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ORIGINAL PAPER

Low-dose rosmarinic acid and thymoquinone accelerate wound healing in retinal pigment epithelial cells

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Abstract

Purpose Thymoquinone (TQ) and rosmarinic acid (RA) are two biologically active compounds found in plants and that possess remarkable anti-oxidant and anti-inflammatory properties. The present study aimed to investigate the potential protective effects of RA and TQ, which have known anti-inflammatory and anti-oxidant effects, on retinal damage by establishing a wound healing model for retinal pigment epithelial cells (ARPE-19).

Method To this end, IC50 doses of RA and TQ in ARPE-19 cells were calculated by MTT assay. Both agents were administered at IC50, IC50/2 and IC50/4 doses for wound healing assay, and wound closure percentages were analyzed. Since the best wound healing was found at IC50/4 dose (low dose) for both agents, other biochemical and molecular analyses were planned to be performed using these doses. Following low dose RA and TQ treatments, the cells were lysed and TGF-β1 and MMP-9 levels were analyzed by ELISA technique from the cell lysates obtained. In addition, the mRNA expression levels of TLR3, IFN-γ and VEGF were calculated by RT-PCR technique.

Results Low dose of RA and TQ dramatically increased wound healing. RA may have achieved this by increasing levels of MMP-9 and TLR-3. In contrast, the mRNA expression level of VEGF remained unchanged. TQ accelerated wound healing by increasing both the protein levels of TGF-β1 and MMP-9. Furthermore, low dose of TQ decreased both TLR3 and IFN-γ mRNA expression levels.

Conclusion Low doses of RA and TQ were clearly demonstrated to have protective properties against possible damage to retinal pigment epithelial cells.

Keywords Retinal damage · Rosmarinic acid · Thymoquinone · Wound Healing

Introduction

Retinal damage can occur in the retina as a result of oxidative stress [1], immune response [2], light [3], arterial obstruction [4], and other factors. In this context, prevention or treatment of retinal damage is important to combat retinal diseases.

Thymoquinone (TQ) is known to possess many beneficial properties such as anti-diabetic [5], anti-oxidant [6], antimicrobial [7], anti-inflammatory [8], anti-allergic [9] and anti-neoplastic [10] which have been well-documented by various studies. In addition, previous studies reported that TQ has positive effects

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on wound healing [11, 12]. TQ is mainly involved in wound healing by inducing angiogenesis, enhanced fibroblast proliferation and subsequent collagen synthesis [13].

TQ has been reported to significantly reduce ocular symptoms in rats with allergic conjunctivitis [14], significantly protect against diabetes-induced changes in rats with its antioxidant, anti-inflammatory and anti-diabetic effects [15], and to be effective in preventing ganglion cell damage that may develop secondary to glaucoma with its ability to lower intraocular pressure in an animal experiment on rabbits [16].

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is a bioactive compound commonly found in plants from the Lamiaceae family including mint, thyme, sage, lavender, rosemary, etc. [17]. In addition to its anti-inflammatory [18], antibacterial [19] antiviral [20], anti-oxidative and anti-apoptotic [21] properties, RA is known to accelerate wound healing [22].

This study aimed to accelerate the healing process in retinal injuries by evaluating the effects of RA and TQ, which are known to have anti-inflammatory and antioxidant effects, on retinal damage by first establishing a model of wound healing in ARPE-19 cells and then, administering them to these cells.

Materials and methods

Chemicals and reagents

RA was purchased from Sigma-Aldrich (Cat. No.: R4033-50MG, Germany), and TQ was purchased from TCI (Cat. No.: T0795, Tokyo, Japan). ARPE-19 retinal pigmented epithelium cell line was purchased from American Type Culture Collection (CRL-2302, Manassas, VA, USA). The TGF-β1 and MMP-9 ELISA kit was purchased from BT-LAB (Bioassay technology laboratory Shanghai, China).

Cell culture

The ARPE-19 retinal pigmented epithelium cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 5% L-Glutamine and 1% penicillin–streptomycin. The cells were maintained at 37 °C into a humidified incubator with 5% CO_2 and 100% humidity.

