EXPERIMENTAL STUDY

Is phenytoin a safe agent for staple line recovery after gastric sleeve surgery in rats?

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ABSTRACT

BACKGROUND: The most challenging and mortal complication of gastric sleeve surgery (SG) is staple line leakage. Although many agents have been used for increasing tissue healing on the stapler line, there is still no consensus on its effectiveness and efficacy. The aim of study is to determine the effect of phenytoin on the healing process of gastric sleeve surgery in rats.

METHODS: On the 10th post-operative day, the effects of phenytoin on bursting pressure in the stapler line were evaluated alongside pathohistological examinations. To investigate the molecular impact of phenytoin on the expression of TGF-β, VEGF, FGF2, and p53 genes, quantitative real-time polymerase chain reaction was utilized. In addition, gene expressions at the protein level were determined by immunohistochemical analysis.

RESULTS: No signs of intra-abdominal leakage were observed in the resected samples. A statistically essential extend in stable line bursting pressure measure was observed between the control group and the group treated with phenytoin application. Pathohistological results indicate that the mean score of collagens of the study group (3.2±0.42) was significantly higher than the control group (2.3±0.48) (P=0.003). In addition, the mean epithelization score of the study group (3.4±0.52) was significantly higher than the control group (2.1±0.57) (P=0.001). mRNA of TGFβ, FGF2, VEGF, and p53 genes drastically increased phenytoin treated group. High FGF2 protein expression levels were determined from phenytoin use compared to the control group.

CONCLUSION: Molecular studies suggest that phenytoin may increase the healing process of Gastric sleeve following SG in rats and may become a new agent for the prevention of human gastric leaks.

Keywords: FGF2; Gastric sleeve surgery; p53; Phenytoin; TGF-β; VEGF.

INTRODUCTION

In recent years, gastric sleeve surgery (SG) has become an increasingly popular bariatric procedure. Factors such as effective weight loss, technical simplicity, and proven positive metabolic benefits have further increased the acceptance and utilization of this procedure. At present, SG constitutes over half of all bariatric procedures performed in the US and various regions of Europe.[1] However, complications of SG still lead to distressing morbidities and even mortality. The most challenging and most mortal complication of SG is staple line leakage that has been reported up to 7%.[2] Many surgeons have applied sutures, several sealants, and substances that increase tissue healing on the stapler line. However, there is still no consensus on its effectiveness and efficacy.[3-5]

Phenytoin (5.5-diphenyl-2-4-imidazolidione, sodium) is one

Cite this article as: Çay F, Duran A, Tokay E, Hacıoğlu N, Köçkar F, Altun E, Kanat BH. Is phenytoin a safe agent for staple line recovery after gastric sleeve surgery in rats? Ulus Travma Acil Cerrahi Derg 2023;29:1321-1328.

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Ulus Travma Acil Cerrahi Derg 2023;29(12):1321-1328 DOI: 10.14744/tjtes.2023.29035 Submitted: 25.07.2023 Revised: 31.07.2023 Accepted: 22.10.2023

of the oldest non-sedating anticonvulsant drugs used in the treatment of partial and tonic-clonic seizures and neuropathic pain, and its chronic side effects are gingival hypertrophy.[6-8] Some side effects of phenytoin have been used in wound and tissue healing studies. Its angiogenic, anti-inflammatory, collagen, anti-microbial, proliferative, and granulation tissue-enhancing properties of phenytoin have been demonstrated in many scar tissues.[9-15] Despite numerous studies demonstrating the beneficial effects of topical or systemic administration of phenytoin on wound healing,[15-17] there is currently an absence of research in the literature that investigates the impact of stapler line wound healing in SG.

In this research, we have, for the first time, the effects of intraperitoneal phenytoin administration on sleeve gastrectomy line healing were investigated at molecular and immunohistochemical levels in rats. The samples underwent assessment based on various parameters, including fibroblastic activity, inflammatory cell infiltration, neo-angiogenesis feature, collagen deposition, as well as the expression levels of TGFβ1, FGF2, VEGF, and p53. These evaluations were conducted using the modified Ehrlich-Hunt scale. In molecular studies, VEGF, FGF-2, TGF-β, and p53 expression levels were analyzed at the mRNA level by Real-Time polymerase chain reaction (PCR) method. The determination of the effect of phenytoin on SG may result in an alternative method that can be used for the elimination of leaks during surgery and the rapid treatment of wounds.

