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Effect of Thymoquinone and Transforming Growth Factor-β1 on the Cell Viability of Nasal Polyp-Derived Fibroblast

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Abstract

BACKGROUND: Nasal polyps are benign masses in the nasal cavity and the abnormal growth of sinonasal tissue due to a chronic inflammatory process. Many fibroblasts populate the nasal polyp stroma release cytokines such as Transforming Growth Factor (TGF) and producing a variety of cytokines resulting in inflammatory cell infiltration. Thymoquinone (TQ) is the main active component in Nigella sativa oil and has the ability to reduces cell viability in many cancer cell line.

AIM: The purpose of this study was to determine the effect of TQ and TGF-β1 on cell viability of Nasal Polyp-Derived Fibroblast.

MATERIALS AND METHODS: Nasal polyp-derived fibroblasts were isolated from nasal polyp specimen and treated with various concentrations of TQ at 1–1000 μM and TGF-β1 at 5 ng/ml to determine the cell viability using the Cell Counting Kit-8 assay after 48 h incubation.

RESULTS: TQ significantly reduced the viability of nasal polyp fibroblast cells to 72.49% at 20 μM and reduced to 5% at 50 μM until 1000 μM with IC50 at 21.93 μM. TGF-β1 at 5 ng/ml significantly reduced the viability of nasal polyp fibroblast cells to 81.96% and TGF-β1 appears to have a dual effect that depends on the concentration of TQ.

CONCLUSION: This study proved that TQ and TGF-β1 were able to reduce the viability of nasal polyp fibroblast cells.

Introduction

Nasal polyps are benign masses in the nasal cavity and the abnormal growth of sinonasal tissue due to a chronic inflammatory process in the nasal mucosa and paranasal sinuses [1], [2]. Nasal polyps marked by inflammatory cell infiltration, structural fibrosis, edematous stromal tissue, and basement membrane thickening. Many fibroblasts populate the nasal polyp stroma, producing a variety of cytokines for polymorphonuclear leukocytes [3]. The majority of fibroblasts in the stroma of nasal polyps release cytokines such as Transforming Growth Factor (TGF), interleukin-6, and matrix metalloproteinases, resulting in inflammatory cell infiltration [1]. TGF-β1 plays a role in regulating the processes of proliferation, differentiation, migration, and apoptosis [4]. TGF-β1 plays an important role in the formation and growth of nasal polyps, triggers cell remodeling, and growth, which causes fibrosis by attracting stromal cells, angiogenesis, and accumulation of extracellular matrix [5]. TGF-β1 is known to trigger the proliferation by increased cell viability, but another reports TGF-β decrease the viability [6], [7].

The main active component of nigella sativa essential oil is thymoquinone (TQ), which has been shown to suppress multi-cancer cell proliferation both *in vitro* and *in vivo* [8]. TQ is a powerful antioxidant in healthy tissues, but it causes the production of reactive oxygen species in tumors [9]. The previous study reported that TQ has been shown to reduces cell survival or cell viability canine osteocarcinoma, human

adenocarcinoma breast cancer (MCF7), and cancer human ovarian adenocarcinoma (BG-1) [10]. Cell viability is a measure of the number of living cells in a population [11].

In the stroma of nasal polyps, there is increase in the number of fibroblast cells in the early phase of nasal polyps and abnormal fibroblast proliferation [12], [13]. TQ and TGF-β1 have been shown to affect viability in various cancer cell lines, but its effect on nasal polyps is unknown. Until now, there has been no report on the effect of TQ and TGF-β1 on nasal polyp fibroblast cell viability and this study was aimed to determine the effect of TQ and TGF-β1 on nasal polyp-derived fibroblast viability.

