Quercetin Inhibits Rat Epidural Fibrosis by Regulating the Biological Behavior of Fibroblasts

Yile Cao  
Yangzhou University

Hui Chen  
Northern Jiangsu People’s Hospital

Zhehao Fan  (✉️ 610417114@qq.com)  
Yangzhou University

Yu Sun  
Northern Jiangsu People's Hospital

Research Article

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Abstract

**Objective:** This study attempted to investigate the effects of quercetin on postoperative fibrosis in the epidural as well as any potentially relevant signaling pathways.

**Methods:** The effect of quercetin on reducing epidural fibrosis was confirmed via histological staining and immunohistochemical analysis *in vivo*. Accordingly, cell counting kit-8 (CCK-8), Western blot analysis, immunofluorescence and EdU staining, TUNEL staining and transmission electron microscopy were used to detect the effects of quercetin on the proliferation and apoptosis of fibroblasts and explore the possible signal transduction pathway.

**Results:** HE staining and Masson staining showed that quercetin could reduce the number of fibroblasts and collagen content. Moreover, LC3 immunohistochemical staining demonstrated that quercetin could induce autophagy, while CCK-8 showed that quercetin inhibited fibroblast viability in a concentration and time-dependent manner. The results of EdU staining, TUNEL staining and Western blot illustrated that quercetin could inhibit the proliferation and promote the apoptosis of fibroblasts. Additionally, immunofluorescence showed that quercetin could inhibit the migration of fibroblasts and reduce collagen content. LC3 immunofluorescence staining, Western blot and transmission electron microscopy demonstrated that quercetin could induce autophagy. Furthermore, following intervention with autophagy inhibitor 3-MA, quercetin was suggested to affect the proliferation and apoptosis of fibroblasts via autophagy, possibly through the PI3K / Akt / mTOR signaling pathway.

**Conclusion:** Quercetin can regulate fibroblast proliferation, apoptosis, migration and other biological behaviors through autophagy, thereby preventing epidural fibrosis. The specific corresponding pathway may be the PI3K / Akt / mTOR signaling pathway.

1. Introduction

Operations involving the spinal canal can inevitably cause damage to the peridural structure, resulting in the formation of epidural fibrosis (EF)[1–2]. During patient recovery and wound repair, local fibroblasts are activated and migrated, leading to spinal canal stenosis and compression of the patient's dural sac and nerve root, which may eventually lead to fibrosis and adhesion and give rise to pain. Therefore, the prevention of EF is of great significance to both surgeons and patients who require surgery[3–4]. Thus far, the pathogenesis of EF and scar adhesion has not been fully studied, however, most scholars believe that the activation, migration and excessive proliferation of fibroblasts in the surgical area are the main causes of the disease[5–6]. Therefore, we believe that adopting effective measures to prevent excessive proliferation of fibroblasts can serve as a reliable means in preventing EF.

Currently, in order to prevent and treat the occurrence of EF and postoperative pain, many methods have been adopted clinically, which mainly include: improved operation to reduce bleeding, drug treatment during and after operation, implantation of biomaterials, and rehabilitation exercise[7–9]. It is of great
clinical significance to identify novel therapeutic and pharmacological targets in order to prevent EF with little side effects[6].

Autophagy is a very conservative biological process in cells[10]. It is believed that the main function of autophagy is to remove damaged cell structures, senescent organelles and various macromolecules that are no longer used in cells[11–13]. Autophagy, which plays an important role in growth and development, is closely connected with cell differentiation and the stress response[14]. This provides a new goal and research direction for our disease prevention and treatment research.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) can be widely found in foods, plants, and beverages[15]. However, whether quercetin can affect cell proliferation, apoptosis and autophagy of fibroblasts in EF has yet to be investigated. Therefore, the present study attempts to explore the influence of quercetin on EF and prove that was resulted by affecting fibroblast autophagy, apoptosis and proliferation. In this study, autophagy was chosen to study the role of quercetin in the prevention of EF.