MATERIALS AND METHODS

Animals

A group of 20 female Wistar Albino rats, with weights ranging from 386 to 401 g, received care in adherence to the guidelines specified in the "Guide for the Care and Use of Laboratory Animals." The experiments carried out on the rats were granted approval by the Balıkesir University Experimental Animal Research Ethics Committee. Each animal's weight was monitored on a daily basis, and they were provided with unrestricted access to a standard laboratory diet and water. There were two groups consisting of 10 rats in one group. In Group 1 (n=10) (control group), animals were anesthetized and applied SG. In Group 2, animals underwent SG and also received 25 mg/kg of phenytoin intraperitoneally for 10 days.

Surgery Protocol

Before the surgery, all rats underwent a 12-h fasting period to ensure they were in a fasted state. The surgery was conducted by the surgeon under sterile conditions. The anesthesia was administrated with 20 mg/kg ketamine hydrochloride/Ketalar ® 100 mg/mL Pfizer, England, and 5 mg/kg xylazine hydrochloride/Rompun ®, 20 mg/mL, Bayer, Canada intraperitoneally as prophylaxis. The surgeon performed a midline abdominal incision, measuring 4 cm in length. During the procedure, the gastroesophageal junction and the pylorus of the stomach were identified by the surgeon. A 3–5 mm incision was made on the stomach, just over pylorus, while leaving a portion of the pylorus entire to keep the continuity of the passage to the duodenum. Suspension sutures were placed on the transection line and gastric fundus. Linear staplers (Linear stapler ECHELON FLEX™ ENDOPATH®) were placed on the marked line and the excised stomach was removed illustrated in Figure 1. No orogastric tube was used during resection. The control group (Group 1) was given 0.5 mL of serum physiology fluid intraperitoneally (IP). After resection, in group 2, 25 mg/kg (0.5 mL) phenytoin sodium (Epitoin) was administered to the stapler line. For the final steps, the abdominal wall was sutured using a 3/0 polyglycolic suture, and the skin was closed with a 4/0 intracutaneous suture to prevent any disturbance or ingestion of the sutures by the rats. Following the surgery, the rats were fasted for an additional 12 h, after which they were provided with a diet and access to drinking water for feeding. Rats were allowed free cage movement immediately after surgery. At day 10 of post-operation, rats were sacrificed. The stomach stapler line was longitudinally cut and divided into two halves. Half of the sample was preserved in 10% formalin solution for histopathological evaluation, while the other half was stored in RNA-safe solution for molecular analysis at a temperature of −80°C. It is essential to mention that all surgical procedures and the care of laboratory animals were conducted in strict accordance with the guidelines set by the ethics committee.

Evaluation of Leakage and Incision Scar

At day 10 after injury, a general anesthesia procedure was applied. Inflammation and edema formation in the abdominal incision scar were investigated (Fig. 1). The previous incision scar was excised, and the abdomen was subsequently opened. Then, the gastric sleeve surgery site was investigated for the presence of mucosal cuts, food residues, and secretions.

Measurement of Gastric Sleeve Burst Pressure

Gastric burst pressure measurements were taken within 3 min of sacrifice of rats. Initially, the abdominal cavity and mediastinal space were exposed to ensure a comprehensive visualization of the upper gastrointestinal tract, encompassing the esophagus and stomach. Then, the stomach segment containing the entire staple line was excised with two incisions, one in the middle of the esophagus and the other 2 cm distal to the pylorus. The stomach was washed with physiological saline solution. 1 cm distal of the pylorus was sutured with 3/0 silk sutures. The infusion pump with gray angiocath was placed from the esophagus to reach the stapler line and was tied with 3/0 silk to prevent leakage. Isotonic methylene blue was continuously administered at a rate of 1 mL/min, and close observation of the stapler line area was conducted. The pressure observed outside the stapler line was documented as the burst pressure.

