

EXPERIMENTAL STUDY

Preventative effect of diclofenac sodium and/or diltiazem in rats with epidural fibrosis

Erdogan B¹, Is M², Aker FV³, Emon ST⁴, Engin T⁵, Akar EA⁴, Sayman E³, Somay H⁴

Sanliurfa Training and Research Hospital, Department of Neurosurgery, Sanliurfa, Turkey.
dr.baris.erdogan@gmail.com

ABSTRACT

OBJECTIVE: Spinal epidural fibrosis is commonly seen after laminectomy. There is not yet proven any agent preventing fibrosis in clinical usage. We used diclofenac sodium and diltiazem, which are fibrosis inhibitors.

METHODS AND MATERIALS: 40 rats were divided into four groups of equal numbers: control, diclofenac sodium, diltiazem, and diclofenac sodium + diltiazem. Laminectomies were performed at L5 and L6. After a 4 week period, the rats were decapitated and the vertebral column blocks were removed for histopathologic examination. Fibrosis percentage, spread of fibrous regions, and fibroblast numbers were evaluated in each group and compared between the groups.

RESULTS: The distribution of epidural fibrosis density, percentage of fibrosis, and distribution of fibroblasts in the diclofenac sodium + diltiazem group were significantly lower than in the other groups. The fibroblast numbers of the diltiazem, and diclofenac sodium + diltiazem groups were significantly lower than in the other groups.

CONCLUSION: Diclofenac sodium + diltiazem used together provided better outcomes because each of them prevented fibrosis via different ways, probably through synergistic action (Tab. 5, Fig. 3, Ref. 43). Text in PDF www.elis.sk.

KEY WORDS: diltiazem, diclofenac sodium, epidural fibrosis, laminectomy.

Abbreviations: CMC/PEO – Carboxy Methyl Cellulose/ Poly Ethylene Oxide, C – Control, COX-2 – Cyclooxygenase-2, DK-Na – Diclofenac sodium, DTZ – Diltiazem, DTZ + DK-Na – Diltiazem + diclofenac sodium, EF – Epidural fibrosis, FBSS – Failed Back Surgery Syndrome, FGF-2 – Fibroblast growth factor-2, IL-6 – Interleukin 6, IL-8 – Interleukin 8, MS – Medulla spinalis, NSAID – Non-Steroidal Anti-inflammatory Drugs, PGE – Prostaglandin, PGE-1 – Prostaglandin E1, PGE-2 – Prostaglandin E2, TGF- β 1 – Transforming Growth Factor Beta 1, TNF – Tumor necrosis factor

Introduction

Epidural fibrosis (EF) is the development of the deposition of dense scar tissue adjacent to the dura mater, which distorts normal tissue architecture following laminectomy (1). Intra-operative

dura mater injury, nerve root damage, excessive intra-operative bleeding cause EF. Epidural fibrosis accounts for approximately 8–14 % of failed back surgery syndrome (FBSS) (2). EF presents either as painless or with a severe pain in the back and legs. When epidural fibrosis tissue is compared to normal tissue, interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- α , and Transforming Growth Factor Beta 1 (TGF- β 1) are observed to increase in the EF tissue. Prostaglandin E1 and E2, and leukotriene B were found to contribute to the accumulation of these substances (3, 4).

Many methods have been used to prevent EF. Non-steroidal anti-inflammatory drugs (NSAIDs) (5), calcium channel blockers (6), pegaptanid sodium (7), mitomycin-C (2), hemostatic polysaccharide agents have been administered (8). All-trans retinoic acid (9), human amniotic fluid-sodium hyaluronate (10), poly (D, L-lactic acid-co-glycolic acid)-b-poly (ethylene glycol)-b-poly (D, L-lactic acid-co-glycolic acid) thermogel (11), etanercept (12), dekorin (13), CMC/PO (14), licofelone (15), temozolamide (16), polymethylmethacrylate (17) and hyperbaric oxygen (18) are examples of other agents that have been studied in the treatment of spinal EF.

