

# Gallic acid liposome and powder gels improved wound healing in wistar rats

Ozkan Karatas<sup>1</sup>, Fikret Gevrek<sup>2</sup>

<sup>1</sup>Tokat Gaziosmanpaşa University, Faculty of Dentistry, Department of Periodontology, Tokat, Turkey

<sup>2</sup>Tokat Gaziosmanpaşa University, Faculty of Medicine, Department of Histology and Embryology, Tokat, Turkey

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## Abstract

**Aim:** Gallic acid is an anti-inflammatory and antioxidant agent which could provide beneficial effects in preventing periodontal inflammation and improve wound healing. The present study aimed to evaluate the effects of gallic acid on experimental palatal wound model in Wistar rats.

**Material and Methods:** 60 female Wistar rats were divided into three main study groups as the control group (C, n=24), gallic acid powder gel administered group (GP, n=18), and gallic acid liposome administered group (GL, n=18). Standardized palatal wounds of 5-mm diameter were created in all rats. Control rats received vehicle gel, and the other groups received either GP or GL gels. Control rats were sacrificed on days 0, 7, 14, and 21. GP and GL groups were sacrificed on days 7, 14, and 21. Hematoxylin-eosin staining was performed, and fibroblast cell counts and inflammatory cell infiltration in the wound area were evaluated. Transforming growth factor (TGF)- $\beta$  expressions were determined via immunohistochemistry.

**Results:** C, GP, and GL groups had a similar decrease in wound area apart from 21<sup>st</sup> day, which GL had significantly lower wound area compared to the control group. Unhealed wound area was significantly lower on the 14<sup>th</sup> and 21<sup>st</sup> days in gallic acid groups. GP and GL groups had significantly higher fibroblast cell counts on all days compared to the C group. GL had significantly highest counts even compared to GP groups. Inflammatory cell infiltration was higher on the 7<sup>th</sup> day compared to 14<sup>th</sup> and 21<sup>st</sup> days in all groups. Gallic acid groups exhibited lower inflammatory cell counts on 7<sup>th</sup> and 21<sup>st</sup> days. TGF- $\beta$  levels significantly increased in GP and GL groups on 7<sup>th</sup> and 14<sup>th</sup> days.

**Conclusion:** Within limitations of the present study, it can be concluded that gallic acid in both powder and liposome forms increased fibroblast cell counts and decreased late inflammation along with increased TGF- $\beta$  expressions in the wound healing process.

**Keywords:** Anti-inflammatory; antioxidants; gallic acid; wound healing

## INTRODUCTION

Thermal, mechanical or surgical trauma deteriorates the integrity of the tissues, and a series of biochemical and cellular activities called the wound healing process begins to restore hemostasis. Wound healing is a well-organized and delicate process and includes three phases as inflammation, proliferation, and maturation. Every step of the process is crucial for optimal wound healing (1,2). The healing process requires anabolic activities after the first phase, inflammation. And the proliferation, differentiation, migration of cells, and collagen production are necessary for this process (1,3,4). Factors related to the patient such as the age, systemic diseases, smoking, malnutrition, drug use can compromise healing and cause scar formation (3,4). On the other hand, the healing can be

improved by additional agents which can be used either systemically or topically (2,5,6).

In addition to being the first step of wound healing, inflammation is involved in the etiopathogenesis of various diseases and conditions as the biological response against bacteria, tissue damage, or irritants (7). In the absence of optimal inflammatory response, pathological conditions arise, and the homeostasis is disrupted (8-10). Furthermore, limitation in the inflammation with adjuvant therapies assists the host defense systems and the elimination of the pathological conditions (9-11). Most of the anti-inflammatory drugs or natural products target the prostaglandin-endoperoxide synthase (usually known as cyclooxygenase, COX) inhibition, which is a significant component of the inflammatory process (12-14). In this respect, antioxidants, especially

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**Corresponding Author:** Ozkan Karatas, Tokat Gaziosmanpaşa University, Faculty of Dentistry, Department of Periodontology, Tokat, Turkey **E-mail:** dtokaratas@hotmail.com

flavonoids, have been shown to exert COX inhibition (15). Gallic acid (GA) is a flavonoid compound that exhibits potent anti-inflammatory effect and is reported to reduce COX-2, nitric oxide, lipid mediators, and 5-LOX expressions (16,17). GA also reduced the inflammatory cell accumulation and chemotaxis through down-regulated nuclear factor  $\kappa$ -B pathway (15,16,18).

