The stereological and histopathological evaluation of the effect of Thymoquinone on peridural fibrosis following laminectomy in rats

Abstract

Background/aim: This study was aimed to investigate the effects of thymoquinone, which is the essential bioactive component of the volatile oil of Nigella sativa on the peridural fibrosis in rats following laminectomy.

Materials and methods: Twenty female Wistar Albino rats were used in our study. The rats were randomly divided into two groups as Sham and Surgery+Thymoquinone. Both groups underwent laminectomy at L1, under general anesthesia. The sham group was not subjected to any drug application. The second group was treated intraperitoneal 10 mg/kg thymoquinone per day for a period of 28 days, following the same surgical procedure. All the groups were sacrificed after four weeks and the laminectomy area was examined in terms of new bone volume, capillary volume and fibrosis volume using stereological approaches.

Results: Statistically significant differences were found between the Sham and Surgery+Thymoquinone groups, in terms of the new bone volume (p=0.01), capillary volume (p=0.01) and fibrosis volume (p<0.001). It was noted that thymoquinone caused significant increase in the new bone volume and vascular volume and a significant decrease in the fibrosis volume.

Conclusion: The results of our study indicate that thymoquinone is effective in decreasing the peridural fibrosis when applied on laminectomy model.

Keywords: Laminectomy, peridural fibrosis, thymoquinone
1. **Introduction**

Annually, a great amount of people undergoes surgery of the lumbosacral region, making this operation one of the most applied treatments for the spinal disorders [1,2]. The failed back syndrome is identified as the persistent pain in lower back, leg, hip, and thigh in the patients who went through the surgical procedure of laminectomy. It is also named “post-laminectomy syndrome” (PLS) [3]. Inappropriate application of the surgical procedure, the disruption of the internal disc, spinal stenosis and peridural fibrosis are among the possible causes of the failed back syndrome [4,6]. The peridural fibrosis is known as one of the most common causes of the failed back syndrome and possesses characteristic features such as the accumulation of scar tissue between the dura mater and the surrounding tissues [7]. The formation of the scar tissue causes the nerve roots to become more prone to be damaged by disc protrusions by restricting their movement. Peridural fibrosis is suggested as one of the primary causes of pain following spinal surgeries [8]. In the literature, there are no established treatment of the peridural fibrosis, and some painkiller drugs are utilized for the alleviation of the painful periods [9]. As the granulation tissue is formed following surgery, peridural fibrosis may increase the incidence of surgical complications [10].

Thymoquinone is the bioactive compound of the volatile oil of black cumin (C_{10}H_{10}O_{2}; 2-isopropyl-5-methyl-1,4-benzoquinone). Thymoquinone is acquired from the seeds of *Nigella sativa* that is bound up Ranunculaceae family [11,12]. Thymoquinone has been used for various purposes such as antioxidant, anti-inflammatory, immunomodulator, anticancer, antimicrobial, antihistaminic, and antineoplastic [13-15]. Thymoquinone inhibits the cyclooxygenase (COX) and lipoxygenase (LO) pathways, which are the enzymes that mediate inflammation [16]. Moreover, it was determined that
thymoquinone inhibit the capacity to produce cellular LO which is synthesized for L-arginine in the presence of the enzyme nitric oxide synthase, is associated with several disorders. Thymoquinone is known to inhibit the NO production with a percentage of %95 [17,18].

Several studies about the peridural fibrosis can be found in the literature. Some previous researches indicated that antineoplastic agents like mitomycin C, 5-Fluorouracil, cyclosporin reduce peridural fibrosis although there are side effects and the cost is high. Furthermore, there are studies focusing on the external particle radiation but no beneficial effect on the peridural fibrosis was determined. The solution for this problem of peridural fibrosis is continuously being researched [19-21], but a standard approach and treatment protocol are not to be established.

In this study, we aimed to investigate the possible effects of intraperitoneal application of thymoquinone, which is the basic bioactive compound of the Nigella sativa volatile oil, on the experimental laminectomy model of rats with histopathological and stereological methods.

2. Materials and methods

2.1. Experimental animals

This study was performed following the ethical approval of XXX University (2014/30), in XXX University Experimental Animals Research Center. In this experimental study, 20 female Wistar Albino rats weighting between 280-300 gr were used. All the subjects were kept in the rooms with stable temperature and humidity, fed with normal tap water and rat pellet without any limitation and additional food. The histopathological and
stereological evaluations of the samples were performed in the Department of Histology and Embryology laboratories of Medical Faculty in Ondokuz Mayis University. Two groups were determined in the experimental design as: Sham group: Ten rats were subjected to operational laminectomy procedure at L1 level, and surgery + Thymoquinone group: Ten rats were subjected to same laminectomy operation. After the operation, Thymoquinone was applied intraperitoneally for 28 days with a dose of 10 mg/kg/day [22].