Histopathological Evaluation

10th day, following the determination of bursting pressure, the stomach specimens were embedded in paraffin wax. Lon-

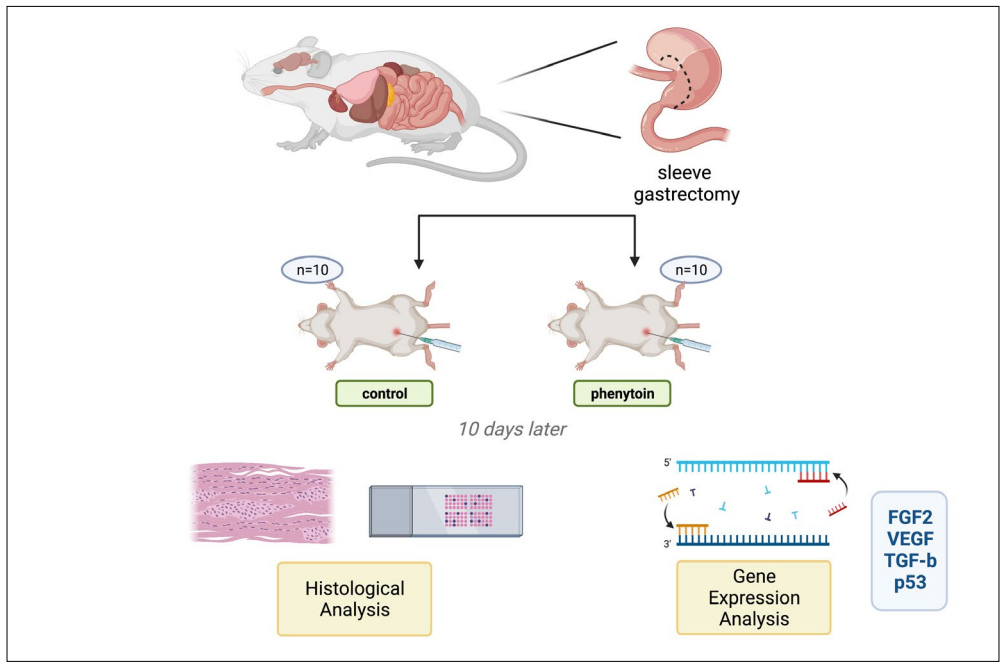

Figure 1. A schematic design of experimental design.

gitudinal sections with a thickness of 3 mm were prepared using a microtome and stained with Hematoxylin and Eosin (H&E) as well as Masson Trichrome (MTC). Subsequently, the stomach specimens were examined under light microscopy at 40× magnification by an experienced pathologist who was unaware of the experimental conditions (blinded examination). Histologically, parameters such as anastomotic line epithelialization, inflammatory cell infiltration, collagen deposition, as well as fibroblast, and vascular proliferation, were evaluated using a numerical grading scale. Traces were designated for stain intensities below 1+, while all other stain intensities were recorded on a four grade scale (0–3).

Real Time Analysis

Tissue samples in RNA-safe solution (Zymo Research®) were disrupted using liquid nitrogen. 1 mg of tissue sample was used for RNA isolation using Trizol Reagent® (Thermo Scientific) as described in the datasheet. Next, 1 µg of RNA was subjected to reverse transcription using the Thermo Scientific cDNA synthesis kit. 1 µL of cDNA was added as a template for qRT-PCR using RealQ Plus 2x Master Mix, Syber green.[18-20] The Real-Time PCR method was performed in a total volume of 12.5 µL, comprising 0.5 µL of 10 pmol/µL primer pair listed in Table 1, 6.25 µL of 2X Master Mix, and 4.25 µL of dH2O. GAPDH primers were used for inter-

Table 1. Primer list used for qRT-PCR study

Name	Sequence of Forward (F) and Reverse (R) (5'-3')	GC % content	Amplicon (bp)
TGF-beta 1	F: 5'GCTCAGTCTGTCTACCTGCA-3'	55	360
	R: 5'-GGCGGGATGGCATCAAGGTA-3'	60	
FGF2	F: 5'-ACTTCGCTTCCCGCACTGC-3'	63	360
	R: 5'-CCAGTTGGTATGTGGCACTG-3'	55	
VEGF	F:5'- CCCATGAAGT GGTGAAGTTC -3'	50	428
	R: 5'- GAACAAGGCTCACAGTGAAC -3'	50	
p53	F: 5'-AGACATTTTCATGCTTATGG-3'	35	524
	R: 5'-ACCATCAGAGCAACGCTCAT-3'	50	
GAPDH	F:5'-CTGGAGAAACCTGCCAAGTATG-3'	50	140
	R: 5'-GGTGGAAGAATGGGAGTTGCT-3'	52	