Cell viability assay

MTT cell viability test and experimental groups

Cells were seeded in 96-well plates with 10,000 cells per well. The cells were treated with different concentrations (1 µM, 10 nM, 50 µM, 100 µM, 250 µM, 500 µM and 1000 µM) of RA and TQ for 24 h. Then, 10 µl MTT solution (5 mg/ml, from SERVA Electrophoresis GmbH, Heidelberg, Germany) was added to each well and incubated for 4 h at 37 °C, with %5 CO_2. Next, 100 µL dimethyl sulfoxide (DMSO) was added to each well and the absorbance of each well was measured at 570 nm using an automated reader (Epoch, Biotek, USA). The IC50 values were calculated for RA and TQ by the GraphPad Prism version 8.0.1 (GraphPad Software, Inc., CA, USA). After the determination of IC50 values, the IC50 doses for RA and TQ were treated in the cells both separately and together. Accordingly, three study groups were formed as follows: control group, RA group and TQ group. Each assay was performed in triplicate. As reported in the literature studies, Dimethyl sulfoxide (DMSO, Millipore Sigma, Germany) was used as a solvent to dissolve both TQ [23] and RA [24] for treatment. Specifically, samples were dissolved in 100% DMSO diluted using Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK) from solutions to IC50 doses, and the final concentration of DMSO in each group was adjusted to be 0.5%.

Wound healing assay

Retinal pigment epithelial cells were seeded in 6-well plates at a number of 3×10^5 and after > 80% coating the bottom of the well with overnight incubation, and the monolayer cell layers were scratched using a 200 µl pipette tip and washed at least twice with PBS. After washing, RA and TQ were applied to the wells at the IC50 dose previously determined by MTT assay, and at half (IC50/2) and quarter (IC50/4) concentrations of the IC50 dose for 24 h. For all groups, the FBS concentration in the medium was reduced to 5%. Cells were photographed at 0 h and 24 h after scratching; % wound closure rates were analyzed by ImageJ software.

Total protein analyses from cell lysates

The cells were washed twice with PBS and then, were harvested using lysis buffer containing protease inhibitor cocktail (Roche Complete, Indianapolis, USA). The lysates were centrifuged at 16.000 g at 4 °C for 15 min. The protein concentration collected from the supernatant was determined by BCA method (TaKaRa, Shiga, Japan).

ELISA analyses

Cells were treated with RA and TQ at a dose of IC50/4, which was the dose that caused the best healing wound for 24 h of incubation. Then, the TGF-β1 and MMP-9 levels were measured in the extracted protein from the cell lysate according to the manufacturer's instructions (BT-LAB, China, MMP-9 and TGF-β1, respectively, Cat No.: E0936Hu and E0134Hu) using ELISA technique. The results were expressed as pg/mg protein.

Total RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Retinal pigment epithelial cells were incubated for 24 h at the IC50/4 dose of both RA and TQ that produced the best wound healing. The cells were collected and washed with PBS after completion of incubations for 24 h. The total RNA for mRNA expression level was isolated by GeneJET RNA Purification Kit (Thermo Scientific Catalog No.: K0731). Quantification and purity of isolated RNAs were analyzed using the Epoch Take3 plate system (Agilent, USA). Then, complementary DNA (cDNA) Synthesis kit (Biorad Cat No.: BR1708891) was generated according to manufacturer's instructions. Shortly, 1 µg of total RNA was used as template in the PCR reaction, which was performed with reverse transcriptase (RT). Then, 1 µl of cDNA of each sample was taken and appropriate amounts of SYBR green PCR Master Mix, forward and reverse primer were added according to the protocols. Expression levels of the target genes were normalized to the housekeeping gene GAPDH. Gene expression values were then calculated based on the $\Delta\Delta Ct$ method using the equation: $RQ = 2^{-\Delta\Delta Ct}$ by REST2009 program. The primer sequences used in PCR reactions and PCR conditions are described in Table 1. Each assay was performed in four replicate.

Statistical analysis

Data on this study were analyzed by GraphPad Prism version 8.0.1 (GraphPad Software, Inc., CA, USA). The conformity of variables to normal distribution was tested with the Kolmogorov–Smirnov test. The descriptive statistics of variables were expressed as mean ± SD deviation for normal distributions. The presence of statistically significant differences between study groups was examined by one-way ANOVA followed by Tukey's Multiple Comparisons test. $p < 0.05$ was considered the threshold of statistical significance level.

