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Original research article

Hemostasis vs. epidural fibrosis?: A comparative study on an experimental rat model of laminectomy

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ARTICLE INFO

Article history:
Received 15 February 2016
Accepted 9 May 2016
Available online 25 May 2016

Keywords:
Epidural fibrosis
Hemostasis
Laminectomy
Rats

ABSTRACT

Aim: The aim of this study was to evaluate the histopathological and biochemical impact and effectiveness of two hemostatic agents, Ankaferd blood stopper (ABS) and Microporous Polysaccharide Hemospheres (MPH), on epidural fibrosis in an experimental rat laminectomy model.

Material and methods: Twenty adult Wistar albino rats were divided into MPH-treated ($n = 6$), ABS-treated ($n = 6$) and control ($n = 8$) groups. Laminectomy of the lumbar spine was performed in all animals and treatment groups were exposed to MPH and ABS while closure was applied in control group as per usual. Epidural fibrosis was evaluated in all groups macroscopically, histopathologically, biochemically and with electron microscopy four weeks later.

Results: Statistically, it was found that MPH-treated group had significantly less epidural fibrosis compared to ABS-treated and control groups.

Conclusion: We compared two hemostatic agents for their propensity to cause adhesions in the present study. Our results show that MPH significantly reduces epidural scar formation and dural adhesion in a rat model of laminectomy while ABS increases postoperative fibrosis.

1. Introduction

In spinal surgery, uncontrolled bleeding may result in loss of neurological function and intractable complaints. Therefore, hemostasis is one of the most important phase of these surgeries. Specific features of the spine prevents rendering mechanical methods of haemostasis such as direct pressure and ligature. For such a long time, bipolar cautery has been the essential apparatus for coagulation of small vessels with

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http://dx.doi.org/10.1016/j.pjnns.2016.05.002

minimizing effect on adjacent tissue. However, chemical haemostatic agents were suggested preferable to bipolar cautery in spinal procedures because of some drawbacks. The complete occlusion of the vessel lumen compromising the perfusion of the neural tissue, thermal injury to adjacent structures induced by dissipiation of heat from the tips of the bipolar forceps and insufficiency in controlling the diffuse capillary bleeding were attributed to bipolar cautery and local haemostatic agents were recommended especially for diffuse capillary oozing which characterizes most intraspinal pathologies [1].

Epidural fibrosis, which is characterized as increased granulation formation and adhesive properties, continues to be a major cause of poor results in spinal surgery [2]. Scar formation increases the risk and technical difficulty of subsequent procedures in addition to persistent pain. Epidural fibrosis prevents nerve roots from gliding within the spinal canal and results in pain postoperatively. Nerve fibers are subject to compression which leads to impaired axoplasmic transport and restricted arterial supply and venous return [3,4].

A great deal of effort has been made to prevent the development of scar formation fibrosis. A variety of materials such as autogenic fat [5], Adcon-L [6], omentum graft [7], amniotic membrane [8], polytetrafluoroethylene [9] and many others have been investigated to prevent or reduce epidural fibrosis. However, results of these studies remain unsatisfactory. It is now widely accepted that, invasion of the postoperative hematoma by the dense fibrotic tissue results with epidural fibrosis. Since residual material of any nature, including blood can serve as nidus for epidural fibrosis, haemostatic agents may play a different role in pathogenesis with a dual mechanism unlike other materials or drugs. For, their presence would reduce the effect of remnants of blood while they act as foreign bodies which provoke scar formation and fibrosis.

Thus, the present study aimed to investigate histopathological and biochemical impacts of Microporous Polysaccharide Hemospheres (HaemoCer™) and Ankaferd blood stopper® on extent of spinal epidural fibrosis in the rats.