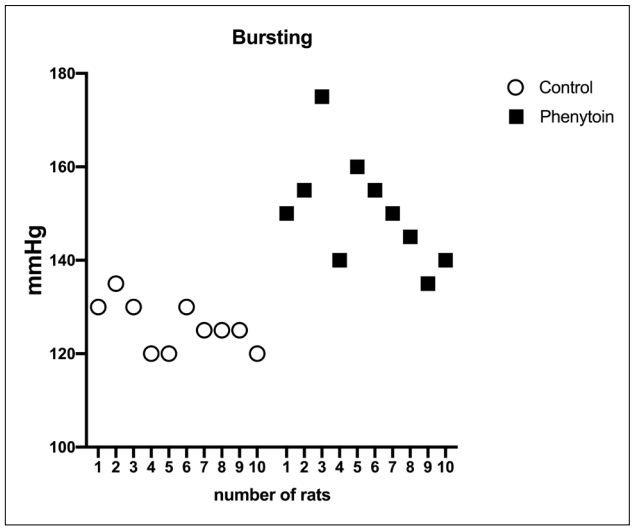

Figure 2. Burst pressures of the stapler line between the control group and the study group.

nal control. Ct values were calculated using Livak method. Graphics were visualized with Graphpad Prism® 8.1.

Immunohistochemical Assessment

The paraffin sections underwent immunohistochemical examination using the following antibodies: FGF2, p53, VEGF, and TGF-β. Throughout these procedures, a hydrogen peroxide receptor blockade was carried out following the washing of all samples with PBS. The samples were then treated with primary antibodies. Afterward, the samples were once again washed with PBS and subsequently incubated with the secondary antibody. Finally, the preparations were stained with chromogen and then counterstained with hematoxylin.

Statistical Analysis

Statistical analysis was conducted utilizing GraphPad Prism 8 software. The findings are presented as mean ± standard deviation (SD). For group comparisons, a one-way ANOVA test was employed, and statistical significance was considered for $P<0.01$.

RESULTS

All surgical procedures resulted in successful stapler closure, without any technical or surgical complications such as mechanical stapling failure or hematoma formation along the stapler line. No signs of intra-abdominal leakage were observed in the resected samples. The effect of phenytoin on burst pressure analysis in stabler line model was determined on the 10th post-operative day. The burst pressure of the stapler line serves as an indicator of the repaired tissue's resistance to breaking under tension and, thus, may partially reflect the quality of the repaired tissue. There were also an important difference in the mean of burst pressure of the stapler line between the control group and the study group namely 126±5.16 and 150.5±11.65, respectively (P=0.001) (Fig. 2).

Pathophysiologic investigations reveal that in the normal gastric wall section, full thickness of all mucosa and gastric wall layers is observed (H&E, 100×) (Fig. 3a). In the control group, only granulation tissue and ulceration are observed in the epithelial area at the gastric anastomosis line wound site (black arrows), epithelialization has not started yet (H&E, 100×) (Fig. 3b). Epithelialization appears to have begun on the granulation tissue in the study group gastric anastomosis line wound site (black arrows), (H&E, 40×) (Fig. 3c). The mean score of collagens of the study group (3.2±0.42) was significantly higher than the control group (2.3±0.48) (P=0.003). In addition, this difference is also evident in the histopathologic figures. In the control group, weak collagen tissue is observed in the granulation tissue of the stomach anastomosis line wound (MTC, 100×) (Figures 3d and e). Significantly increased collagen tissue is observed at the wound site of the gastric anastomosis line in the control group (MTC, 100×) (Figures 3d and e). In addition, the mean epithelization score of the study group (3.4±0.52) was significantly higher than the control group (2.1±0.57) (P=0.001) (Figures 3b and c).