Gallic acid is a flavonoid also named as trihydroxybenzoic acid and was reported to modulate inflammation through down-regulating major pro-inflammatory cytokines such as interleukins (IL), tumor necrosis factor (TNF)- $\alpha$ , nitric oxide (NO), inducible NO synthase (iNOS), and nuclear factor  $\kappa$ B (NF- $\kappa$ B) (15,16,19). Along with its anti-inflammatory effects, GA can also modulate anabolic activities, especially promote bone formation and prevent metabolic bone diseases (19-22). In this respect, Chauhan et al. reported the elevated osteogenic activity with increased osteoblast proliferation, alkaline phosphatase (ALP) expression, and calcium and phosphorus metabolism (20). GA also reduced inflammatory pathways and inhibited inflammation-driven bone destruction both in vitro and in vivo (22). Jin et al. also revealed increased bone metabolism via increased osteoblast cell viability, proliferation, and mineralization (21). Regardless of the tissue, resolution of inflammation, cell proliferation, differentiation and migration, and collagen synthesis are required to achieve optimal wound healing. GA might promote wound healing via its positive effects on anabolic activities mentioned above (3,23). Therefore, the present study aimed to evaluate the effects of gallic acid in either powder or liposome forms on experimental palatal wound model in Wistar rats. Wound closure, unhealed wound area, fibroblast cell counts, inflammatory cell infiltration, and TGF- $\beta$  expressions were evaluated.

## MATERIAL and METHODS

All experimental designs and procedures were approved by the local ethics committee of animal studies of Tokat Gaziosmanpasa University, and the study was conducted at Tokat Gaziosmanpasa University Faculty of Dentistry and Faculty of Medicine. All experimental procedure was performed following the guidelines of the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the manuscript were created according to the 'NC3Rs ARRIVE Guidelines, Animal Research: Reporting of In Vivo Experiments'.

A power analysis was performed, and the power of the study was calculated as 90%. Healthy, 8-10 week old, weighing 250-300 grams, female Wistar rats (*Rattus norvegicus*) were used in this study. Rats showing disabilities or disorders have excluded from the study. The study consisted of three major groups as control group, gallic acid powder gel group, and gallic acid liposome gel group.

The study groups were;

1. Control groups (C, n=24)
  - a. Sacrificion on day 0 (Group 1, Control day 0)

- b. Sacrificion on day 7 (Group 2, Control day 7)
- c. Sacrificion on day 14 (Group 3, Control day 14)
- d. Sacrificion on day 21 (Group 4, Control day 21)

2. Gallic acid powder gel groups (GP, n=18)
  - e. Sacrificion on day 7 (Group 5, GP day 7)
  - f. Sacrificion on day 14 (Group 6, GP day 14)
  - g. Sacrificion on day 21 (Group 7, GP day 21)

3. Gallic acid liposome gel groups (GL, n=18)
  - h. Sacrificion on day 7 (Group 8, GL day 7)
  - i. Sacrificion on day 14 (Group 9, GL day 14)
  - j. Sacrificion on day 21 (Group 10, GL day 21)

Standardized 5mm soft tissue defects were created via a metal punch in all rats under general anesthesia. All procedures were carried out by an experienced researcher, and all rats had topical treatment in either vehicle gel or gallic acid gels.

General anesthesia was provided via intraperitoneal injection of ketamine (Eczacibasi Ilac Sanayi, Istanbul, Turkey) (100 mg/kg) and xylazine (Eczacibasi Ilac Sanayi, Istanbul, Turkey) (0.5 mg/kg). After stabilization of the rats, mouths were opened via a retractor and the palatal gingiva was marked. A metal punch of 5mm diameter was used to remove a circular full-thickness flap, and the exposed bone area was left uncovered. Bleeding was controlled with sterile gauzes, and rats were observed every day for three days in case of any complication such as postoperative bleeding. Rats were kept in individual cages in a room with 12 hours of light/dark cycles and received food and water ad libitum.

### Gallic acid gel preparation

Glycerin and alcohol (v/v, 5/1) were used as the carrier and benzocaine (Anestezin, Botafarma, Ankara, Turkey) with 5% concentration was added to the mixture to relieve pain after surgical procedures. Gallic acid powder (Sigma Aldrich, Missouri, USA) as 5% concentration was added to the mix, and a homogenous colorless gel was formed by a pharmacist before application. Gallic acid liposomes were prepared according to a liposome protocol described by Cui et al. briefly, gallic acid, soy lecithin, and cholesterol were dissolved in chloroform and subjected to an ultrasound shaker for 10 minutes. After a homogeneous solution was achieved, the solvent was evaporated with a rotary evaporator. The thin film obtained after evaporation was treated with phosphate buffer solution and stirred with an ultrasound shaker for 10 minutes. The mixture was then centrifuged at 5000 rpm for 10 minutes and filtered with a standard filter paper. After the filtration, 5% of liposome was added to the carrier with benzocaine addition (24).