Results

IC50 doses of the agents

It was found that IC50 values of RA and TQ were 178.9 µM and 87.06 µM for 24 h' treatment, respectively (Fig. 1).

Table 1 Oligonucleotide primer sequences and RT-PCR programs

Genes	Primer sequences (5′→3′)	RT-PCR programs	Cycle
GAPDH	F-5′GATTTGGTCGTATTGGGCGC3′ R-5′AGTGATGGCATGGACTGTGG3′	95 °C-30 s/59 °C-1 m/72 °C-30 s	35
TLR3	F-5′TGCACGGGCTTTTCAATGTG3′ R-5′ACGAAGAGGCTGGAATGGTG3′	95 °C-30 s/57 °C-1 m/72 °C-30 s	35
IFN-γ	F-5′GTGATTATCGGCAGCTGGTG3′ R-5′TCCCTTTGTTTCTCCCCTGG3′	95 °C-30 s/57 °C-1 m/72 °C-30 s	35
VEGF	F-5′TGCAAAAACACAGACTCGCG3′ R-5′CCCTCCCAACTCAAGTCCAC3′	95 °C-30 s/57 °C-1 m/72 °C-30 s	35

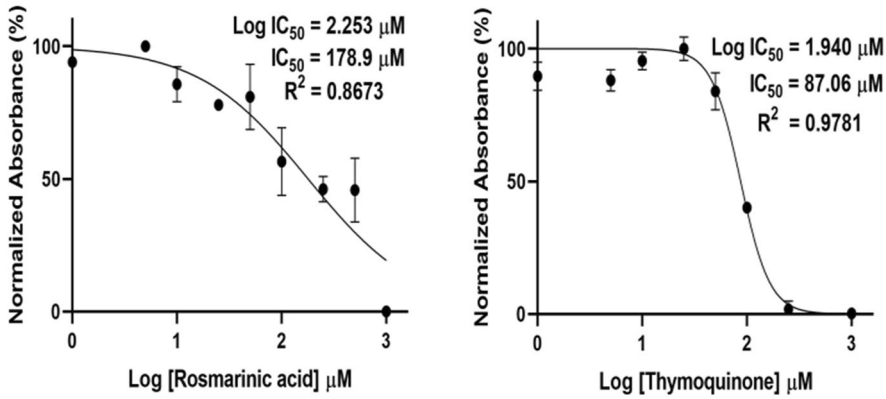

Fig. 1 IC50 doses of RA and TQ

Table 2 Comparison of % wound healing data between groups

Doses	% Wound healing assay		
	Control	Rosmarinic acid	Thymoquinone
IC_{50}	58.55 ± 3.532^{a}	29.48 ± 6.967^{b}	1.159 ± 0.8573^{c}
$IC_{50/2}$	58.55 ± 3.532^{a}	69.22 ± 13.34^{a}	65.83 ± 12.12^{a}
$IC_{50/4}$	58.55 ± 3.532^{a}	79.82 ± 2.104^{b}	94.77 ± 0.7039^{c}

There is a statistical difference between the values represented by different superscript the rows. ($p < 0.05$) IC50 dose for RA: 178.9 µM; IC50/2 dose: 89.45 µM; IC50/4 dose: 44.72 µM. IC50 dose for TQ: 87.06 µM; IC50/2 dose: 43.53 µM; IC50/4 dose: 21.76 µM

Low doses of RA and TQ accelerated wound healing

While both RA and TQ delayed wound healing at IC50 concentrations, wound healing at IC50/2 concentrations was comparable in all groups, but wound healing at IC50/4 concentrations was far more rapidly in the RA group than in the control group, and in the TQ group than in both RA and control groups (Table 2; Figs. 2, 3). The wound area was virtually entirely closed by RA and TQ treatments at the IC50/4 dose, with 79.82% and 94.77% closure, respectively.

