

# Topical quercetin gel application improved wound healing in Wistar rats

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## Abstract

**Aim:** Quercetin is an antioxidant agent with proven beneficial effects in preventing inflammation. Aim of present study was to evaluate effects of quercetin gel on a secondary intention wound healing model in Wistar rats.

**Material and Methods:** 42 Wistar rats were divided into two groups, one group administered quercetin gel (Q, n=18) while other administered vehicle (C, n=24). 5-mm diameter palatal wounds were created in each rat and all rats were given either quercetin gel or vehicle daily until sacrifice days. Gel contained 5% quercetin, 5% benzocaine and glycerin, and each rat administered 0.2 ml of gel every day. Rats were sacrificed at 0th day, 7th day, 14th day, and 21st day. Fibroblast cell counts and inflammatory cell infiltration were evaluated on hematoxylin-eosin stained sections. Collagen type I and III were evaluated via immunohistochemistry.

**Results:** Wound area decreased from day 0 to day 21 in both groups. Significant differences in wound contraction and unhealed wound area were observed between 7th and 21th days in both groups. Improvement in wound area in morphological measurements was similar in both quercetin and control groups except for 21st day on which quercetin exhibited higher wound closure. Histological findings showed significantly higher fibroblast cell counts on 7th, 14th, and 21st days in the quercetin group and difference in both quercetin. Inflammatory cell counts significantly decreased from day 0 to 21 in both groups. Collagen I synthesis were lowest on the 0th day and significantly increased on all other times in both groups. As for collagen III, the levels were highest on 7th day in both groups and significantly decreased on the 14th and 21st day.

**Conclusion:** Present results revealed an improvement in wound healing after quercetin administration which was indicated by decreased inflammatory cells and increased fibroblast cells. However, collagen synthesis was found similar.

**Keywords:** Antioxidants; collagen; quercetin; wound healing.

## INTRODUCTION

Wound healing is a complex series of events which starts with inflammation and continues with proliferation and maturation of the wounded tissue (1,2). Inflammation is the very first step of the healing process (1,3,4) and the proliferation and maturation phases follow inflammatory phase (3,4). Factors which could affect the inflammation might compromise the healing process and implementation of additional agents such as antiseptics, antioxidants or nutritional products can improve the healing process (2,5,6).

Several factors contribute to the inflammation, a major one being the oxidative stress which is caused by an

imbalance in the reactive oxygen species (ROS) and antioxidant defenses (7). Increase in ROS might disrupt the wound healing by inducing apoptosis of fibroblast cells, up-regulating matrix metallo proteinase (MMP) production, increasing necrosis and decreasing collagen synthesis (8,9). ROS also increase inflammatory mediators and delay anabolic reactions in wound healing process (10). Achieving oxidative equilibrium may lead to deviant inflammation process, increased cell migration and synthesis of growth factors, and accelerated new tissue formation (10). Therefore, antioxidants might provide significant improvements in reducing tissue damage and promoting the healing process (9,11).

One of the most potent antioxidants with anti-

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inflammatory and anti-microbial efficacy is quercetin which is a plant-derived bioflavonoid compound (12-14). Quercetin modulates several signal molecules and kinases such as extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, p38, and protein kinase B phosphorylation and suppress inflammation by down-regulating major pro-inflammatory cytokines such as interleukin (IL)-1 beta (15). In addition, quercetin also modulates major pro-inflammatory pathways such as mitogen-activated protein kinase (MAPK) pathway and induces inhibitor of kappa B-alpha (I $\kappa$ B- $\alpha$ ) which regulates majority of pro-inflammatory events in the body (16,17). The antioxidant and anti-inflammatory features of the quercetin was also reported to provide a scar free wound healing (18,19). The effects of quercetin in wound healing were manifested with stimulation of extracellular matrix formation, up-regulation of vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)- $\beta$  synthesis (18,19). Quercetin was also found to be effective in disrupted wound healing process in persistent wounds associated with diabetes after topical quercetin implementation (20).

Based on the favorable effects of quercetin as an antioxidant and anti-inflammatory agent, the aim of the present study was to evaluate the effects of quercetin gel on a secondary intention wound healing model in Wistar rats. For this purpose, the wound closure via stereomicroscopic evaluation, fibroblast and inflammatory cell counts and Collagen I and Collagen III expressions in histological slides were determined.