2. Material and methods

2.1. Experimental design

Twenty adult Wistar albino rats weighting 300–350 g were used in this study. The study was conducted at the Maltepe University, Faculty of Medicine, Laboratory for Experimental Animals with the approval of Animal Experiments Local Ethics Committee. All rats received human care as outlined in the ''Guide for the care and use of laboratory animals'' (National Research Council). The rats were divided in three groups randomly: MPH-treated group ($n = 6$), ABS-treated group ($n = 6$) and control group ($n = 8$). They were subjected to food deprivation 24 h before surgery, but were allowed free intake of water. Prophylactic antibiotics were not applied pre- or postoperatively.

2.2. Surgical procedure

All rats were anesthetized via the intramuscular route by 60 mg/kg ketamine (Ketalar, Pfizer, Istanbul) and 10 mg/kg xylazine (Rompun, Bayer, Istanbul). After the animals were stabilized on the operation table in prone position, surgical area was shaved and cleaned with povidone iodine solution (Drogsan, Istanbul). Following a vertical midline incision from Th11 to L3 to expose L1 vertebra, the lumbar fascia was opened bilaterally from the midline and paravertebral muscles were detached in a subperiosteal manner. L1 total laminectomy was performed under the operating microscope (Möller-Wedel, Wedel, Germany). Exposure of dura mater was carried out by the removal of ligamentum flavum and epidural fat tissue. Hemostasis was obtained with the topical application of MPH, ABS and saline in first, second and third groups respectively. Similar appearance of the surgical area was detected in all rats with regard to hemorrhage. The closure of the wound was achieved with 3/0 vicryl as per usual. The rats were sacrificed at the 30th postoperative day (4th week) with intra-peritoneal high dose (75–100 mg/kg) of Tiopental Sodium (Pentothal Sodium, Abbott, Italy). Finally, each vertebral column was resected in an en bloc fashion.

2.3. Macroscopic assessment

Macroscopic assessment was performed blindly by selecting samples randomly. Animals with dural tear, nerve root injury and infection were excluded from the study. The results were classified according to the Rydell classification (Table 1) [10].

2.4. Histopathological examination and grading

Histopathological examinations were done in Medical Biology and Histology Department Laboratory of Cerrahpasa Medical Faculty at Istanbul University. Tissue samples were fixed in 10% buffered formalin solution for 4 days and decalcified with 5% hydrochloric acid solution for 5 days. Three consequent sections from middle, proximal and distal parts of laminectomy region were taken and placed in sampling casettes. After they were washed with tap water for 3 h to eliminate acidic remnants, routine follow-up process was carried out. Afterwards, each segment was embedded in paraffin blocks and sectioned in 3 μm thick coronal slices by microtome. Hematoxylin–Eosin (H&E) and masson trichrome dye were performed for histopathological examination and sections were evaluated blindly by the histopathologist with Olympos BX61 light microscope (Olympos, Tokyo, Japan) and photographed with Olympos camera DP71 (Olympos, Tokyo, Japan). Evaluation of epidural fibrosis was performed and graded according to the definitions of He et al. [11] as summarized in Table 2. Many studies that evaluate the efficacy of the drugs or materials against epidural fibrosis, histopathological grading. The interrater reliability of this classification was tested and it

Table 1 – Macroscopic evaluation according to Rydell classification [10].

Grade 0	No scar tissue in the duramater
Grade 1	Scar tissue in the duramater but dissected easily
Grade 2	Scar tissue in the duramater, difficult dissection together with impaired duramater
Grade 3	Adhered scar tissue in the duramater and cannot be dissected

Table 2 – Histopathological classification of epidural fibrosis, according to the criteria of He et al. [11].

Grade 0	No fibrosis influencing the duramater
Grade 1	Fine fibrous bands between fibrous tissue and duramater
Grade 2	Continuous adhesion in less than 2/3 of the laminectomy defect
Grade 3	Adhesion of the fibrous tissue in more than 2/3 of the laminectomy defect and/or fibrous tissue reaches to the nerve roots

Table 3 – Fibroblast classification in reference to the Hinton criteria [13].

Grade 1	<100 fibroblast
Grade 2	100–150 fibroblast
Grade 3	>150 fibroblast

showed perfect agreement among the assessors who independently evaluated four hundred slides in a recent study [12].

