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The Effect of *Trans*-Resveratrol on The Viability of Human Trabecular Meshwork Cells.

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ABSTRACT

In vitro models using cell culture are frequently used to investigate cellular effects of *trans*-resveratrol as it has been shown to possess chemotherapeutic, anti-aging, neuroprotective, anti-apoptotic, and anti-oxidant properties. Since *trans*-resveratrol was shown to lower intraocular pressure in oculonormotensive rats and rats with steroid-induced oculohypertension, use of normal and steroid-treated human trabecular meshwork cells (HTMCs) will provide a useful *in vitro* model for further studies. However, the effect of *trans*-resveratrol on the viability of HTMC has not been investigated. Therefore, the present study investigated the concentration- and time-dependent effect of *trans*-resveratrol on viability of HTMCs. HTMCs were treated with *trans*-resveratrol (3.125-50 µM) for 2, 5 and 7 days in the presence and absence of dexamethasone. The viability was assessed using MTS assay and the 50% cytotoxic concentration (CC_{50}) was calculated using a regression analysis. A significant decrease in cell viability was observed when cells were treated with 50 µM *trans*-resveratrol, both in the presence or absence of dexamethasone. This effect of *trans*-resveratrol was independent of the duration of treatment. The CC_{50} of *trans*-resveratrol in the presence of dexamethasone was 1.47, 1.60 and 1.47 folds higher compared to that in the absence of dexamethasone. In conclusion, this study demonstrated that incubation of HTMCs with *trans*-resveratrol up to a concentration of 25 µM does not affect the viability but at 50 µM, it significantly reduces viability both in the presence or absence of dexamethasone. This effect of trans-resveratrol on the viability of HTM cells is dose-dependent but not time-dependent.

Keywords: CC_{50}, cell viability, human trabecular meshwork cells, MTS assay, *trans*-resveratrol

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INTRODUCTION

Resveratrol (3,4',5 tri-hydroxystilbene) is a polyphenol naturally found in grapes, peanuts, berries and chocolates. It exists as *trans*- and *cis*- resveratrol. Between the two, *trans* isoforms is more biologically active compared to *cis* isoform and has been shown to possess chemotherapeutic, anti-aging, neuroprotective, and anti-oxidant properties [1-3]. Hence, its therapeutic applications in several diseases have been investigated.

We earlier reported that topical application of *trans*-resveratrol reduces intraocular pressure (IOP) in oculonormotensive rats and rats with steroid-induced ocular hypertension [4-5]. However, the mechanisms underlying the IOP lowering effect of *trans*-resveratrol remain unclear. Elevated IOP is a major risk factor in the pathogenesis of glaucoma [6], a leading cause of irreversible blindness worldwide [7]. Maintenance of normal IOP depends on the critical balance between the inflow and outflow of aqueous humor from the anterior chamber of eye. Increased resistance to aqueous outflow particularly in the trabecular meshwork (TM) has been implicated in primary open angle glaucoma (POAG), the most common type of glaucoma. TM forms the major outflow pathway for the drainage of aqueous humor from the anterior chamber of eye and the molecular pathways in this tissue that cause increased resistance to aqueous outflow are often the focus of investigation. Therefore, *in vitro* studies using human TM cells (HTMCs) are most appropriate to investigate involvement of molecular pathways underlying the changes in TM tissue of glaucomatous eyes.

Normal HTMCs have been widely used in glaucoma-related research. The activity of several molecular pathways, however, may differ in glaucomatous TM compared to that in normal TM. Hence, to induce glaucomatous changes in HTMC *in vitro*, treatment with steroids such as dexamethasone has been widely used [8-10]. Steroid-treated HTMCs are a direct representation of steroid-induced glaucoma in human. Since changes in TM seen in steroid-induced glaucoma largely resemble those seen in POAG, steroid-treated HTMCs are also a useful model for POAG-related investigations [11].

