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Effects of Topical Phenytoin on Nasal Wound Healing After Mechanical Trauma: An Experimental Study

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Objectives/Hypothesis: Impaired postoperative wound healing is the second most common morbidity after synechia formation in endoscopic sinus surgery. The aim of this experimental study was to investigate the potential effects of topical phenytoin on wound healing after nasal mucosal trauma in rats.

Study Design: An experimental study at the Inonu University Faculty of Medicine.

Methods: Twenty-four rats were randomized into three groups: 1) phenytoin group (n = 8), 2) control group (n = 8), and 3) vehicle group (n = 8). After damaging the right nasal cavity, in the phenytoin group, 1% topical phenytoin cream was applied for 7 days. The rats in the control group did not receive any treatment. The vehicle group was treated with daily topical cold cream for 1 week. The rats were sacrificed at the end, and the nasal cavities were excised. Tissue edema and inflammatory cell infiltration were compared among the groups. Additionally, proliferating cell nuclear antigen (PCNA) and cluster of differentiation 31 (CD31) immunoexpression levels were evaluated. Furthermore, in biochemical analysis, the tissue levels of vascular endothelial growth factor and (EGF) of the groups were investigated.

Results: In the phenytoin group, tissue edema and inflammatory cell infiltration were significantly decreased, and PCNA and CD31 immunoexpression levels were more prominent ($P < .001$) and the tissue EGF levels were significantly higher ($P < .01$).

Conclusions: Topical phenytoin treatment may alter the nasal wound healing after mechanical trauma. The potential beneficial effects of topical phenytoin on nasal mucosa should be investigated by further experimental and human trials.

Key Words: Nasal mucosa, wound healing, phenytoin, intranasal administration, epidermal growth factor, vascular endothelial growth factor, proliferating cell nuclear antigen, cluster of differentiation 31, rats, animal experimentation.

Level of Evidence: NA

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INTRODUCTION

The pseudostratified columnar epithelium of sinonasal cavities protects the upper airway against exogenous agents and ensures normal ventilation through its action as a physical barrier and mechanical clearing system.[1] The maintenance of the normal aeration of sinus spaces depends on the normal functioning of the sinonasal mucosa. Damage to this protective epithelium may occur because of diverse etiologies including congenital defects, allergy, infections, physical trauma caused by nose-picking, accidents, chemical trauma caused by smoking or noxious gases, and surgery.[1–4]

Endoscopic sinus surgery (ESS) is a superior surgical method for treating recurrent acute sinusitis, chronic sinusitis, obstructive nasal polyposis, extramucous fungal sinusitis, periorbital abscess, rhinoliquorrhea, antrochoanal polyp, mucocele, and choanal atresia; for foreign body extraction; for dacryocystorhinostomy; for the excision of various tumors of the sinuses, nose, and anterior, middle, and posterior cranial fossa; for epistaxis control; for optic nerve decompression; and for orbit decompression.[5] ESS is a common procedure, accounting for more than 50% of all ear, nose, and throat operations performed.[6] The delayed mucosal healing of nasal cavity is one of the most frequent problems after ESS.[7–9] These complications may block the normal mucociliary drainage pathways of the sinuses, causing the recurrence of chronic sinusitis. Although there are plenty of data to support ways of improved healing after nasal surgery, there is no current established standard of care to accelerate the healing of the nasal cavity after ESS.

Since 1938, 5.5-diphenyl-2–4-imidazolidione, sodium (phenytoin) has been used as an anticonvulsant. It was later noticed that half the patients treated with phenytoin developed gingival overgrowth.[10] Histological investigation of gingival tissues treated with oral phenytoin revealed increased neovascularization.[11] Local use of phenytoin has proved effective in promoting the healing process of cutaneous wounds without significant systemic absorption.[12,13] Studies have shown that phenytoin

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stimulates fibroblast proliferation, decreases collagenase activity, increases epidermal and keratinocyte growth factor receptors, accelerates initial inflammatory responses, induces new vessel formation, speeds the decrease of microbial colonies, and improves healing.[14,15]

Based on this background, we aimed to investigate the potential effects of topical phenytoin on nasal wound healing in rats by examining the tissue vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) levels and pathological findings of damaged nasal mucosa in an experimental model.