Grading of histopathological epidural fibrosis was also done according to the predominance of fibroblasts. Hinton et al. defined their grading system as grade 1 (less than 100 fibroblasts), grade 2 (between 100 and 150 fibroblasts) and grade 3 (more than 150 fibrobrasts) (Table 3). Average number of fibroblasts was calculated by counting of three regions under light microscope at 40× magnification.

2.5. *Ultrastructural imaging by electron microscopy*

A solution of 4% glutaraldehyde (G5882; Sigma, St. Louis, MO, USA) in a 0.1 M phosphate buffer solution is used for fixation. After tissue samples were post-fixed in 1% OsO_4 prepared in the same buffer, they were dehydrated with graded ethanol (Merck, Darmstadt, Germany), and embedded in araldite (G4901; Sigma, St. Louis, MO, USA). Ultra-thin sections of 50-nm thick were taken using an ultramicrotome (Reichert UM 2 and UM 3, Austria) and positioned on cupper grids (200 mesh), stained with uranyl acetate and lead citrate. Sections were analyzed with a transmission electron microscope (JEM-1011, Jeol Tokyo, Japan) and Olympos Soft Imaging camera system (Tokyo, Japan).

2.6. *Biochemical evaluation*

Biochemical evaluations were carried out in Biochemistry Department Laboratory of Cerrahpasa Medical Faculty at Istanbul University. The tissues were weighed and washed in 0.9% NaCl. A piece of tissue sample were stored at −80 °C until assayed for levels of myeloperoxidase (MPO) and hydroxyproline (HP).

2.7. *Preparation of tissue samples*

About 190–200 mg of each tissue sample was weighed and diluted 20%w/v in 20 m Mice-cold Tris–HCl, pH 7.4, and homogenized with a Bosch Scintilla SA (Switzerland). The homogenate was centrifuged at 5000 × *g* for 10 min, and biochemical parameters were performed in the supernatant fraction. All biochemical parameters were studied on the same day.

2.8. *Measurement of myeloperoxidase (MPO) activity*

Measurement of MPO activity was done with a commercially available kit (MPO, Rat, ELISA kit Catalog no: HK105-02, Hycult Biotech, Frontstraat 2A5405 PB, Uden, The Netherlands). The coefficients of intra-and inter-assay variations were 4.0% ($n = 15$) and 5.1% ($n = 15$), respectively.

2.9. *Measurement of hydroxyproline (HP) levels*

Measurement of HP levels was done with a commercially available kit (Hydroxyproline Assay Kit, Catalog No: MBS162747, My Bio Source, San Diego, CA 92126, USA). The coefficients of intra- and inter-assay variations were 3.6% ($n = 15$) and 4.8% ($n = 15$), respectively.

2.10. *Statistical analysis*

All values are expressed as mean ± SD. Levels of hydroxyproline, myeloperoxidase activity and fibroblast counting of groups were compared using Kruskal–Wallis one-way ANOVA and Friedman two way ANOVA tests using UNISTAT 5.0 for Windows (Istanbul University). $p < 0.05$ was considered as statistically significant. Multiple post hoc comparisons were also carried out among the groups, and each group and the Dunn-test was performed.

3. Results

3.1. *Macroscopic evaluation*

Postoperative period of all rats was uneventful and none of them had wound infection, neurological deficit or cerebrospinal fluid leakage. In MPH-treated group, weak fibrotic adhesions were observed in epidural region where laminectomy was performed. It did have loose texture permitting dissection easily. In control and ABS-treated groups, rigid and intensive epidural adhesions were detected and dissection of them was difficult. According to Rydell classification [10], the difference between MPH-treated group and the other groups was found to be statistically significant ($p < 0.01$) (Table 4).