Low doses of TQ increased both TGF-β1 and MMP-9 levels and low doses of RA and TQ increased MMP-9 levels

TGF-β1 protein levels were measured as 10.98 pg/mg protein in the control group and 14.28 pg/mg protein in the RA group, which were comparable to the control group. TGF-1 protein levels in the TQ group were found to be greater than in the control and RA groups, with an average of 25.83 pg/mg protein (respectively, $p < 0.001$, $p = 0.004$). MMP-9 protein levels in the control group were 13.44 pg/

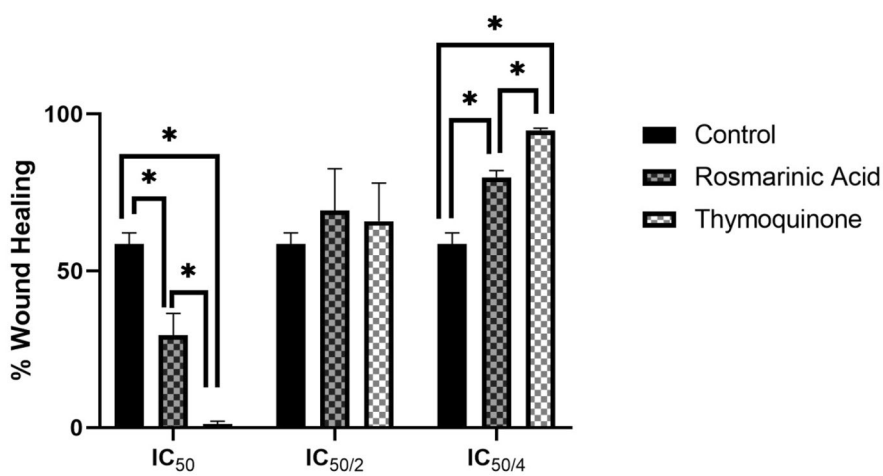

Fig. 2 Comparison of decreasing doses of RA and TQ in terms of % wound healing

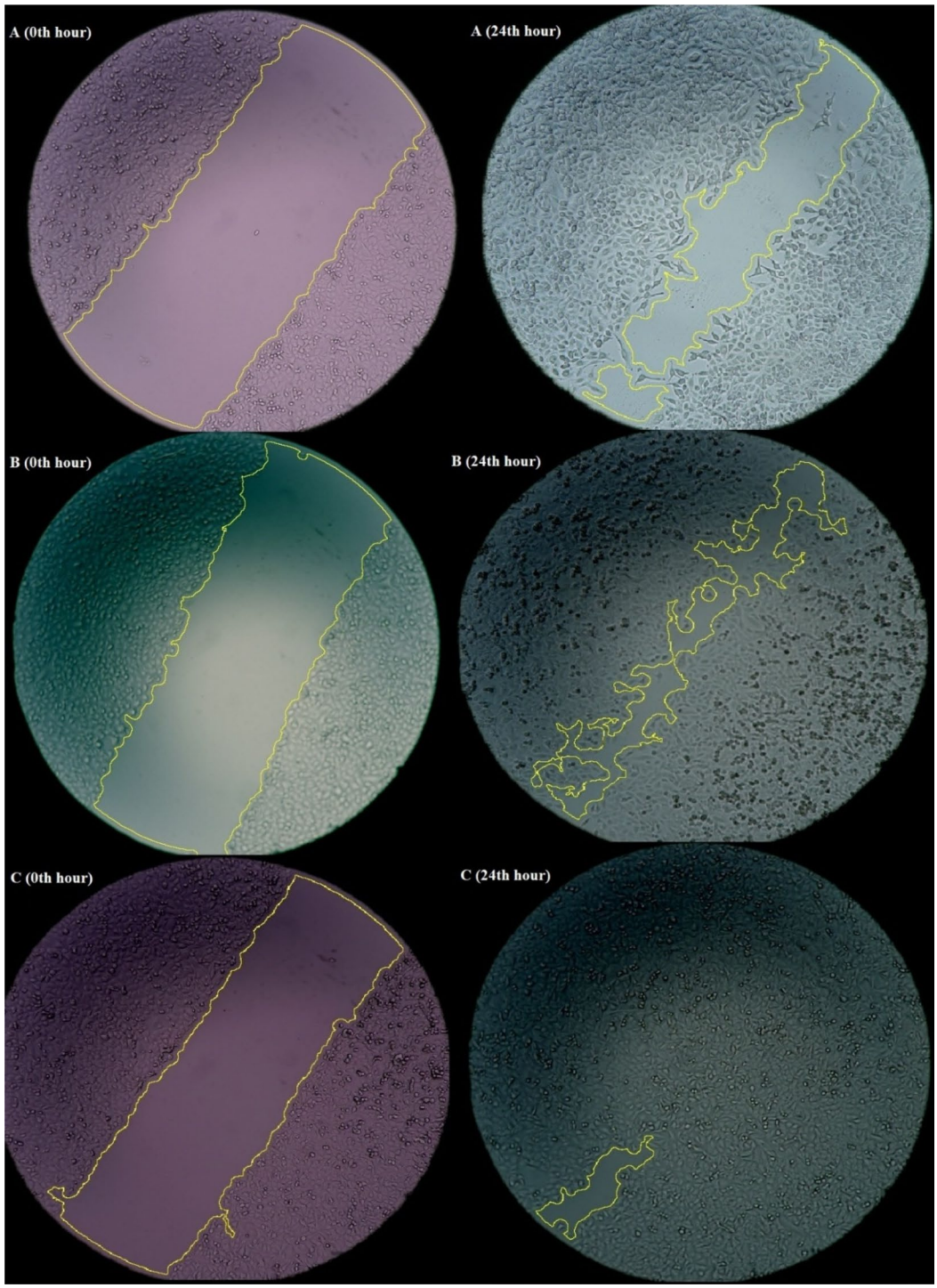

Fig. 3 The effect of RA and TQ at IC50/4 doses (44.72 µM and 21.76 µM, respectively) on wound healing. **a** Control group, **b** RA group, **c** TQ group. 0th hour on the left, 24th hour on the right in all groups. Wound healing measurements were made using ImageJ software

mg protein, 16.37 pg/mg protein in the RA group, and 30.62 pg/mg protein in the TQ group. MMP-9 protein levels were found to be greater in the RA group than in the control group, and higher in the TQ group than in both the control and RA groups (Table 3; Fig. 3). MMP-9 protein levels were found to be mean 13.44 pg/mg protein in the control group, mean 16.37 pg/mg protein in the RA group, and mean 30.62 pg/mg protein in the TQ group. According to these findings, MMP-9 protein levels were higher in the RA group than in the control group and higher in the TQ group than in both the control and RA groups (respectively, $p = 0.024$, $p < 0.001$, $p < 0.001$) (Table 3; Fig. 4).

