Effects of phenytoin injection on vocal cord healing after mechanical trauma: An experimental study

Introduction: Phenytoin is an anticonvulsant drug and also causes fibroblast proliferation, collagen synthesis and increase in epidermal growth factor. Therefore the aim of the present study is to evaluate the effect of phenytoin injection in the wound healing process in rats with vocal cord injury by histopathological methods.

Material and methods: After damaging the vocal cords of ten Albino-Wistar rats bilaterally, left vocal cord is kept as the control group. Phenytoin was injected to right vocal cord. Ten rats sacrificed. Histopathologically, thickness of lamina propria, density of fibroblast and collagen were evaluated.

Results: Thickness of lamina propria was 18,0±7,1 μm in the control group, 65,5±10,7 μm in phenytoin group. The density of fibroblast and collagen were statistically lower in the control group, compared with the phenytoin group (p<0,05).

Conclusion: Phenytoin injection in rats after vocal cord injury significantly increases the thickness of lamina propria and density of fibroblast and regular and matur collagen in lamina propria. The findings in our study provide a feasible scientific view, for adding phenytoin treatment to vocal cord surgeries in otolaryngology practice, but further studies are needed in order to evaluate the use of phenytoin in preventing the formation of scar tissue and possible effects on vocal cord vibration in humans after vocal cord injury.

Keywords: Phenytoin, vocal cord, wound healing, rat
Introduction

Vocal cord injury is a significant problem which affects the quality of life and causes severe voice disturbances [1]. Scar tissue usually occurs after the surgery, radiotherapy, and inflammation [2]. This leads to decreased viscoelasticity of the vocal fold lamina propria (increased stiffness), which reduces the vibration potential of the vocal folds [3]. Voice is often breathy or aphonic and the phonation threshold pressure is elevated [4].

There isn't any recently used, effective treatment preventing vocal cord injury. The treatment is usually difficult and may include voice therapy by a speech and language therapist and injection augmentation. Many substances have been tried. In order to discover an effective treatment modality, incidents occurring in early stages after vocal cord injury should be known. Fibrin coat is seen in the injury site on the first day; fibrin coat leaves its place to cellular aggregation on the third day of the wound healing process after trauma. New collagen production occurs on the fifth day, and mature collagen aggregation occurs on the seventh day [5,6].

Agents like autologous fat, hepatocyte growth factor, fascia, collagen, hyaluronic acid, and hydroxyapatite and platelet-rich plasma have been used to treat vocal cord injury in literature. However, the efficacy of these treatments is usually limited, hence these treatments are unable to recover the normal distribution of extracellular matrix components [7-10].

Phenytoin (Diphenylhydantoin Sodium – DpH) is an anticonvulsant drug used in grand mal and psychomotor epilepsy, but its positive effects on wound healing are mentioned in recent publications. In a review, it was found that phenytoin causes fibroblast proliferation, collagen synthesis and increase in epidermal growth factor, with the same mechanism causing gingival hypertrophy, as a side effect of systemic phenytoin use and induces new vessel formation, speeds the decrease of microbial colonies, reduced pain and inflammation and improves healing [11]. In various studies, it was found that topical phenytoin has important positive effects in the recovery period of skin ulcerations, burns and diabetic wounds [12]. Furthermore, it was reported that phenytoin decreases wound contraction and development on scar tissue [13].

In this study, we aimed to evaluate the effect of phenytoin injection in the recovery of vocal cord injury in rats.
Material and Methods:

Study design:

Ethical approval for animal experimentation was obtained from Ankara University, Animal Experimentation Ethics Committee with approval number 2011-119-461. Ten adult male healthy Albino-Wistar rat weighted 250-270 gram from Hıfzısiah Laboratory were used. Rats were rested under the appropriate temperature and humidity for 48 hours in order to adapt after transportation. Rats were kept in an environment with humidity of 65-70%, and temperature of 22±2° C, under 12 hours of light and 12 hours of dark and, free food and water supplement were provided in Ankara University, Faculty of Veterinary, Surgery Clinic. Every clause in Helsinki Final Act consisting experimental studies was complied.