Diclofenac sodium acts by inhibiting cyclooxygenase-2 (COX-2) (19, 20). Some studies have shown it to modulate collagen synthesis by inhibiting prostaglandin E2 (PGE-2) and reducing fibroblast proliferation in colon cells. Diclofenac sodium reduces the numbers and mechanical strength of fibroblast in local wound healing (21–23). Diltiazem was shown to inhibit fibroblast growth factor-2 (FGF-2), reduce numbers of heart fibroblast cells, pro-

¹Sanliurfa Training and Research Hospital, Department of Neurosurgery, Sanliurfa, Turkey, ²Health Sciences University, Fatih Sultan Mehmet Training and Research Hospital, Department of Neurosurgery, Istanbul, Turkey, ³Health Sciences University, Haydarpasa Numune Training and Research Hospital, Department of Pathology, Istanbul, Turkey, ⁴Health Sciences University, Haydarpasa Numune Training and Research Hospital, Department of Neurosurgery, Istanbul, Turkey, and ⁵Health Sciences University, Mehmet Akif İnan Training and Research Hospital, Department of Neurosurgery, Sanliurfa, Turkey

Address for correspondence: B. Erdogan, MD, Sanliurfa Training and Research Hospital, Department of Neurosurgery, Yenice yolu- Eyyübiye, Sanliurfa, Turkey.
Phone: +904143171717

Tab. 1. He et al's grading system.

Grade 0	Dura free of scar tissue
Grade 1	Only thin fibrous bands between the scar tissue and dura
Grade 2	Continuous adherence in less than two-thirds of the laminectomy defect
Grade 3	Significant scar tissue adherence affecting more than two-thirds of the laminectomy

According to their system; grade 0 = dura is free of scar tissue, grade 1 = only thin fibrous bands between the scar tissue and dura, grade 2 = continuous adherence in less than two-thirds of the laminectomy defect, and grade 3 = significant scar tissue adherence affecting more than two-thirds of the laminectomy defect

liferate human tendon fibroblasts and reduce fibroblast linkage, increase the amount of superoxide dismutase in heart fibroblast cells and assist remodeling after a cardiac injury (24–28). In light of these studies, we considered that diclofenac sodium and/or diltiazem might reduce EF. It was planned to use diclofenac sodium and diltiazem together and separately in our study.

Materials and methods

Our experimental study was conducted after receiving the approval from the Marmara University Animal Experiments Local Ethics Committee (protocol code: 044.2016 date: 06/06/2016) at Marmara University Experimental Animal Laboratory (DEHAM). Histopathologic examinations were performed in the Haydarpaşa Numune Training and Research Hospital Pathology clinic.

The rats were divided into 4 groups: control (C), diltiazem (DTZ), diclofenac sodium (DK-Na) and diltiazem + diclofenac sodium (DTZ + DK-Na). The rats were anesthetized using an intraperitoneal injection of ketamine hydrochloride (Ketalar 100 mg/kg (ip), Pfizer, Istanbul) and xylazine hydrochloride (Rompun 10 mg/kg (ip), Rompun 2 %, Bayer, Istanbul, Turkey). The rats were placed on an operating board in the prone position. After the L5 and L6 skin level was detected, the skin was shaved. The surgical field was disinfected using povidone-iodine (POVIDO 10 % polyvinyl pyrrolidone-iodine complex, Saba, Turkey) and

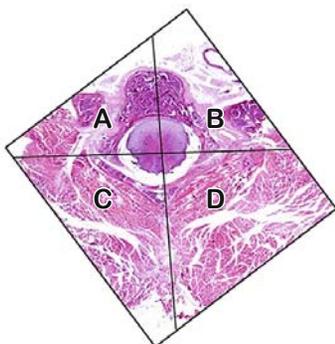


Fig. 1. We modified Lubina et al.'s grading system. We used a light microscope instead of MRI. We divided an axial vertebra into four regions. One line between the middle of the corpus vertebra to the spinous process of the vertebra, and another line between the two transverse processes of the vertebra. We named the regions as A, B, C, and D. We calculated the percentage of fibrosis for each region and as a total.

