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Wound Healing Potential of Quinic Acid in Human Dermal Fibroblasts by Regulating the Expression of FN1 and COL1α Genes

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Abstract: Quinic acid (QA) is an alicyclic organic acid widely found in plants. It accumulates in varying concentrations of plant species and is actively metabolized throughout the plant's life cycle. Wound healing after skin injury involves a complex interaction of many cells, fibroblasts, endothelial cells, and regenerated immune cells and their interrelating extracellular matrix. In this study, it was aimed to investigate the healing effect of QA on scar tissue, changes in oxidative stress parameters, FN1 and COL1A1 gene levels. For this purpose, fibroblast cells were seeded in 24, 96 and well plates for wound healing, MTT analysis and Real-Time PCR testing (respectively). Wells were drawn with a 100 µL pipette tip for wound line. As a conclusion of our study, it was determined that cell viability increased significantly, especially in the QA 20 μg^{-ml} group at the end of 48 hours. Increased cell viability and antioxidant capacity resulted in increased cell proliferation. Both FN1 and COL1A1 gene expression levels were up regulated in the QA groups compared to the control group. Our findings show for the first time that QA promotes migration and/or proliferation of fibroblasts by regulating oxidative stress and the FN1A and COL1A1 genes. This activity may be related to the production of FN1A and COL1A1, which are considered important targets for modulation of the tissue repair process.

Kinik Asit'in FN1 ve COL1A1 Gen Ekspresyonunu Düzenleyerek İnsan Dermal Fibroblastlarında Yara İyileştirme Potansiyeli

Anahtar Kelimeler
FN1A, COL1A1, 8-OHdG

Öz: Kinik asit (QA), bitkilerde yaygın olarak bulunan bir alisiklik organik asittir. Bitki türlerinin değişen konsantrasyonlarında birikir ve bitkinin yaşam döngüsü boyunca aktif olarak metabolize edilir. Cilt yaralanmasından sonra yara iyileşmesi, başta keratinositler, fibroblastlar, damarların endotel hücreleri ve rejenere bağışıklık hücreleri ve bunlarla ilişkili hücre dışı matris olmak üzere birçok hücrenin karmaşık bir etkileşimini içerir. Bu çalışmada, QA'nın yara dokusu üzerindeki iyileştirici etkisinin oksidatif stres parametreleri, FN1 ve COL1A1 gen düzeylerindeki değişimler incelenmesi amaçlanmıştır. Bu amaçla, fibroblast hücreleri yara iyileşmesi için 24 oyuklu plakalara, MTT analizi için 96 ve Real-Time PCR testi için 6 oyuklu plakalara ekildi. Kuyucuklar, yara hattı için 100 µL pipet ucu ile çizildi. Çalışmamız sonucunda 48 saat sonunda özellikle QA 20 μg^{-ml} grubunda hücre canlılığının önemli ölçüde arttığı belirlendi. Artan hücre canlılığı ve antioksidan kapasitesi, hücre çoğalmasının artmasına neden oldu. Hem FN1 hem de COL1A1 gen ekspresyon seviyeleri, kontrol grubuna kıyasla QA gruplarında yukarı regüle edildi. Bulgularımız ilk kez kinik asidin oksidatif stresi ve FN1A ve COL1A1 genlerini düzenleyerek fibroblastların göçünü ve/veya çoğalmasını desteklediğini göstermektedir. Bu aktivite, doku onarım sürecinin modülasyonu için önemli hedefler olarak kabul edilen FN1A ve COL1A1 üretimi ile ilgili olabilir.