Table 3 Comparison of TGF-β1 and MMP-9 data between groups

Groups	TGF-β1 (pg/mg protein)	MMP-9 (pg/mg protein)
Control	10.98 ± 2.559^{a}	13.44 ± 1.686^{a}
RA	14.28 ± 3.024^{a}	16.37 ± 0.2051^{b}
TQ	25.83 ± 4.258^{b}	30.62 ± 1.983^{c}

There is statistical significance between different superscripts. $p < 0.05$ was considered statistically significant

The MRNA expression levels of TLR3, IFN-γ and VEGF

The mRNA expression level of IFN- γ in the RA group was found to be 1.012-fold compared to the control group and was comparable to the control group ($p = 0.962$). The RNA expression levels of TLR3 and VEGF were found to be 1.793-fold and 2.424-fold, respectively, and to be statistically significantly upregulated in the RA group compared to the control group ($p = 0.029$, $p < 0.001$, respectively). The mRNA expression level of VEGF was found to be suppressed 0.877-fold in the TQ group compared to the control group; however, this suppression was not statistically significant ($p = 0.621$). The mRNA expression levels of TLR3 and IFN-γ were found to be downregulated 0.166-fold and 0.24-fold in the TQ group compared to the control group (Table 4, Fig. 5). This result was significant ($p < 0.001$ and $p < 0.001$, respectively).

