USA). All surgical procedures were done under x4 optical magnification (Carl-Zeiss Opmı 9-FC 293191, Germany) and performed by the first author (RO). The rats had been then released for free food and water consumption for six weeks. After six weeks, the rats were sacrificed with the administration of intraperitoneal high dose thiopental sodium (Pental, Ulagay, İstanbul-Turkey, 2007) solution (10 mg/kg). The vertebral columns of the rats (L4-6) were removed en bloc, including the whole laminectomy area, dural sacs, nerve roots, and paravertebral soft tissues. The specimen was then placed into 10% buffered formalin.

Experimental groups

Adult male Sprague-Dawley rats weighing 250–300g were used in this study.

Control group (n=6): A laminectomy was performed and the surgical site was irrigated with saline solution (Eczacıbaşı, Turkey). Hemostasis had been achieved using cotton pad compression for several minutes. Any other hemostatic materials and bipolar cauterization were not applied.

ABS group (n=6): ABS (Ankaferd Blood Stopper®; ABS; Ankaferd Health Products Ltd., Istanbul, Turkey) 1 cc (Urtica dioica: 0.6 mg, Vitis vinifera: 0.8 mg, Glycrrhiza glabra: 0.9 mg, Alpinia officinarum: 0.7 mg, Thymus vulgaris: 0.5 mg) was poured on the surface of the laminectomy site for hemostasis.
and any other hemostatic materials and bipolar cauterization were not applied.

MPH group (n=6): MPH (Arista™; Medafor Inc, Minneapolis, MN) powder was filled adequately the surface of the laminectomy site for hemostasis. Any other hemostatic materials and bipolar cauterization were not applied.

**Histological analysis**

The specimen had been decalcified in a De Castro solution (mixture: 300 ml of ethanol, 50g of chloral hydrate, 670 ml of distilled water, 30 ml of 70% nitric acid) for 10 days. After complete decalcification, the specimens were dehydrated and embedded in paraffin 5 μm thick sections obtained from previously prepared paraffin blocks that were subjected to hematoxylin and eosin (H&E) staining and Masson’s trichrome. The specimens were examined under a light microscope. Grading of EF and arachnoidal involvement was performed according to the definition of He et al. (Table 1). The histological examination was conducted in a blinded manner by an experience examiner.

**TABLE 1 - Grading of the epidural fibrosis and arachnoidal involvement.**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Arachnoidal involvement</th>
<th>Epidural fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>Undetectable</td>
<td>The dura is free of scar tissue</td>
</tr>
<tr>
<td>Grade 1</td>
<td>Minimal</td>
<td>Only thin fibrous bands are observed between the scar tissue and dura</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Moderate</td>
<td>Continuous adherence is observed in less than two-thirds of the laminectomy defect</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Severe</td>
<td>Scar tissue adherence is large, affecting &gt; two-thirds of the laminectomy defect</td>
</tr>
</tbody>
</table>

To determine the number of fibroblasts taken from the three separate sections on each laminectomized spine, three distinct regions on the surface area were determined as 100.000 μm². Six different areas were counted on each animal, and stereological analyses of fibroblasts were conducted according to the studies described previously. A stereological workstation composed of a digital camera (mbf/Bioscience, Qimaging), automatic controlled specimen stage, a light microscope (Leica, DM400B), and a software program (mbf Bioscience, Stereo investigator, version 9) were used to count fibroblasts. Therefore, we chose an area fraction approach with an area of an unbiased counting frame of 900 μm². Meander sampling of each sectioned fibroblast was done in a 70 μm × 70 μm step size in a systematic-random manner. For each animal, the number of fibroblasts in the selected areas listed was adapted by one mm².

**Statistical analysis**

Data analysis was performed by using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normally or not was determined by using Kolmogorov-Smirnov test. Data were shown as mean ± SD, where applicable.

The mean differences among groups were analyzed by One-Way ANOVA. When the p value from One-Way ANOVA are statistically significant, post hoc LSD were used to know which group differ from others. Chi-squared test was used for analysis of nominal (categorical) data. If p value less than 0.05, it was considered as statistically significant.

**Results**

**Wound healing and complications**

ABS and MPH did not affect wound healing in any rats. We did not observe any wound infection, abnormal foreign-body reaction, abscess formation, hematoma, or CSF leakages. All animals were ambulatory at the time of sacrifice.