2. Materials And Methods

2.1. Fibroblast culture and treatment

In line with the procedure put forward by Li et al. (2017), a main fibroblast cell line was acquired. Using Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY), cultured cells were attained at 37°C and 5% CO2. The medium was supplemented with penicillin with a concentration of 100 U/ml, 15% fetal bovine serum (FBS; Gibco), and streptomycin with a concentration of 100 mg/ml (PS; Thermo, Rockford, IL). The cells were cultured to 3–6 generations and those exponentially growing were used as the experimental materials. The fibroblasts were seeded in 96-well plates, 6-well plates or 10-cm culture dishes overnight. After the cell density reached 50–80%, 7.4-pH phosphate buffered saline (PBS) was used to wash the cells, which were then treated with Sigma-brand Quercetin (St. Louis, MO, USA).

2.2. Cell viability assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8,Dojindo, Tokyo, Japan). Cells were seeded in 96-well plates in triplicate and treated separately with 5 to 320µmol/L quercetin dilutions. In another group, cells were cultured in DMEM for 0, 12, 24, 36, 48, 60, and 72 hours, after which the cells were incubated with 10µL of WST-8 (Dojindo Laboratories, Kumamoto, Japan) for 1 hour at 37°C. Cells positive for WST-8 staining were considered to be viable cells.

2.3. EdU incorporation assay

Cell proliferation was measured using Cell-Light KFluor488 EdU Kit (Ribobio, Guangzhou, China). Fibroblasts with a density of 1 x 10^5 were seeded into 6-well plates and incubated at 37 C for 24 hours. They were then treated with quercetin at different concentrations for 24 hours. Then, 50 µM EdU was added to each well for 2 hours. The cells were immobilized with 4% paraformaldehyde for 10 minutes
and then infiltrated with 0.5% Triton X-100 for 15 minutes. The nuclei were stained with Hoechst 33342 and were then observed under an inverted fluorescence microscope.

2.4. TUNEL assay in fibroblasts

In order to assess human fibroblast apoptosis, TUNEL staining was conducted (KeyGEN, Nanjing, China). Trial procedures strictly complied with the directions given by the manufacturer. In order to perceive the apoptosis attributes, a fluorescence microscope was utilized. It was deemed apoptotic if the fibroblasts were TUNEL-stained. Through DAPI staining, the overall fibroblast count was determined.

2.5. Western blot analysis

Using a radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), the total protein was acquired. In addition, a BCA Protein Assay Kit (Beyotime, Shanghai, China) was utilized in the determination of total protein concentration. Through a 5–12% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), 50-µg protein extracts were detached from being transmitted into membranes containing polyvinylidene difluoride (Millipore, Bedford, MA, USA). The membranes were blocked for two hours at room temperature using 5% skim milk before being nurtured using primary and secondary antibodies, in line with the provided directions. Eventually, the protein bands were noted through an improved chemiluminescence determination (ECL- Plus kit, Beyotime), which were then digitally taken (Bio-Rad ChemiDoc XRS+, California, America). Blotting with β-actin regulated the differences in protein loading. Using the densitometry of the unsaturated captures and an exclusion of the background density, immunoreactive bands were then counted (Image J, NIH, Bethesda, USA).

2.6. Immunofluorescent staining

Cells were treated with 0 and 10-6M quercetin, respectively, then fixed with 4% paraformaldehyde for 10 minutes. After washing, permeation was conducted for 15 minutes using 0.1% Triton X-100.Cells were incubated overnight at 4 C using anti-α-SMA (1:100) and anti-LC3 antibody (1:100). It was performed using an antibody made of anti-rabbit IgG H&L 1 hour after washing (Alexa Fluor 555) (1:200) (Abcam, British).

2.7. Rat grouping

We researched 36 adult male SD rats (Yangzhou, China) weighing 280 ± 20g who were then purchased from the laboratory animal center of Yangzhou university. Accordingly, 48 Sprague-Dawley adult male rats (Yangzhou, China) weighing 280 ± 20g were purchased from the laboratory animal center of Yangzhou university for analysis. All animals were duly cared for in accordance with experimental animal protection guidelines, and this study was sanctioned by Yangzhou’s Animal Ethics Committee. The samples were arbitrarily separated so as to comprise three groups (12 rats per group): quercetin (100 mg/kg), quercetin (200mg/kg) and the control group (saline).