3.2. *Histopathological evaluation*

In control group; compact connective tissue formed by the collagen fibers which were arranged in different directions had much irregular blood vessels in it. Dura mater was comparatively thick at laminectomized side. Compression to spinal cord was detected (Fig. 1A). In the ABS-treated group same diagnosis were observed as control group (Fig. 1B and C). Partially adhesion of compact fibrotic tissue was seen in MPH-treated group and compression to spinal cord in laminectomized region was supposed to be less according to light microscopy (Fig. 1D and E).

According to the specifications of He et al. [11], three rats had grade I, two rats had grade II, and one rat had grade III epidural fibrosis in MPH-treated animals. In control groups, one rat was at grade II, seven rats were at grade III. Two rats were at grade II, and four rats were at grade III in

Table 4 – The grades of epidural fibrosis on macroscopic evaluation according to Rydell classification.

Grade	Control group ($n = 8$)	MPH-treated group ($n = 6$)	ABS-treated group ($n = 6$)
Grade 0	0 (0%)	1 (16.6%)	0 (0%)
Grade 1	2 (25%)	2 (33.3%)	1 (16.6%)
Grade 2	2 (25%)	2 (33.3%)	2 (33.3%)
Grade 3	4 (50%)	1 (16.6%)	3 (50%)

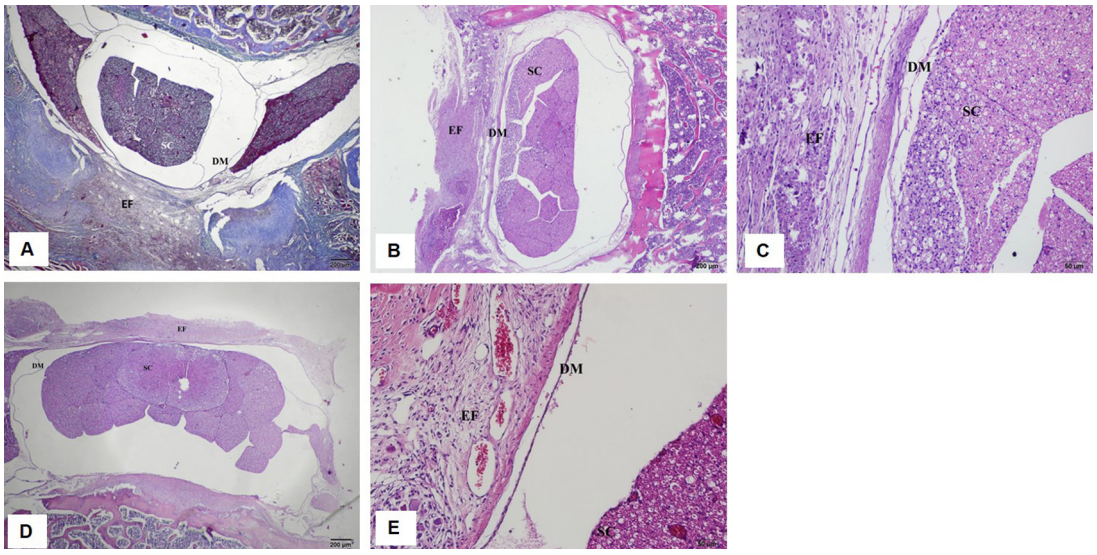

Fig. 1 – Histopathological examination of the epidural fibrosis. Control (A), ABS-treated groups (B and C) and MPH-treated groups (D and E). SC, spinal cord; DM, dura mater; EF, epidural fibrosis. H&E staining. Bar: A, B, D; 200 μm–C, E; 50 μm.

Ankaferd-treated animals. The variation of histopathological grades was statistically significant between MPH-treated group and others ($p < 0.001$). Thus, MPH was found to significantly reduce epidural fibrosis at laminectomy sites.

According to Hinton criteria [13], number of fibroblasts was calculated. Among the members of control group, two rats were at grade II and six of them were at grade III while two rats were at grade II and four rats were at grade III in Ankaferd-treated group. However, three rats were at grade II, two rats were at grade III and one rat was at grade I in MPH-treated group. Decrease of fibroblast numbers in MPH-treated group was found statistically significant when compared to Ankaferd-treated and control groups ($p < 0.05$) (Table 5).