## MATERIAL and METHODS

The present study protocol was approved by the local ethics committee of animal studies of Tokat Gaziosmanpasa University (approval number-HADYEK-44). The study was designed and 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 was created according to the 'NC3Rs ARRIVE Guidelines, Animal Research: Reporting of In Vivo Experiments'. Two main study groups and three sub-groups were created based on the sacrifice days. Palatal wounds of 5mm diameter were created on 42 female Wistar rats. All surgical procedures and maintenance of the animals were carried out at Tokat Gaziosmanpasa University Animal Research Center. All rats were kept in a special room with 12 hours dark-light cycles, under controlled temperature and humidity in individual cages. All rats achieved water and standardized rat chow ad libitum.

The study groups were;

1. Control groups (C, n=24)
  - a. Sacrifice on day 0 (Group 1, Control day 0)
  - b. Sacrifice on day 7 (Group 2, Control day 7)
  - c. Sacrifice on day 14 (Group 3, Control day 14)

- d. Sacrifice on day 21 (Group 4, Control day 21)
2. Quercetin groups (Q, n=18)
    - e. Sacrifice on day 7 (Group 5, Q day 7)
    - f. Sacrifice on day 14 (Group 6, Q day 14)
    - g. Sacrifice on day 21 (Group 7, Q day 21)

All surgical procedures were performed by an experienced researcher under general anesthesia with an intraperitoneal injection of ketamine (Eczacibasi Ilac Sanayi, Istanbul, Turkey) (100 mg/kg) and xylazine (Eczacibasi Ilac Sanayi, Istanbul, Turkey) (0.5 mg/kg). Firstly, rats were stabilized, and mouths were opened using a retractor. The gingiva in the palatal region of maxillary molar teeth was removed with a disposable punch with five-millimeter diameter. The bone was exposed and the wound was left uncovered to mimic a secondary intention wound healing model, and the bleeding was controlled.

### Vehicle and Quercetin implementation

Quercetin powder (Sigma Aldrich, Missouri, USA) gel was freshly prepared by a pharmacist before application. 5% quercetin (21,22) was dissolved in glycerin/alcohol (v/v 5:1) and 5% benzocaine (Anestezin, Botafarma, Ankara, Turkey) was also added to the mixture. The control rats received the gel containing only 5% benzocaine. Each rat had 0.4 ml gel and the gel application was performed at 9.00 am every day until the sacrifice day. 0.4 ml gel of quercetin contained 20 $\mu$ g quercetin and studies reported that the phenolic compounds cause no toxicity or tissue damage under the doses of 50 $\mu$ g (23,24).

Six rats in each subgroup were euthanized via anesthetic overdose in the 0th, 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days. The maxilla was removed and fixed in neutral buffered formalin solution with 10% concentration.

### Stereomicroscopic evaluation

All maxilla were photographed under 12.5x magnification via a stereomicroscope (Stemi 2000 and Axiovision 4.8, Carl Zeiss, Jena, Germany). The wound borders, unhealed area and the difference as the newly formed epithelization area were measured via a program integrated with a stereomicroscope (Stemi 2000 and Axiovision 4.8, Carl Zeiss, Jena, Germany). All measurements were performed by a blinded experienced researcher.

### Histological evaluation

After stereomicroscope photographs were taken, the palatal mucosa was dissected and dehydrated through an ethanol series. After dehydration, tissues were cleared with xylene and embedded in paraffin. All histological procedures were performed by an experienced researcher (F.G.) who was blind to the identities of the samples. Serial sections of 5  $\mu$ m were obtained from paraffin blocks. Hematoxylin-eosin (H&E) staining and immunohistochemistry for Collagen I and Collagen III were performed.

Fibroblast and inflammatory cells were counted in H&E-

stained slides. Measurements were performed in a cell counting frame of 10000µm<sup>2</sup> under 1000x magnification. Three analyses were performed for each animal, and a mean value was recorded. Fibroblast, endothelial, and inflammatory cells which can be easily differentiated (25-29) was observed, and fibroblast and inflammatory cells within the frame was counted. Inflammatory cells counted were macrophage, neutrophil, eosinophil, T lymphocytes and plasma cells (25-30). The cells which were not easily differentiated were not counted.