MATERIALS AND METHODS

Care of Animals and Treatment

This study was carried out at the Inonu University Experimental Research Laboratory of the Faculty of Medicine. It had the approval of the ethics committee and complied with the guidelines for the care and use of experimental animals. Twenty-four young adult male Wistar rats, each weighing between 150 and 200 g, were purchased from the animal laboratory. All the rats were examined by a veterinarian and determined to be in good health.

Drug Preparation and Treatment

The 24 rats were randomized (using random number tables) into three groups: 1) a phenytoin (study) group (n = 8), 2) a control group (n = 8), and 3) a vehicle group (n = 8).

Nasal damage was induced mechanically using the method described by Khalmuratova et al.[16] Each animal was intraperitoneally anesthetized with 10 mg/kg xylazine hydrochloride (Alfazyne; Alfasan International B.V., Woerden, the Netherlands) and 60 mg/kg ketamine hydrochloride (Ketalar; Eczacibaşi Parke-Davis, Istanbul, Turkey). Unilateral wounds in the nasal mucosa were induced using the brushing technique. Mechanical injuries were performed with an interdental brush (10 mm) inserted through the right nostril.

For the preparation of 1% phenytoin cream, 1 g of phenytoin powder was added to 99 g of cold cream, and it was then applied into the right nasal cavity of the rats in the phenytoin group once daily for 1 week. The control group received no drug treatment. The vehicle group received the topical application of cold cream daily for seven days. All of the drug applications to the rats in the phenytoin and vehicle groups were conducted under general anesthesia. At the end of the 7 days, the rats were euthanized, and en block excision of the nasal cavities, nasal septum, and superior/middle/inferior turbinates were performed. Histological samples were taken from the nasal septum of the middle segment of the sinonasal cavity in coronal sections. The remaining tissue was kept at −80°C until they were used for EGF and VEGF analysis.

EGF- and VEGF-Level Analysis

To determine the EGF and VEGF levels, 100 mg of tissue (the nasal septum of the middle segment of the sinonasal cavity in coronal sections) were rinsed with phosphate-buffered saline (PBS), homogenized in 1 mL of PBS, and stored overnight at −20°C. After two freeze-thaw cycles were performed to break the cell membranes, the supernatant was centrifuged for 5 minutes at 5,000*g* at 2°C to 8°C. The supernatant was assayed and removed immediately.

EGF and VEGF levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits according to the manufacturer's instructions. EGF and VEGF were measured using anti-rat ELISA kits from CUSABIO (Wuhan, China; cat no: CSB-E08029r and CSB-E04757r). The plates were read at 450 nm using a CA-2000 ELISA microplate reader (CIOM Medical Co. Ltd., Zhejiang, China). The EGF and VEGF quantities in the samples were calculated from standard curves of recombinant cytokines using a linear regression method.

Histopathological Investigation

The excised nasal tissues were processed for histological and immunohistochemical studies. Formalin-fixed specimens were embedded in paraffin, cut into 4 μm-thick sections, and stained with hematoxylin and eosin for evaluation with light microscopy. Morphological evaluation of the nasal tissues was performed blindly. A modified semiquantitative grading system that was previously reported by our research team was used to score the degree of tissue damage to the nasal mucosa.[17] The major histopathological findings of the wound healing in the nasal mucosa after mechanical trauma, including edema and inflammatory cell infiltration were evaluated and compared among the groups.

The sections were also stained for proliferating cell nuclear antigen (PCNA) and cluster of differentiation 31 (CD31) via immunohistochemistry. All proliferating nuclei in the mucosal cells were stained with mouse monoclonal antibody against PCNA (Clone PC10; Sigma-Aldrich Co., St. Louis, MO), which is a nuclear protein that has peak expression during the S phase of the cell cycle and was used previously to identify proliferating cells' monoclonal anti-PCNA clone PC10 from mouse ascites fluid. Clone PC10 was diluted to 1/1,000 and applied to five paraffin sections deparaffinized in xylene using the labeled streptavidin biotin method. Endothelial cells were stained using mouse monoclonal antibody against CD31 antigens, a glycoprotein expressed on the luminal surface of endothelial cells (ready to use, clone QBEnd/10; Novocastra, Newcastle, UK). Antigen retrieval was performed with enzyme digestion using trypsin. The slides were quenched in Super Block (ScyTek Laboratories, Logan, UT) for five minutes at room temperature. Immunodetection was performed using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories). In each case, the final products were visualized by aminoethylcarbazole chromogen, and counterstaining was performed with hematoxylin. The rat peritoneal vessel served as a positive internal control for CD31. For PCNA, human tonsil tissue served as a positive control. Negative controls (primary antibody was omitted) were routinely performed on adjacent serial sections.