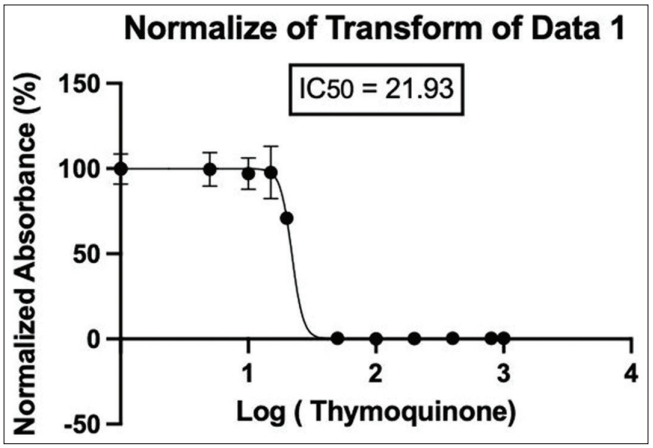

Figure 1: IC50 value of Thymoquinone on nasal polyp-derived fibroblast cells

Materials and Methods

Reagent

Dulbecco's Phosphate Buffer Saline, trypsin EDTA, enzyme collagenase, and antibiotic-antimycotic mixture were purchased from Gibco (Grand Island, NY, USA). TQ >98% powder, cell counting Kit-8 (CCK-8), and TGF-β1 human were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dimethyl sulfoxide was purchased from (AppliChem).

Isolation of primary nasal polyp fibroblasts

Nasal polyp specimen taken from 1 patient with non-eosinophilic chronic rhinosinusitis with nasal polyp by endoscopic simple polypectomy in the Department of Otorhinolaryngology Dr. Cipto Mangunkusumo Hospital, Jakarta. Subjects were

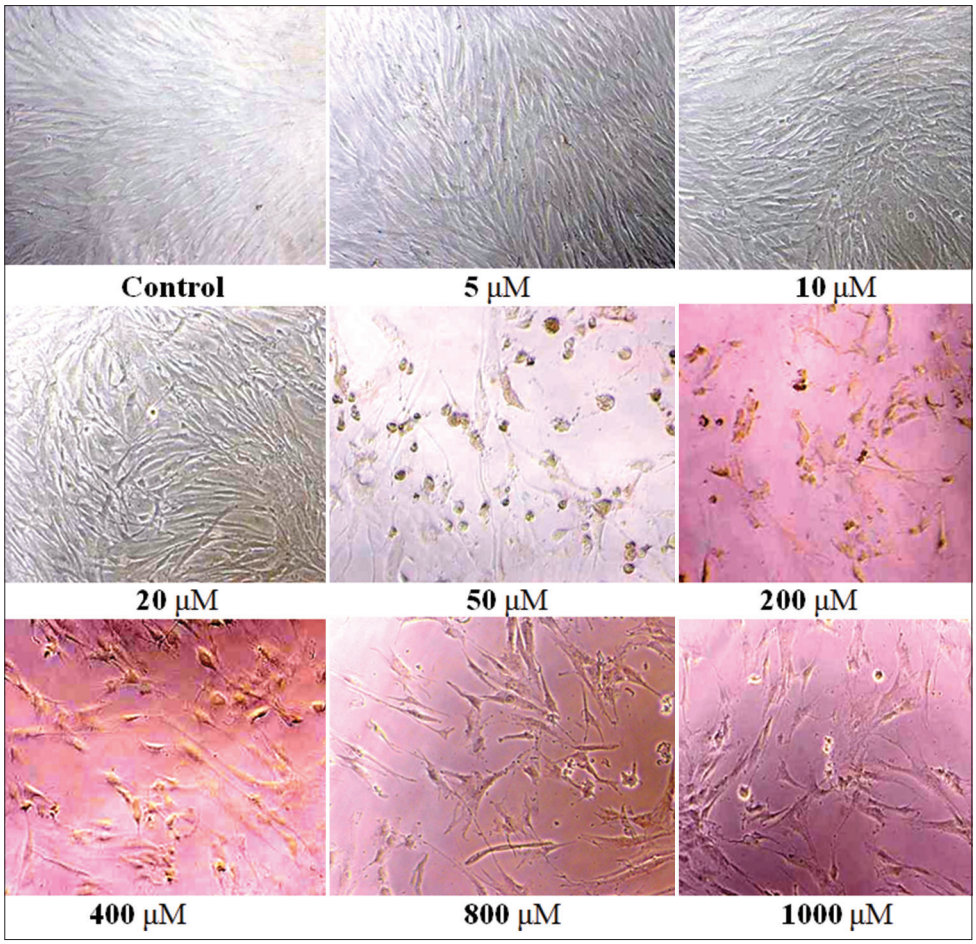

Figure 2: Morphology of nasal polyp-derived fibroblast cells was observed by inverted microscope