### Collagen I and Collagen III immunohistochemistry

Three slides per animal were chosen for immunohistochemical staining for each parameter. All selected sections were rehydrated and submerged to hydrogen peroxide (3%) for suppressing endogenous peroxidase activity. Afterward, sections were washed with phosphate buffered solution (PBS) thrice for five minutes (3x5) and incubated with rabbit serum for 30 minutes. After this incubation, primary antibodies were applied, and sections were incubated overnight at 4°C in a humidified dark room. The primary antibodies and dilutions were Goat polyclonal anti-Collagen I antibody (Abcam plc, Cambridge, UK) (1:250) and anti-Collagen III antibody (Abcam plc, Cambridge, UK) (1:250). Then, sections were washed 3x5 with PBS and incubated with biotinylated secondary antibodies immunoglobulin G for 30 minutes. After washing 3x5 with PBS again, sections were treated with streptavidin-horseradish peroxidase conjugated reagent and incubated for 30 minutes. After washing again 3x5 with PBS, 3-Amino-9-Ethylcarbazole chromogen was applied to visualize specific immunoreactivity. After last wash with 3x5 PBS, sections were counterstained with Gill's hematoxylin and washed with distilled water and then mounted.

Immunoreactivity reactions were observed as red staining in different shades from pale red to bright red color. All sections were evaluated with light microscopy under 400x magnification (Nikon, Tokyo, Japan). A cell counting frame was marked, and cells within the frame were counted considering their staining densities as no staining-0, slight staining-1, mild staining-2, and severe staining-3. To convert the stained cells and the staining density to a numeric value, an H score was calculated based on a formula,  $[\sum Pi(i+1)]$ . i: shows the intensity score of the staining and Pi: shows the percentage of the stained cells. All immunohistochemistry evaluations were performed by an experienced blinded examiner (O.K.) Immunohistochemistry analysis was performed from three different areas, and a mean value was recorded for each evaluated parameter (27,28).

### Statistical Analysis

A power analysis was performed, and the power of the study was calculated as 90%. All data were analyzed with IBM SPSS program (SPSS v.20.0, 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

The wound area results exhibited a significant difference in all tested durations in both groups ( $p < 0.05$ ). The wound closure on the 21<sup>st</sup> day was significantly higher than those of the 7<sup>th</sup> day in both groups ( $p < 0.05$ ). However, quercetin group showed higher improvement from day 14 to 21 compared to the control day 14 to control day 21 ( $p < 0.05$ ) (Figure 1, Figure 2, Table 1). 7<sup>th</sup> and 14<sup>th</sup> days exhibited similar wound closure in both groups ( $p > 0.05$ ) (Table 1). The only significant difference between quercetin and control groups was found on the 21<sup>st</sup> day ( $p < 0.05$ ). Both quercetin and control groups had wound healing with no complications.

**Table 1. Measurements of the palatal wound area, unhealed wound area and wound closure**

Groups/ Parameters	Palatal wound area	Unhealed wound area	Wound closure
Control Day 0	19.60±0.10	19.60±0.03	0.01±0.00
Control Day 7	12.89±2.07 <sup>a</sup>	9.52±5.31 <sup>a</sup>	2.94±0.95
Control Day 14	9.57±2.32 <sup>ab</sup>	7.51±5.42 <sup>a</sup>	3.09±1.89
Control Day 21	6.05±2.01 <sup>abc</sup>	2.25±2.18 <sup>abc</sup>	6.85±4.94 <sup>a</sup>
Test Group Day 7	12.76±1.98 <sup>a</sup>	7.12±3.61 <sup>a</sup>	5.08±1.43
Test Group Day 14	9.00±1.87 <sup>ae</sup>	3.31±3.13 <sup>ae</sup>	6.81±3.73 <sup>a</sup>
Test Group Day 21	3.00±0.75 <sup>a,ef</sup>	1.79±2.47 <sup>a,ef</sup>	13.96±6.36 <sup>a,d,ef</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>e</sup> $p < 0.05$  vs. test group day 7, <sup>f</sup> $p < 0.05$  vs. test group day 14.

The unhealed area in the center of the wound was also measured. Both the wound contraction and the unhealed area decreased from day 0 to day 21 ( $p < 0.05$ ). However, a significant difference in the unhealed area was observed between the day 7 and 21 in both groups ( $p < 0.05$ ). The healing from the day 7 to 14 and day 14 to 21 were similar in both groups ( $p > 0.05$ ). All groups showed similar decrease in the unhealed areas on all tested days ( $p < 0.05$ ) and the amount of decline was similar ( $p > 0.05$ ) (Figure 1, Figure 2, Table 1).