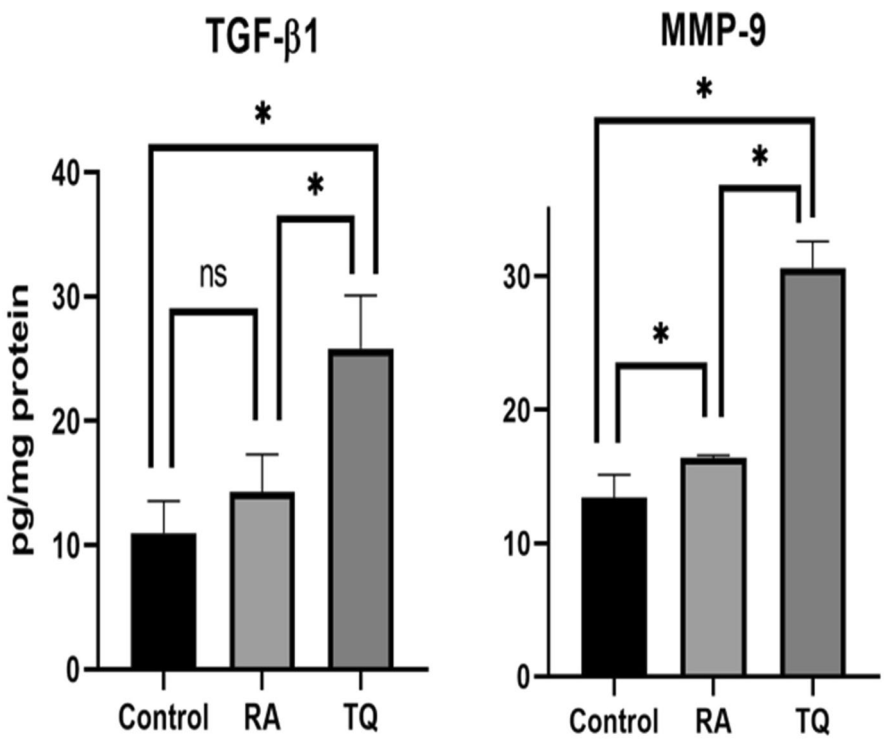

Fig. 4 TGF-β1 and MMP-9 protein levels comparison between groups. $p < 0.05$ was considered statistically significant (*$p < 0.05$; ns, non-statistical)

Table 4 Comparison of mRNA Expression Levels of Genes

Genes	Rosmarinic acid			Thymoquinone		
	Gene expression	*p* value	Up/down regulation	Gene expression	*p* value	Up/down regulation
TLR3	1.793	0.029	UP	0.166	< 0.001	DOWN
IFN-γ	1.012	0.962	NS	0.240	< 0.001	DOWN
VEGF	1.189	0.549	NS	0.877	0.621	NS

NS, non-statistical ($p > 0.05$); UP, fold increase; DOWN, fold decrease; $p < 0.05$ was considered statistically significant. All expression levels were compared to the control group