Accordingly, to investigate the mechanisms underlying the IOP lowering effect of *trans*-resveratrol, normal as well as steroid-treated HTMC are appropriate *in vitro* model. However, the effects of treatment with different concentrations of *trans*-resveratrol up to different time points on the viability of HTMC are not known. Hence, the objective of this study was to evaluate the dose- and time- dependent effects of *trans*-resveratrol on the morphology and viability of HTMC in the presence and absence of dexamethasone.

EXPERIMENTAL

Cell culture

Primary HTMCs from ScienCell Research Laboratories (CA, USA) were maintained in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, CA, USA). Cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. After the cells became 80% confluent sub-culturing was done. Cells from 5th passage were used for this study. In a 96 well plate, 2×10^3 cells were seeded and they were divided into 4 groups. In group 1, cells were maintained in DMEM. The group 2 was further subdivided into 5 groups that were cultured in media containing *trans*-resveratrol (Sigma Aldrich, Missouri, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO) (Sigma Aldrich, Missouri, USA) at concentrations of 3.125, 6.25, 12.50, 25.00 and 50.00 µM, respectively. Similarly cells in group 3 were subdivided into 5 groups that were incubated in media containing 5 concentrations of *trans*-resveratrol along with 100 nM dexamethasone (Enzo Life Science, New York, USA). The stock solution of *trans*-resveratrol was prepared at 500 µM in 0.1% (DMSO) and stored at -20°C, protected from light. Cells in group 4 and 5 were incubated in DMEM containing 100 nM dexamethasone and 0.1% DMSO, respectively. Incubation was done for 2, 5 and 7 days. At the end of each time point, cells were examined for morphological changes using light microscope and MTS assay was done to estimate the cell viability. All experiments were repeated 3 times and for each set of experiment estimations were done in triplicate.

MTS assay

MTS assay (Promega, Wisconsin, USA) was done based on manufacturer's instructions. Briefly, after each time point, 20 µL of CellTiter 96® AQ_{ueous} One Solution Reagent was added into each well containing the cells in 100 µL of culture medium. After incubation at 37°C for 1 hour, the absorbance was recorded at 490 nm

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using a 96-well plate reader. The data were expressed as the percentage of viable HTM cells. Subsequently, CC_{50} of *trans*-resveratrol was calculated using linear regression.

RESULTS

Effect of *trans*-resveratrol on HTM cell morphology

The cell cultured with dexamethasone (100 nM) appeared elongated in shape with thickened margins compared to those cultured in DMEM or DMSO whereas the cells in DMSO group were morphologically similar to those in DMEM group. This effect was visible after 2 days of incubation and was more prominent after 7 days of incubation. In all resveratrol treated groups also cells were elongated in shape compared to those cultured in DMEM of DMSO (Figures 1a, 1b and 1c).

Figure 1(a) Representative images of cells after 2 days treatment with i. DMEM; ii. DMSO (0.1%); iii. dexamethasone (100 nM); iv, v, vi, vii, viii. Increasing concentrations of *trans*-resveratrol (3.125-50 µM); ix, x, xi, xii, xiii. Co-treatment of 100 nM dexamethasone and increasing concentrations of *trans*-resveratrol (3.125-50 µM).