Statistical Analysis

For the statistical analyses, the values were expressed as the mean ± standard deviation whenever appropriate. The normality of the distributions was tested using the Kolmogorov-Smirnov test. The one-way analysis of variance test, followed by the post hoc Tukey test and Fisher exact test, were carried out to compare the groups. The homogeneity of the groups was confirmed by Levene's test. Data analyses were performed using a statistical software package (SPSS version 15.0; SPSS, Inc., Chicago, IL). For all comparisons, statistical significance was defined as $P < .05$.

RESULTS

The histopathological changes in the nasal mucosa of each group are presented in Table I. Topical phenytoin treatment was associated with significantly decreased tissue inflammation, shown by decreased edema and

TABLE I.
Comparison of Histological Changes of Nasal Mucosa After Treatment.

	Phenytoin Group, n = 8, n (%)	Control Group, n = 8, n (%)	Vehicle Group, n = 8, n (%)	*P*
Edema				.004
None	3 (37.5%)	0 (0%)	0 (0%)	
Minimal	5 (62.5%)	0 (14.3%)	2 (25%)	
Moderate	0 (0%)	6 (75%)	5 (62.5%)	
Severe	0 (0%)	2 (25%)	1 (12.5%)	
Inflammatory cell infiltration				.004
None	3 (37.5%)	0 (0%)	0 (0%)	
Minimal	5 (62.5%)	1 (12.5%)	2 (25%)	
Moderate	0 (0%)	3 (37.5%)	5 (62.5%)	
Severe	0 (0%)	4 (50%)	1 (12.5%)	

diminished inflammatory cell infiltration compared to those of control and vehicle groups ($P < .05$).

The immunohistochemical detection of PCNA and CD31 revealed the proliferation of many mucosal cells and endothelial cells in samples of the phenytoin group, indicating increased wound healing and angiogenesis (Table II, Fig. 1). In contrast, nasal mucosal samples of control and vehicle groups developed only a few proliferating cells and exhibited significantly decreased staining for CD31 (Fig. 1).

The mean VEGF levels of the nasal mucosa of rats were found to be 2.11 ± 0.02 pg/mL in the phenytoin group, 2.06 ± 0.02 pg/mL in the control group, and 2.03 ± 0.04 pg/mL in the vehicle group ($P > .05$). VEGF levels were not significantly different among the groups. The measured tissue EGF levels in the groups were as follows: 198.8 ± 9.2 pg/mL in the phenytoin group, 150.8 ± 5.08 pg/mL in the control group, and 160.6 ± 5.08 pg/mL in the vehicle group.

The phenytoin-treated rats had significantly increased tissue EGF levels compared to the other groups ($P < .05$). The intergroup comparison of the mean EGF levels of the animals is presented in Figure 2.

DISCUSSION

In the current study we demonstrate a clear relationship between topical phenytoin application and altered nasal wound healing through use of novel methods including investigation of PCNA and CD31 expression and EGF levels. Our results show that phenytoin-treated rats have elevated tissue levels of EGF and markedly increased PCNA and CD31 immunohistochemical expression in the nasal mucosa. To the best of our knowledge, this is the first time that such observations have been reported in an experimental model.

ESS is an effective treatment modality for patients with sinonasal pathologies that are unresponsive to medical therapy and has been reported to provide both immediate and long-term symptom reduction and improvement in quality of life in 85% of patients.[18,19] Although the efficacy of ESS is clearly described within the past 2 decades, delayed nasal mucosal healing and adhesion formation after ESS is a potential cause of surgical failure. Previous studies have shown that the overall incidence of impaired wound healing after ESS is about 20%, and a majority of these cases requires additional surgical interventions.[9,20]

TABLE II.
Comparison of PCNA and CD31 Immunostaining Among the Groups.