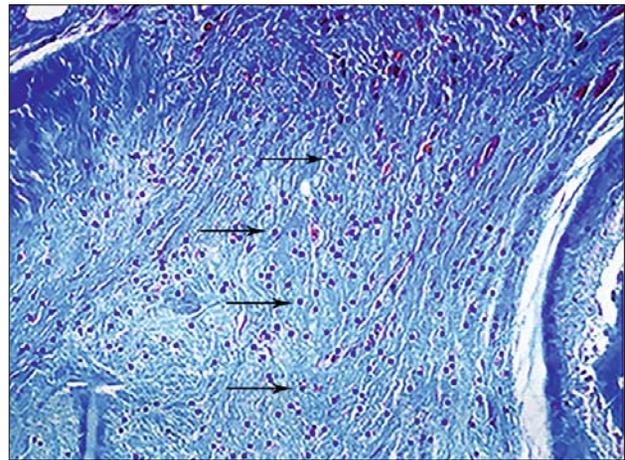


Fig. 2. We calculated the number of fibroblasts in digital system, which stained with Masson's trichrome examples. The arrows show fibroblasts.

draped with sterile towels. A midline dorsal skin incision was made using a number-15 blade and continued down to the spinous process. The paravertebral muscles were bluntly dissected. L5 and L6 lamina were exposed. Laminectomy was performed on the L5 and L6 vertebrae. The dura mater was exposed. In the control group, the dura mater was only irrigated with saline. In the diltiazem group, cotton pads (4 x 4 mm²) soaked with DTZ 5 mg/mL were applied to the exposed dura mater for 5 minutes. The cotton wool was then removed and the laminectomy site was irrigated with saline. In the DK-Na group, cotton pads (4 x 4 mm²) soaked with DK-Na 25 mg/mL were applied to the exposed dura mater for 5 minutes. The cotton wool was removed and the laminectomy site was irrigated with saline. In the DTZ + DK-Na group, cotton pads (4 x 4 mm²) soaked with 5 mg/mL DTZ and 25 mg/mL DK-Na were applied to the exposed dura for 5 minutes. The cotton wool was then removed and the laminectomy site was irrigated with saline. Following hemostasis, the wound was closed in accordance with the anatomy using staples. There were no complications or adverse effects from the surgery. The rats were taken to their cages.

The rats were closely monitored for dehydration, infection, body weight, feeding, and wounds in the post-operative period. Post-dressings were made daily until the 7th day and staples were removed on the 7th day. Intra-peritoneal saline was administered to dehydrated animals. Seven rats were lost in the postoperative period. The study continued with the remaining 33 rats. The rats were decapitated on post-op day 28 using a guillotine. The rats were examined at the Haydarpaşa Numune Training and Research Hospital Pathology Clinic for histopathologic changes.

Histopathological examination and classification

The vertebral colon was removed as a block containing the L5 and L6 levels. The entire sampling/lesion area was sampled in coronal planar sections. Samples were fixed in 10 % buffered formaldehyde for 48 hours. Afterwards, the samples were taken to a "short decalcification solution" and the bone tissue was allowed to soften. Samples with an appropriate softness were taken