**Assessment of the count of fibroblasts**

The count of fibroblast results were calculated as 984.166 ± 245.996 in the control group, 706.666 ± 163.666 in the ABS group, and 545.833 ± 183.014 in MPH group. The differences of fibroblasts count between MPH and control groups were
The grading of EF was detected as 2.33 ± 0.51 in the control group, 1.66 ± 0.81 in the ABS group, and 1.33 ± 0.51 in the MPH group. Epidural fibrosis in the MPH group was markedly lower than the control group and showed statistically significant differences (p=0.036). However, there was no statistically significant difference between the ABS and control groups (p=0.135) (Table 2) (Figures 1B, 2B and 3B).

### TABLE 2 - Result of histological analysis; the count of fibroblasts, epidural fibrosis score and arachnoidal involvement.

<table>
<thead>
<tr>
<th>Histological analysis</th>
<th>Control group</th>
<th>MPH group</th>
<th>ABS group</th>
<th>P¹:0.002, P²:0.030, P³:0.065</th>
</tr>
</thead>
<tbody>
<tr>
<td>The count of fibroblasts</td>
<td>984.16±245.996</td>
<td>545.83±183.014</td>
<td>706.66±163.666</td>
<td></td>
</tr>
<tr>
<td>Epidural fibrosis score</td>
<td>2.33±0.51</td>
<td>1.33±0.51</td>
<td>1.66±0.81</td>
<td>P¹:0.036, P²:0.135, P³:0.465</td>
</tr>
<tr>
<td>Arachnoidal involvement score</td>
<td>2.33±0.51</td>
<td>1.33±0.75</td>
<td>1.50±0.54</td>
<td>P¹:0.036, P²:0.076, P³:0.423</td>
</tr>
</tbody>
</table>

**Assessment of EF**

The grading of EF was detected as 2.33 ± 0.51 in the control group, 1.66 ± 0.81 in the ABS group, and 1.33 ± 0.51 in the MPH group. Epidural fibrosis in the MPH group was markedly lower than the control group and showed statistically significant differences (p=0.036). However, there was no statistically significant difference between the ABS and control groups (p=0.135) (Table 2) (Figures 1A, 2A and 3A).

**Assessment of arachnoidal involvement**

The gradings of arachnoidal involvement were detected as 2.33 ± 0.51 in the control group, 1.50 ± 0.54 in the ABS group, and 1.33 ± 0.75 in the MPH group. The differences in the grading of arachnoidal involvement between the MPH and control groups were statistically significant (p=0.036). Although there was no statistically significant difference, ABS group has a lower grade of AI compared to control group (p=0.076). These results indicate that both the MPH and ABS groups had an attenuated grade of arachnoidal involvement compared to control group (Table 2) (Figures 1A, 2A and 3A).

**FIGURE 1** – A. Photomicrographs demonstrating fibrosis in the control group. Direct contact between the underlying spinal cord (SC) and the epidural fibrosis tissue (F) is evident. L: Lamina; D: Dura mater, A: Arachnoid mater, Hemotoxylin & Eosin, Bar: 200 µm. B. Fibroblast density, note the increased number of fibroblast cells, Black Arrow: Fibroblast, H&E Bar: 50 µm
Discussion

Quantitative features of the formal characteristics at the fibrous tissue, such as number of fibroblasts, are usually parameters for determining density of epidural fibrosis. Stereological methods are commonly used in research of the fibroblast regarding to experimental peripheral nerve injuries model, and it was showed that the stereological analysis techniques can supply correct and trustworthy estimates of fibroblast count and epidural fibrosis. Previous studies suggested that the fibroblast cells in the fibrous tissue should be counted in three different areas, expressed as a mean value, and the fibroblast densities should be graded. In our study, to determine the number of fibroblasts, we performed a stereological morphometric analysis of the fibroblast densities for three separate sections and six different areas were counted on each animal. To the best of our knowledge, this is the first report about the stereological analyses of fibroblasts for the evaluation of the EF after laminectomy in the literature.