Control group received a gel containing glycerin/alcohol and benzocaine. The topical applications were performed every morning at 9:00 am until the sacrificion day.

Six rats were sacrificed immediately after surgical procedure and considered as control day 0. Six rats in each

major group were sacrificed on 7th, 14th, and 21st days. Maxilla was removed and fixed in 10% formalin solution. Palatal mucosa was evaluated under 12.5 x magnification via a stereomicroscope (Stemi 2000 and Axiovision 4.8, Carl Zeiss, Jena, Germany) and standardized photographs were taken from each rat.

### Stereomicroscopic evaluation

Standardized photographs were evaluated via an image analysis program (Stemi 2000 and Axiovision 4.8, Carl Zeiss, Jena, Germany). The outer area of the wound and the unhealed wound area were measured by an experienced blind researcher and recorded.

### Histopathological evaluation

After standardized photographs were taken, palatal mucosa was dissected, and histological tissue processing was performed. Firstly, all tissues were rehydrated with ethanol series and then cleared with xylene series. Then tissues were embedded in paraffin, and serial sections were obtained. Hematoxylin-eosin (H&E) staining and TGF- $\beta$  immunohistochemistry were performed. An experienced blinded researcher performed all histological analysis. Total inflammatory cells and fibroblast cell counts in the wound area were determined on H&E-stained sections. Cell counting was performed under 1000x magnification in a cell counting frame of 10000 $\mu$ m<sup>2</sup>. Three measurements were performed for each section, and a mean value was calculated and recorded. Fibroblast and inflammatory cells were counted based on their morphology. Inflammatory cells counted were macrophage, neutrophil, eosinophil, T lymphocytes, and plasma cells (25-27).

### TGF- $\beta$ immunohistochemistry

TGF- $\beta$  expressions were determined via immunohistochemistry. Firstly, three sections were selected from each animal and rehydrated through ethanol series. Then all sections were cleared with xylene, and endogenous peroxidase activity was suppressed with hydrogen peroxide (3%). After washing three times for five minutes (3x5) with phosphate-buffered solution (PBS) sections were incubated with rabbit serum for 30 minutes. After serum incubation, sections were washed 3x5 with PBS and incubated with primary antibodies overnight at 4°C in a humidified dark room. The antibody (Abcam plc, Cambridge, UK) dilution was 1:250. After primary antibody incubation, sections were rewashed 3x5 with PBS and incubated with biotinylated secondary antibody immunoglobulin G for 30 minutes. Then, all sections were rewashed and treated with a streptavidin-horseradish peroxidase-conjugated reagent for 30 minutes. After washing again with 3x5 PBS, 3-Amino-9-Ethylcarbazole (AEC) chromogen was applied to visualize immunoreactivity for 5 minutes. After AEC treatment, sections were rewashed with 3x5 PBS and counterstained with Gill's hematoxylin and washed with distilled water and then mounted.

AEC provided a red color in the slides with different shades from pale red to the dark red. Immunohistochemistry was

evaluated by 400x magnification (Nikon, Tokyo, Japan). A cell counting frame was created, and all cells within was marked according to their staining density. The staining density was recorded from 0 to 3 as no staining-0, slight staining-1, mild staining-2, and dense staining-3. To compare the results, all stained and non-stained cells were converted to a numeric value, H score, which provided a statistical comparison. The conversion was performed based on a formula,  $[\sum Pi(i+1)]$ . In the formula i: presents the intensity score of the staining and Pi: shows the percentage of the stained cells. An experienced blinded researcher performed all immunohistochemistry evaluations from three different points on each slide, and a mean value for each animal was recorded (26,27).

### Statistical Analysis

All data were analyzed with IBM SPSS program (IBM, New York, USA) and presented as mean and standard deviation. All data were firstly analyzed with the Kolmogorov-Smirnov test for normality. The immunohistochemistry results were analyzed with non-parametric tests, Mann Whitney U and Kruskal Wallis tests. The stereomicroscope measurements, fibroblast, and inflammatory cell counts were analyzed with parametric tests, One Way ANOVA, and Tukey.  $p < 0.05$  was considered statistically significant.