Histological examinations were conducted to assess collagen deposition, inflammatory cell infiltration, epithelialization, fibroblast activity, and neovascularization. These scoring evaluations were aimed at detecting the significant impact of phenytoin on the wound healing of the stapler line (Fig. 4). Statistically significant differences were observed in all histopathological results between the 1st day and 10th day of the post-operative period (P<0.0001) (Fig. 4). Inflammatory cell infiltrate, fibroblast, and neovascularization score of phenytoin-treated groups were not important compared to the control group of 10th day of post-operative day as shown in Figure 4.

After analyzing the histological changes in the stomach stapler line following phenytoin treatment, the impact of phenytoin on wound healing was further investigated by assessing the mRNA expression of FGF, VEGF, p53, and TGFβ genes in both phenytoin and control groups. These genes are crucial regulators in matrix remodeling and angiogenesis. On the 10th day post-wounding in rats, mRNA expressions were

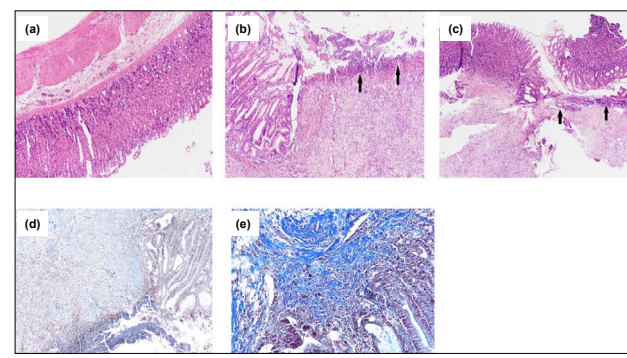

Figure 3. Pathophysiologic investigations of stabler line after SG **(a)** the normal gastric wall section **(b)** Control group of the gastric anastomosis line wound site and **(c)** Phenytoin treated group of gastric anastomosis line wound site.

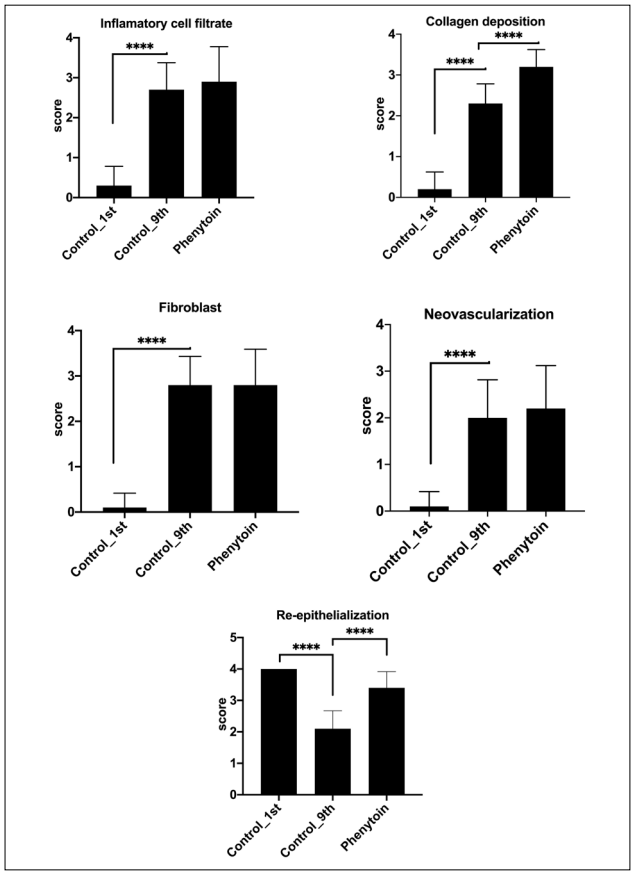

Figure 4. Histological examinations, Inflammatory cell infiltrate, Fibroblast, Collagen deposition, Fibroblast, neovascularization, and epithelialization scores in the control and phenytoin-treated rats from the stapler line. Means ± SD are shown. **** represent P<0.0001.