Table 5 – Number of fibroblasts according to Hinton criteria.

Groups	Hinton fibroblast counting
Control	204.66 ± 20.36
ABS-treated group	207.45 ± 20.36
MPH-treated group	139.29 ± 11.07*

(Mean ± SD). The MPH-treated group showed the least fibroblast counting level.
* $p < 0.001$, compared with control and ABS-treated group.

3.3. *Ultrastructural assessment by electron microscopy*

Proper lineage of mature collagen fibers and active fibroblasts were observed in the control group (Fig. 2A). The collagen fibers of Ankaferd group were less mature and there were edematous areas between the collagen fibers (Fig. 2B). The collagen fibers of the HaemoCer group compared to Ankaferd group were more immature and edematous areas between the fibers were pronounced better. In the fibrous area, more polymorphonuclear cells were detected in the MPH-treated group when compared to other groups (Fig. 2C).

3.4. *Evaluation of hydroxyproline (HYP) and myeloperoxidase (MPO)*

HYP and MPO levels were determined in epidural scar tissue for all groups (Fig. 3). It was detected that HYP level in MPH-treated group was 11,297.14 ± 1131.1 ng/gr while it was 17,993.50 ± 2394.1 ng/gr in ABS-treated group and 15,002.97 ± 3013.1 in control group. When compared to control group, results which were lower in MPH-treated group and conversely higher in ABS-treated group, showed statistically significant differences for both comparisons ($p < 0.05$). Lower myeloperoxidase levels were detected in both MPH-treated and ABS-treated groups as 14,326.76 ± 1726.9 and 12,543.30 ± 3977.7 respectively while it was 17,984.75 ± 4599.0 for control group. These decrements were also found

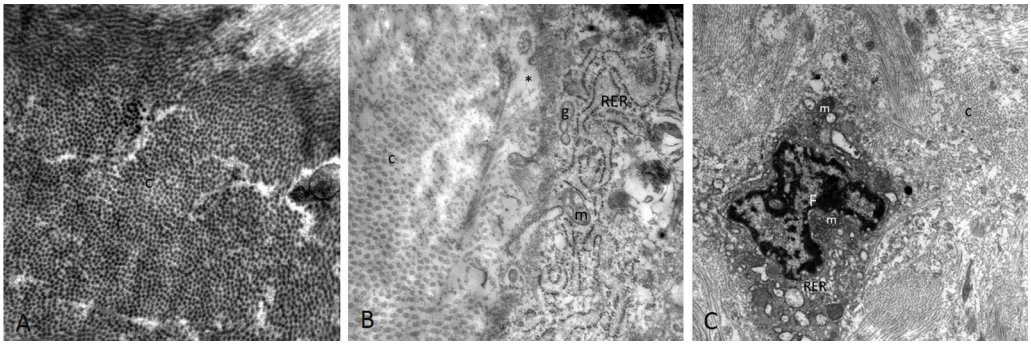

Fig. 2 – Ultrastructural evaluation of the epidural fibrosis. Properly located mature collagen fibers in the Control group (A), Expanded RER, golgi and swollen mitochondria with the absence of crista in the active fibroblast cytoplasm. Edematous areas between the collagen fibers in the ABS-treated group (B), Expanded RER, golgi and swollen mitochondria with the absence of crista in the active fibroblast cytoplasm. Collagen fibers are located properly and various directions in the MPH-treated group (C). (Magnification: A, B ×40,000, C ×15,000.) C, collagen fibers; RER, rough endoplazmic Reticulum; m, m itochondria; g, Golgi; *, edematous areas; F, fibroblast.