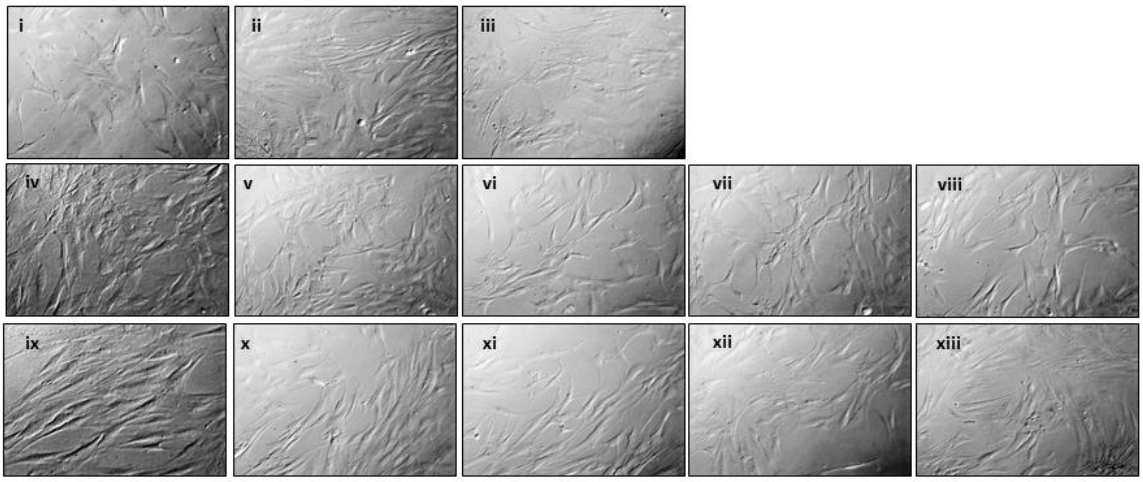

Figure 1(b) Representative images of cells after 5 days treatment with i. DMEM; ii. DMSO (0.1%); iii. dexamethasone (100 nM); iv, v, vi, vii, viii. Increasing concentrations of trans-resveratrol (3.125-50 µM); ix, x, xi, xii, xiii. Co-treatment of 100 nM dexamethasone and increasing concentrations of trans-resveratrol (3.125-50 µM).