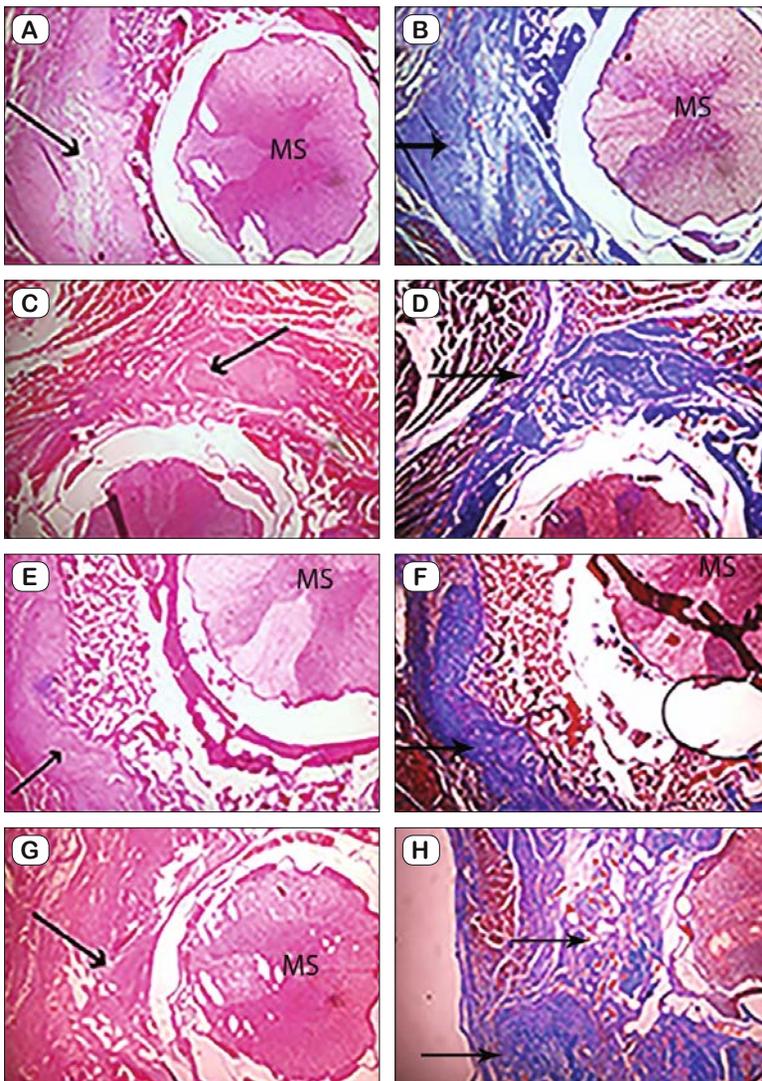


Fig. 3. A) The pictures show an example of DTZ. The picture is stained with hematoxylin and eosin. The arrows show fibrosis, which occur after trauma. B) The pictures show an example of DTZ. The picture is stained with Masson's trichrome. The arrows show fibrosis, which occur after trauma. C) The pictures show an example of control. The picture is stained with hematoxylin and eosin. The arrows show fibrosis, which occur after trauma. D) The pictures show an example of control. The picture is stained with Masson's trichrome. The arrows show fibrosis, which occur after trauma. E) The pictures show examples of DTZ + DK-Na. The picture is stained with hematoxylin and eosin. The arrows show fibrosis, which occur after trauma. F) The pictures show examples of DTZ + DK-Na. The picture is stained with Masson's trichrome. The arrows show fibrosis, which occur after trauma. G) The pictures show examples of DK-Na. The picture is stained with hematoxylin and eosin. The arrows show fibrosis, which occur after trauma. H) The pictures show examples of DK-Na. The picture is stained with Masson's trichrome. The arrows show fibrosis, which occur after trauma. MS – Medulla spinalis. Hematoxylin-eosin and Masson's trichrome X40.

following a routine tissue examination. After 18 hours of routine tissue processing, the tissue was embedded in paraffin blocks and cut to a thickness of 4–5 microns. It was stained using hematoxylin and eosin. In addition, a special histochemistry stain, Masson's trichrome, was used in selective blocks that represented the best of

the lesion area in order to assess fibrosis. Sections were evaluated by a light microscopy.

We modified Lubina et al.'s grading system. We used a light microscope instead of magnetic resonance imaging (MRI) for epidural fibrosis assessment. We evaluated EF using He et al.'s grading system and our modified Lubina grading system (29, 30) (Tab. 1, Fig. 1). In addition, we calculated the number of fibroblasts in each group and compared the findings between the groups (Fig. 2).