		Phenytoin Group, n = 8, n (%)	Control Group, n = 8, n (%)	Vehicle Group, n = 8, n (%)	*P*
PCNA					.002
	None	0 (0%)	2 (25%)	1 (12.5%)	
	Minimal	0 (0%)	6 (75%)	6 (75%)	
	Moderate	4 (50%)	0 (0%)	1 (12.5%)	
	Diffuse	4 (50%)	0 (0%)	0 (0%)	
CD31					.001
	None	0 (0%)	0 (0%)	0 (0%)	
	Minimal	0 (0%)	7 (87.5%)	6 (75%)	
	Moderate	3 (37.5%)	1 (12.5%)	2 (25%)	
	Diffuse	5 (62.5%)	0 (0%)	0 (%)	

CD31 = cluster of differentiation 31; PCNA = proliferating cell nuclear antigen.

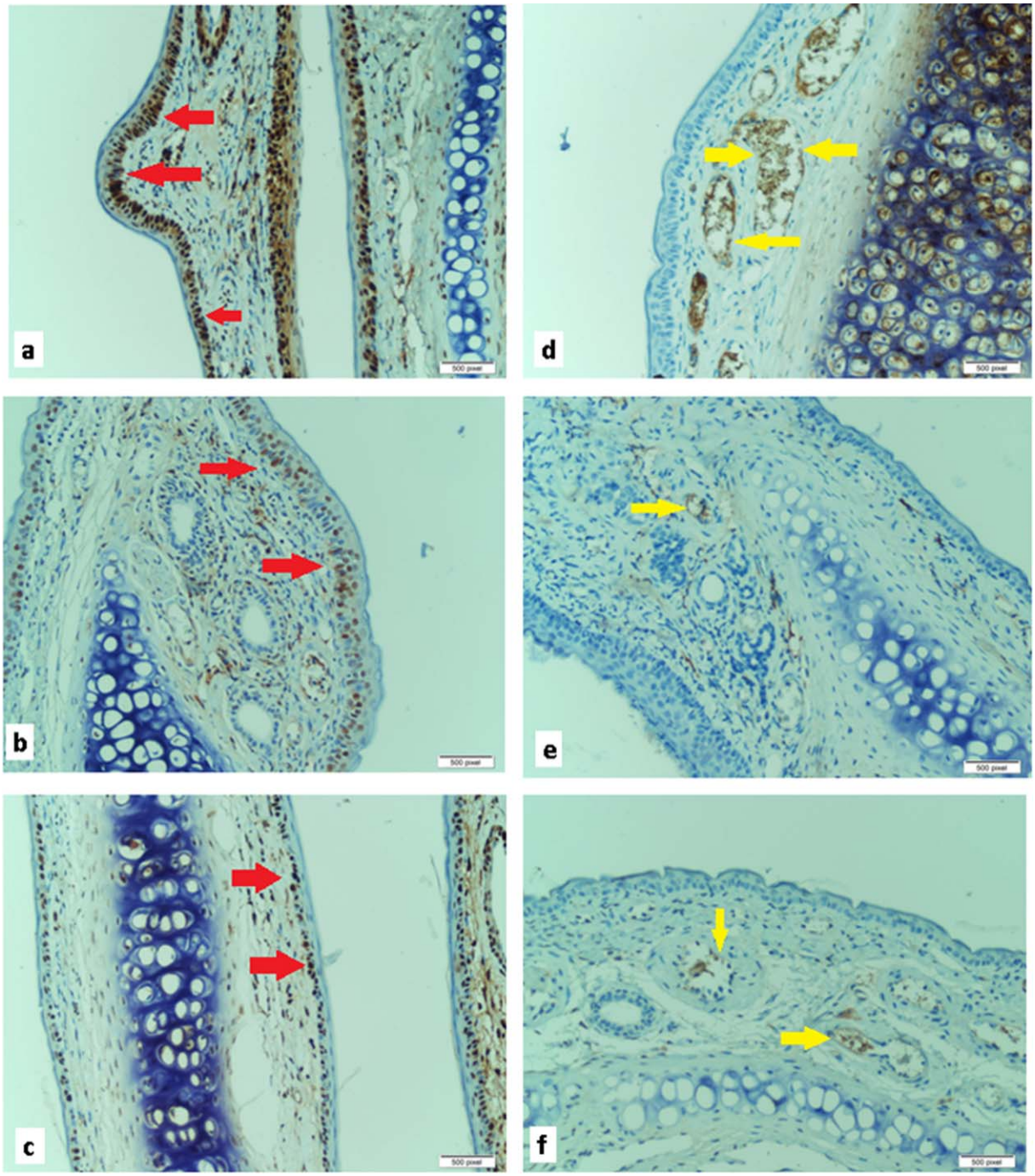

Fig. 1. Representative photomicrographs of the nasal tissue sections obtained from phenytoin (a, d), control (b, e) and vehicle (c, f) groups after the treatment. In phenytoin-treated rats, PCNA (a) and CD31(d) expressions were significantly increased as compared with control (b, e) and vehicle (c, f) groups. The arrows indicate PCNA- (red arrows) and CD31- (yellow arrows) stained mucosal and endothelial cells. CD31 = cluster of differentiation 31; PCNA = proliferating cell nuclear antigen.

To prevent postoperative bleeding and adhesions after ESS, it is common practice to place nasal or sinus packing material in the surgical area. Many different materials have been used, including polyvinyl acetate sponges, and ribbon gauze.[21,22] However, these have the disadvantage of having to be removed at some time during the postoperative period, thus reopening the wound and possibly contributing to poor healing. Patients have also described this as the worst aspect of their treatment.[23] In addition, these methods have had relatively limited success in promoting nasal wound healing and preventing adhesions.[23,24]

Wound healing is determined by a careful equilibrium between the synthesis and breakdown of collagen

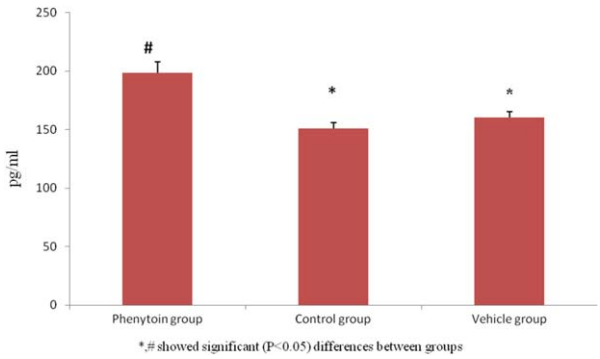

Fig. 2. Tissue epidermal growth factor levels in rats. *#Significant differences between groups ($P < .05$).

and by effective vascular support.[15,25] Previously, Turan et al. reported that topical phenytoin treatment was associated with increased fibroblastic proliferation, epithelialization, and vascularization in the rat skin incisional regions.[15] As a key mediator in wound healing, EGF is an effective mitogenic agent in fibroblasts, vascular endothelial cells, and epithelial cells.[26] In another interesting experimental study, Brown et al. reported that repeated treatment with EGF increases epithelial cell proliferation in a dose-dependent manner and accelerates the wound healing process.[27] Similarly, in the present study, topical phenytoin treatment was associated with a significantly increased tissue level of EGF in the nasal mucosa.