1. INTRODUCTION

Tissue healing process after damage is one of the main characteristics of all organisms due to the preservation of homeostasis of the body [1]. The skin is the body's most important barrier against the harmful effects of external factors. Therefore, complex mechanisms are involved in the self-defense of the tissue and the preservation of the completeness of the skin after damage [2-4]. Wound healing after skin injury contains a complex interaction of many cells, primarily keratinocytes, endothelial cells of vessels, and regenerated immune cells and their extracellular matrix [2, 5]. These processes are regulated by a variety of mediators, including cytokines, growth factors, inflammatory cells, platelets, matrix metalloproteinases [6]. Conversely, some wounds do not heal on time and regularly, therefore it results in chronic non-healing wounds [7]. Since cytokines, growth factors, proteases, and cellular and extracellular elements all play important roles at different stages of the healing process, changes in one or more of these components may explain the impaired healing sighted in chronic wounds [8].

Fibronectin (FN) is an extracellular matrix (ECM) protein that reorganizes the cell's cytoskeleton, facilitating cell movement, dictating cell adhesion, spreading, migration, proliferation, and apoptosis [9]. FN, which has different structures and different roles in wound healing, contributes to the formation of fibrin clots. As a result, they take a role in wound healing by suppressing leukocytes and endothelial cells [10]. Cellular FN is vital for wound sites, like cellular proliferation, fibroblast polarization, cell migration, collagen complex regulation (I/III complex), and regulating neovascularization. A member of the FN family, Fibronectin 1 (FN1) has a critical role in cellular growth and tissue repair and is expressed highly in human dermal fibroblasts (HDFs) [11].

QA is one of the types of organic acids commonly found in plants. This substance, which is found in different concentrations according to the plant species and the developmental stages of the plants, is actively metabolized throughout the life cycle of the plant [12-14]. Some plants have intrinsically high amounts of QA, such as tea, coffee, and some fruits [15, 16]. QA derivatives have many advantageous effects, including antioxidant [17], anti-inflammatory [18], hypoglycemic and hepatoprotective effects, and inhibition of mutagenesis and carcinogenesis.

In this study, the cellular wound repair mechanism was tried to be elucidated by QA and the changes in the FN1 and Collogen1A1 gene levels were considered. In this respect, our study will provide new information to the literature.

2. MATERIAL AND METHOD

2.1. Chemicals & Reagents

QA, Phosphate buffer solution (PBS), Dulbecco Modified Eagles Medium (DMEM), Trypsin (with EDTA), dimethylsulfoxide (DMSO), fetal bovine serum (FBS), and antibiotics were purchased from Sigma (St. Louis, MO, U.S.A).

2.2. Cell Culture

For our study, Primary Dermal Fibroblast Normal; Human, Neonatal (HDFn) was purchased ATCC. The cell was resuspended by fresh medium, 10% FBS, and antibiotic 1% (penicillin, amphotericin B, and streptomycin). The cells were cultured in 6, 24, and 96 well plates and stored at an incubator in optimal condition (5% CO2; 37 °C) [19].

2.3. Wound Assay

With the wound assay, it was evaluated the migration rate of QA in the fibroblast cell line. Fibroblast cells were seeded in a 24-well plate and were incubated until 100% confluency. At the end of day 5, each well was wound with a sterile plastic pipette tip (yellow tip-100 µl). Cell debris was aspirated with PBS. Cells were then exposed to various concentrations of QA (2.5, 5, 10, and 20 μg^{-ml}). Images from the central area of the wound were photographed at 0-48 hours by an invert microscope (Leica Microsystems, Wetzlar, Germany) at ×20 magnification to assess cell migration. All experiments were done in triplicate.

2.4. Drug Administration

After the cells reached 85% confluency, they were seeded into 6 (for Real Time PCR), 24 (for wound healing assay) and 96 well plates (for MTT). Experimental groups were determined as control, wound control, QA (2.5, 5 ,10, and 20 μg^{-ml}) were administered. It was incubated under optimum conditions until the first wound was closed in the experimental groups.