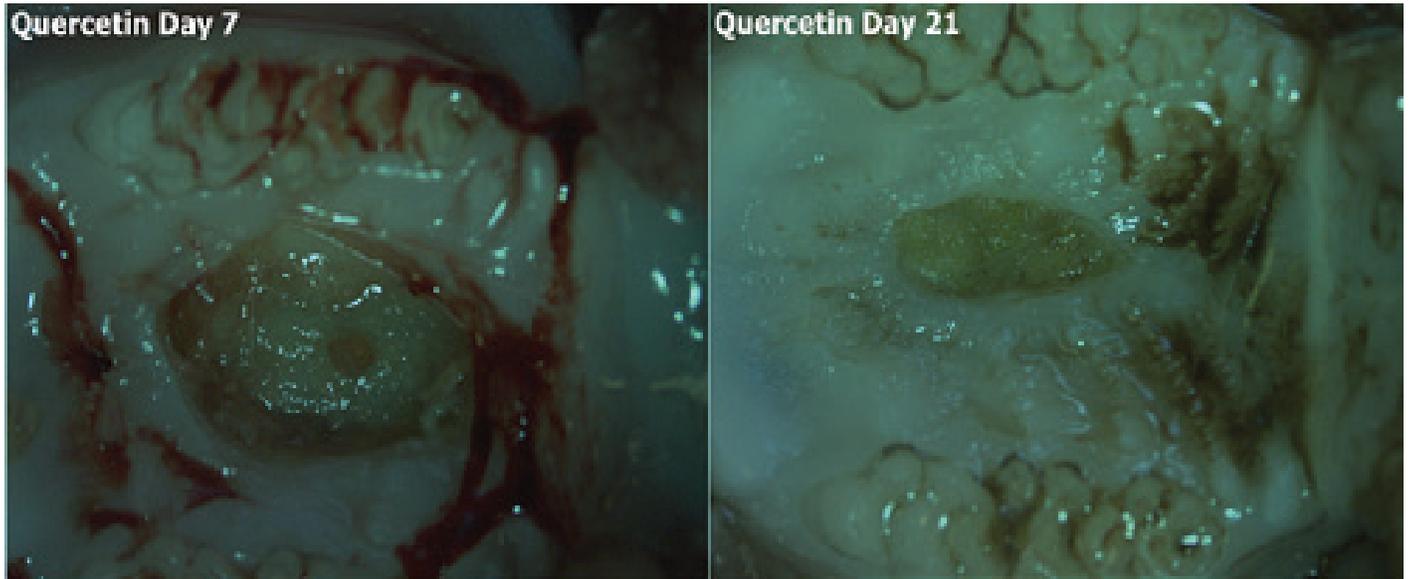


Figure 1. Representative Stereomicroscope images of the Quercetin day 7 and 21 groups under 12.5x magnification