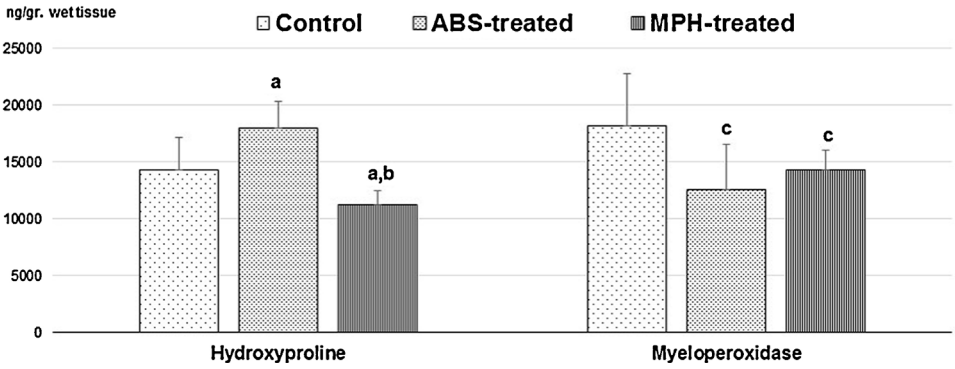

Fig. 3 – HYP and MPO levels in epidural scar tissue for all groups. $^{a,c}p < 0.05$, $p < 0.01$ vs. control group, $^{b}p < 0.001$ vs. ABS-treated group.

statistically significant when compared to control group ($p < 0.001$).

4. Discussion

Effective hemostasis is mandatory for spinal surgery. Because of thermal side effects of electric tissue coagulation and difficulty in management of diffuse hemorrhage, hemostatic agents are needed. Fibrin was used topically as a hemostatic agent for the first time in surgery by Bergel [14]. Until now, several topical hemostatic agents have been commonly used by neurosurgeons. Additionally, they are often used for tissue adhesion and tissue engineering.

In this study, multiple parameters were used to evaluate the effects of two haemostatic agents on epidural fibrosis in rat model after laminectomy. MPH is a new powder hemostat, composed of tightly engineered microporous particles with closely controlled porosity. Although not well-documented in the literature, absorption of the fluid components of blood and concentration of platelets and clotting factors were thought to be the mechanisms of hemostasis by MPH. Murat et al. who were the first to describe MPH also stated that it activates clotting cascade and hyperconcentrates platelets and coagulation proteins, while enhancing a hemostatic plug [15]. Without depending on coagulation cascade, a gel matrix, produced by the blood cells and proteins results in accelerated blood clotting [16].

Effects of MPH were evaluated in several experimental studies [17]. It has been reported that MPH does not have severe side effects on rabbit brain tissue [18]. Hemostatic effects of MPH in brain were evaluated in a preliminary report [19] and Tschan et al. have investigated the safety and efficacy of MPH in patients with brain tumors and suggested that it allows effective hemostasis without adverse reactions [16].

Ankaferd blood stopper® (ABS) (Ankaferd Drug Cosmetics Inc. Co., Istanbul, Turkey) which has been used as a local haemostatic agent against various types of bleeding, is composed of the plant extracts; Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum and Urtica dioica. It is thought to show effects on endothelium, blood cells,

angiogenesis, cellular proliferation, vascular dynamics and cell mediators instead of coagulation factors [20]. Formation of an encapsulated protein network that allows for erythrocyte aggregation is stimulated by ABS. In our study, its effects on epidural fibrosis were compared with HaemoCer™ (BioCer, Bayreuth, Germany), a novel plant-based MPH which are manufactured from herbal-based polysaccharides.

It has been suggested that postoperative hemorrhagic collection at he operation site and formation of seroma give rise to epidural fibrosis [21,22]. LaRocca and Macnab postulated that fibroblasts originating from traumatized paraspinal muscles infiltrate and replace the epidural hematoma with granulation tissue formation [3]. Granulation tissue then matures into epidural fibrosis due to extensive organization [23]. A prospective, randomized study which was conducted over 10 years from 2002 to 2012 has revealed that suction drains improve patient outcome and reduce epidural fibrosis significantly even when they are used without any medication [24]. In the same study, the amount of postoperative hematoma and its transformation into fibrous tissue were suggested as main targets to minimize the risk of epidural fibrosis. In this regard, hemostasis would be the main subject to take into consideration, if we plan to deal with epidural fibrosis.

