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Vitamin D3 has antifibrotic effects on rats with epidural fibrosis

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ABSTRACT

Objective: One unfavorable outcome of spinal operations is epidural fibrosis. Recurrent back pain and radiculopathy could negatively impact the patient's quality of life. In this study, using a rat laminectomy model, we sought to examine any potential antifibrotic benefits of vitamin D3.

Patients and Methods: Control, local, and systemic vitamin D3 groups (n = 6 rats per group) were randomly assigned. Laminectomy alone was carried out in the control group. Vitamin D3 application onto the dura was done in the local D3 group. Intraperitoneal delivery of the systemic vitamin D3 was carried out in the systemic vitamin D3 group. Epidural fibrosis was studied macroscopically and histopathologically four weeks later.

Results: The macroscopic epidural fibrosis score was lower in the local and systemic vitamin D3 groups compared to the control group ($p<0.001$, for both groups). Comparing the local and systemic vitamin D3 groups to the control group, fibrosis thickness was significantly decreased ($p<0.01$, for both groups).

Conclusion: Vitamin D3 could be an alternative treatment for preventing spinal epidural fibrosis.

Keywords: Epidural fibrosis, Laminectomy, Vitamin D3

1. INTRODUCTION

Laminectomy is a widespread surgical procedure used to treat patients with spinal stenosis, and partial laminectomy is carried out during lumbar disc herniation operations [1]. However, epidural fibrosis (EF) following laminectomy can result in back and leg pain that is resistant to treatment because of strain on the dura mater and nerve roots [2]. EF is still not always preventive or treatable, despite studies to develop antifibrotic drugs [3-5].

A physiological process called fibrosis development frequently takes place during normal wound healing. However, several situations, both before and after laminectomy, could result in more fibrosis [6]. Inflammatory cells enter the surgical field due to oxidative stress and inflammatory mediators brought on by surgical trauma and tissue damage [7]. Because these inflammatory cells release cytokines, local fibroblasts grow as a result. Components of the extracellular matrix, such as fibronectin and collagen, are produced when fibroblast proliferative activity and stimulation are too high [8]. These overly-produced compounds alter the natural tissue structure, which promotes the growth of a significant amount of scar tissue and compresses neurological structures [9].

Vitamin D3 is a neuroactive steroid, that controls glutathione metabolism, cytokine release, and antioxidant activity, and reduces nitric oxide synthase expression [10-12]. Vitamin D3 may also have a positive impact on each of the four stages of fibrosis development, including tissue remodeling, wound healing, the inflammatory phase, fibroblast migration and differentiation, and fibrosis [13]. Vitamin D3 has antioxidant, anti-apoptotic, anti-inflammatory, and antifibrotic properties; thus, using the rat laminectomy-EF model, we investigated the antifibrotic efficacy of the local and systemic vitamin D3 treatment.

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2. MATERIALS and METHODS

Experimental Animals

Each of the experimental methodologies used in this investigation was reviewed and approved by the University of Health Sciences' Regional Animal Research Ethics Committee. (20.11.2020, 06.06). All aspects of animal care and testing were conducted under the European Union Council Directive (86/609/EEC), which addressed the protection of animals used in research. 18 female Sprague-Dawley rats weighing between 150 and 250 g were used in the investigation. Each rat was kept in a room that had a 12-hour light cycle, adequate humidity, and temperatures that were kept between 22 and 25 °C.

Rats were placed into three groups at random and given unrestricted access to food and water as follows:

Laminectomy (Control) Group (n = 6): Below is a description of the laminectomy surgery that was performed on the rats. After achieving adequate site hemostasis, the procedure was completed without administering any topical or systemic medications to the rats in this group. On the 30th day following the operation, subjects were sacrificed using the methods described below.

Local Vitamin D3 Group (n = 6): Following laminectomy and adequate site hemostasis, the rats in this group received 0.5 µg of vitamin D3 (Ostriol, VEM Ilaç, Istanbul, Türkiye) topically applied to the dura at the laminectomy site. The procedure was completed once it was confirmed that the administered solution had lingered in the area for at least five minutes. On the 30th day following the procedure, subjects were sacrificed using the methods described below.