## RESULTS

### Morphometric evaluation

Both gallic acid and control groups had wound healing with no complications. The wound area was 19.60 mm<sup>2</sup> baseline and decreased from day 0 to 21 in all groups ( $p < 0.05$ ). The wound contraction was significant on all tested durations; significant differences were observed in comparisons of 7th vs. 21st days in all groups ( $p < 0.05$ ) (Figure 1, Table 1). The outer measurements of the wounds were lower in the GL group compared to the control group on day 21 ( $p < 0.05$ ). Other comparisons revealed no significant differences ( $p > 0.05$ ). The unhealed area in the center of the wound was also measured. The unhealed area was also decreased from day 0 to 21. Significantly lower values were observed on the 14th and 21st days in the GP and GL groups compared to the control group ( $p < 0.05$ ) (Table 1). The healing was similar in all groups on day 7 ( $p > 0.05$ ) (Table 1).

### Histopathological evaluation

The wound healing, the healed and unhealed areas, and epithelization were evaluated on the histological slides. Total inflammatory cells and fibroblast cells were also counted (Figure 2). Inflammatory cell count was higher in control 7th day compared to the other durations ( $p < 0.05$ ). The 0th-day control value was significantly different compared to the other durations and groups ( $p < 0.05$ ). Inflammation decreased from day 7 to 21 in all groups; however, significant differences were found between the control group, GP, and GL groups on day 7 ( $p < 0.05$ ). On day 21 control group also had significantly higher inflammatory cell counts compared to the GP and GL groups ( $p < 0.05$ ).

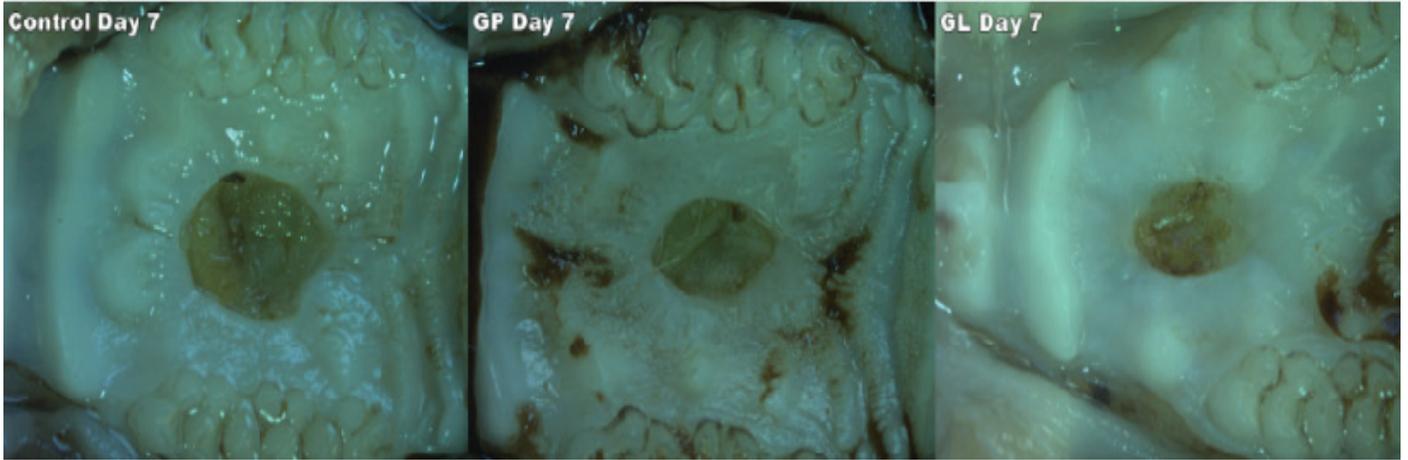


Figure 1. Representative Stereomicroscope images of the study groups under 12.5 x magnification.