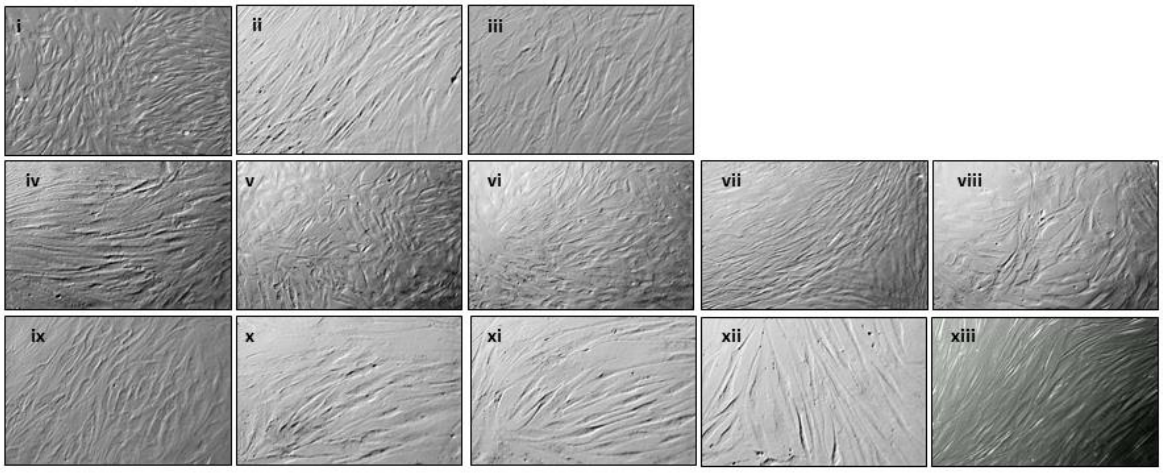

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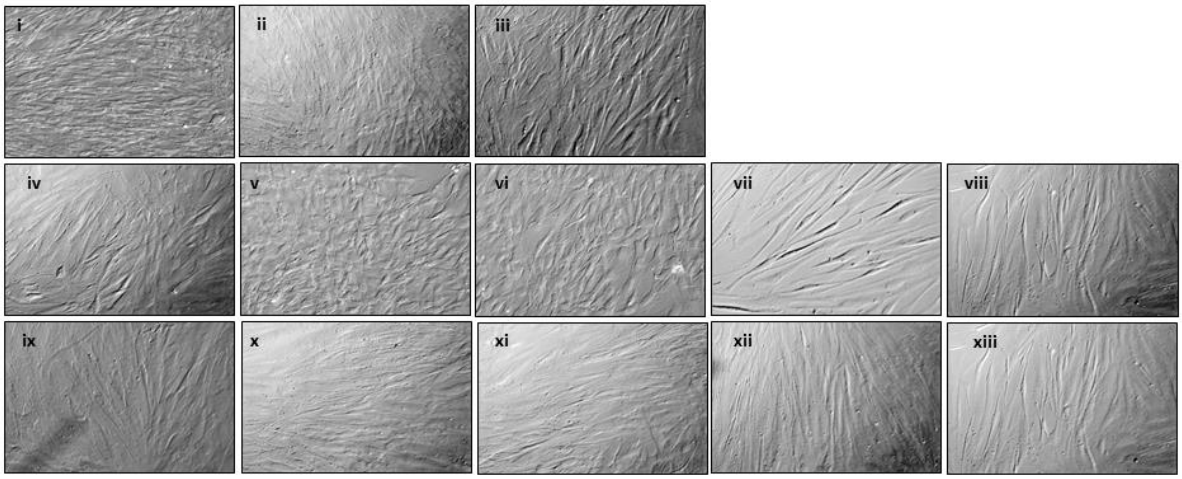
Figure 1(c) Representative images of cells after 7 days treatment with i. DMEM; ii. DMSO (0.1%); iii. dexamethasone (100 nM); iv, v, vi, vii, viii. Increasing concentrations of *trans*-resveratrol (3.125-50 µM); ix, x, xi, xii, xiii. Co-treatment of 100 nM dexamethasone and increasing concentrations of *trans*-resveratrol (3.125-50 µM).



Effect of *trans*-resveratrol on HTM cell viability

Effect of *trans*-resveratrol on the viability of HTMCs was estimated by using MTS assay. The viability of cells treated with dexamethasone remained close to 100% at all 3 time points as was also the case with DMSO treated group. Cells that were incubated with 3.125 -25 µM *trans*-resveratrol both in the presence and absence of dexamethasone also showed close to 100% viability after 2 , 5 and 7 days of incubation. However, at 50 µM concentration *trans*-resveratrol significantly reduced HTM cell viability. In the absence of dexamethasone, cell viability in 50 µM *trans*-resveratrol treated group was 1.86, 1.93 and 1.89 folds lower compared to DMEM group after incubation for 2, 5 and 7 days. In the presence of dexamethasone, cell viability in 50 µM *trans*-resveratrol treated group was reduced by 1.44, 1.43, and 1.45 folds compared to DMEM group at the same time points (Figures 2a, 2b and 2c).

Figure 2a. Effect of trans-resveratrol in the presence and absence of dexamethasone on the viability of HTMC after 2 days of incubation. *p<0.001 vs DMEM; #p<0.001 vs DMSO; @p<0.001 vs Dexa; $p<0.0001 vs corresponding R 3.125, 6.25, 12.5 and 25. R: *trans*-resveratrol; Dexa: dexamethasone