2.2. Procedure
For prophylactic purposes, a single dose of 50 mg/kg ceftriaxone (Rocephine, Roche, Turkey) was applied intraperitoneally 30 minutes before the operation. The general anesthesia was established in rats, which were not given any food one-night prior, with the intraperitoneal injection of the mixture of Ketamine (50 mg/kg) (Ketalar, Eczacıbaşı, Turkey), Xylazine (10 mg/kg) (Rompun 2% Bayer, Turkey). The anesthesia was regulated to keep rats unresponsive to painful stimuli without interrupting their spontaneous respiration and additional doses were given when required.

The formation of the laminectomy model:
The rats were fixed by their extremities and tails with prone position, on specially manufactured wooden platforms. Then the surgical areas were shaved, the disinfection was performed with povidone iodine solution (POVİOD; % 10 polyvinylpyrrolidone-iodine complex, Saba, Turkey). The operational area was covered with sterile coverings. A three-cm skin incision was made on the spinous processes with lumbar 1 vertebra in the middle of the incision and then the paraspinous muscles were removed with blunt dissection. Small automatic retractors were used to uncover laminas and spinous
processes. With the help of the surgical microscope (Leica RM 2135, Leica Instruments, Nussloch, Germany), duramater was exposed by making a total laminectomy to L1 vertebra (Figure 1, A, B). The layers were properly closed in all groups following the hemostasis [23].

**The intraperitoneal application of thymoquinone:**

The surgery day was noted as day zero and immediately after intraperitoneal thymoquinone was applied to Surgery + Thymoquinone group subjects with a dose of 10 mg/kg/day for 28 days.

Sham and Surgery + Thymoquinone groups were both handled in their own cages. At the end of the four weeks, they were sacrificed with intraperitoneal high dose (75-100 mg/kg) Thiopental sodium (Pentothal Sodium, Abbott, Italy). During the collecting of the samples, the animals were checked for dura tear, nerve damage and infection. There were no spotted dura tear or infection in the studied rats. The vertebral columns were removed and were put in 10 % formaldehyde for histological tissue processing.

**2.3. Histological analysis**

The vertebrae samples obtained from the subjects were kept in 10% formaldehyde solution for post fixation for one week and then decalcified in 5% formic acid solution for 21 days. The light microscopical routine histological tissue processing steps were performed of the samples following decalcification. Ten micrometer sections in thickness were taken from every paraffin block in accordance with the stereological approaches as the results of the pilot study and stained with Hematoxylin and Eosin.

The section sampling fraction was determined as ¼ and the sections were taken in 10 µm thickness. The point density of the grid was determined according to the coefficient of error [24]. The volume estimations of the new bone, new capillary and fibrosis in the
laminectomy area were performed according to Cavalieri method (Figure 2, A-D) using
the following formula [25].
Area = a/p (µm x µm) x (ΣP) µm²
a/p: area between two points
ΣP: the total number of points intersecting with the area of interest
Volume=t x a/p x ΣP

2.4. Statistical Analysis
The SPSS 20 (IBM Corporation for Mac) program was used for the statistical
evaluation of the data and the data analyzed by using student’s t test. p values lesser
than 0.05 was accepted statistically significant.

3. Results
The new bone volumes of the Sham and Surgery+Thymoquinone groups are found as
1.495±0.075 (Mean±SEM) µm³ and 2.265±0.133 µm³, respectively. There is significant
difference between the groups (p= 0.01) (Figure 3, A, B).
Regarding the new capillary volume, it is found as 0.780±0.131 µm³ in Sham and
1.897±0.09 µm³ in Surgery+Thymoquinone groups, respectively. There is statistically
significant difference between the groups (p=0.01) (Figure 3, A, B).
The mean fibrosis volume is found as 3.151±0.147 µm³ in Sham group and 1.936±0.095
µm³ in Surgery+Thymoquinone group, respectively. Resemble to the previous
parameters, there is also significant statistical difference between the groups (p<0.001)
(Figure 3, A, B).
3. **Discussion**