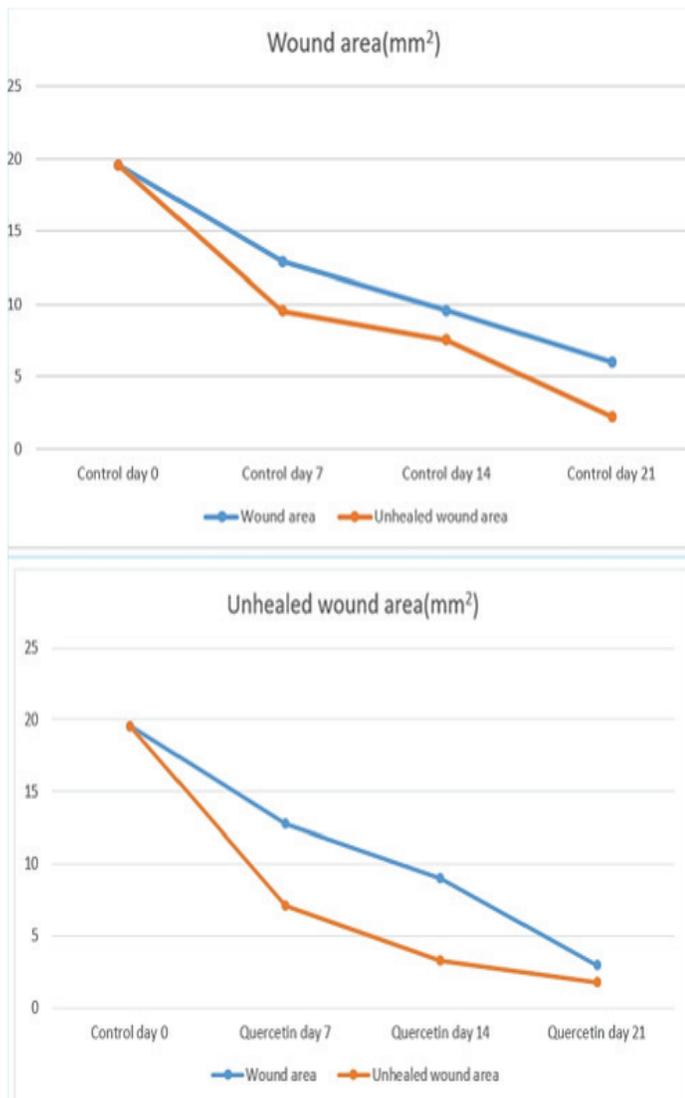


Figure 2. Wound area and unhealed wound area in the study groups

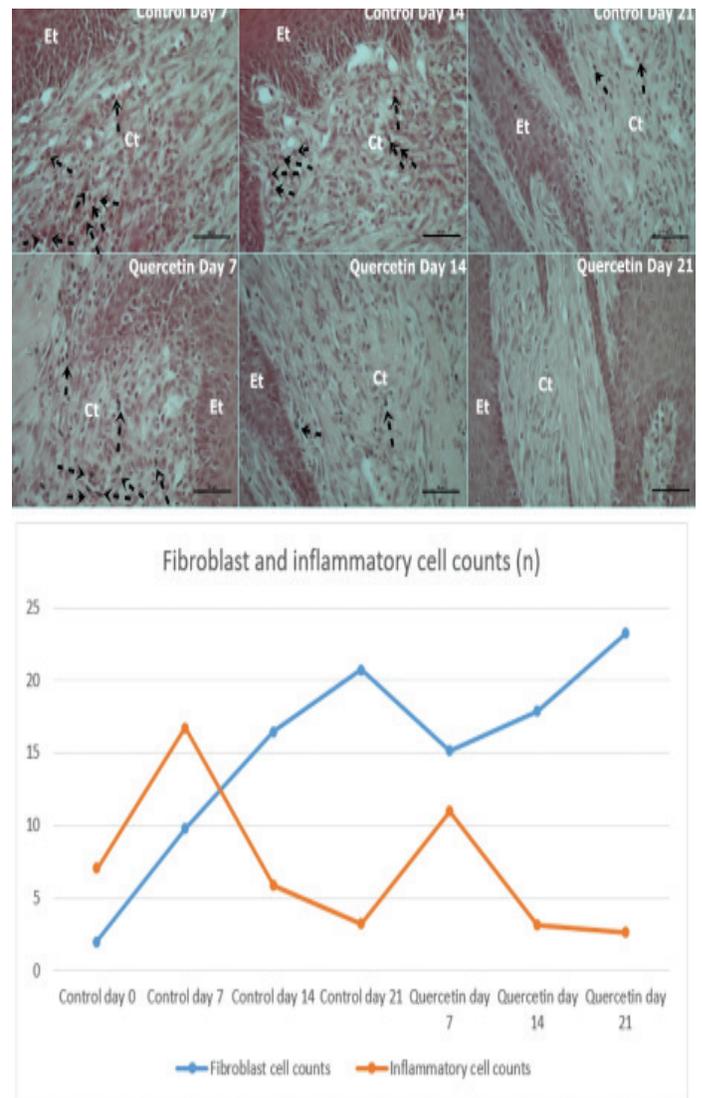


Figure 3. Representative Hematoxylin-eosin staining images, fibroblast cell counts and inflammatory cell counts of the study groups. Interrupted black arrows indicate inflammatory cells. Ct: Connective tissue, Et: Epithelial tissue

Table 2. Immunohistochemistry results and fibroblast and inflammatory cell counts

