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Original Article

In vitro study: thymoquinone inhibits the proliferation and migration of keloid fibroblasts and increases their apoptosis

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A B S T R A C T

Background: No treatment can eliminate keloids. Thymoquinone (TQ) is hypothesized to play a pivotal role in treating keloids by modulating cellular processes such as proliferation, migration, and apoptosis. However, the existing studies investigating its effects on these mechanisms in keloid fibroblasts are limited and require further exploration.

Objective: This study aims to investigate the effects of TQ on the proliferation, migration, and apoptosis of keloid fibroblasts in vitro.

Methods: This experimental study was conducted using keloid fibroblast cultured in vitro. Cells were seeded in a 96-well plate at a density of about 5x103 cells per well with 100 µl of culture medium, and cells were cultured for 24, 48, and 72 hr for each concentration of TQ. Cell proliferation was assessed using a CCK-8 Kit, measuring optical density with a microplate reader. Apoptosis was measured using the TUNEL assay. Cell migration following TQ treatment was evaluated using the Scratch assay. The statistical test used a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test.

Results: TQ inhibited the proliferation of keloid fibroblasts at a dose of 5 µM after 48 hours of incubation and 10 µM after 24 hours of incubation. The inhibitory effect of TQ on fibroblast proliferation increased in a dose- and time-dependent manner. Treatment at 5 and 10 µM doses increased apoptosis in keloid fibroblast cultures. The TQ5 µM group achieved 60% closure, while the 10 µM group showed 55% closure. Migration was significantly inhibited in the 25 µM and 50 µM groups, with only 30% and 10% closure, respectively, at 72 hours.

Conclusion: Thymoquinone inhibits the proliferation and migration of keloid fibroblast cells while promoting apoptosis. These properties suggest that TQ could be developed as a potential treatment for keloid-related skin issues.

INTRODUCTION

Keloids are a type of scar formed due to the excessive growth of fibrous tissue, where collagen production becomes abnormally high after skin injury.[1] This condition can cause significant discomfort, such as pain, itching, and cosmetic concerns. A study reported that, among 121 keloid patients, at least 28.9% experienced pruritus, and 26.4% reported pain.[1,2] Currently, no treatment has been able to eliminate keloids.[2-3] Available keloid therapies include laser therapy, cryotherapy, silicone gel or plaster application, radiation therapy, surgical removal, and injections of corticosteroids, flavonoids, interferon, or 5-fluorouracil.[4] Treatments for keloids, such as corticosteroid therapy, surgery, and radiation, often have limited efficacy and can lead to undesirable side effects, such as infection or hypopigmentation.

However, these treatments are costly and painful for many people, are only practical for smaller keloids, and require repeated procedures, resulting in low success rates.[5] Therefore, alternative research is needed, such as the use of herbal ingredients and research using herbal extracts that can reduce cell viability and increase apoptosis in keloid fibroblasts.[6] The use of natural substances for treating various skin conditions has gained significant attention due to their lower side effects and higher biocompatibility.

TQ is a natural substance that offers therapeutic potential in keloid treatment, particularly for regulating biological processes such as inflammation, cell proliferation, and

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apoptosis without damaging surrounding healthy tissues.[8] TQ has gained attention for its diverse pharmacological properties, including anti-inflammatory, antioxidant, and anticancer activities.[6–8] Although TQ's anticancer and proapoptotic properties are reported in many studies, their effects on keloids have not been fully explored. TQ is used in this research due to its demonstrated ability to modulate cellular processes such as proliferation, apoptosis, and migration, which are critical in the pathogenesis of keloids.[8-9]

Previous research has extensively studied TQ in various contexts. In cancer studies, TQ has been shown to inhibit tumor growth by inducing apoptosis and suppressing proliferation in breast, prostate, and lung cancer cells. It modulates key signaling pathways such as NF-κB, PI3K/AKT, and p53, which are also relevant in fibroblast-mediated scarring.[11-13] TQ's anti-inflammatory effects have been documented in autoimmune and inflammatory diseases, where it reduces pro-inflammatory cytokines and oxidative stress.[14] Furthermore, studies in wound healing have highlighted TQ's role in regulating fibroblast activity and promoting balanced tissue repair. No research proves the effect of TQ on keloid therapy.