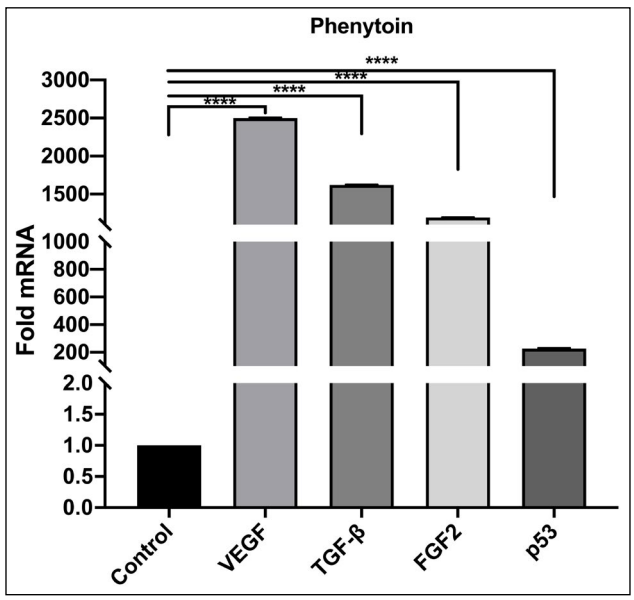

Figure 5. The mRNA expression p53, VEGF, FGF, and TGF-β by Real-Time PCR in the control and phenytoin-treated groups from the stapler line. Means ± SD are shown. **** represent P < 0.0001.

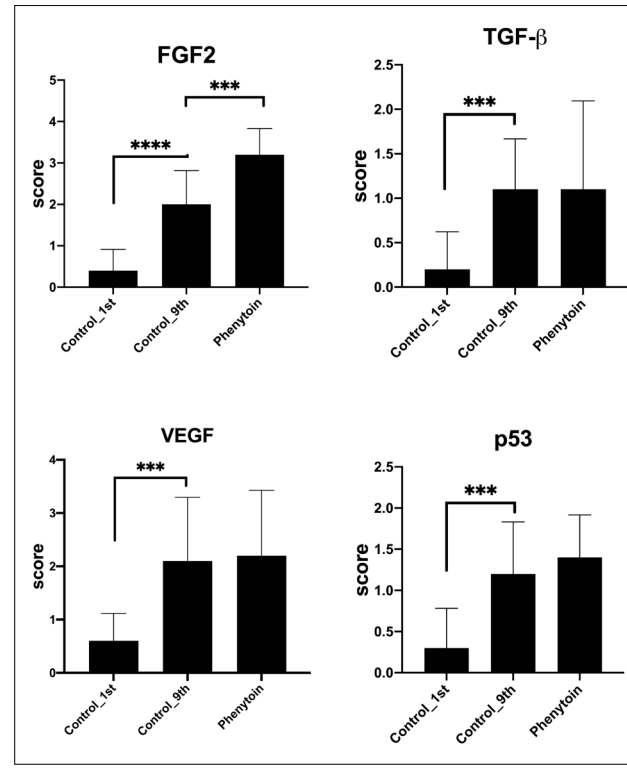

Figure 6. The protein expression FGF2, TGF-β, VEGF, and p53 and by immunohistochemistry in the control and phenytoin-treated groups from the stapler line. Means ± SD are shown. ***P<0.0001 and ****P<0.00001.

determined using Real-time PCR, and the expression of the target gene was normalized using the GAPDH gene as a reference. The results demonstrated a noteworthy increase in the expression of TGF-β, VEGF, p53, and FGF genes in the phenytoin group compared to the control group on day 10 (P<0.001). The most affected gene was VEGF whereas the least affective gene was p53 gene (Fig. 5).

To assess the protein effect of these markers in the stomach, immunohistochemistry was conducted. The protein expression of VEGF, TGF-β, FGF-2, and p53 gene scores on the 10th day was found to be statistically upper than 1st day of the control group. While there was a slight increase in the protein expression of TGFβ, VEGF, and p53 in the phenytoin-treated group compared to the 10th day control group, these increases did not reach statistical significance. However, on the 10th day, a importantly higher FGF protein effect was observed in the stomach stapler line of the phenytoin-treated group when compared to the control group (P<0.0001) (Fig. 6). As shown in Figure 7, in the control group, low FGF immunoreactivity is observed on the granulation tissue at the gastric anastomosis line wound site (FGF, 200×). In the study group, significantly increased FGF reactivity was observed in the granulation tissue at the wound site of the gastric anastomosis line (FGF, 200×).