2.8. laminectomy modeling and intragastric administration of quercetin
Rat laminectomy models were established according to the procedure outlined by Sun et al. (2007). After intraperitoneal ketamine injection (100 mg/kg) as anesthesia, the samples’ fur were shaved within L1 and L2, and the visible skin was disinfected using iodophor. Applying an incision on the midline skin and a detachment of the paravertebral muscles, the L1 vertebral plate was made visible. By detaching the spinous process and L1 vertebral plate using rongeur forceps, the dura mater was uncovered. According to this method, L1 laminectomy was performed in rats, after which the experimental model was obtained. Penicillin (50 mg/kg) was injected intramuscularly in each rat to prevent incision infection, once a day for three days.

From day 1 after laminectomy, the rats were gavaged with quercetin by intragastric administration in different doses (100 mg/kg and 200 mg/kg) or the control group (saline) once a day for 1 month. In this month, all rats were carefully cared for.

2.9. Histological analysis

A month later, histological examination was conducted on the remaining six rats in each group. Anesthesia was administered on the samples prior to applying 4% paraformaldehyde as an intracardiac perfusion. Overall, the L1 spinal column, alongside epidural fibrous tissue and paravertebral muscles, were detached for soaking using a 10% formalin buffer, which was then softened and paraffin-embedded. Through the L1 vertebra, continuous 4-µm transverse incisions were carried out. Masson and hematoxylin-eosin (H&E) were used for dyeing. Employing a ×400 magnification on a light microscope, the degree of epidural scar adhesion and epidural fibrosis was then determined.

2.10. Immunohistochemistry

The expression of LC3 in epidural fibrosis was detected via immunohistochemical staining. The sample was treated for endogenous peroxidase blockage by applying 3% H2O2. PBS was then applied thrice on the portions, which were then incubated at 4°C overnight using an anti-LC3 antibody. Then, it was incubated with anti-mouse IgG for 1.5 hours at room temperature, and antibody binding was detected using a DAB staining solution. Eventually, the samples were de-stained using hematoxylin for 3 minutes, after which the fibroblasts were determined through an optical microscope.

2.11. Statistical analysis

In this study, SPSS 22.0 was used in the analysis of the experimental data. Using one-way ANOVA, the differences in every set were determined, and all results were presented as mean ± standard deviation (SD). In regard to the determination of statistical significance, P should be less than 0.05.

3. Results

3.1. Macroscopic and histological evaluation of EF
Cell counting of the HE stained sections demonstrated that as the concentration of quercetin increased, fibroblast counts decreased. This results suggested that the local application of quercetin can reduce EF by reducing the number of fibroblasts (p < 0.05, Fig. 1(a,c)). Masson staining showed that the collagen content in the 200mg/kg group was the lowest, while decreasing collagen I and collagen III trends were related to the rise in drug concentration. These results indicated that the collagen content in the operation area decreased along with the increase in quercetin concentration (p < 0.05, Fig. 1(b,d,e,f,g,h)).

3.2. Quercetin induces autophagy in the surgical area

In order to explore the effect of quercetin on autophagy, the expression of p-mTOR and LC3 in epidural tissue sections were tested using immunohistochemistry. The results illustrated that the expression levels of LC3 gradually increased while the expression of p-mTOR decreased along with the concentration of quercetin, exhibiting a concentration-dependent manner (p < 0.01, Fig. 2(a,b,c,d)). It can be concluded that quercetin can induce autophagy following epidural surgery, for which the mTOR mediated signaling pathway is involved.

3.3. Quercetin affects the biological behaviors of fibroblasts such as apoptosis, proliferation and migration

In regard to the investigation of the specific effect of quercetin on fibroblasts, cytological experiments were carried out. CCK-8 showed that the viability of fibroblasts decreased with the increase in quercetin concentration (p < 0.05, Fig. 3(a)). Fibroblasts were continuously treated with 20µmol/L quercetin, and their cell viability was detected at different times. Accordingly, the viability of fibroblasts was found to decrease with time in a time-dependent manner (p < 0.05, Fig. 3(b)). Edu staining showed that the number of positive cells decreased significantly (p < 0.05, Fig. 3(e,f)), indicating that quercetin had a significant inhibition on cell proliferation, while Western blot analysis yielded similar results. The expression of PCNA and Cyclin D1 decreased (p < 0.05, Fig. 3(c,d)), which further demonstrated the inhibitory effect of quercetin on the proliferation of fibroblasts.