excluded if they had active allergy, inflammation, aspirin hypersensitivity, and previous sinonasal surgery or if they had received antibiotics, antihistamine, steroids, or other medications for at least 4 weeks preceding surgery. Allergy status was defined using the skin prick test. This study was approved by the ethics committee University of Indonesia and Dr. Cipto Mangunkusumo Hospital. Nasal polyp tissue was placed in a sterile tube containing 25 ml of PBS (Gibco) and 1% antibiotic-antimycotic combination (Gibco) and stored in a cooler at 4°C and immediately transported to the integrated cell laboratory foundation of YARSI University, Jakarta. Decontamination was performed by inserting pieces of tissue into a tube containing betadine for 2 min, wash with 70% alcohol for 2 min, and do it 3 times. Transfer the tissue to a culture dish containing sterile PBS plus antibiotics-antimycotics. Pieces of lower tissue cleaned of fat and blood vessels until clean. The tissue was cut with scissors and a razor blade with a size of 1 cm × 2 cm. Transfer the tissue pieces to a culture dish containing collagenase/dispase and stored in a sterile container and then placed in the freezer. Transfer the tissue pieces to a new culture dish containing sterile PBS. Using sterile tweezers, carefully separate the epidermis and dermis. The dermis was taken and cut into small pieces using sterile scissors and then put into a 15 ml tube containing Trypsin-EDTA. Vortex for 5 min then incubate for 1 h and repeat the vortex again. Filter using a 70 m cell strainer, put in a 15 ml tube containing DMEM growing medium. Centrifuge for 10 min at 1500 rpm, then discard the supernatant, and dissolve the pellet with complete growth medium and DMEM plus 10% FBS. Plant in a culture plate and then incubate in a 37°C, 5% CO_2 and change the medium every 3 days. After visible solid cell growth and reaching confluence, the cells were sub-cultured or passages the third to fourth passages of fibroblasts which were used for subsequent experiments. The purity of obtained nasal polyp-derived fibroblasts (NPDFs) was confirmed microscopically by characteristic spindle-shaped cell morphology [14], [15].

Cytotoxic assay

A CCK-8 assay (Sigma aldrich) were used to measure the cytotoxicity of TQ on NPDF. The CCK-8 assay was used to measure cytotoxicity under starved conditions. NPDF (1×10^4 cells/ml) were grown in 96-well plates for 24 h and treated with TQ over a range of concentrations 1, 5, 10, 15, 20, 50, 100, 200, 400, 800, and 1000 μM and followed by incubation for 48 h at 37°C, 5% CO_2. To determine the effect TQ combine with TGF-β1, NPDF (1×10^4 cells/ml) was grown in 24-well plates for 24 h and treated with TGF-β1 5 ng/ml 2 h before TQ over a range of concentrations 5, 10, 15, and 20 μM and followed by incubation for 48 h at 37°C, 5% CO_2. After 48 h, the NPDF was washed, and the cell viability was assessed using a CCK-8 assay (Sigma aldrich). CCK-8 solution 10 μl was added to each well, followed by incubation for 2 h at 37°C, 5% CO_2. The absorbance at 450 nm was determined by a multiplate reader (Lambda Bio-20; Beckman). Cell viability was expressed as a percentage of that of the control (untreated) cells. For each concentration of TQ, mean values of the mean absorbance rates from two wells were calculated. The mean optical density (absorbance) of two wells was used to calculate the percentage of cell viability as follows: Percentage of cell viability = (OD drug/OD control) × 100 [16].

Statistical analysis

The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) with $p < 0.001$ and by Bonferroni for *post hoc* test with $p < 0.05$. All experiments were performed in at least two replicate. The IC50 was determined using the GraphPad Prism V.5 software.

Results

Cytotoxicity test

The CCK-8 assay demonstrated a dose dependent toxic effect with increasing concentrations of thymoquinone on NPDF under starved conditions. The doses of TQ given to nasal polyp fibroblast cells in this study were 1, 5, 10, 15, 20, 50, 100, 200, 400, 800, and 1,000 μM and incubated for 48 h. No significant toxicity was observed at concentrations <20 μM. However, significant toxicity on NPDF was observed at concentrations ≥ 20 μM ($p < 0.001$) as seen from the cell morphology and IC50 value. Using prism nine application, the IC50 value was obtained 21,93 μM so that the TQ dose of 21,93 μM showed that it was able to inhibit 50% of the biological activity of fibroblast cells derived from nasal polyps (Figure 1).