Administration of Drug and Anesthetics:

Each rat received 60mg/kg intraperitoneal (i.p.) ketamine hydrochloride (Ketalar®, Eczacıbaşı Parke-Davis, İstanbul, Türkiye) and 10 mg/kg i.p. Xylazine HCl (Alfazyne®, Alfasan International B.V. Woerden, Netherlands). Rectal temperature was measured regularly under anesthetics, and they were replaced onto warm blankets in order to keep their body temperature at 35 °C. Endoscopic direct laryngoscopy was performed with 30 degrees 2.7 mm pediatric telescope, the right vocal cord was considered as phenytoin group and left vocal cord was considered as control group (Figure 1.) The lesion was made with Richards 1 mm cup forceps by the same surgeon on the 1/3 front, 2/3 back meeting point of both vocal cords (Figure 2). Consequently, phenytoin was administered with 0.05 ml ppd injection to lateral of the lesion made to the upper side of the right vocal cord. 15 days later, rats were sacrificed for histopathological examination.

Histopathological Examination

Histopathological evaluation was performed by the pathologists who were blinded for study groups. Vocal cords were removed by dissection while preserving integrity (Figure 3). After fixation in 10% formaldehyde solution, it was decalcified in 10% formic acid solution for 24 hours. Macroscopic sampling was performed with the supervisor. The material was processed as a whole in order to evaluate the vocal cord. After Routine paraffin process, parameters mentioned below
were evaluated in hematoxylin-eosin (H & E) and Masson-Trichrome staining with Olympus BX 50 ® light microscope using 5 mm thick sections. The density of collagen and fibroblast in lamina propria was measured by dividing each vocal cord cross-section into 20 unit areas and calculated as a percentage.

**Statistical analysis**

Data were analyzed with SPSS for Windows 11.5 package program. Shapiro Wilk test was used to determine whether the distribution of continuous variables was close to normal. Descriptive statistics were shown as mean ± standard deviation or median (minimum-maximum) for continuous variables. The difference of the mean thickness of lamina propria between the control and study groups was evaluated with the dependent t-test, and the difference of median values of collagen and fibroblast densities was evaluated by Wilcoxon Sign test. Results with p <0.05 were considered statistically significant.

**Results**

Thickness of lamina propria was 18,0±7,1 µm in the control group, 65,5±10,7 µm in phenytoin group. The thickness of lamina propria was significantly higher in the phenytoin group than the control group (p <0.001; Figure 4). Density of collagen was 36.5%(25,0-40,0) in the control group, 77,0% (72,0-83,0) in phenytoin group and density of fibroblast was 30,0%(25,0-35,0) in the control group, 60,0% (60,0-70,0) in phenytoin group. There was a statistically significant difference between the groups regarding both collagen and fibroblast density, and the intensity levels in the control group were found to be lower than the phenytoin group (p = 0.005; Figure 5; Table ).

**Discussion**

In adults, vocal cords have a structure of three layers, epithelium, lamina propria (superficial, intermediate and deep layer) and musculus vocalis. The superficial layer of lamina propria and epithelium are essential for vibration and phonation. In particular, surgical trauma affects these two layers. Acute response after injury is vital in the healing process. Proliferative phase starts 2-3 days after injury and ends after 2-3 weeks [8]. Interfering with the formation of neomatrix by altering the cell phenotype can change the functional results of damaged vocal cord.
Different animal models were used in studies about vocal cord scar. Rats have advantages over other models; it has been shown that the rat vocal cord lamina propria has similar characteristics with the human vocal cord lamina propria. Lamina propria of rats is divided into three layers, and the deep layer contains more collagen fibers than the superficial layer [14].

Various agents have been used to accelerate the wound healing after vocal cord injury. Yıldız et al. used estradiol and dexamethasone in the rat model with vocal cord injury. They didn’t find a significant difference when compared with the group receiving saline [15]. In another study, basic fibroblast growth factor was used, and it was shown to reduce vocal cord scar formation [16]. Hirano et al. found increased viscoelastic properties and less scar tissue formation after Hepatocyte Growth Factor injection [8].

Phenytoin accelerates wound healing with fibroblast proliferation, collagen deposition, collagenase enzyme inhibition, and antibacterial activity pathways. Fibroblast proliferation, the production of the extracellular matrix and its proteins, and the activity of growth factors and their mediators may be increased within the wound. Ultimately collagen production and deposition are increased, resulting in enhanced wound strength [12,17]. Furthermore, the effects of neural membrane stabilization and local inflammatory reaction limitation are other useful properties which are shown in the literature [11,18]. Acceleration of wound healing after surgical treatment in vocal cord pathologies is a direct contributor to the success and patient satisfaction. In cases where wound healing is poor, even after a successful surgery negative functional results may be seen. Thus, the effects of phenytoin, inducing fibroblast proliferation and collagen deposition, may play an essential role in the treatment of surgical vocal cord injury. The efficacy, safety, ease of use and low cost of phenytoin as an antiepileptic drug suggests the possibility that it can also be used prophylactically in adjuvant therapy after nasal surgery [19]. In various studies and reviews, it has been reported that phenytoin can be used topically in wounds in skin and soft tissues and various types of ulcers [17, 20, 21].