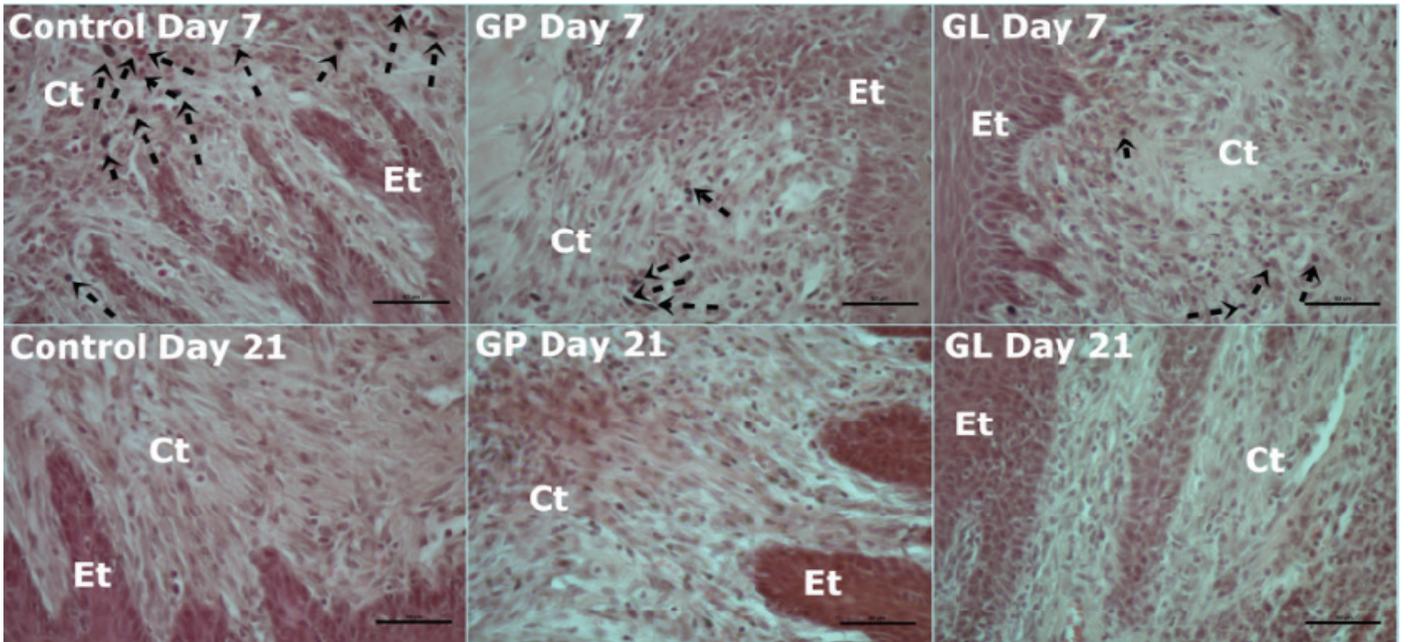


Figure 2. Representative Hematoxylin-eosin staining images of the study groups. Ct: Connective tissue, Et: Epithelial tissue.