Morphology

Effect of TQ on cell viability

Based on the optical density value from CCK-8 assay, it was seen that the increase in TQ dose gradually affected the viability of nasal polyp-derived fibroblast cells (Figure 2). Using the formula for cell viability, it was found that the percentages of viable cells were as follows: untreated group at 100%, TQ 1 μM 92.14% ± 4.86; 5μM 94.93% ± 4.84; 10 μM 96.08% ± 4.94; 15 μM 98% ± 16.78; 20 μM 72.49% ± 4.67; and 50 μM until 1000 μM between 5.21% ± 0.13–5.81% ± 0.65 (Table 1). Using the one-way ANOVA test showed that there was a significant difference in mean cell viability fibroblasts in the treatment group ($p<0.001$) and using Bonferroni for *post hoc* test, there was a significant decrease in

Table 1: The results of cell viability assessment in the control and thymoquinone group

Groups	n	Mean (SD) (%)	p[a]	*Post-hoc*[b]										
				TQ (1 μM)	TQ (5 μM)	TQ (10 μM)	TQ (15 μM)	TQ (20 μM)	TQ (50 μM)	TQ (100 μM)	TQ (200 μM)	TQ (400 μM)	TQ (800 μM)	TQ (1000 μM)
Control	2	100	<0.001	1.000	1.000	1.000	1.000	0.024	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (1 μM)	2	92.14 (4.86)			1.000	1.000	1.000	0.286	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (5 μM)	2	94.93 (4.84)				1.000	1.000	0.115	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (10 μM)	2	96.08 (4.94)					1.000	0.080	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (15 μM)	2	98 (16.78)						0.044	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (20 μM)	2	72.49 (4.67)							<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TQ (50 μM)	2	5.6 (0.62)								1.000	1.000	1.000	1.000	1.000
TQ (100 μM)	2	5.21 (0.13)									1.000	1.000	1.000	1.000
TQ (200 μM)	2	5.44 (0.46)										1.000	1.000	1.000
TQ (400 μM)	2	5.78 (0.54)											1.000	1.000
TQ (800 μM)	2	5.81 (0.65)												1.000
TQ (1000 μM)	2	5.62 (0.26)												

[a]ANOVA, [b]Bonferroni. SD: Standard deviation, TQ: Thymoquinone.

the viability of nasal polyp-derived fibroblast cells at a concentration of 20 μM – 1000 μM (Figure 3).

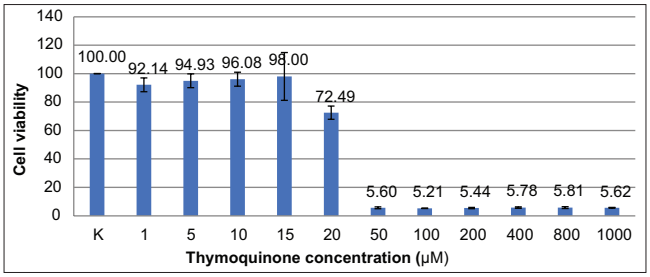

Figure 3: Bar graphs of nasal polyp-derived fibroblast cell viability at various concentrations of TQ and negative control

Effect TQ and TGF-β1 on cell viability

TGF-β1 5 ng/ml also decreases the viability of nasal polyp-derived fibroblast cells to 81.96% ± 6.13. In the combine group with TQ at 5 μM, cell viability was decreased to 86.65% ± 0.35; 10 μM to 84.86% ± 0.57; 15 μM to 86.35% ± 0.29; and 20 μM to 88.46% ± 6.82 (Table 2). Using the one-way ANOVA test showed that there was a significant difference in all groups ($p < 0.05$) and using Bonferroni for *post hoc* test, there was only the TGF-β1 group made a significant decrease in the viability of nasal polyp-derived fibroblast cells (Figure 4).

Figure 5 shows comparison the action of TQ with or without TGF-β1 5 ng/ml which caused a decrease in viability at all groups. In the groups with concentration 5, 10, and 15 μM, TGF-β1 caused a more decrease in cell viability when compared to group without TGF-β1, whereas in the 20 μM group, TGF-β1 caused an increase in cell viability when compared to group without TGF-β1.

Discussion

Nigella sativa also known as Black Seed, Alhabahat Alsawda, and Alkamoun Alaswad [17]. TQ is the main active component in Nigella sativa essential oil, which inhibits multi-cancer cell proliferation and development both *in vitro* and *in vivo* [8]. TQ is a terpenoid molecule with the chemical formula (2-isopropyl-5-methylbenzo-1,4-quinone) and the greatest TQ level in black cumin was discovered in Ethiopia (3.098.5 mg/kg) [18].