Statistical investigations

Statistical analysis was performed using the NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA) program. The Kruskal–Wallis test was used for comparison of non-normally distributed data using descriptive statistical methods (mean, standard deviation, median, frequency, ratio), and the Mann-Whitney U test was used to determine the differences between the groups. The Fisher–Freeman–Halton test was used to compare qualitative data. The results were evaluated with a confidence interval of 95 % and a significance level of $p < 0.05$.

Results

The He grade of all animals in the DTZ + DK-Na (Fig. 3 E, F) group were grade 1 and significantly lower than those of the control (Fig. 3 C, D), DK-Na (Fig. 3 G, H), and DTZ groups (Fig. 3 A, B, Tab. 2). The modified Lubina grading system percentages in the DK-Na + DTZ group were significantly lower than those of the control, DK-Na, and DTZ groups ($p = 0.004$, $p = 0.013$, and $p = 0.038$, respectively) (Tabs 3 and 4). The numbers of fibroblasts in the g DTZ + DK-Na group were significantly lower than in the control and DK-Na groups ($p = 0.033$, $p = 0.003$, respectively). The number of fibroblasts in the DTZ group was significantly lower than in the DK-Na group ($p = 0.028$, $p < 0.05$) (Tab. 5).

Discussion

Epidural scar tissue develops following a spinal surgery, when epidural fat is replaced by a hematoma. This hematoma is absorbed and replaced by granulation tissue (31, 32). Dense EF causes nerve root irritation, entrapment, restriction of nerve root mobility, and a direct dural compression, which is the main reason for pain in the back and leg (33). Postoperative epidural fibrosis following a lumbar disc surgery significantly increases the hazards of the revision of spine surgery and contributes to the occurrence of FBSS. FBSS can be seen in 10–40 %

of lumbar spine operations. Postoperative pain is correlated to EF amounts (34, 35).

Several methods were used to prevent EF. These methods include microdiscectomy, anti-inflammatory drug use, various biologic and synthetic materials (oil graft, amniotic membrane,

synthetic polymeric materials, silk-polyethylene glycol hydrogels, sodium hyaluronate, pegaptanid sodium, gelatin sponge + dexamethasone, fibrin glue, hybrid chitosan membrane, Adcon-L and mitomycin C) (7, 36–40). Despite many studies, most have been found to be only mildly or not at all effective. However, given that laminectomy is the most common neurosurgery procedure, studies are ongoing.

In our study, DK-Na potent analgesic agent widely used in clinical practice, and DTZ, which has been successfully used in coronary artery disease and hypertension, were used separately and together. No studies in literature have used DTZ and DK-Na to prevent EF. Diclofenac sodium and DTZ were used for the first time in this study. Diclofenac sodium inhibits the conversion of arachidonic acid to prostaglandins (PG) by inhibiting COX-2. The PG family, PGE-2 in particular, leads to cell proliferation, apoptosis, angiogenesis, and inflammation. PGE-2 is a potent pro-inflammatory. PGE-2 has also been shown to play a role in the etiopathogenesis of cancer (41–44). Diltiazem reduces the

Tab. 2. Assessment of groups according to the He grading system.

		Groups			
		Control	DK-Na	DTZ	DTZ + DK-Na
He	Grade 1	3 (37.5)	4 (57.1)	3 (37.5)	10 (100)
	Grade 2	4 (50.0)	2 (28.6)	4 (50.0)	0
	Grade 3	1 (12.5)	1 (14.3)	1 (12.5)	0

p 0.031*

Fisher-Freeman-Halton test *p < 0.05

There were statistically significant differences between the groups according to He grading system (p < 0.05). We compared the groups with each other to determine, which group or groups caused the differences. The DTZ + DK-Na group had only grade 1 and it was found significantly less than in the other groups.

Tab. 3. Distribution according to modified Lubina Grading system.