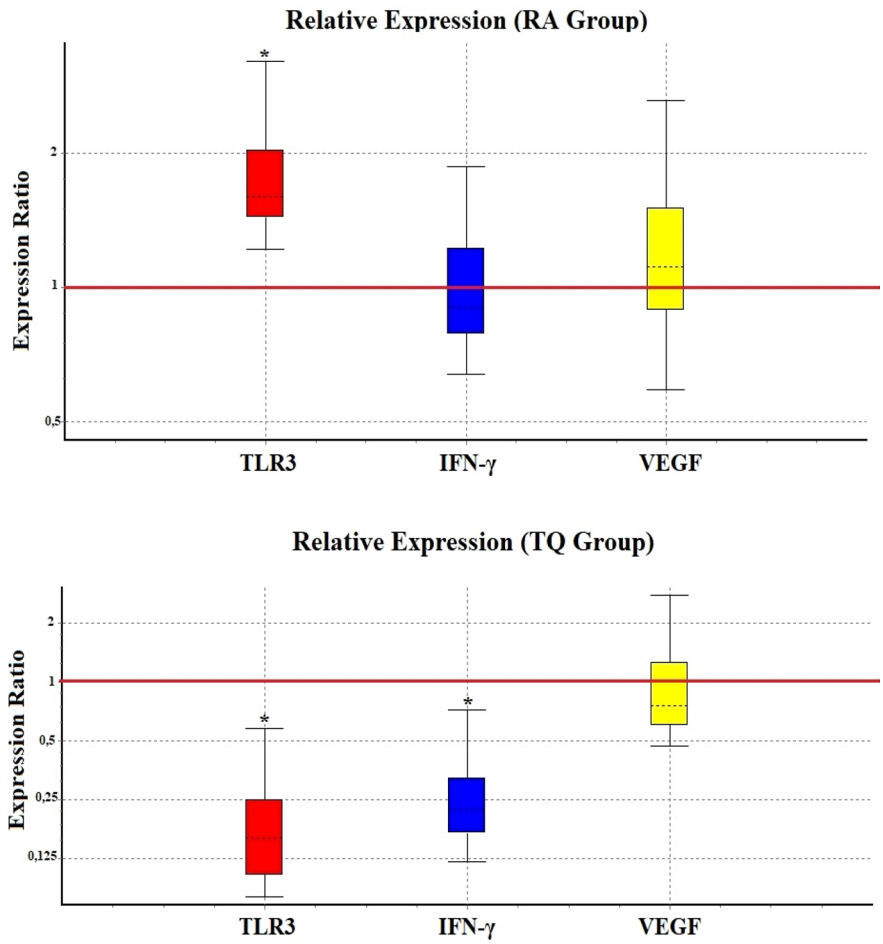

Fig. 5 Relative mRNA expression levels of genes. Values are expressed as the mean ± SD. All groups were compared with the control group, and the results were given as fold increase/decrease. The REST 2009 software (Qiagen) was used for statistical analysis and graphing. The red line parallel to the *x*-axis shows the position of the control group. < 0.05 was considered statistically significant *$p < 0.05$

Discussion and conclusion

Vascular endothelial growth factor (VEGF) is an important agent for retinal and choroidal neovascularization, age-related macular degeneration (AMD), and events associated with diabetic retinopathy. The retinal pigment epithelium (RPE), strategically located between the retina and choroid, plays a critical role in retinal disorders. Oxidative stress in retinal pigment epithelium (RPE) cells may contribute to the progression of age-related macular degeneration. Thymoquinone (TQ) is an active compound obtained from *Nigella sativa* that has antioxidant effect. Previous studies have highlighted that RA has protective

effects on RPE cells via VEGF inhibition and TQ has also protective effects on RPE cells due to its antioxidant effect [25, 26]. In the current study, unlike other studies, it was found that TQ and RA improve and protect the retinal pigment epithelium through different gene expressions and different components.

MMPs play an important role in the regulation of ECM remodeling. Several studies have demonstrated that MMP-1 is present in normal retina, whereas MMP-2 and MMP-9 are found in epiretinal and subretinal membranes [27]. Gonzalez-Avila [28] noted obvious increases in MMP-2 and MMP-9 expression in the subretinal fluid of PVR patients. Also, a previous study conducted regarding TGF-β1 reported that it induced VEGF expression [29]. In the present study, although both RA and TQ significantly increased TGF-β1 and MMP-9 levels in ARPE-19 cells, this was not reflected in VEGF expression and it was observed that RA and TQ did not increase VEGF expression. It should also be noted that, interestingly, when ARPE-19 cells were treated with low doses (IC50/4) of TQ and RA, increased MMP-9 and TGF-β1 accelerated, rather than delayed, wound healing.

Toll-like receptors (TLRs) are important components of innate immunity that participate in host defense against microbial pathogens. Previous studies have shown that TLR-3 is expressed in retinal pigment epithelial (RPE) cells [30]. Research has highlighted the presence of TLRs in RPE cells, and the resulting TLR signaling in RPE cells may play an important role in innate and adaptive immune responses of these molecules within the retina [30]. Furthermore, it has been reported that wound healing is significantly delayed in TLR-3 gene deleted mutant rats compared to the control group [31]. In the present study, it was observed that RA caused an increase in TLRs while TQ inhibited them significantly. While VEGF was comparable to the control group in both TQ and RA groups, TLRs and IFN-γ were suppressed especially in RPE cells treated with low dose TQ, which explains the acceleration of wound healing in ARPE-19 cells. This finding suggested that the healing of RPE cells may be associated with a mechanism other than VEGF.

When the pathogenesis of AMD, which is the leading cause of severe visual loss in elderly individuals, is examined, prolonged inflammation appears to be an important factor [32]. In this sense, in the current study, both the RNA expression levels of TLR3 and IFN-γ were decreased 0.166-fold and 0.24-fold, respectively, in the TQ group compared to the control group. In addition, TQ accelerated wound healing, which was considered as a positive result in terms of AMD pathogenesis.