Systemic Vitamin D3 Group (n = 6): After laminectomy and achieving adequate site hemostasis, the procedure was completed without giving any topical treatment to the rats in this group. Starting immediately after the procedure, a single intraperitoneal dose of 0.5 µg of vitamin D3 (Ostriol, VEM Ilaç, Istanbul, Türkiye) was administered for ten days. The dosage of calcitriol was selected from a previously published study [12]. The approaches described below were used to sacrifice subjects on the 30th day after the surgical procedure.

Surgical procedure

To prevent surgical site infections, intramuscular cephazolin sodium 20 mg/kg was given before surgery. The rats received intraperitoneal injections of 10 mg/kg of xylazine (Rompun, Bayer, Turkiye) and 50 mg/kg of ketamine (Ketalar, Parke Davis, Turkiye) to induce anesthesia. With a rectal probe installed and then lying on a heating pad, the animals' body temperatures were kept at 37°C.

On the operating table, the rats were kept flat. The surgery sites were cleansed with povidone after shaving their lower backs. A longitudinal midline skin incision over the L1-L3 levels was made following sterile isolation. The lumbosacral fascia was incised, the L2 laminae were revealed, and the paravertebral muscles were subperiostally dissected. At the L2 level, a laminectomy was performed to expose the dura mater and epidural spaces, and the ligamentum flavum and epidural fat tissue were removed from the surgical site. The wounds were anatomically repaired with 4-0 prolene polypropylene sutures after topical medications were administered in local treatment group. In the control and systemic treatment groups, no topically administered treatments were used. Under a surgical microscope, each treatment was meticulously carried out to safeguard the neurological structures by identical surgeons (BG, PKB).

Following surgery, the rats were given unlimited access to food and liquids for four weeks. The rats were terminated by an intraperitoneal injection of 50 mg/kg ketamine (Ketalar, Parke Davis, Turkiye). The laminae, dural sacs, nerve roots, and paravertebral tissues, such as the muscles and skin, were removed from the first to third lumbar vertebrae.

Macroscopic assessment of epidural fibrosis

A blinded neurological surgeon used the Rydell classification to evaluate the EF after precisely reopening the surgical sites for a macroscopic evaluation from 0 to 3, the grade increase means more epidural fibrosis. This classification scheme includes the following grades [14]:

Grade 0	No scar tissue in the dura mater,
Grade 1	Scar tissue in the dura mater but dissected easily,
Grade 2	Scar tissue in the dura mater, difficult dissection and impaired dura mater,
Grade 3	Adhered scar tissue in the dura mater and cannot be dissected.

Histopathological Evaluation

The upper L1 to lower L3 levels of the spine were divided axially to isolate the laminectomy area for histological examination. The detailed procedure was described previously [15]. A blinded pathologist performed the histopathological evaluations. The extent of any inflammation, fibrosis, and other histological changes and the development of scar tissue were all determined. The parameters for evaluating EF microscopically were presented by Bozkurt et al. in 2019 [4]. The grading scale number increased with fibroblast cell density increase [4].

Statistical analysis

GraphPad Prism 8.0 was used to analyze the data. (GraphPad Software Inc., La Jolla, CA, USA). The differences between the groups were compared using a one-way analysis of variance, followed by the Tukey posthoc test. A p-value of <0.05 was used to determine statistical significance. Confidence interval was 95%.

3. RESULTS

Wound healing and complications related to the procedure

Within the local vitamin D3 group, one fatality occurred. There were no hematomas, erythema, infections, or leaks of cerebrospinal fluid. No neurological deficit was observed at 4 weeks.

Macroscopic evaluation of epidural fibrosis

In the laminectomy group, severe epidural adhesions (n = 5 grade 3 and n = 1 grade 2) were observed. Mild and moderate epidural adhesions (n = 3 grade 1, n = 2 grade 2) were observed in the local vitamin D3 group. Mild and moderate epidural adhesions (n = 5 grade 1, n = 1 grade 2) were observed in the systemic vitamin D3 group. The EF severity score was decreased statistically significantly in the local and systemic vitamin D3 groups when compared with the laminectomy group ($p<0.001$ for each comparison) (Figure 1).