In a study by Tateya et al. [14] in rats with vocal cord injury, collagen type I, peaks 2 weeks after the lesion formation and then gradually decreases. Similarly, fibronectin density synthesized by fibroblasts peaks and decreases gradually after 2 weeks. In our study; in the phenytoin group, fibroblast and collagen density, but collagen fibers were more regular and matur than the control group. Phenytoin has also been reported to enhance the maturation of collagen in
both normal skin and granulation tissue in rats, possibly by promoting collagen cross-linking [22].

Previous studies have shown that the thickness of lamina propria is higher in the scarred rabbit vocal cord than the normal vocal cord [23,24]. In our study; in the phenytoin group, thickness of lamina propria were significantly more than the control group. Although phenytoin has anti-inflammatory properties, the reason for the high thickness of lamina propria in the phenytoin group is due to increased collagen density. There was no study that showed the effects of phenytoin on vocal cord lamina propria in the literature. In our study, rats were sacrificed on the 15th day for histopathological evaluation. The thickness of the lamina propria can be followed by longer follow-up for the evaluation of the effects of phenytoin.

Surgical treatment is the primary treatment option in vocal cord diseases. In order to recover the patient's voice fast and with good quality, and to preserve the mucosal wave, diligence is essential. The best contribution of this study to the literature is that it was the first study to evaluate phenytoin injection in vocal cord injury. However, the effect of phenytoin on vocal cord vibration was not determined hence an experimental model showing vocal cord functions could not be established.

This experimental study has several limitations, such as the small group sizes. The small size issue occurred due to our ethical concern regarding the “principle of reduction” in animal experiments; however, larger numbers would be needed to reach a clear conclusion on the topic. The second limitation is that we used a subjective scoring system to evaluate the histopathologic changes in the vocal cord samples. It would be more accurate to use an image analysis program that allows for objective/automated interpretation of the scarred vocal cord healing and histopathological changes and will facilitate a clear conclusion.

As a result, it has been shown in this study that phenytoin, which improves wound healing process in various tissues, has positive effects in rat vocal cords. It is relatively easy to handle and inject, although, as with all other materials, there is variability to the final location of the injectate. New collagen and fibroblast synthesis is stimulated in response to injection of phenytoin. The phenytoin was not shown to improve the biomechanical properties of the scarred vocal fold. According to these findings, phenytoin may be more appropriately used to medialize a stiffened
scarred vocal fold that is contributing to glottic insufficiency, rather than to restore optimal viscoelastic properties of a deficient lamina propria. The findings in our study provide a feasible scientific view, for adding phenytoin treatment to vocal cord surgeries in otolaryngology practice. The rapid improvement of the patient's voice quality may increase patient satisfaction and improve the results of the operation. Other experimental and clinical studies should support the data we obtained.
References


**Table:** Lamina Propria Thickness, Collagen and Fibroblast Density According to Control and Phenytoin Groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Group</th>
<th>Phenytoin group</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Thickness of lamina propria (µm)</td>
<td>18,0±7,1</td>
<td>65,5±10,7</td>
<td>&lt;0,001</td>
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<tr>
<td>Density of collagen (%)</td>
<td>36,5 (25,0-40,0)</td>
<td>77,0 (72,0-83,0)</td>
<td>0,005</td>
</tr>
<tr>
<td>Density of fibroblast (%)</td>
<td>30,0 (25,0-35,0)</td>
<td>60,0 (60,0-70,0)</td>
<td>0,005</td>
</tr>
</tbody>
</table>
Figure 1: Superior view of the rat larynx through the endoscope
Figure 2: A wound was surgically created on bilateral vocal cord
Figure 3: The view of larynx and the vocal cords after dissection
Figure 4: Comparison of thickness of lamina propria according to groups
Figure 5: Comparison of collagen and fibroblast density according to groups