In order to investigate the specific effect of quercetin on fibroblast apoptosis, fibroblasts with different concentrations of quercetin were stimulated for 24h. Afterward, TUNEL staining was used to detect whether quercetin had an effect on fibroblast apoptosis (p < 0.05, Fig. 3(j)). Next, Western blot assay was performed to detect the apoptotic proteins, which demonstrated the promotional effect of quercetin on the fibroblast apoptosis. (p < 0.05, Fig. 3(g,h,i)).

Finally, immunofluorescence staining was used to detect the expression of migration protein α - SMA, collagen I and collagen III (p < 0.05, Fig. 3(k,l,m)). The results showed that the expression of α - SMA decreased after quercetin, and collagen I and collagen III content were significantly inhibited. All of the corresponding results indicated that quercetin can inhibit the proliferation, migration and collagen secretion of fibroblasts while promoting their apoptosis.
3.4. Quercetin regulates fibroblast apoptosis and proliferation through autophagy, and mTOR mediated signaling pathway is involved

In order to study the specific mechanism of quercetin on the proliferation and apoptosis of fibroblasts, further experiments were performed. First, the expression of autophagy related proteins following quercetin were detected at different concentrations. With the increase in drug concentration, the results illustrated that the expression of Beclin 1, Atg5, and LC3-II/LC3-I increased, while that of p62 decreased ($p < 0.05$, Fig. 4(a,b)). This trend was closely related to drug concentration, which exhibited a concentration dependent manner. Moreover, LC3 immunofluorescence images demonstrated the presence of a significantly increased number of LC3 dots in fibroblasts following treatment of quercetin ($p < 0.05$, Fig. 4(c,d)). To further explore the role of autophagy, autophagy inhibitor 3-MA was used. However, following the stimulation of 3-MA, the low expression levels of Cyclin D1 and PCNA were partly reversed after quercetin induction. Additionally, the high expression levels of cleaved-PARP and LC3-II/LC3-I were found to be partially inhibited ($p < 0.05$, Fig. 4(e,f)). Meanwhile, the results of TUNEL, EdU assay and LC3 immunofluorescence yielded the same results ($p < 0.05$, Fig. 4(g,i,k,h,j,l)), which indicated that autophagy was inhibited after application of 3-MA.

Finally, proteins related to the classical pathway of autophagy were then detected. To further investigate its mechanism, the expression levels of related proteins in the mTOR mediated signaling pathway were detected. As the concentration of quercetin rose, the expression levels of p-PI3K, p-mTOR, and p-AKT decreased, while that of p-AMPK increased in a concentration-dependent manner ($p < 0.05$, Fig. 4(m,n)). The above results demonstrated that quercetin-induced negative regulation of mTOR mediated signaling pathway played an important role in the action of quercetin.

4. Discussion

Surgery-induced extensive epidural fibrosis following laminectomy often results in negative effects on outcome and even causes failed back surgery syndrome (FBSS)[1–2]. Meanwhile, fibroblast proliferation plays an important role in the formation of EF[16]. Following surgical laminectomy, fibroblasts in the bone defect site are activated by inflammatory cytokines and growth factors to produce collagen and extracellular matrix. This activation produces excessive fibrous connective tissue to repair the local defects, eventually leading to epidural fibrosis[17].