This research differs from prior studies in that it specifically focuses on the impact of TQ on keloid fibroblasts. Previous investigations primarily explored TQ's anticancer properties or its effects on non-pathological wound healing, this study examines its role in suppressing fibroblast proliferation and migration while enhancing apoptosis.[15] This study aims to evaluate the impact of TQ on keloid fibroblasts in vitro, focusing on its ability to regulate their proliferation, migration, and apoptosis.

METHOD

Study Design

This research was an in vitro experiment with a post-test-only control group design.[16]

Study Site

This research was conducted from July 2024 to September 2024 at the Stem Cell Laboratory, Integrated Research Laboratory of YARSI University in Jakarta.

Materials

The materials used were Thymoquinone, Dulbeco's Modified Essential Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), Antibiotics-Antimycotics (AA), Dimethyl sulfoxide (DMSO), Trypsin 0,25%, Trypan Blue, CCK-8 KIT and Tunnel Biotium Kit.

In Vitro Procedure

Cell Preparation

Cryotubes containing keloid fibroblast cells were removed from nitrogen storage. The cryotubes were then thawed in a water bath for 1-2 minutes until thoroughly melted, and the entire cell suspension was transferred to a 15 ml tube containing 9 ml of complete DMEM + FBS + AA. The cells were then centrifuged at 1500 RPM for 7 minutes. After discarding the supernatant, the pellet was resuspended in 1 ml of complete medium, followed by cell counting. Cell expansion was performed in a T75 flask. Once the cells reached over 80% confluency, they were harvested and seeded into 96- and 24-well plates for treatment application.[17]

Experimental Procedure

The treatment in this research involves five groups of keloid fibroblasts consisting of a control group (K) and treatment by TQ groups in various doses. These groups are TQ5 (TQ 25 μM), TQ10 (TQ 10 μM), TQ25 (TQ 25 μM) and TQ50 (TQ 50 μM). Keloid fibroblast cells are cultured in 96 healthy plates for the cell proliferation test and 24 well plates for the cell migration and apoptosis test. After the cells attach and grow stable for the next 1×24 hours, cells can be given treatment. We weighed as much as 0,164 grams of powder Thymoquinone (Sigma-Aldrich) and dissolved it into 10 ml of complete DMSO to obtain a dose stock of 10.000 μM. Then dilute as much as 25x so the dose stock to 400 μM and can dilute return to get the dose TQ under treatment on each well that contains 100 μl of cells.

Proliferation Assay

The CCK-8 cell proliferation assay protocol involves seeding cells into a 96-well plate at an appropriate density and allowing them to adhere overnight. After treatment or incubation, 10 μL of the CCK-8 solution is added directly to each well containing 100 μL of culture medium, and the plate is incubated for 1-4 hours at 37°C. After incubation, the absorbance is measured at 450 nm using a microplate reader. The absorbance (optical density) is directly proportional to the number of viable cells, allowing assessment of cell proliferation.[19]

Cell Migration Assay

Cells were cultured until they reached 80% confluency in 24-well plates. A sterile yellow tip was then used to create a scratch in the cell colonies, disrupting their connections. The cells were rinsed with PBS before being treated, and their migration ability was assessed by measuring the gaps and capturing images using a digital microscope. Migration measurements were taken at 0, 24, 48, and 72 hours.[18] Images were consistently captured from the same area across all test samples, and the scratch areas were analyzed using the ImageJ application.[19-20]

TUNNEL Apoptosis Assay

The TUNEL apoptosis assay by Biotium involves the following steps: First, cells are fixed using 4% paraformaldehyde in PBS for 15 minutes at room temperature, followed by washing with PBS. Cells are then permeabilized with 0.2% Triton X-100 for 5 minutes. After another wash, the cells are incubated with the TUNEL reaction mixture, which includes TdT enzyme and a fluorescent-labeled dUTP, at 37°C for 60 minutes in a humidified chamber. Following incubation, the cells are rewashed with PBS to remove unincorporated nucleotides.

The samples are then analyzed using fluorescence microscopy to assess the degree of DNA fragmentation, indicating apoptosis.[20]

Statistical Analysis

The data obtained were expressed as mean±standard deviation (SD). The analysis was performed using a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test.[21]

Ethical Considerents

This research has been approved by the research ethics committee of YARSI University, number No: 066/KEP-UY/EA.10/III/2024.

RESULTS

Table 1 shows that proliferation in TQ groups decreased compared to control in the TQ group with doses of 10, 25, and 50 µM at 24-hour incubation. At 48-hour incubation, proliferation decreased lower than at 24-hour incubation. Figure 1 shows that the decrease at 48-hour incubation was much lower than at 24 hours with a dose of 5 µM, proliferation was only 27.58%, and continued to decrease with TQ 10 µM to 6.53% at 50 µM T administration. The ability of TQ to reduce proliferation in keloid fibroblast cell culture was significantly significant p <0.05.