Fibroblasts derived from nasal polyps are a valid *in vitro* model for research, because fibroblasts are an important component of the ground substance in nasal polyps and are involved in various inflammatory responses associated with disease pathogenesis [19].

The cytotoxic test is a biological screening method that uses tissue cell samples *in vitro* to examine the impact of chemicals on cell growth. IC50 parameters and cell morphology were used to estimate cytotoxic potential [20]. This is the first study to show that TQ has an effect on nasal polyp-derived fibroblast cells. The cytotoxic potential of TQ was determined by observing cell morphology and IC50 parameters [20]. Using prism nine application, the IC50 value in this study was 21.93 μM. IC50 TQ has also been reported in various cell line viability studies, including in rat hepatic stellate cell lines with an IC50 of 28.91 μM, and HepG2 cell proliferation with an IC50 of 46 μM [21], [22]. From another study reported, an IC50 TQ value of 25 μM in breast cancer cell lines was the minimum dose that has been able to inhibit the proliferation of breast cancer cells by stopping the S phase significantly in the cell cycle [23]. From this study, we find that TQ at 21.93 μM is the minimum concentration that has been able to inhibit 50% biological activity of cells. The limitations of our study were that we did not observe the proliferation and cell cycle of nasal polyp fibroblasts.

Cell viability is a measure of the proportion of live and healthy cells in a population [24]. Cell viability assays are used to determine how a cell responds to a pharmacological or chemical stimulus. This assay is used to test the efficacy of newly developed cancer-targeting therapies [24], [25]. Various compounds can cause toxicity to cells through different mechanisms such as the destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors, and polydeoxynucleotide inhibition [26]. In our study, we also report the effect of TQ with or without TGF-β1 on the cell viability of nasal polyp-derived fibroblast. Using the CCK-8 assay, it was demonstrated that a TQ 20 μM concentration for 48 h was able to significantly reduce the viability of nasal polyp fibroblast cells to 72.49% or a decrease of 27.51% compared to the

Table 2: The results of cell viability assessment in the control, transforming growth factor-β1, and thymoquinone groups

Groups	*n*	Mean (SD) (%)	p[a]	*Post-hoc*[b]				
				Control (+)	TQ (5 μM) + TGF-β1 (5 ng/ml)	TQ (10 μM) + TGF-β1 (5 ng/ml)	TQ (15 μM) + TGF-β1 (5 ng/ml)	TQ (20 μM) + TGF-β1 (5 ng/ml)
Control	2	100	0.03	0.045	0.180	0.103	0.164	0.327
TGF-β1	2	81.96 (6.13)			1.000	1.000	1.000	1.000
TQ (5 μM) + TGF-β1 (5 ng/ml)	2	86.65 (0.35)				1.000	1.000	1.000
TQ (10 μM) + TGF-β1 (5 ng/ml)	2	84.86 (0.57)					1.000	1.000
TQ (15 μM) + TGF-β1 (5 ng/ml)	2	86.35 (0.29)						1.000
TQ (20 μM) + TGF-β1 (5 ng/ml)	2	88.46 (6.82)						

TGF-β1: Transforming growth factor-β1, SD: Standard deviation, TQ: Thymoquinone. a is anova . ; p value <0.05; b is Bonferroni ; p value is < 0.001

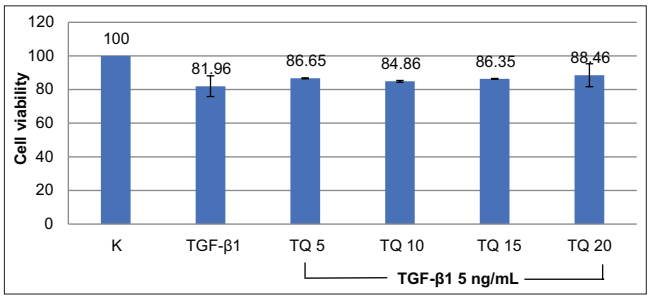

Figure 4: Bar graphs of nasal polyp-derived fibroblast cell viability at various concentrations of TQ influenced by transforming growth factor-β1 5 ng/mL

control and progressively decreases at a concentration of 50–1000 μM to 5% (Figure 3). Similar results were reported by Kus *et al*. which proved that 50 μM TQ for 48 h was able to reduce nasal polyp fibroblast cell viability to 18% and increase apoptosis in human prostate cancer cell line (LnCaP) by activating caspase-9 [10]. Khan *et al*. (2017) who proved that the TQ concentration of 5 μM–50 μM was able to reduce the viability of MDA-MB-435 cells, Hela cells, and BT-549 cells [27].