Quercetin, a kind of major flavonoid found in many traditional Chinese medicines, is an effective substance for treatment[18]. Specifically, one study demonstrated that quercetin, through its free radical scavenging and antioxidant properties, attenuates radiation-induced DNA damage and apoptosis in the kidney and bladder tissues of rats, suggesting that quercetin may possess potential benefits in radiotherapy by minimizing adverse effects[19].
In this study, the preventive effect of quercetin on epidural fibrosis scar adhesion was studied. The results showed that quercetin can effectively prevent the formation of EF, exhibiting a concentration dependent manner. The results of HE and Masson staining showed that quercetin could reduce the number of fibroblasts and collagen content in the operation area. Here, fibroblasts acted as the main effector cells of scar formation, while the amount of collagen determined the degree of fibrosis. These results fully explain that quercetin can reduce the number of fibroblasts and collagen content in the operation area to inhibit fibrotic scar adhesion in rats following epidural surgery, which is concentration dependent. The mechanism of quercetin that produces this effect is not clear, hence, immunohistochemical staining was carried out to detect the expression of LC3 and p-mTOR. The results showed that with a rise in drug concentration, LC3 expression increased significantly while that of p-mTOR decreased. LC3 is the characteristic protein of autophagy; therefore, we can speculate that autophagy plays an important role in the prevention of epidural fibrosis scar adhesion by quercetin.

Autophagy is an important biological behavior of organisms, which is extremely conservative in evolution[20]. The regulation of autophagy is a very complex process, and existing research is incomplete. The regulation of autophagy can be divided into mTOR dependent and mTOR independent regulation. mTOR is a mammalian target of rapamycin[21]. As the most important protein in autophagy regulation, mTOR can sense multiple signals and regulate autophagy negatively[22]. mTOR plays an important role in cell biological processes, such as proliferation and apoptosis. mTOR is involved in many developmental processes, such as nerve regeneration and T lymphocyte activation [23]. In general, mTOR inhibits the activity of autophagy initiation molecule ATG 1, controlling the occurrence of autophagy[24]. Activated mTOR participates in many cellular functions by phosphorylating certain factors (4E-BP1 and P70S6K) in protein translation[25]. When a tumor occurs, its expression will be upregulated, and autophagy will be inhibited[26]. Most autophagy signal transduction pathways are conducted via the mTOR pathway; hence, as an important inhibitory pathway, it plays an irreplaceable role in autophagy[27].

In the cytological experiments, CCK-8 showed that quercetin could effectively inhibit the viability of fibroblasts in a concentration and time-dependent manner. Afterward, Edu and TUNEL staining demonstrated that quercetin could inhibit the proliferation of fibroblasts and increase its apoptosis. Western blot analysis of proliferation and apoptosis related proteins yielded similar results. Subsequently, the expression of migration associated protein α - SMA, collagen type 1 and collagen type 3 were detected. The results illustrated that quercetin could reduce the expression of α - SMA, type 1 collagen and type 3 collagen and that quercetin has a regulatory effect on the biological behavior of fibroblasts. Quercetin can inhibit the viability and reduce the number of fibroblasts through the regulation of proliferation, apoptosis, and migration.

In order to further clarify the role of autophagy in the process of quercetin, LC3 immunofluorescence detection was done, which found that the number of fluorescent spots increased significantly in the drug group, indicating that autophagy was activated after quercetin. The autophagy related proteins beclin-1, LC3, ATG5 and p62 were then detected by Western blot. Accordingly, beclin-1, LC3-II / I ratio and ATG5
expression increased with a rise in quercetin concentration, while p62 decreased in a concentration-dependent manner, further suggesting the occurrence of autophagy. Quercetin can induce autophagy in fibroblasts. In order to further explore the relationship between autophagy and the effects of inhibiting proliferation and inducing apoptosis, autophagy inhibitor 3-MA was used. The cells were divided into groups, pretreated with 3-MA, intervened by quercetin, and detected by LC3 immunofluorescence staining. The results showed that the number of fluorescent spots in fibroblasts pretreated with 3-MA was lower than that in the control group after quercetin intervention. The expression of LC3 protein was then detected by Western blot. The results also showed that the LC3-II / I ratio in fibroblasts pretreated with 3-MA was lower than that in the control group. These results fully indicate that 3-MA can effectively inhibit quercetin induced autophagy in fibroblasts. Following 3-MA pretreatment of fibroblasts, Western blot was used to detect the proliferation and apoptosis of the related proteins. As autophagy in fibroblasts was inhibited, quercetin's effect on cell viability and proliferation was found to be effectively reduced, while its ability to induce apoptosis of fibroblasts was partially reversed. Finally, the protein expression of the key upstream pathways of mTOR were detected, which illustrated that the expression levels of p-PI3K and p-AKT decreased while p-AMPK increased, indicating that the PI3K / Akt and AMPK/mTOR pathways were inhibited, thus inhibiting the activity of mTOR.