Groups/Parameters (H score)	Collagen I	Collagen III	Fibroblast cell counts	Inflammation cell counts
Control Day 0	19.90±9.05	35.32±20.50*	2.00±0.11	7.00±0.92*
Control Day 7	65.60±19.80*	63.65±17.32*	9.75±2.31*	16.72±1.30*
Control Day 14	76.78±5.65*	25.92±13.95 <sup>†</sup>	16.50±2.92 <sup>†</sup>	5.87±2.41 <sup>†</sup>
Control Day 21	70.58±5.82*	12.33±6.17 <sup>†</sup>	20.75±1.03 <sup>†,‡</sup>	3.25±1.03 <sup>†</sup>
Q Day 7	76.87±6.18*	68.12±18.09 <sup>†</sup>	15.12±0.83 <sup>†,‡,§</sup>	11.00±1.87 <sup>†,‡,§,¶</sup>
Q Day 14	74.62±4.47*	36.73±27.55 <sup>†,§</sup>	17.87±2.58 <sup>†,‡,§</sup>	3.12±1.55 <sup>†,§</sup>
Q Day 21	70.09±3.77*	21.05±19.44 <sup>†,§</sup>	23.25±1.90 <sup>†,‡,§,¶</sup>	2.62±1.47 <sup>†,‡,§,¶</sup>

\*p<0.05 vs. control day 0, <sup>†</sup>p<0.05 vs. control day 7, <sup>‡</sup>p<0.05 vs. control day 14, <sup>§</sup>p<0.05 vs. control day 21, <sup>¶</sup>p<0.05 vs. quercetin day 7, <sup>††</sup>p<0.05 vs. quercetin day 14

### Histological evaluation

In the HE-stained slides, the healed and unhealed wound area and newly formed epithelial and connective tissues were examined. Fibroblast and inflammatory cell counts in the groups were determined (Figure 3, and Table 2). Inflammatory cell counts in both groups significantly increased on the 7<sup>th</sup> day and decreased on the 14<sup>th</sup> and 21<sup>st</sup> days. The inflammatory cell counts were the highest in the 7<sup>th</sup> day control group and the differences were statistically significant (p<0.05). Quercetin caused significantly lower inflammation on the day 7<sup>th</sup> compared to the control group (p<0.05). The decrease of the quercetin inflammatory cell counts from day 7 to 14 and 14 to 21 in the group were found significant (p<0.05) while the differences in the control group were not found significant (p>0.05). Quercetin decreased early inflammation.

For the fibroblast cell counts, values exhibited a reverse pattern compared to the inflammatory cell counts. Cell counts in the control group showed a statistically significant increase from 0<sup>th</sup> day to the 21<sup>st</sup> day (p<0.05). Fibroblast on the day 0<sup>th</sup> was significantly lower than those of the other groups (p<0.05). Even though the cell counts increased on the day 7<sup>th</sup> in both groups, the increase in the quercetin group was more evident (p<0.05). Quercetin treated rats exhibited significantly higher fibroblast cell counts on 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days compared to the equivalent control groups (p<0.05) (Figure 3, Table 2).

### Collagen I and Collagen III immunohistochemistry

The immunohistochemistry results of the Collagen I and Collagen III expressions in the study groups were presented in Figure 4, Figure 5, and Table 2.