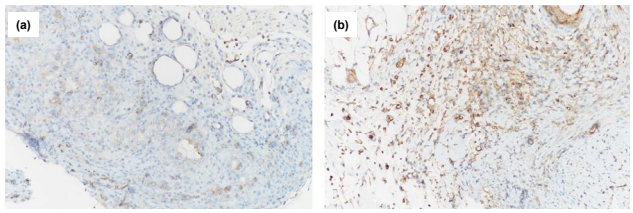

Figure 7. FGF2 protein expression in the control **(a)** and phenytoin treated group **(b)** after 10th of SG.

DISCUSSION

Risk factors for post-SG (sleeve gastrectomy) leaks include high body mass index, the type of staple cartridges, male gender, concomitant sleep apnea, open surgery, the use small plugs, and prolonged operative time. Studies have suggested that reinforcing the stapler line can help prevent post-operative bleeding and leakage following sleeve gastrectomy.[21] For this purpose, various reinforcement methods have been described, including overstitching, reperitonealization, non-absorbable strips, sealants, omental dressing, bracing with bioabsorbable materials, and tissue-healing agents.[22] Despite extensive research, a consensus has not yet been reached on the most dependable reinforcement procedure or tissue-healing agents that can effectively prevent stapler line leakage.

Gentileschi et al. conducted a study comparing buttressed transection with polyglycolic acid-trimethylene carbonate, staple-line roofing with a gelatin fibrin matrix, and oversewing, but they found no significant differences in preventing stapler line leakage.[23] Derici et al. investigated the practice of overstitching the stapler line and concluded that although it increased operative time, there was no evidence supporting its effectiveness in reducing leakage.[24] A reverse study reported that staple line support with bovine pericardium reduced staple line leakage.[25] In another study, Gagner and Buchwald compared non-reinforced, non-absorbable bovine pericardial strips, over-stitched, and absorbable polymer membranes, and found that the most effective approach for preventing leakage was the application of absorbable polymer membrane.[26] It has also been reported that suturing the staple line with a continuous suture is more successful than reinforcing the staple line with fibrin glue.[27] They compared the efficacy of different modalities such as a bovine pericardium and no supplementation, biocompatible glycolide copolymer, and supplementation with bovine pericardium was found to be the most successful method.[28] Coskun et al. used fibrin glue to strengthen the stapler line in their study and they did not find any leakage.[29] Sepúlveda et al. reported that the stapler line did not leak when using overlapped stitches.[30]

Epithelialization is a crucial process that occurs during the proliferative phase of wound healing, where the epithelium progresses over the granulation tissue. In vivo and clinical studies have demonstrated that topical phenytoin accelerates the inflammatory process, promotes granulation tissue formation, and enhances re-epithelialization.[31,32] Our study aligned with these findings from the literature, as we discovered a importantly increase in epithelialization in the phenytoin-treated group. This positive effect on wound healing in the staple line highlights the beneficial impact of phenytoin in our research.

In the process of wound healing, collagen plays a central role, as collagen fibers are responsible for enhancing wound strength. Studies have reported that phenytoin exerts positive effects on wound healing by inhibiting collagenase release and promoting favorable collagen distribution.[33] In a study by Tokgöz et al., they found an increase in the amount of collagen in the phenytoin group.[34] Similarly, in their wound healing study, Turan et al. observed a notable and importantly extance in collagen accumulation within the phenytoin-treated group.[35] In our study, consistent with the literature, we believe that phenytoin increased collagen in the study group and this increased the burst pressure by strengthening the staple line.

Wound healing process is usually controlled by growth factors such as FGF, VEGF, TGF- β, EGF, and interleukin family.[36] Thus, this study also examined the impact of phenytoin treatment on FGF, VEGF, and TGF expression in the healing of the gastric staple line. In addition, the previous reports have indicated that FGF enhances the healing of gastrointestinal injuries in animal models.[37] Similarly, it was shown by Turan et al. that FGF was increased.[35] In this study, we observed an important increase in FGF levels in the study group compared to the control group, indicating that phenytoin administration elevated FGF expression at both the mRNA and protein levels. This upregulation of FGF contributed to the strengthening of the staple line, as evidenced by the increased burst pressure. In addition, VEGF and TGF expressions were importantly upregulated in phenytoin group compared to the control group. This increasing expression may play an important role in gastric line healing. At the same time, studies have been found that p53 is indirectly effective in the wound-healing process.[38] Therefore, p53 expression was investigated in phenytoin-administered groups after SG. p53 expression was found to be significantly increased at mRNA and protein levels in the phenytoin-treated groups compared to the control group. This relationship was demonstrated for the first time in this study.