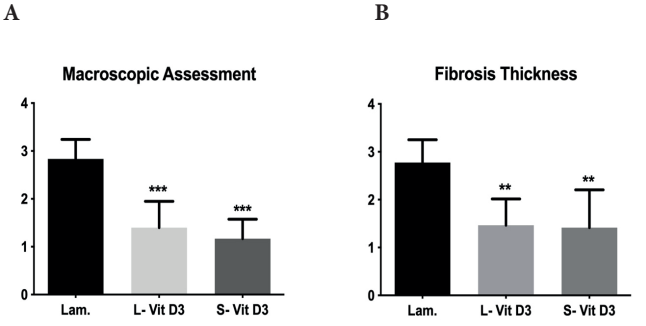

Figure 1. *Macroscopic assessment of epidural fibrosis score of study groups. Data are presented as mean ± SD. After laminectomy, severe epidural adhesions were observed in the laminectomy group. A. Macroscopic epidural fibrosis score was decreased statistically significantly in the local and systemic vitamin D3 groups when compared with the laminectomy group (***: p<0.001). B. Fibrosis thickness was significantly lowered in the local and systemic vitamin D3 treatment groups (**: p<0.01). (L: local, Lam: Laminectomy, S: Systemic, Vit D3: vitamin D3).*

Histopathologic Assessment

Microscopic EF scores were not different among local and systemic vitamin D3 groups when compared with the laminectomy group ($p>0.05$, for both comparisons). Fibroblast cell density classification scores were not different among local and systemic vitamin D3 groups when compared with the laminectomy group ($p>0.05$, for both comparisons). Mean scores for fibrosis thickness were decreased in local and systemic vitamin D3 groups when compared to the laminectomy group ($p<0.01$, for both comparisons). (Table I). Cartilage destruction was seen in one rat in the local vitamin D3 group, and bone destruction was seen in one rat in the systemic vitamin D3 group. Foreign body and mild chronic inflammation responses were also not significantly different among groups ($p>0.05$) (Figure 2).

Table I. *Microscopic evaluation results of experimental groups*

	Control	L-Vit D3	S – Vit D3
Epidural fibrosis grade	Grade 3:n=4 Grade 2:n=2	Grade 1:n=1 Grade 2:n=2 Grade 3:n=3	Grade 1:n=1 Grade 2:n=3 Grade 3:n=2
Mean fibroblast grade	Grade 3:n=3 Grade 2:n=3	Grade 2:n=5	Grade 2:n=5 Grade 3:n=1
Fibrosis thickness (mm)	2.8±0.48	1.5±0.55	1.4±0.79
Cartilage destruction	none	n=1	none
Bone destruction	none	none	n=1
Foreign body reaction	n=3	n=0	n=4
Mild chronic inflammation	n=2	n=1	n=1

Abbreviations: L: local, S: Systemic, Vit D3: vitamin D3.