2.5. MTT Tetrazolium Assay Concept

10 µL MTT (5 mg^{-ml} concentration) was added to each well and incubated for 4 hours (5% CO2; 37 °C) to perform the MTT assay. To dissolve formazan crystals, after 4 hours the medium was removed, and 100 µL DMSO was put in. Cell viability was measured by optical density read at 570 nm using the Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA) and the cell viability calculated as a %.
Viability Rate (%) = (O.D of groups/Control O.D) X 100

2.6. Total Antioxidant Capacity (TAC) Assay

The antioxidant capacity was investigated to determine the TAC level. For this purpose, 500 µL reagent 1 (Buffer) solution and 30 µL sample were added to the well, the initial absorbance was measured at 660 nm. 75 µL Reagent 2 (ABTS Radical Cation) solution was added and the second measurement was made at 660 nm. TAC values were calculated as Trolox Equiv/mmol L^{-1}, according to the formula below.

A2-A1= ΔA absorbance (Standard, sample, or H2O)

Result =(H2O ΔA bs-Sample ΔA bs)/(H2O Δbs-standard Δbs)

2.7. Total Oxidant Status (TOS) Assay

500 µL of Reagent1 (Buffer) solution was added to the wells containing 75 µL of a sample, and the initial absorbance value was read at 530nm, for evaluated spectrophotometrically TOS assay. Then 25 µL of Reagent 2 (Pro chromogen) solution was added to the same well. After 10 minutes at room temperature, the second absorbance value was read. TOS values were calculated as H2O2 Equiv mmol/L^{-1}, according to the formula below.

A2-A1= Δ Absorbance (Standard or sample)
Results= (Sample Δ Abs)/(Standard ΔAbs) x10

2.8. Lactate Dehydrogenase (LDH) Assay

According to the manufacturer's instructions (Cayman Chemicals, USA), LDH was determined with the LDH detection kit. Cells were seeded in 96 well plates. 6 wells were used for each concentration. Triton X-100 (10%) and 20 µL Assay buffer were added and incubated at room temperature for one hour. The cells were centrifuge 400Xg for five minutes. 100 µL of cell supernatant were transfered to a new 96- well assay plates. LDH reaction solution was added to each well and incubated the plate with gentle shaking on an orbital shaker for 30 minutes at 37 °C. LDH levels was measured the absorbance OD value at 490 nm [20].

((Experimental Value A490)-(Spontaneous Release A490))/((Maximum Release A490)-(Spontaneous Release A490)) X 100

*Maximum release: All cells were killed by adding Triton X-100. Spontaneous release: Control group with nontoxic materials (cell medium) and Experiment value

2.9. Glutathione (GSH) Assay

GSH was investigated with the GSH detection kit (Elabscience, USA). In this method, the principle was based on the formation of a yellow complex due to GSH reaction with dinitrobenzoic acid. The experiment was conducted according to the kit protocol and the results were read at 450 nm wavelength. The obtained results were given as % value.

2.10. 8-hydroxy-2'-deoxyguanosine (8-OHdG) Assay

The Oxidative DNA Damage ELISA kit is an ELISA for the quantitative measurement of 8-OHdG. DNA in each sample was obtained according to the manufacturer's (Cell Biolabs, USA) instructions and the kit protocol was applied according to the manufacturer's instructions.

2.11. Gene Expression

Total RNA was extracted from neuronal cells. Total RNA was used for synthesizing complementary DNA (cDNA) using cDNA Reverse Transcription Kit. The sequences of gene-specific PCR primers are listed below. Results are stated as relative fold compared with the control group. We normalized gene expressions to beta-actin using ΔΔCt method (x) and states as fold change to control.

βactin
Forward: 5'-CCAACCGCGAGAAGATGA-3'
Reverse: 5'-CCAGAGGCGTACAGGGATAG-3'
FN1
Forward:5′-CCAACTACCAGTAGCGAAAA −3′
Reverse: 5′-GCAGGGAAAGGAAAGAAA-3′
COL1A1
Forward: 5′-GACCAGGAATTCGGCTTCGAAGT-3′
Reverse: 5′-CATTGGATCCTGTGTCTTCTGGG-3′

2.12. Statistical Analyses

Statistical analyses were utilized using One-way ANOVA and Tukey HSD method (SPSS 26 software). P<0.05 and P<0.01 were considered a statistically significant differences in all tests. Results are submitted as mean and standard deviation (mean±SD).