Lumbar pain is one of the most common complaints and is the second most common reason of seeking professional medical help [26]. It is estimated that approximately 200,000-350,000 patients in USA undergo lumbar surgery due to reasons such as discogenic back pain, trauma, tumor, spondylolisthesis, and spinal stenosis [20]. In the laminectomy area, the fibroblast population increases due to activation of the inflammatory cytokines and growth factors for the repair of local defective vertebral area and large amounts of collagen fibers are formed. With the occurrence of the collagen fibers, fibroblasts become fibrocytes. The scar tissue is formed from fibrous connective tissue [27]. The compression of the nerve cord due to peridural fibrosis may lead to the occurrence of an unwanted clinical result following the surgery [4,28]. The scar tissue that might follow the surgical operation is possible to cause constriction of the nerve root and stenosis [29]. Although it is not thoroughly enlightened how the clinical symptoms and adhesion formation are related, peridural fibrosis is associated to many cases of failed back surgery [28]. Beside the radicular symptoms due to the nerve constriction, the scar formation may also cause increased durotomy during revision surgeries [30]. The process of fibrosis is regulated by the myofibroblast cells as they produce collagen fibers [31]. The exact mechanism behind the formation of peridural fibrosis after surgery is not yet clearly uncovered. A previous study reported that the laminectomy membrane is formed due to the invasion of fibroblasts from spinal muscles [32]. Peridural adhesion following the spinal surgery might lead to the
secretion of vasoactive factors and inflammation which consequently result in collagen
formation and the infiltration of fibroblasts and macrophages.

Thymoquinone, which is the basic component of the volatile oil of *Nigella sativa*, has
the formula of \((C_{10}H_{10}O_2; 2\text{-isopropyl-5-methyl-1,4-benzoquinone})\) and is being used
for years as an antioxidant, anti-inflammatory and antineoplastic agent [13,14]. In their
study, Awad et al. stated that thymoquinone is promising agent for defense against
fibrosis, hepatic steatosis, oxidative stress and inflammatory apoptosis [33]. Moreover,
it was also previously suggested that thymoquinone is a scavenger of free radicals and
superoxide radicals and it protects the functionality of various antioxidant enzymes
[34]. In their study, Pourgholamhossein et al. demonstrated that thymoquinone is
protective against the lung fibrosis as it displays features of oxidative stress inhibition
and profibrotic gene down regulation [35]. In addition, thymoquinone is being used for
cancer treatment [36]. Ahmad et al. found that thymoquinone has potential to be a
chemo preventive agent in cancer studies and use in anti-tumor therapeutic paradigms
[37].

Thymoquinone is used in the management of inflammatory and autoimmune diseases,
as it inhibits NO production approximately 95% [17]. Also, thymoquinone has an
inhibition effect on COX and LO pathways in the arachidonic acid metabolisms of rat
peritoneal leucocytes [38]. Besides, in an allergic airway inflammation model in rats,
thymoquinone resulted inhibition of PGD2 and COX-2, resulting an anti-inflammatory
effect [39].

In the current study, the aim was to assess the effect of thymoquinone application
following laminectomy on the new bone formation. When the results evaluated, it was
detected that thymoquinone application causes significant increase in new bone volume
and vascular volume (p=0.01) whereas it causes significant decrease in the fibrosis volume (p<0.001). These results are in agreement with the results of the previous study of Wirries et al. in which they suggested that thymoquinone may be induce osteogenesis [40]. In a previous study, Kirui et al. showed that sustained thymoquinone application increases bone healing [41]. Also, there are several studies demonstrated that thymoquinone application has protective and beneficial features against the spinal cord ischemia/reperfusion injury as it decreased the concentration of the oxidative products, as well as the reduction of the neuropathic pain in parallel to our results [42,43].

Masson’s Trichrome staining is one of the best ways to show peridural fibrosis. However, in this study, we showed quantitative evaluation of peridural fibrosis stereologically, therefore we prefer H&E staining as this staining method is feasible for the stereological estimation.

Although further physiological, biochemical and electron microscopical examinations such as investigating hydroxyproline level are required, our study may guide future studies and contribute to the scientific literature. It is also important to compare the effect of thymoquinone with a standard drug or investigate the local use of thymoquinone as a different group in future studies. We have the opinion that presented results may enlighten the way for researchers studying in this area.

Conflict of Interest

Authors declare no conflict of interest.

References


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Figure 1: Photo of the paravertebral muscle and fascia (A) and exposure of the spinous process after the incision (B).
Figure 2: The application of the Cavalier’s principle on the sections. Point counted grid (A) was used to perform stereology. Every point represents a certain area (A and B) and after putting the point counted grid on the section, the counting was performed to obtain total area of the related parameters (C and D).
**Figure 3:** The representative histological images show the laminectomy areas in Sham (A) and Surgery+Thymoquinone (B) groups respectively (Arrowheads: Capillaries exist in the fibrosis area (F), Black arrow: New bone, L: Lamina).