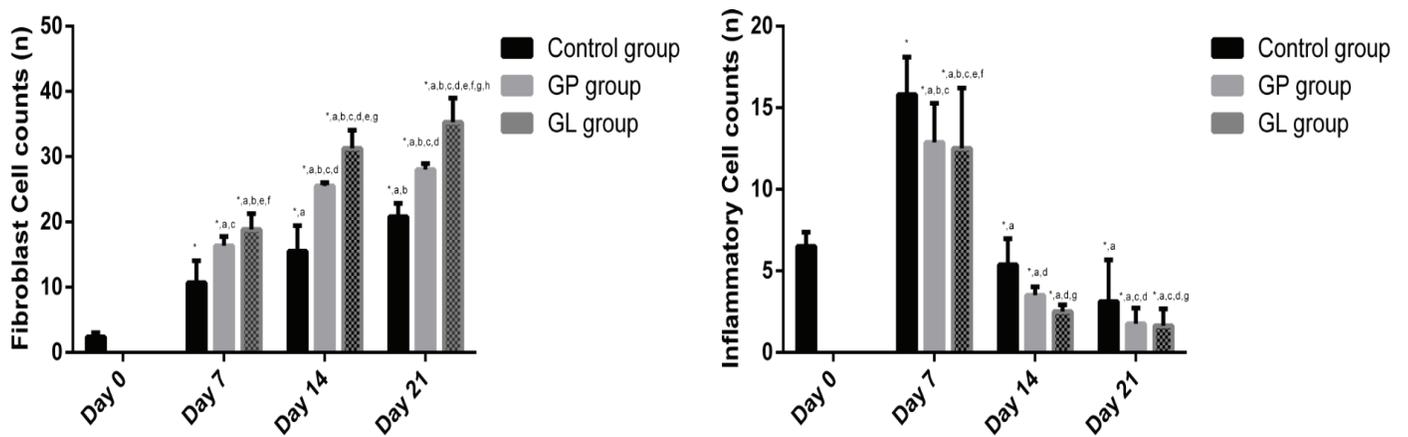
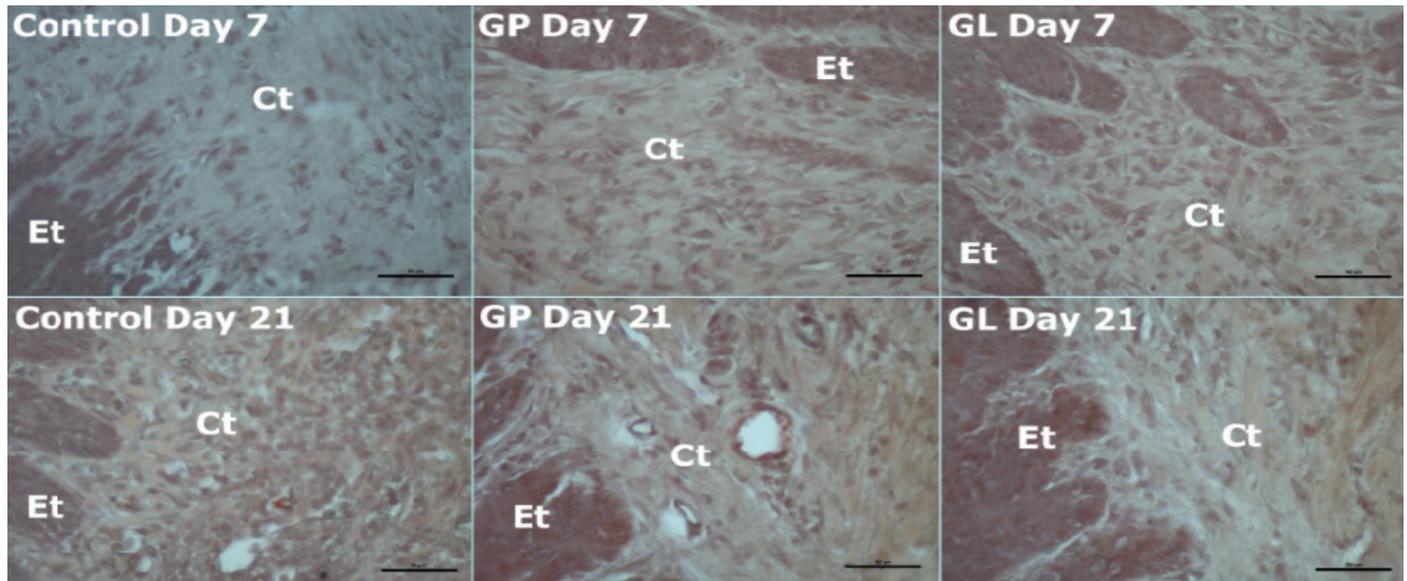


Figure 3. Fibroblast cell counts and inflammatory cell counts in the study groups. \* $p < 0.05$  vs. Control day 0, <sup>a</sup> $p < 0.05$  vs. Control day 7, <sup>b</sup> $p < 0.05$  vs. Control day 14, <sup>c</sup> $p < 0.05$  vs. Control day 21, <sup>d</sup> $p < 0.05$  vs. Gallic powder day 7, <sup>e</sup> $p < 0.05$  vs. Gallic powder day 14, <sup>f</sup> $p < 0.05$  vs. Gallic powder day 21, <sup>g</sup> $p < 0.05$  vs. Gallic liposome day 7, <sup>h</sup> $p < 0.05$  vs. Gallic liposome day 14.



**Figure 4.** Representative immunohistochemistry staining of TGF-beta in the study groups

Fibroblast cell counts exhibited a reverse pattern than inflammatory cells. Fibroblast cell counts were the lowest on the control day 0 ( $p < 0.05$ ). Cell counts significantly increased from day 0 to 21 in all groups ( $p < 0.05$ ). Also, the control group in all durations (7th, 14th, and 21st days) exhibited significantly lower fibroblast cell counts when compared to the equivalent gallic acid groups ( $p < 0.05$ ) (Figure 3). Gallic acid in both powder and liposome forms provided similar increase compared to the control group.