3. RESULTS

3.1.MTT Assay

The wound line closed at the end of 48 hours in the 20 µg^{-ml} group and the experiment was terminated at that hour. Cell viability results of all groups after 48 hours of application were revealed by the MTT test and the cell viability graph is given in Figure 1. Cell viability was considered as 100% in the control group (negative control). In the positive control group (wound), this rate was found to be 66.00% at the end of 48 hours. The viability rate of QA 1 µg^{-ml} was found to be 79.81%. While this rate was QA 2.5 µg^{-ml} 97,65% at the end of 48 hours in the QA 2 µg^{-ml} group, it increased by nearly 45.13% at the QA 20 µg^{-ml}. The cell viability level increased with wound healing. the highest cell viability level was seen in the 20 µg^{-ml} group. When MTT results are examined, it is clearly seen that QA increases cell viability. The results were statistically significant (P<0.05, P<0.01).

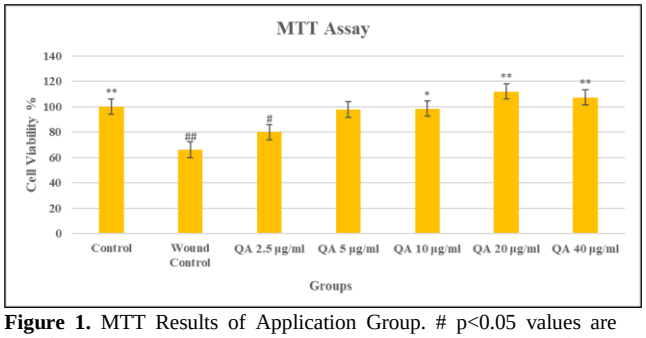

Figure 1. MTT Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

3.2. Wound Healing

The wound line was created with a 100 µl pipette tip. There was no change at the 12th hour. For this reason, 24-

36- and 48-hours images were used. When our results were examined, it was determined that the wound line was closed from the 36th hour in the QA 20 and 40 μg^{-ml} groups. In the control wound group, the wound line was observed even at the 48th hour. When compared with this group, it was designated that the cell proliferation increased, and the wound line was completely closed in the QA 20 and 40 μg^{-ml} groups (Figure 2).

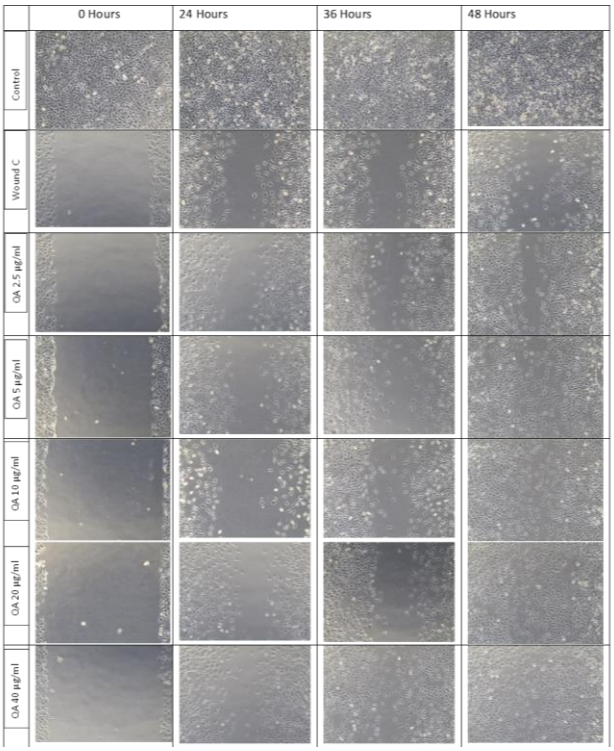

Figure 2. Wound line images of 24, 36 and 48 hours.