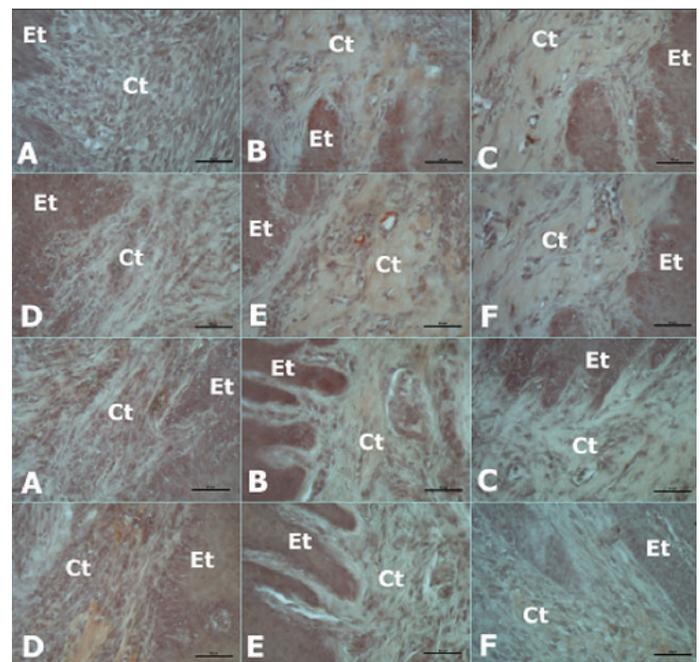
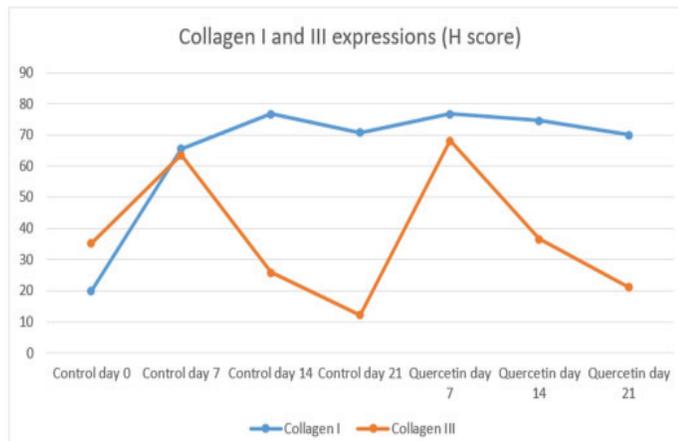


Figure 4. Representative immunohistochemistry staining of Collagen I and Collagen III in the study groups. Ct: Connective tissue, Et: Epithelial tissue

Collagen III levels increased from day 0 to day 7 and the highest levels were observed on the day 7<sup>th</sup> in both groups (p<0.05). The decrease in the collagen III levels from the day 7 to 14 and 7 to 21 were significant in both groups however the differences from the day 14 to 21 in quercetin and control groups were not found significant (p>0.05).



**Figure 5.** Immunohistochemistry staining results of Collagen I and Collagen III in the study groups

As for the collagen I synthesis, the significantly lowest value was observed in the 0th day ( $p < 0.05$ ). The expressions increased from the day 0 to 7, 14, and 21. The highest collagen I expressions were in the 14th day in both groups however, the differences in comparisons 7<sup>th</sup> vs. 14<sup>th</sup>, 14<sup>th</sup> vs. 21<sup>st</sup>, and 7<sup>th</sup> vs. 21<sup>st</sup> days were insignificant in both groups ( $p > 0.05$ ).

Both quercetin and control groups exhibited similar alterations in the collagen I and III expressions in all tested times.

## DISCUSSION

The effect of quercetin on wound healing was evaluated in the present study through a secondary intention wound healing model in Wistar rats. The results revealed that quercetin significantly improved wound healing on the day 21 by decreased wound area. Quercetin also reduced the early inflammation and increased fibroblast cell density. However, collagen I and III synthesis in the quercetin treated groups compared to the control rats were similar in all tested times.

Inflammation is the first stage of wound healing and anabolic activities such as cell migration, proliferation, extracellular matrix production begin after the inflammatory phase (3). Quercetin is a bioflavonoid compound with potent anti-inflammatory efficacy through suppressing inflammatory mediators such as nitrogen reactive species or cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  (31). Anti-inflammatory effect occurs mainly through modulating various signal molecules such as MAPK pathway and blocking of TNF- $\alpha$  mediated inflammation of macrophage cells and inhibition of cyclooxygenase and lipoxygenase productions (16,32-36). Down-regulation of cyclooxygenase production was also associated with the antimicrobial effect of quercetin which is manifested by blockage of lipoteichoic acid signal transduction and related NF-KB activation (12,16,32). Decrease in TNF- $\alpha$

and nitric oxide production was also reported in vivo (13). The present study evaluated the inflammation through the inflammatory cell infiltration in the wound area and the results revealed a significant decrease in the inflammatory cell counts in accordance with previous reports (19,32-36). Quercetin gel implementation reduced inflammatory cell infiltration, especially on the early period of the healing which was observed on the 7<sup>th</sup> day. The decreases in the inflammatory cell infiltrate on the 14<sup>th</sup> and 21<sup>st</sup> days were similar to those of the control groups.