		Modified Lubina Grading system						p	
		Negative	1	2	3	4	Mean+SD		Median
C Region	Control	0	1	3	4	0	2.38+0.74	2.5	0.005**
	DK-Na	0	1	4	1	1	2.29+0.95	2	
	DTZ	0	3	4	1	0	1.75+0.71	2	
	DTZ+DK-Na	2	6	2	0	0	1+0.67	1	

*Kruskal–Wallis test **p < 0.01

Our modified Lubina grading system showed region C had a statistically significant difference (p < 0.05). We compared the groups with each other to determine, which group or groups caused the differences. The DTZ + DK-Na group's median value was statistically significantly lower than the control, DK-Na, and DTZ groups (0.003; p = 0.007; p = 0.042, respectively)

Tab. 4. Assessment of percentage values according to groups in the modified Lubina Grading system.

		Groups			
		Control	DK.Na	DTZ	DTZ+DK.Na
Modified Lubina Percent (%)	Mean+SD	32.03+12.25	29.54+12.36	27.41+16.02	15.63+6.75
	Min-Max	12.5-50	12.5>50	12.5–62.5	6.25–25
	Median	31.25	31.5	25	12.5
^a p					0.016*
^b Control- DTZ+DK-Na					0.004**
^b DK-Na – DTZ+DK-Na					0.013*
^b DTZ- DTZ+DK-Na					0.038*

*Kruskal–Wallis test, ^bMann–Whitney U test *p < 0.05 **p < 0.01

The modified Lubina system's percentage measurement showed a statistically significant difference (p < 0.05). We compared the groups with each other to determine, which group or groups caused the differences. The DTZ + DK-Na group has a statistically significantly lower modified Lubina system's percentage than the control, DK-Na, and DTZ groups (p = 0.004; p = 0.013; p = 0.038, respectively)

Tab. 5. Assessment of fibroblast number according to groups.

		Groups			
		Control	DK-Na	DTZ	DTZ+DK-Na
Fibroblast number	Mean+SD	474.13+180.43	483.14+110.22	330.5+104.71	310.4+64.9
	Min-Max	195–705	296–648	234–538	234–393
	Median	489.5	506	284.5	305.5
^a p					0.014*
^b Control – DTZ+DK-Na					0.033*
^b DK-Na – DTZ					0.028*
^b DK-Na – DTZ+DK-Na					0.003**

*Kruskal–Wallis test, ^bMann–Whitney U test, *p < 0.05, **p < 0.01

Fibroblast numbers showed a statistically significant difference (p < 0.05). We compared the groups with each other to determine, which group or groups caused the differences. DTZ + DK-Na group's fibroblast number was statistically significantly lower than in the control and DK-Na groups' (p = 0.033; p = 0.003). The DTZ group's fibroblast numbers was statistically significantly lower than the DK-Na group (p = 0.028; p < 0.05)

reaction of inflammation in the damage of certain tissue. Histochemical analysis of tissue has shown a reduction of TNF- α and IL-8, both of which have pro-inflammatory effects (4). Thus, DTZ reduces inflammation. DTZ and DK-Na reduced inflammation in different ways, showing synergistic action; used together, both provided better outcomes.

In our study, the combined DTZ + DK-Na group had a significantly lower EF density, had a significantly lower fibrosis percentage, and had significantly less fibrosis in the C region of the vertebra than in other groups (Fig. 1). We determined that the DTZ + DK-Na group and the DTZ group had significantly lower fibroblast numbers than the DK-Na group and the control group.

We believe that DTZ and DK-Na were more effective when they were used together because they inhibited inflammation in different ways.

Conclusion

Laminectomy is the most commonly used method in neurosurgery practice. Post-operative persistent back and leg pain is recognized as FBSS. FBSS, caused by EF, adversely affects the quality of patients' lives socially, as well as economically and psychologically.

We assessed DK-Na and DTZ in the prevention of EF. Both are cheap and readily available. We determined that by the administration of these drugs prior to closing the surgical area, the formation of EF was significantly reduced. However, neither DK-Na nor DTZ have been used in the treatment of EF. Our findings are based on experiments performed on a limited number of animals only; any effect on humans at this stage is unknown. More studies of this area are required before there may be any potential clinical use of these entities with the objective of reducing EF.

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