3.3. LDH Results

The activity of LDH was rated according to the standard solution (pure-%100) of the commercial kit (Figure 3). The results of the control group were found as 20.61±1.03 % and 10 at the end of 48 hours and the cellular death rate increased as the LDH level increased. The LDH level increased sharply in the wound group (97.96 ± 4.92 %). The wound group was found to be the group with the highest LDH level. Almost 4 times increase was observed compared to the control group. A significant difference was found when compared with the control group. This indicates the extent of cellular damage. However, in the groups treated with QA, there was a decrease in LDH levels at increasing concentrations in parallel with the healing of the scar tissue. The findings were compared with the wound group. Especially in the 20 μg^{-ml} group (42.01 ± 2.11), the LDH level approached the control group. These results correlate with MTT. It was determined that cytotoxicity was eliminated because of QA repairing the scar tissue and contributing to cell proliferation (P<0.05, P<0.01).

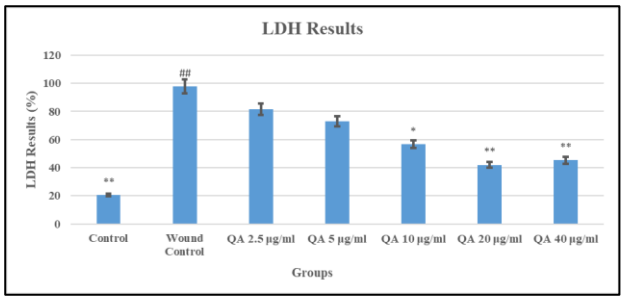

Figure 3. LDH Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

3.4. Redox State in Fibroblast Cells Treated with QA

TAC level and the findings are given in Figure 4. According to our data, the total antioxidant activity values of the control group were determined as 11.21±1.008 mmol Trolox equivalent/L. When the control group and the wound group were compared, the TAC value of the wound group was determined as 7.73±0.21 mmol Trolox equivalent-L. A significant increase in TAC level occurred in the QA group because it is a molecule with antioxidant activity. The highest increase was observed in the QA group 40 μg^{-ml} (13.15±0.678 mmol Trolox equiv.-L). This value was higher than the control group. We attribute the reason for this to the antioxidant property of the QA molecule. QA caused increased cell proliferation and decreased cellular oxidation (P<0.05, ** P<0.01).

Contrary to the TAC level, the TOS level was the lowest in the control group (5,2±0.08) (Figure 5). TOS level was determined as 20.41±0.234 in the wound group. There was a decrease in the QA group depending on the dose increase. The results were found to be significant when compared with the control group.