Along with reduced inflammation, quercetin administration promotes the wound healing by also inducing fibroblast proliferation in rats in early period of wound healing (19) which occurred through stimulation of VEGF, TGF- $\beta$ , IL-10, and CD31. Wang et al. also reported the effect of quercetin on connective tissue metabolism via reducing expression of MMP-2, MMP-9, cathepsin B, and cathepsin K and increased tissue inhibitor of MMP-1 gene expression (14). Increased VEGF expressions were also reported along with decreased MMP production (37). On the other hand, Doersch et al. found no impact of quercetin on fibroblast cell growth or wound contraction (18). In contrast to Doersch et al. fibroblast numbers increased from day 0 to 21 in both groups (18). Furthermore, in the present study, quercetin-treated rats had significantly higher fibroblast cells compared to the control group on all tested durations. This result was in accordance with Gopalakrishnan et al. who also reported increased fibroblast cell counts in the wound area from day 0 to day 14 (19).

In addition to the fibroblast proliferation, the healing process also requires the increase in fibroblast function and the synthesis of extracellular matrix molecules such as hydroxyproline and glucosamine and Ahmad et al. demonstrated that quercetin induced hydroxyproline and glucosamine in rats even with the presence of diabetes (20). Gomathi et al. reported promoted wound healing after quercetin-incorporated collagen matrices in dermal wound healing (38). They also indicated elevated collagen production with increased hydroxyproline synthesis (38). However, the most pronounced effect of quercetin on wound healing and connective tissue metabolism is the prevention of scar formation (39). This effect occurs in a dose-dependent manner and causes a decrease in collagen production (39,40). However, the effect of quercetin on MMPs result in an increase in the collagen in the connective tissue due to the decreased collagen destruction (14,37). Collagen synthesis is a crucial component of wound healing since collagen III production begins earlier in the inflammatory phase while collagen III synthesis begins later on the proliferation phase. Both collagen I and III synthesis were reported to peak on days from 7 to 14 (41). Furthermore, Shukla et al. reported that quercetin increased collagen synthesis, fibroblast function and new blood vessels (42). However, the present results showed similar collagen I and III synthesis when control

and quercetin groups were compared. Furthermore, the highest collagen III levels were observed on the day 7 in both groups while the highest collagen I expressions were on the day 14 in the control group and on the day 7 in the quercetin group. Collagen I significantly increased from day 3 to day 14, and at day 21<sup>st</sup>, a slight decrease was observed in the control group. On the other hand, quercetin caused an increase in the collagen I on the day 7 and a slight decrease on the days 14 and 21 were observed which could indicate an early activation of proliferation phase in wound healing process.

Fibroblast count, collagen and extracellular matrix production and inflammation are histological indicators of wound healing. The most prominent clinical feature of wound healing is wound closure. In this regard, Jangde et al. reported accelerated wound healing and decreased wound closure time after quercetin gel implementation (43). Ambiga et al. also showed that quercetin-containing flavonoids promoted wound healing and provided a better wound closure in skin wounds in rats (44). Furthermore, Gopalakrishnan et al.(19) used a topical gel of quercetin with a concentration of 0.1% and reported smaller wound area and greater wound closure. The wound healing was morphologically evaluated in the present study and the present results showed reduced wound area and increased wound closure from day 0 to day 21. The unhealed wound area exhibited a similar pattern and decreased from day 0 to 21 in both quercetin and control groups. The improvement in the wound area was more evident in the quercetin groups. In contrast with Gopalakrishnan et al.(19), quercetin did not provide significant improvements on the days 14<sup>th</sup> and before in wound closure in morphometric measurements. However, the wound closure of the quercetin group on the day 21 was higher than the control group. The concentration of the quercetin gel was used in the present study was 5% with a dose of 20µg for each rat which was reported to be effective in previous studies (21,22) with no toxicity or tissue damage (23,24).

## CONCLUSION

The present study was designed and conducted as an experimental study evaluating the effect of quercetin on a secondary intention wound healing model in rats. The wound closure and unhealed area was slightly better in the quercetin treated rats. A possible explanation was the increased fibroblast cell count after quercetin administration and early reduction in the inflammatory cell infiltration. Nonetheless, immunohistochemistry analysis showed no difference in Collagen, I and Collagen III synthesis after quercetin administration. The present results should be interpreted considering limitations of animal studies as the animal biology differs from human biology in terms of physiologic processes. Performing further biochemical analysis would have enlightened the

effects of quercetin on wound healing better. Therefore, studies with further analysis like Western-blot, cell-culture or PCR should be performed.

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

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