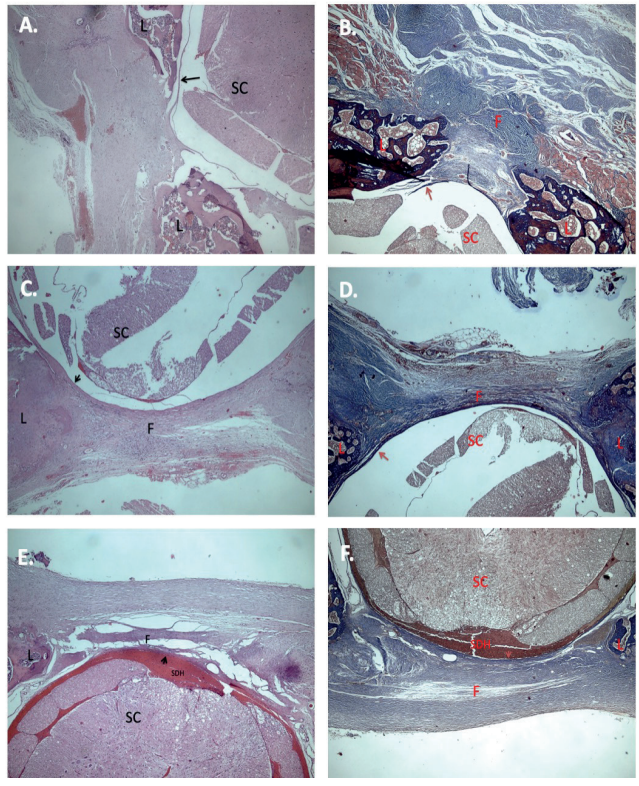

Figure 2. *Representative photomicrograph of the epidural fibrosis analysis of the study groups.* ***A-B:*** *In the control group moderate (A), and severe (B) epidural fibrosis is observed (Fibrosis grade: 2 and 3). L: Laminae, F: Fibrosis, SC: Spinal Cord, Arrow: Dura Mater (A) hematoxylin and eosin: H&E, x40. (B) MT: Masson trichrome, x40.*
C-D: *In the local vitamin D3 group mild epidural fibrosis is observed (Fibrosis grade:1). L: Laminae, F: Fibrosis, SC: Spinal Cord, Arrow: Dura Mater. (C) H&E, x40, (D) MT, x40.*
E-F: *In the systemic vitamin D3 group moderate epidural fibrosis is observed as a thin layer of fibrosis band (Fibrosis grade:2). L: Laminae, F: Fibrosis, SC: Spinal Cord, SDH: Subdural Hemorrhage, Arrow: Dura Mater (E) H&E, x40, (F) MT, x40.*

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4. DISCUSSION

In this study, the local and systemic vitamin D3 treatment lowered EF, as shown by macroscopic analysis. Both the local and systemic vitamin D3 groups showed a significant decline in fibrosis thickness scores. In the literature, this is the first time vitamin D3 was reported to exert antifibrotic effect in rat model of EF.

The nerve root is compressed by sticky scar tissue after laminectomy in EF, which results in recurrent radicular pain and physical disability [2]. Up to 50% of individuals who underwent laminectomy, experienced long-term poor symptom relief or a recurrence of symptoms [5]. According to Erbayraktar et al., EF operations have a high rate of complications and a low rate of success [16]. As a result, research on prevention has garnered interest in favor of surgical treatment, and several procedures and drugs to stop the fibrosis-causing process have been investigated [3-5, 16-23]. Reduced tissue cellularity and increased extracellular matrix component deposition (including collagen, dermatan sulfate, and fibronectin) are two contributing causes in the etiopathogenesis of EF [24]. The etiology of EF involves adhesions that form as a result of the fibrotic process, reparative inflammation, and exceptionally severe cicatrization [25]. Transforming growth factor-β1 (TGF-β1) production is one of the main processes that initiates the production of EF [8, 26,27].

Vitamin D is a fat-soluble vitamin, and it performs important functions in the body's calcium and phosphate absorption and bone-building mechanisms. However, vitamin D is not just a simple vitamin, its pleiotropic activity has been demonstrated in many diseases. It has effects on cell proliferation, differentiation, immunomodulation, apoptosis, intracellular signal transduction, oxidative stress, and inflammation from many different pathways [28-31]. Its inhibitory effects on fibrosis have been shown in previous studies [13, 32-35]. It has been shown that vitamin D has a reducing effect on TGF-β1 levels [36-38]. The primary isoform of the TGFβ superfamily is TGF-1, which acts as a pro-fibrotic switch to activate myofibroblasts [39]. Additionally, it controls the expression of α-SMA (smooth muscle actin) and its targets, including extracellular matrix components [39]. Thus, this mechanism may explain its potent antifibrotic activity. In a recent idiopathic pulmonary fibrosis study, it was also stated that TGF-β1 was the main regulator when vitamin D3-related antifibrotic actions were investigated [40].

In our study, we showed that vitamin D3 has antifibrotic actions when given locally and systemically in a rat model of EF. The strength of this study is that the histopathological data clearly show the ameliorative effect of vitamin D3 in the treatment groups. However, there are also some limitations to this study. Our ability to conclude is constrained by the small number of rats per group, notably in the local vitamin D3 group. Our conclusions are also limited because no biochemical tests were conducted. Further investigation with biochemical parameters could enhance our understanding of the mechanism of action of vitamin D3 in EF.

Conclusions

Macroscopic EF formation was inhibited, and fibrosis thickness scores were decreased in the local and systemic vitamin D3 groups. Our findings offer the first experimental proof of the protective abilities of vitamin D3 against EF.

Compliance with Ethical Standards

Ethical approval: Each of the experimental methodologies used in this investigation was reviewed and approved by the University of Health Sciences' Regional Animal Research Ethics Committee (approval number: 20.11.2020, 06.06). All aspects of animal care and testing were conducted under the European Union Council Directive (86/609/EEC), which addresses the protection of animals used for experimental purposes.

Conflict of interest: The authors declare that there is no conflict of interest.

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Authors contibutions: PKB, BG: Conceived and planned the experiments, PKB, JH, AS, AB and BG: Carried out the experiments, PKB and AS: Contributed to sample preparation, PKB, AS and BG: Contributed to the interpretation of the results, PKB: Took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors approved the final version.

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