Table 1. Effect of TQ on the Proliferation of Keloid Fibroblasts Cultured for 24 and 48 Hours

Time (h)	C (Control)	TQ 5 µM	TQ 10 µM	TQ 25 µM	TQ 50 µM
24	100 ± 5.49	97.99 ± 4.15	84.93 ± 13.37	79.5 ± 8.27	67.27 ± 9.78
48	100 ± 8.57	27.58± 1.46	12.48 ± 1.19	11.2 ± 1.38	6.53 ± 0.6

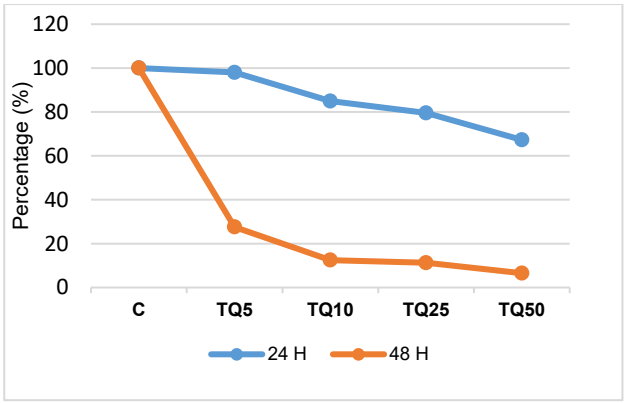

Figure 1. Thymoquinone inhibits keloid fibroblast proliferation. C (control), TQ5 (Thymoquinone 5 µM), TQ10 (Thymoquinone 10 µM), TQ25 (Thymoquinone 25 µM), TQ50 (Thymoquinone 50 µM).

Figure 2 shows a microscopic picture of apoptosis due to TQ exposure which was seen in the TQ 10, TQ 25, and TQ 50 µM dose groups, while in the control and TQ5 groups, no apoptosis was seen as indicated by cells that fluoresce green.

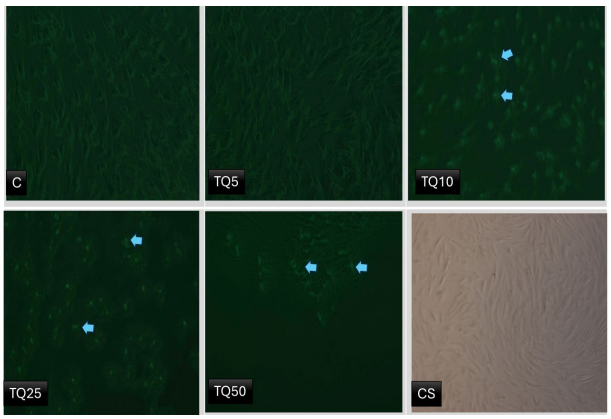

Figure 2. Microscopic images of apoptosis in keloid fibroblast cells following treatment with varying doses of TQ. C (control), TQ5 (Thymoquinone 5 µM), TQ10 (Thymoquinone 10 µM), TQ25 (Thymoquinone 25 µM), TQ50 (Thymoquinone 50 µM), CS (normal cell culture without staining).

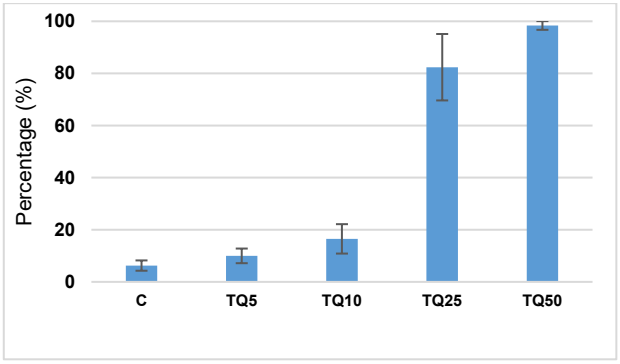

Figure 3. Percentage of apoptosis in keloid fibroblast cells following treatment with varying doses of TQ. C (control), TQ5 (Thymoquinone 5 µM), TQ10 (Thymoquinone 10 µM), TQ25 (Thymoquinone 25 µM), TQ50 (Thymoquinone 50 µM)

Figure 3 illustrates apoptosis percentages in keloid fibroblasts following TQ exposure, with error bars representing the standard deviation of five replicates. One-way ANOVA ($p < 0.05$) followed by LSD comparison showed a significant increase in apoptosis in the TQ-treated groups compared to the control (K). Table 2 details apoptosis rates, increasing from 6.25% in the control to 16.5% at 10 µM and peaking at 98.4% at 50 µM. Apoptosis at 25 µM and 50 µM was significantly higher ($p < 0.05$).