The first step for decreasing EF is achieving adequate hemostasis in surgical site. Bipolar coagulation and cotton are broadly using for hemostasis. However, cotton and coagulation on the surgical site may cause additional fibrosis and adhesion formation. Although both the introduction of microsurgical techniques and improvements in bipolar coagulation technology ease to hemostasis, EF after laminectomy can still occur. To overcome this problem, several topical hemostatic agents were developed to prevent bleeding and achieve adequate hemostasis. Hemostatic agents are more useful especially in the case of ooze bleeding with the risk of postoperative hematoma related to extensive EF. Hemostatic agents using in neurosurgical
interventions must have a strong hemostatic efficacy to provide durable hemostasis and also be degraded rapidly without tissue reaction or inflammation.\footnote{21,23,26-29.}

Microporous Polysaccharide Hemospheres (Arista; Medafor, Inc., Minneapolis, MN) is a novel hemostatic agent made from plant starch. It is formed into tightly engineered microporous particles with porosity and spherical diameters and was recently approved by the Food and Drug Administration for internal use. When applied to bleeding, these particles behave as a molecular screen that fastly absorb the fluid and small molecular components of blood (molecular weight, < 40,000 Da), as a result of concentrating platelets, thrombin, fibrinogen, and other proteins on the outer surfaces of the particles. This localized dehydration of blood activates endogenous clotting processes and accelerates the development of a platelet fibrin plug.\footnote{22,24} MPH has excellent efficacy for hemostasis, a rapidly cleared (by α-amylase at 6 or 12 h after its application) profile from the surgical site, and no negative effects on healing, such as fibrosis. Furthermore, it is shown that MPH reduces the incidence of surgical site infection\footnote{23-25.} and has excellent effects on healing, such as fibrosis. MPH has been mostly used in surgeries on parenchymatous organs\footnote{23,24}, but there is no information about the application of MPH after laminectomy for the prevention of EF. In the present study, we investigated the effect of MPH on fibrosis formation. Our results showed that the local application of MPH significantly diminished epidural scar formation and prevented arachnoidal involvement (control and MPH; \( p < 0.05 \)). In addition, we also found MPH treatment was associated with a significantly decreased number of fibroblast cells in the epidural scar formation at the laminectomy site (control and MPH; \( p < 0.05 \)). This effect mostly related to its active hemostatic property. We can conclude that MPH can be applied safely for hemostasis in the case of oozing bleeding into the laminectomy site with the risk of postoperative EF.

Ankaferd Blood Stopper® (ABS) (Trend Teknoloji İlaç AS, Istanbul, Turkey) is a folkloric medicinal plant extract product, which has historically been used in Turkish practical medicine as a hemostatic agent.\footnote{26-30.} It is a standardized mixture of the agent containing various ratios of five herbal extracts: Thymus vulgaris, Glycyrrhiza Glabra, Vitis Vinifera, Alpinia Officinarum, and Urtica Dioica. Each herb has effects on the endothelium, blood cells, angiogenesis, cell proliferation, and other physiologic mediators.\footnote{26-29.} It was shown that the ABS-induced network formation is related to the functions of blood proteins and red blood cells without destroying individual coagulation factors.\footnote{26-27.} The basic mechanism of action for ABS is the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation and forming a fibrin plug.\footnote{27,28.} Previous studies have performed its safety, efficacy, sterility, and non-toxicity for external usage for hemostasis.\footnote{26-29.} However, its effects on the formation of the fibrous tissue are still controversial. Some studies suggest that the topical application of ABS increased pericardial adhesion and fibrosis.\footnote{28.} In another experimental study, ABS application did not increase intra-abdominal adhesion formation.\footnote{28.} Our study demonstrated that the topical application of ABS did not increase EF after laminectomy in a rat model. Although the occurrence of fibrosis was statistically similar in both groups, the ABS-treated group showed a lower EF and AI score than the non-treated control group (For EF and AI, \( P \) value respectively: 0.135; 0.076). Furthermore, we also found significantly decreased number of fibroblast cells in the epidural scar formation with ABS treatment (control and ABS; \( p < 0.05 \)). Our results were similar with the previous studies which demonstrated the decreasing of adhesion formation after ABS application for hemostasis in the intra-abdominal surgeries.\footnote{28.} This beneficial effect is mostly related to the hemostatic and anti-inflammatory activity of ABS.

Conclusions

The topical application of microporous polysaccharide hemospheres and ankaferd blood stopper significantly reduces EF formation, dural adhesion and fibroblast cell density in an experimental rat laminectomy model. MPH and ABS, which have been previously used safely in humans for wound healing and parenchymatous organs hemostasis, could be safely used for preventing epidural fibrosis after laminectomy in humans.

References

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