5. Conclusion

In conclusion, the corresponding experiments fully demonstrate that quercetin can effectively prevent the occurrence of epidural fibrosis scar adhesion, which may occur through the mTOR mediated signaling pathway in order to regulate the proliferation, apoptosis, migration and other biological behaviors of fibroblasts. Although this study is still relatively basic with various limitations, it provides novel insight and research directions for the clinical prevention of epidural fibrosis scar adhesion.

Abbreviations

3-MA: 3-Methyladenine
CCK-8: cell counting kit-8
HE: hematoxylineosin
PARP: poly ADP-ribose polymerase
DMEM: Dulbecco's Modified Eagle's medium
FBS: fetal bovine serum
PBS: phosphate buffer saline
RIPA: radioimmune-deposition
Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Contributions

Yile Cao, Yu Sun, and Zhehao Fan designed and performed the whole experiments. Yile Cao and Zhehao Fan contributed to the preparation of the manuscript. Hui Chen helped in the performance of animal surgeries and the interpretation of data. All authors read and approved the final manuscript.

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Figures
Figure 1

Macroscopic and histological evaluation of EF. (a,c) Fibroblast counts in images of H&E staining (magnification, ×400). (b,d) Representative images of Masson staining and the OD value of collagen (magnification, ×400). (e,f) Representative images of Masson staining and the relative levels of collagen. (g,h) Representative images of Masson staining and the relative levels of collagen.

Figure 2
Immunohistochemical staining of LC3 and p-mTOR in epidural fibrosis tissues. (a,c) LC3 immunohistochemistry staining results showed that Quercetin treatment reduced the number of LC3-expressing cells in a dose-dependent manner in rats. (b,d) p-mTOR immunohistochemistry staining results showed that Quercetin treatment reduced the number of p-mTOR-expressing cells in a dose-dependent manner in rats.

Figure 3
Quercetin affects the biological behaviors of fibroblasts such as apoptosis, proliferation and migration. (a,b) The CCK-8 results indicated that quercetin could reduce the survival rate of fibroblasts in a time- and concentration-dependent manner. (c,d) Western blotting indicated that quercetin downregulated the expression levels of PCNA and Cyclin D1 with increasing concentrations. (e,f) EDU test showed that the percentage of EDU positive cells decreased significantly after the treatment with quercetin for 24 h (20μmol/L). (g,h,i) Western blot analysis showed that quercetin significantly increased the expression of apoptotic marker protein Cleaved-PARP and Bax, and then decreased the expression of anti-apoptotic protein Bcl-2 in a concentration-dependent manner. (k,l) Immunofluorescence staining was used to detect the expression of migration protein α-SMA. (m) Immunofluorescence staining was used to detect the expression of collagen I and collagen III.
Figure 4

Quercetin regulates fibroblast apoptosis and proliferation through autophagy, and mTOR mediated signaling pathway is involved. (a,b) Western blotting was used to analyze the effect of quercetin on the expression of autophagy-related proteins in fibroblasts. (c,d) Immunofluorescence study of anti-LC3 antibody showed that after 20μmol/L quercetin treatment of fibroblasts for 24 h, flake LC3 fluorescence was formed in the cytoplasm. (e,f) After treated with Autophagy inhibitors 3-MA, the expression of cell proliferation-related protein increased, while the expression of cell apoptosis and autophagy-related
protein decreased. (g,h) Quercetin increased expression of LC3 in fibroblasts via inhibiting PI3K/AKT/mTOR pathway. (i,j) EdU staining showed that after treated with Autophagy inhibitors 3-MA, the proliferation limitation of fibroblasts induced by quercetin was reversed to a certain extent. (k,l) TUNEL staining was used to detect the apoptosis of fibroblasts. It indicated that there was significant difference between quercetin group and quercetin mixed 3-MA group. (m,n) Western blot results showed that the phosphorylation levels of PI3K, AKT, MAPK and mTOR decreased in a dose-dependent manner after quercetin acted on fibroblasts.