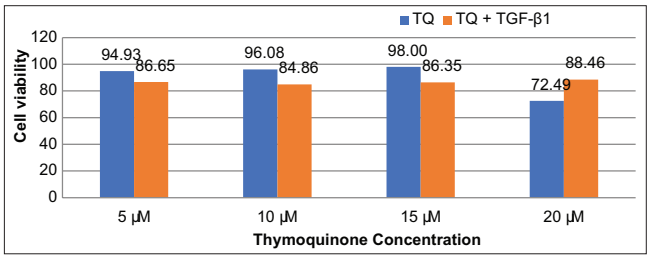

Figure 5: Comparison bar graphs of nasal polyp-derived fibroblast cell viability due to TQ and the combination of TQ and transforming growth factor-β1 5 ng/mL

Samarghandian *et al.* (2019) proved that TQ 25, 50, and 100 μM for 72 h were able to reduce cell viability to about 60% and increase apoptosis of A549 lung tumor cells by increasing caspase-3 and caspase-9 but did not decrease the viability of normal control cell MRC-5 fibroblast cells (human fetal lung cell line)[28]. In this study, TQ was able to reduce the viability of nasal polyp fibroblast cells, but some compounds such as 1,25(OH)2D3 (active compound of vitamin D) or calcitrol which were shown to have anti-proliferative, pro-apoptotic and pro-differentiation properties, and anti-cancer properties were not able to reduce the viability of nasal polyp fibroblast cells up to a dose of 1000 nM with incubation for 72 h [13]. Fucoxanthin which is able to trigger apoptosis in various cancer cell lines has also been shown to be unable to reduce the viability of nasal polyp fibroblast cells [29]. In this study, we found that TGF-β1 5 ng/ml caused a significant decrease in nasal polyp fibroblast cell viability compared to controls (Figure 4). Until now, there has been no report on the effect of TGF-β1 on the viability of fibroblast cells derived from nasal polyps. Viability and proliferation are two different characteristics of cells. Viability is a measure of the number of living cells in a population, while proliferation is a measure of cell division or the number of cells that divide, and not all living cells divide. Although proliferation can be interpreted as viability, the absence of proliferation is not automatically considered a sign of cell death [11]. Some researchers suspect that the proliferation that occurs in nasal polyp fibroblasts depends on the amount of TGF-β1 concentration given. A decrease in the concentration of TGF-β1 will trigger proliferation and an increase in the concentration of TGF-β1 will inhibit proliferation [30]. Another report states that low levels of TGF-β1 stimulate fibroblast proliferation and increase profibrotic factor release, whereas higher levels of TGF-β1 promote myofibroblast development [31]. In our study, TGF-β1 appears to have a dual action that depends on the concentration of TQ. TGF-β1 reduced cell viability at TQ < 15 μM, but TGF-β1 increase cell viability at TQ 20 μM (Figure 5). The dual action of TGF-β was also reported by Zhang *et al*. who reported that low concentrations of TGF- β (0.1 ng/ml) in benign cells were able to induce proliferation, whereas at high concentrations (10 ng/ml)/ml)/ml), they stop growth in the same cells. The effect of TGF-β on benign cells does not necessarily result in growth arrest. In normal physiological circumstances, TGF-β regulates cellular activity with precision. Erk signal activation differences appear to play a central role in this regulation. Cells activate Erk and stimulate TGF-β expression when TGF-β levels in the local environment are low and when the TGF-β concentration is more than sufficient, the cells have the ability to stop ERK activation to prevent TGF-β expression. These findings demonstrate an important effect of TGF-β, specifically in advanced cancer cells TGF-β triggers tumor development but in benign or early cancer cells TGF-β offers a homeostatic mechanism [32].

Conclusion

This study concluded that TQ and TGF-β1 can reduce cell viability of nasal polyp-derived fibroblast.

The limitation of this study is that we did not identify the signaling pathway involved in the effect of TQ and TGF-β1 on the viability of nasal polyp-derived fibroblast cells.

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