TGF-β1 acts as a strong chemotactic signal to recruit neutrophils to the wound, especially in keratinocyte and fibroblast tissue damage [33, 34]. In the present study, it was observed that low dose TQ, but not low dose RA, increased TGF-β1 protein levels in retinal pigment epithelial cells in direct proportion to wound healing.

It is known that severe and prolonged inflammation impairs wound healing and increases scar formation [35]. In this context, in accordance with the literature, TQ was found to accelerate wound healing by lowering both the mRNA expression levels of TLR3 and IFN-γ in our study. In contrast to TQ, RA increased the mRNA expression levels of TLR3 by 1.793-fold compared to the control group, but IFN-γ mRNA expression levels were found to be comparable to the control group, which means that it was thought that it did not trigger inflammation even if it did not decrease it.

In the current study, low dose TQ and RA were found to contribute to wound healing at different rates and this contribution was observed to be mediated through different biochemical pathways. TQ promoted wound healing by increasing TGF-β1 and MMP-9 protein levels, whereas RA caused a very minimal increase in both TGF-β1 and MMP-9, whereas its main effect was achieved by increasing TLR-3 mRNA expression. In the current study, both TQ and RA had no effect on VEGF mRNA expression. This result was found to be different from previous studies supporting the anti-angiogenesis effect of TQ and RA through VEGF inhibition [26]. In the current study, neither TQ nor RA had any effect on VEGF mRNA expression. This result was found to be different from previous studies supporting the anti-angiogenesis effect of TQ and RA through VEGF inhibition [26].

Vascular endothelial growth factor (VEGF, VEGF-A) is a major regulator of both physiological and pathological angiogenesis [36]. Experimental data support that VEGF stimulates epithelialization and collagen deposition in a wound [37]. Recent studies on wound healing have clearly confirmed that VEGF activation accelerates wound healing

[38–40]. Nevertheless, VEGF signaling in retinal cells is known to be associated with retinal angiogenesis [41–43].

In the present study, VEGF levels, which accelerate wound healing and are closely associated with retinal angiogenesis, were found to be comparable in the TQ and RA treated group compared to the control group, whereas TQ significantly reduced TLR3 and IFN-γ gene expression, thus accelerating wound healing and not causing angiogenesis. This suggested that wound healing was not only related with VEGF levels but also with inhibition of TLR3 and IFN-γ gene expression in this study.

One of the limitations detected for our study was the use of retinal pigment epithelial cell migration stimulation in in vitro experimental models developed for proliferative vitreoretinopathy (PVR) [44]. In these experimental models, ARPE cells were incubated with multiple combinations of epidermal growth factor (EGF) + fibroblast growth factor-2 (FGF-2) or EGF + FGF-2 + TGF-β1 and the application times of these agents were 120 h [45] to 7 days [46]. In this study, a sufficient application time and stimulating agents were not applied in order to observe and evaluate also the effects of experimental PVR models. In this respect, it is not thought that the agents that were applied in this study for the healing of the wound model will pose a risk in terms of PVR. Nonetheless, we believe that it would be appropriate to be careful about the use of RA and TQ in patients with PVR and to conduct further scientific research in this area.

The valuable findings of this study are that RA and TQ delayed wound healing at full IC50 concentrations, wound healing was better at IC50/2 concentrations but similar to the control group, whereas wound healing at IC50/4 concentrations was very successful in the group given RA and TQ. The reason for this result may be the cytotoxic effect of these agents on RPE cells in overdose. With these findings, this study becomes the first to investigate the dose–dependent effects of TQ and RA on RPE cells and to prove their efficacy at low doses. In other words, it was concluded that TQ and RA contributed to wound healing in RPE cells at proper dosage, but in overdose, on the contrary, they delayed wound healing. These results suggest that, particularly in AMD cases, if TQ and RA are administered at proper doses, it will shed light on future studies in regards to repair of RPE damage without the need for VEGF inhibition.

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Author contributions All the authors listed participated in the manuscript and have read and approved the final submission. Specifically, MK and SS designed research, performed research, analyzed data and wrote the paper.

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Data availability The data's that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors claim no conflict of interest and certify that they have no association or participation with any organization or individual with any financial interest or non-financial interest in the subject matter or materials discussed in this article.

Consent to participate and consent for publication All the coauthors have approved the manuscript for submission and publication in your journal, and I am the corresponding author.

Ethics approval Not applicable.

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