**Table 1.** Measurements of palatal wound area, unhealed wound area, and TGF- $\beta$  expressions among groups

Groups/ Parameters	Palatal wound area	Unhealed wound area	TGF- $\beta$ Expressions (H score)
Control Day 0	19.60 $\pm$ 0.03	19.60 $\pm$ 0.01	12.08 $\pm$ 1.49
Control Day 7	12.99 $\pm$ 2.67 <sup>a</sup>	9.48 $\pm$ 4.52 <sup>a</sup>	52.65 $\pm$ 2.29 <sup>a</sup>
Control Day 14	9.70 $\pm$ 2.35 <sup>a,b</sup>	7.67 $\pm$ 3.47 <sup>a,b</sup>	54.18 $\pm$ 2.79 <sup>a</sup>
Control Day 21	6.28 $\pm$ 3.98 <sup>a,b,c</sup>	3.21 $\pm$ 2.09 <sup>a,b,c</sup>	62.74 $\pm$ 2.36 <sup>a</sup>
GP Day 7	12.00 $\pm$ 2.00 <sup>a</sup>	7.05 $\pm$ 0.88 <sup>a</sup>	69.03 $\pm$ 1.40 <sup>a</sup>
GP Day 14	8.25 $\pm$ 1.16 <sup>a</sup>	1.37 $\pm$ 1.76 <sup>a,c</sup>	71.71 $\pm$ 8.97 <sup>a</sup>
GP Day 21	5.01 $\pm$ 2.13 <sup>a</sup>	1.02 $\pm$ 1.19 <sup>a,d</sup>	63.34 $\pm$ 5.16 <sup>a</sup>
GL Day 7	9.12 $\pm$ 3.44 <sup>a</sup>	7.00 $\pm$ 1.06 <sup>a</sup>	82.53 $\pm$ 1.67 <sup>a,b</sup>
GL Day 14	6.87 $\pm$ 2.64 <sup>a</sup>	0.95 $\pm$ 0.67 <sup>a,c</sup>	73.18 $\pm$ 1.08 <sup>a,c</sup>
GL Day 21	3.37 $\pm$ 2.13 <sup>a,d</sup>	0.56 $\pm$ 0.37 <sup>a,d</sup>	67.74 $\pm$ 7.08 <sup>a</sup>

<sup>a</sup> $p < 0.05$  vs. control day 0, <sup>b</sup> $p < 0.05$  vs. control day 7, <sup>c</sup> $p < 0.05$  vs. control day 14, <sup>d</sup> $p < 0.05$  vs. control day 21. No statistical difference was observed between GP and GL groups in all tested durations.

### TGF- $\beta$ immunohistochemistry

The immunohistochemistry results of the TGF- $\beta$  expressions and representative immunohistochemistry staining images in the study groups were presented in table 1, figure 4. TGF- $\beta$  expressions were significantly

lower in the control day 0 compared to the other days and groups ( $p < 0.05$ ). The values increased from day 0 to 21 in all groups. Values of TGF- $\beta$  levels on the 7th and 14th days in the control group were significantly lower compared to the gallic acid groups ( $p < 0.05$ ). However, on the 21st day, the values in the groups were similar ( $p > 0.05$ )

### DISCUSSION

Gallic acid is a potent antioxidant and anti-inflammatory agent. The present study evaluated the effects of two different gallic acid gel forms on a secondary intention wound healing model in Wistar rats. The results revealed that morphological improvement in the wound area was better in the gallic acid groups on day 14 and 21, and the wound closure increased from day 0 to 21 in all groups. Gallic acid liposome gel provided better closure on the day 21 compared to the control group. Increased fibroblast cell counts were observed in all tested times in both gallic acid groups; however the inflammatory cell counts were similar on day 14 while gallic acid caused a higher decrease in the inflammation on the 7th and 21st days in both GP and GL groups. TGF- $\beta$  expressions also exhibited a substantial increase on the 7th and 14th days in the gallic acid administered group. However, the levels were similar in the 21st day in all groups.

Inflammation is a biological response which is regulated by mainly NF-KB pathway (10,13,22). In addition to pathological conditions, inflammation is also crucial in wound healing, which is followed by proliferation and maturation phases (3,23). After the initial inflammatory period, proliferation, differentiation, migration of cells to the wound area, and collagen deposition occur (1). The faster the inflammation is resolved, the quicker and better the wound healing occur. In the inflammatory process, major pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  are up-regulated with activation of NF-KB signaling and blockage of this pathway results in inhibition of inflammation (11,15,18,19). In this respect, gallic acid

was reported to block NF-KB, prevent inflammation and associated pathologic alterations (15,18,22). In addition to NF-KB, iNOS, adhesion molecules of neutrophils, and COX-2 production also decreased after gallic acid treatment (18,28). Rong et al. showed that gallic acid successfully reduced IL-6 and TGF $\beta$ 1-smad2 signaling and reduced inflammation in vivo (29). The decrease in inflammation and increase in collagen deposition with increased fibroblast cell counts and activity accelerate the healing process, and gallic acid was reported to reduce inflammatory cell infiltration and cell counts in vivo (30,31). The present results also revealed reduced inflammation as reported in previous recent studies which demonstrated decreased inflammatory markers and inflammatory cells (30-32). In addition to these studies, the present study evaluated the liposome form of gallic acid and found that both powder and liposome were effective in terms of anti-inflammatory effect. Total inflammatory cell counts on 7th and 21st days, which are early and late periods of wound healing were lower in the gallic acid treated groups. However, the inflammatory cell counts on the 14th day were similar in all groups.