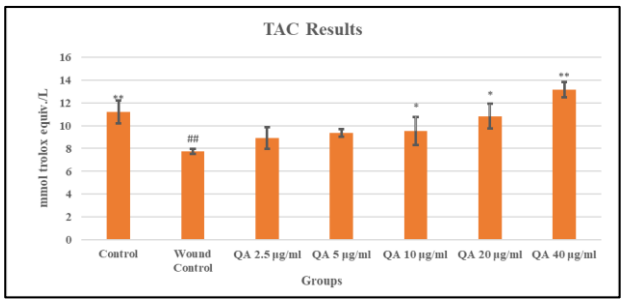

Figure 4. TAC Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

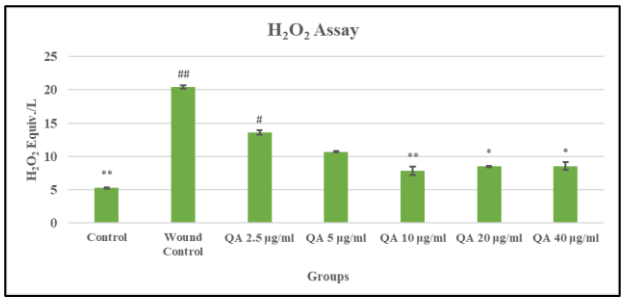

Figure 5. TOS Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

3.5. GSH Results

Another intracellular enzyme for determining cellular oxidative damage is GSH. GSH activity was measured, and the results were statistically evaluated and presented in Figure 6. The GSH level of the control group was 42.89% after 48 hours. As the GSH level decreases, the cellular death rate increases. After the injury in the wound group, the GSH level was determined as 23.14% and decreased approximately 2 times compared to the control group. In the QA group, the GSH level started to increase gradually, and the results approached the control group in the QA 40 μg^{-ml} group. A significant difference actuarially was observed according to the results obtained (P<0.05 and P<0.001).

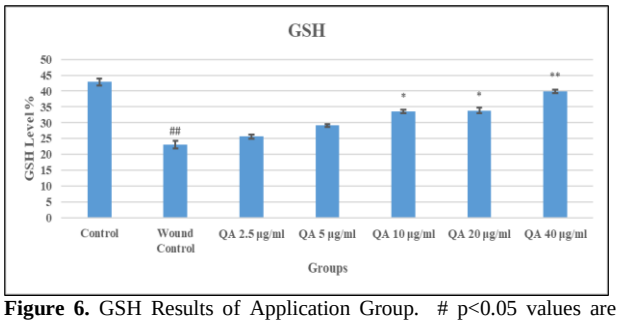

Figure 6. GSH Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

3.6. 8-OHdG Levels of Fibroblast Cells Treated with QA

Prevalently used as a biomarker for oxidative stress, 8-OHdG is one of the predominant forms of reactive oxygen species (ROS) lesions. In our study, oxidative DNA damage caused by the wound in fibroblast cells was demonstrated using 8-OHdG biomarker. While the 8-OHdG level was found to be 0.289 pg^{-ml} in the control group, cellular damage gradually increased in the wound group. The most significant result was observed in the QA 40 μg^{-ml} group, as in other data. The results were found significant (P<0.05 and P<0.001) (Figure 7).

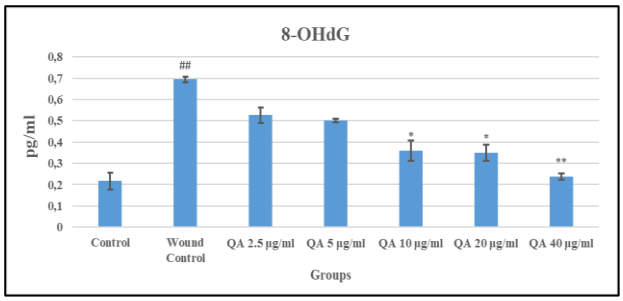

Figure 7. 8-OHdG Results of Application Group. # p<0.05 values are significant wound group compared to the control * p<0.05= significant, ** P<0.01= very significant

3.7. Real- Time PCR Results

There was no change in the COL1A1 gene level in the control and wound groups. With the onset of healing in the wound group, an increase in the regulation of COL1A1 gene expression started in the QA groups. COL1A1 is co-expressed with repair of scar tissue. When the results we obtained were examined, it was determined that there was a 1.6-fold increase in QA 20 and 40 μg^{-ml} concentrations. The control group was accepted as 1, and the other groups were compared to the control group. The data obtained were found to be statistically significant (p<0.05, ** P<0.01) (Figure 8a-b). Similarly, there was no change in the FN1 gene level in the control and wound groups. It was determined that there was a approximately 1.5-fold increase in QA 20 and 40 μg^{-ml} concentrations.