CONCLUSION

This experimental study presents compelling evidence that intraperitoneal administration of phenytoin, the first of its kind in the literature, significantly mitigates the deleterious effects of sleeve gastrectomy staple line leakage. By providing a safer and more effective staple line for wound healing in the sleeve gastrectomy procedure compared to stapling alone, intraperitoneal phenytoin holds great potential. Although further research is necessary to elucidate the mechanism underlying its beneficial effects, our findings pave the way for promising clinical applications of intraperitoneal phenytoin to

enhance the safety of sleeve gastrectomy procedures.

Ethics Committee Approval: This study was approved by the Faculty of Medicine, Balıkesir University Ethics Committee (Date: 24.09.2020, Decision No: 2020/6-2).

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Design: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Supervision: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Resource: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Materials: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Data collection and/or processing: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Analysis and/or interpretation: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Literature search: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Writing: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.; Critical review: F.Ç., A.D., E.T., N.H., F.K., E.A., H.B.K.

Conflict of Interest: None declared.

Financial Disclosure: The author declared that this study has received no financial support.

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DENEYSEL ÇALIŞMA - *ÖZ*

Fenitoin sıçanlarda tüp mide ameliyatından sonra zımba hattının iyileşmesi için güvenli bir madde midir?

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AMAÇ: Tüp mide ameliyatının (SG) en zorlu ve ölümcül komplikasyonu stapler hattının kaçmasıdır. Stapler hattında doku iyileşmesini hızlandırmak için birçok ajan kullanılmasına rağmen hala etkinliği ve etkinliği konusunda fikir birliği yoktur. Çalışmanın amacı, fenitoinin sıçanlarda tüp mide ameliyatının iyileşme sürecine etkisini belirlemektir.
GEREÇ VE YÖNTEM: Patohistolojik incelemelerin yanı sıra postoperatif 10. günde fenitoinin stabiler hatta patlama basıncı analizine etkisi belirlendi. Fenitoinin VEGF, TGF-β, FGF2 ve p53 genlerinin ekspresyonu üzerindeki moleküler etkisi qRT-PCR ile araştırıldı. Ayrıca immünohistokimyasal analiz ile protein seviyesindeki gen ifadeleri belirlendi.
BULGULAR: Rezeke edilen örneklerde intraabdominal kaçak bulgusuna rastlanmadı. Stabil hat patlama basıncı değerlerinde kontrol ve fenitoin uygulama grupları arasında istatistiksel olarak anlamlı artışlar olmuştur. Patohistolojik sonuçlar, çalışma grubunun ortalama kollajen skorunun (3.2±0.42) kontrol grubuna (2.3±0.48) göre anlamlı derecede yüksek olduğunu göstermektedir (p=0.003). Ayrıca çalışma grubunun ortalama epitelizasyon skoru (3.4±0.52) kontrol grubuna göre (2.1±0.57) anlamlı olarak yüksekti (p=0.001). VEGF, TGF-β, FGF2 ve p53 genlerinin mRNA'sı fenitoin verilen grupta önemli ölçüde arttı. Fenitoin kullanımında kontrol grubuna göre yüksek FGF2 protein ekspresyon seviyeleri belirlendi.
SONUÇ: Moleküler çalışmalar, fenitoinin sıçanlarda SG'yi takiben mide tüpünün iyileşme sürecini artırabileceğini ve insan mide kaçaklarının önlenmesi için yeni bir ajan olabileceğini düşündürmektedir.

Anahtar sözcükler: Fenitoin; tüp mide ameliyatı; VEGF; TGF-β;FGF2; p53.

Ulus Travma Acil Cerrahi Derg 2023;29(12):1321-1328 DOI: 10.14744/tjtes.2023.29035