In addition to the anti-inflammatory effect, gallic acid also enhances collagen I and extracellular matrix synthesis and osteoblast proliferation (33). Yang et al. recently demonstrated that gallic acid successfully promoted wound healing through focal adhesion kinases (FAK), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (Erk) and also increased fibroblast and keratinocyte proliferation and migration. The effect of gallic acid was also evident even in diabetic conditions (34). Thanikachalam et al. revealed that gallic acid increased cell migration from wound borders and promoted wound healing both in vitro and in vivo (35). Furthermore, Pellenz et al. showed decreased apoptosis in the keratinocyte and fibroblast cells after gallic acid treatment (36). In terms of matrix degradation, Wang et al. reported that gallic acid down-regulated MMP-1 and -3 expressions while up-regulating tissue inhibitors with the inhibition of AKT and ERK1/2 signaling (37). Increased angiogenesis, collagen deposition, and regeneration were also reported after gallic acid administration (38). Luo et al. also reported faster wound contraction, epithelialization, increased hydroxyproline content, high tensile strength, collagen deposition, and vascularization with decreased IL-6, NO, and TNF- $\alpha$  levels (32). Supporting the beneficial effect of gallic acid on wound healing, both forms of gallic acid successfully increased fibroblast cell counts in the wound area and improved the wound contraction in the present study.

Furthermore, Alves Barros et al. showed increased fibroblast proliferation, angiogenesis, and collagen production in vivo (30). Reduction in inflammation, along with increased fibroblast activity, is the most critical effect of gallic acid contributing to wound healing which is also reported after gallic acid administration in skin wounds in rats (30). Improved wound healing results in shorter healing period and faster epithelialization as Tsala et al. and Upadhyay et al. reported by shortened healing time

with increased epithelialization rate, fibroblast proliferation, and decreased inflammatory cell infiltration (31,39). The results of the present study also supported the previous results (30,31,39) with higher fibroblast cell counts in gallic acid administered groups in all tested durations. The effect of gallic acid liposome on fibroblast cell counts was higher than the gallic acid powder, especially on the later periods of the healing process.

14th and 21st days showed better wound contraction and smaller unhealed area in the gallic acid-treated groups, and the improvement was more significant in the GL group on the day 21. TGF- $\beta$  expressions on the wound area were higher in the early period of wound healing in the GL group while GP and control groups exhibited similar expression patterns. Also, the TGF- $\beta$  levels were similar among the groups on the 21st day.

The liposome is a spherical vesicle composed of phospholipids and can be used to encapsulate drugs or nutrients to prolong the efficacy duration or avoid molecular destruction before functioning (40). The most significant benefit is the protection of the active agent encapsulated within the liposome. However, in the topical use of encapsulation, the release of the agent might be slower than the free form, and therefore, the efficacy might decrease (40). In the present case, the gallic acid confined to the liposome did not have a superior effect than the free gallic acid, and the results were similar in all the parameters evaluated. However, regardless of the encapsulation, gallic acid provided significant improvement in the measured parameters in the present secondary intention wound healing model.

## CONCLUSION

In conclusion, gallic acid improved wound healing by decreasing wound area, increasing fibroblast cells, reducing inflammatory cell infiltration, and inducing TGF- $\beta$  expressions. Besides, the most evident effects of gallic acid were increased fibroblast cell counts and decreased inflammation. However, there are certain limitations of the present study. Firstly, this study is a histological study performed on animals. Secondly, no biochemical analysis was performed. Thirdly, gallic acid powder and liposome were used as 5% concentration. Further studies involving different doses, further biochemical analysis, and different study protocols might be beneficial in revealing the effect of gallic acid on connective tissue metabolism and wound healing.

*Competing interests: The authors declare that they have no conflict of interest.*

*Financial Disclosure: There are no financial supports.*

*Ethical approval: This study was approved by the Institutional Ethics Committee and conducted in compliance with the ethical principles according to the Declaration of Helsinki.*

*Ozkan Karatas ORCID: 0000-0001-9891-7172*

*Fikret Gevrek ORCID: 0000-0002-3722-2542*

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