Table 3 presents cell migration inhibition over 24, 48, and72 hours. The control group showed faster scratch closure (58.33%, 39.00%, and 12.33%, respectively), while the slowest closure was observed at 50 µM (96%, 93%, and 89%). These findings indicate that TQ inhibits keloid fibroblast migration dose-dependently. Figure 4 illustrates the migration assay results, showing complete wound closure in the control group within 72 hours, with the TQ5 group also demonstrating initial closure. In contrast, the TQ25 group exhibited significantly reduced cell migration, leaving a substantial unclosed area after 72 hours.

Table 2. Effect of TQ on the Apoptosis of Keloid Fibroblasts cultured in five replicates

Sample	C (Control)	TQ5 µM	TQ10 µM	TQ25 µM	TQ50 µM
1	7	14.28	11.11	62.5	98
2	3.57	9.3	11.11	87.5	100
3	5.7	11.11	22.5	77.78	96
4	6	7.5	15.6	88.89	100
5	9	7.7	22.2	95.23	98
Mean±SD	6.25 ±1.98	9.98 ±2.81	16.5 ±5.64	82.38 ±12.75	98.4 ±1.67

Table 3. Effect of TQ on the Migration of Keloid Fibroblasts Cultured for 24, 48 and 72 Hours

Time (h)	C (Control)	TQ5 µM	TQ10 µM	TQ25 µM	TQ50 µM
0	97.67 ± 2.52	96.00± 5.29	97.67± 2.08	97.00 ± 2.65	97.00 ± 2.65
24	58.33 ± 1.53	62.33 ± 2.08	77.6±2.52	90.33 ± 4.51	96.00 ± 1.73
48	39.00 ± 3.61	54.67 ± 4.16	60.67 ± 4.04	82.67 ± 3.05	93.00 ± 3.61
72	12.33 ± 2.51	45.00 ± 5.00	52.33 ± 2.51	75.00 ± 5.00	89.67 ± 5.51

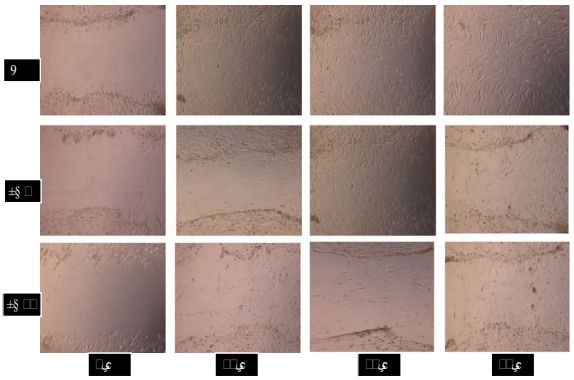

Figure 4. Microscopic images of keloid fibroblast migration after treatment with various doses of Thymoquinone (TQ). C (control), TQ5 (Thymoquinone 5 µM), TQ25 (Thymoquinone 25 µM).

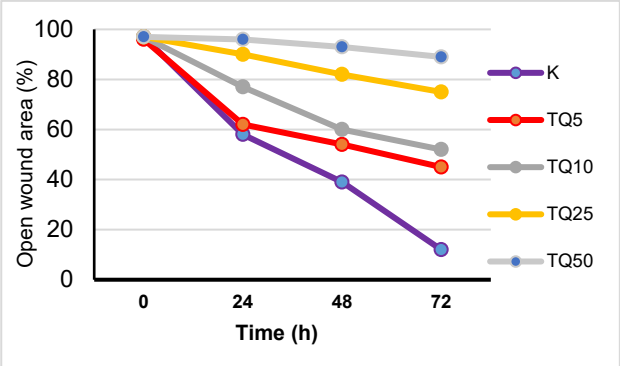

Figure 5. The Effect of Thymoquinone on Keloid Fibroblast Migration. Legend: C (control), TQ5 (Thymoquinone 5 µM), TQ10 (Thymoquinone 10 µM), TQ25 (Thymoquinone 25 µM), TQ50 (Thymoquinone 50 µM). Dose-response curves of the Thymoquinone after the administration for 24, 48, and 72 hours of treatment. Cell migration is quantified by the percentage (%) of an open wound assuming that the wound area reduces linearly over time.