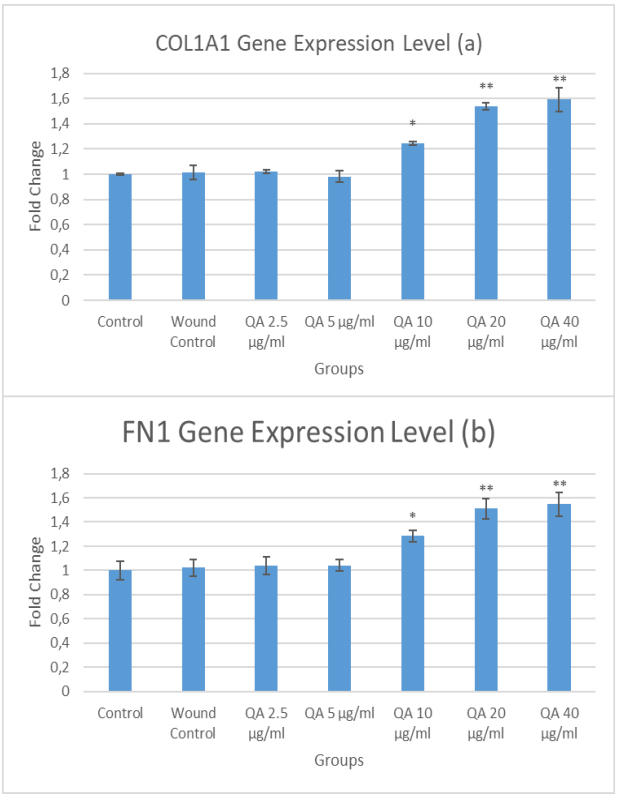

Figure 8. Gene Expression of Application Group. a) COL1A1 gene level; b) FN1 gene level* p<0.05= significant, ** P<0.01= very significant

4. DISCUSSION AND CONCLUSION

In our study, the effect of QA on the healing process of fibroblast cells was investigated. When the findings we obtained were analyzed, it was determined that the increased oxidative stress and cellular cytotoxicity in the wound area were eliminated by QA and the wound line was closed faster than the normal process. Wound healing is a biological process that includes many important stages such as hemostasis, cell proliferation, inflammatory events, and tissue remodeling [1, 21]. Increased oxidative stress at the wound site plays an important role in regulating normal wound healing by inflammation, angiogenesis, facilitating hemostasis, wound closure, granulation tissue formation, and development and maturation of the extracellular matrix [22]. For this reason, the increase in the amount of ROS in that region takes a central place in the wound healing processes. However, various substances with antioxidant activity have been proven to be beneficial in wound healing [23, 24]. However, various substances with antioxidant activity have been proven to be beneficial in wound healing. The antioxidant effect of QA showed a healing effect on the wound. It provided the closure of the wound line in a shorter time compared to the wound group. In addition, the oxidative stress, which increased in the wound group, decreased gradually in the QA group, which triggered the wound area to heal in a shorter time.
During wound healing, FN is one of the first and most abundant ECM components to accumulate at that site [25, 26]. Fibronectin triggers fibril formation with ECM formation. Fibronectin matrix deposition in wounds stimulates collagen and contributes to wound contraction. In addition, fibronectin can link to other cells to further stabilize the ECM [27]. In vitro studies have shown that Fibronectin polymerization leads to the composition and stability of the ECM and cell matrix adhesion [27, 28]. Fibronectin polymerization enables collagen types I and III to be deposited into the ECM and results in stabilization of collagen I matrix fibrils11. A study conducted by Shi and Sottile showed that membrane-type matrix metalloproteinase 1 (MT1-MMP/MMP) promotes ECM fibronectin conversion by regulating the division of large fibronectin fibrils and subsequently regulating endocytosis of α5β1 integrin [29]. They also showed that inhibiting fibronectin polymerization accelerated myofibroblast migration.

Our findings show for the first time that QA promotes migration and/or proliferation of fibroblasts by regulating oxidative stress and the FN1A and COL1A1 genes. This activity may be related to the production of FN and COL, which are considered important targets for modulation of the tissue repair process and play an important role in the wound healing process. These results are the first evidence to support the traditional use of this substance to treat wounds.

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