However, haemostatic agents may act as precipitating factors for epidural fibrosis because of foreign body reaction. This view leads to a dilemma of using them also to prevent epidural fibrosis or avoiding their usage for less scar formation.

There are several studies reporting the effects of MPH and ABS on epidural fibrosis. It was shown that ABS decreased the occurence of inflammation and necrosis while increasing new bone formation. But it did not decrease fibrosis significantly [25]. ABS increased postoperative pericardial adhesions and fibrosis scores in experimental rabbit model [26]. Likewise, Behcet et al. have reported that ABS is not efficient in preventing intraabdominal adhesions. It was suggested that adhesions were increased with the amount of ABS used, on the contrary [27]. In a rat peritoneal model, activated starch microspheres revealed lower adhesion scores when compared with other hemostatic agents [28]. It was also reported that residual presence of MPH was markedly decreased contrasting with the other agents in another study on rat brain [29].

MPH was found to be more effective than ABS in terms of hemostasis time and amount of bleeding in a study of experimental rabbit epistaxis model [30]. Another study of partially nephrectomized rat model has revealed that HaemoCer leads less tissue reaction when compared with ABS [31]. Herbal-based hemostatic agents cause no inflammation and foreign body reaction while other chemical hemostatic agents do. For, MPH could be degraded completely by alpha amylase approximately 6 h after the application [32]. This short lived clot may account for the results, making it a more suitable agent to prevent epidural fibrosis. Rapid degradation of MPHs was supposed to be another advantage compared with other topical agents [16]. In a recent study, Emon et al. have demonstrated that MPH (HaemoCer) has preferential effects on epidural fibrosis after laminectomy. The results of their study have suggested that MPH does not enhance epidural fibrosis when compared to laminectomized mice without hemostatic material [33].

Multiple parameters including Rydell classification, number of fibroblasts according to Hinton criteria, the histopathological analysis and fibrosis grading according to He et al. were used to evaluate the effects of MPH and ABS in the present study. It is well known that collagen plays an important role in every stage of wound healing with regulatory and stabilizing functions on the forming tissue [34]. Amount of hydroxyproline increases in this process and hydroxyproline (HYP) level is known to be a significant parameter for determination of fibrosis such as myeloperoxidase activity (MPO) [35,36]. In our study HYP and MPO levels were determined in epidural scar tissue for all groups in addition to ultrastructural evaluation with electron microscopy.

Macroscopic examinations revealed that 50% of the rats in ABS-treated and control groups showed grade 3 fibrosis according to Rydell classification while the ratio was only 16.6% in MPH-treated rats for the same grade ($p < 0.01$). In the histopathological examinations, it was detected that fibrous tissue developed over duramater was thinner in MPH-treated group when compared to others and number of fibroblasts were decreased in MPH-treated group significantly as well. Ultrastructural assessment by electron microscopy have demonstrated that fibrous tissue formation was significantly prevented in the MPH-treated group, supporting the results obtained from other similar studies. The hydroxyproline levels of the MPH-treated group were very low at the end of the 4th week, while they were increased in the In the ABS-treated group. Interestingly, MPO was decreased in both treatment groups when compared with control group.

These data show that MPH significantly reduces epidural scar formation and dural adhesion in a rat model of laminectomy while ABS increases postoperative fibrosis. Our results were valuable in terms of MPH's strong hemostatic effects and reduced foreign body reaction. The main standpoint of our study is the absolute neccessity of hemostasis. We suppose that it would be better to prevent epidural fibrosis by avoiding primary factors rather than applying special drugs or materials.

5. Conclusion

Our study reveals that MPH might be a better agent than ABS in spinal surgery considering epidural fibrosis. We suppose that these data could help to improve the design of new hemostatic agents in the future.

Conflicts of interest

The authors have no the conflict of interest about that article.

Acknowledgement and financial support

None declared.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals.

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