DISCUSSION

This study highlights the potent effects of TQ, the primary bioactive component of Nigella sativa, in modulating critical cellular processes such as proliferation, apoptosis, and migration in keloid fibroblasts.[22] These findings offer compelling evidence of TQ's therapeutic potential, consistent with its efficacy in other pathological models, including cancer and fibrotic disorders.[12] The ability of TQ to regulate these key cellular mechanisms underscores its promise as a potential therapeutic agent for fibroproliferative conditions.

Furthermore, TQ has been shown to inhibit fibroblast growth in nasal polyp cultures, further validating its role in suppressing abnormal cell proliferation. Similar inhibitory effects have been observed with green tea extract on keloid fibroblast growth, suggesting shared mechanisms of action. Significant suppression was observed at 25 µM and 50 µM concentrations, consistent with earlier reports on the anti-proliferative properties of TQ in cancer cell lines, including multiple myeloma.[11] TQ's ability to reduce proliferation likely involves modulating key inflammatory pathways, particularly those involving cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), which have been implicated in tumorigenesis and fibrosis.[24]

Moreover, the migration assays revealed a significant inhibition of keloid fibroblast mobility at higher doses of TQ. The wound closure process, typically achieved within 72 hours in untreated cells, was markedly delayed in TQ-treated groups. This anti-migratory effect mirrors findings in renal carcinoma studies, where TQ suppressed migration by downregulating EP2/EP4 receptors involved in PGE2 signaling.[24] These results suggest that TQ may target similar molecular pathways in keloid fibroblasts, positioning it as a promising candidate for controlling fibroblast-mediated tissue remodeling in keloid pathology. We observed that TQ inhibits the migration of keloid fibroblasts. TQ holds potential as a complementary natural chemotherapeutic agent for treating keloids.

These results indicate that TQ may play a significant role in controlling keloid formation by modulating cell growth, inducing apoptosis, and reducing cell migration.[23] However, additional research is necessary to fully elucidate the molecular pathways involved and explore TQ's clinical potential in treating fibroproliferative disorders like keloids. These results align with previous findings that reported

similar apoptotic effects in multiple myeloma cells.[25] Fluorescence microscopy images revealed characteristic apoptotic changes, further supported by elevated caspase-3 levels. Caspase-3, a critical executioner in the apoptotic cascade, indicates intrinsic apoptotic activation.[11]

These results are consistent with prior research on TQ's pro-apoptotic effects in various cancer models, including multiple myeloma and renal carcinoma, where TQ was shown to modulate apoptotic pathways through STAT-3 and other signaling cascades.[11] TQ inhibits cell proliferation and induces apoptosis in squamous cell carcinoma. Combining TQ and DG (diosgenin) is a potential antineoplastic therapy in this common skin cancer.[26] This apoptotic effect is significant, as aberrant regulation of apoptosis has been identified as a central mechanism in keloid formation. The overproduction of extracellular matrix proteins and resistance to apoptosis are key drivers of keloid fibroblast proliferation, leading to pathological scarring. TQ may help restore the balance between cell survival and death by inducing apoptosis, which is often disrupted in keloid formation.[11]

Thymoquinone can synergistically reduce cell viability with anticancer drugs, such as cisplatin, which can cause damage.[27] Observed anti-proliferative, anti-migratory, and pro-apoptotic effects of TQ position it as a compelling therapeutic option for keloid management. Given the limitations of existing keloid treatments, which often involve invasive procedures with suboptimal outcomes, TQ offers a natural and multi-targeted approach to mitigating keloid pathology. Its ability to modulate inflammation, suppress fibroblast proliferation, and induce apoptosis could make it a compelling alternative or adjunct to conventional therapiess.

CONCLUSIONS AND RECOMMENDATION

Thymoquinone effectively inhibits keloid fibroblast proliferation, induces apoptosis, and reduces migration dose-dependently. These findings highlight its potential as a promising treatment for keloid scars and fibroproliferative disorders. However, further research is warranted to fully elucidate the molecular mechanisms underlying TQ effects on keloid fibroblasts. Additionally, in vivo studies and clinical trials must confirm TQ efficacy and safety in keloid therapy.

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