As an endothelial specific growth factor, VEGF is strongly angiogenic in vivo and is produced mainly from fibroblasts and inflammatory cells.[25] VEGF transcription and secretion are elevated in partial- and full-thickness skin wounds.[28] Additionally, in the wounds of diabetic mice, impaired neovascularization is accompanied by diminished VEGF mRNA and protein levels.[29] Although tissue VEGF levels were comparable between the groups in our study, the immunoexpression of CD31, a glycoprotein expressed on the luminal surface of endothelial cells, was found to be significantly higher after local phenytoin treatment. CD31 is involved in the adhesion of lymphocytes to the endothelium and transmigration into the extravascular space.[30] Several studies have also suggested that CD31+ lymphocytic infiltration is linked to increased angiogenesis in tumors and other conditions.[31,32] Therefore, according to the results of the present study, it is possible to postulate that phenytoin may increase vascularization of the damaged nasal mucosa in a VEGF-independent pathway. In line with these findings, Pitiakoudis et al. suggested that phenytoin, by inducing local cytokine release, contributes to the accumulation of lymphocytes and angiogenesis, thus accelerating would healing.[33]

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 histological changes in the nasal mucosal samples. Using an image-analysis program that allows for the objective/automated interpretation of nasal wound healing and histological changes would be more accurate and would make it easier to draw a clear conclusion.

CONCLUSION

This research shows that intranasal phenytoin treatment after mechanical trauma to the nose significantly alters wound healing via increased epithelial proliferation and angiogenesis. Further research should be performed to study how this affects synechiae formation after sinus surgery.

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