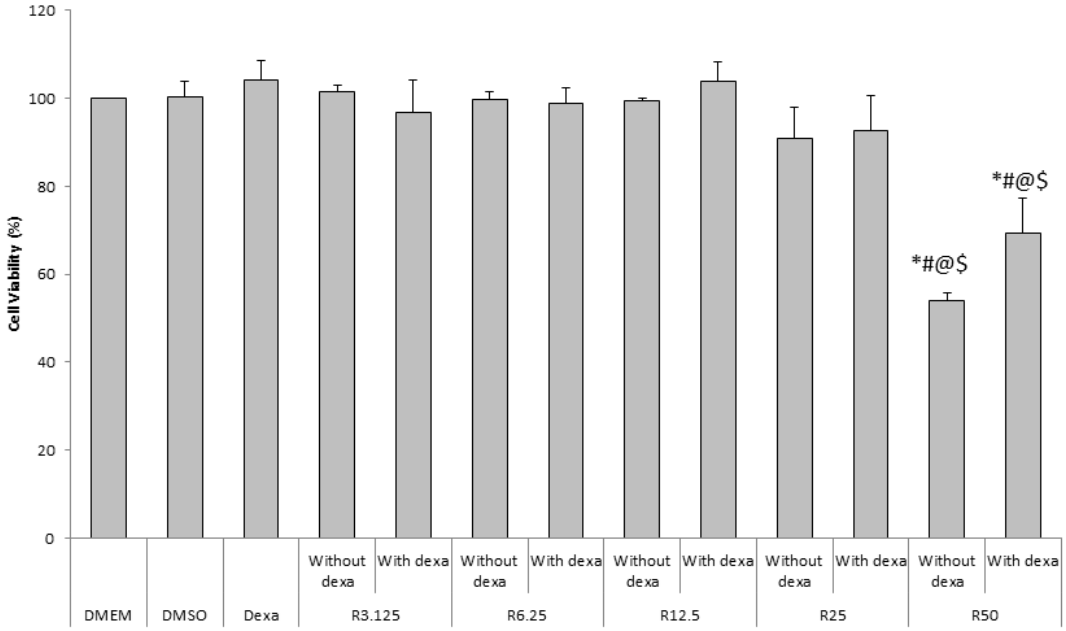


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Figure 2b. Effect of trans-resveratrol in the presence and absence of dexamethasone on the viability of HTMC after 5 days of incubation. *p<0.001 vs DMEM; #p<0.001 vs DMSO; @p<0.001 vs Dexa; $p<0.0001 vs corresponding R 3.125, 6.25, 12.5 and 25. R: *trans*-resveratrol; Dexa: dexamethasone

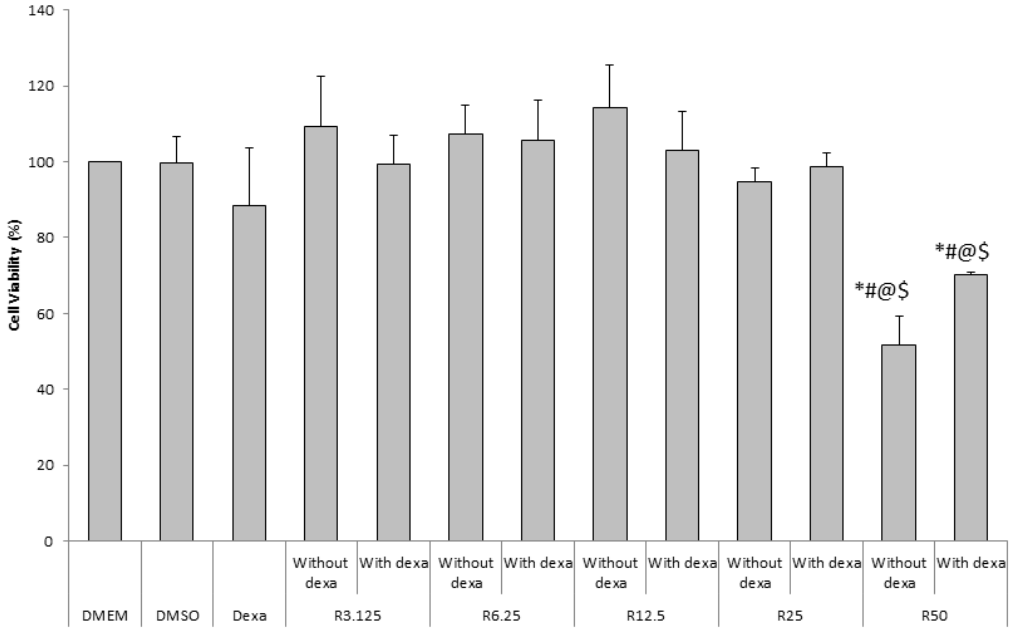
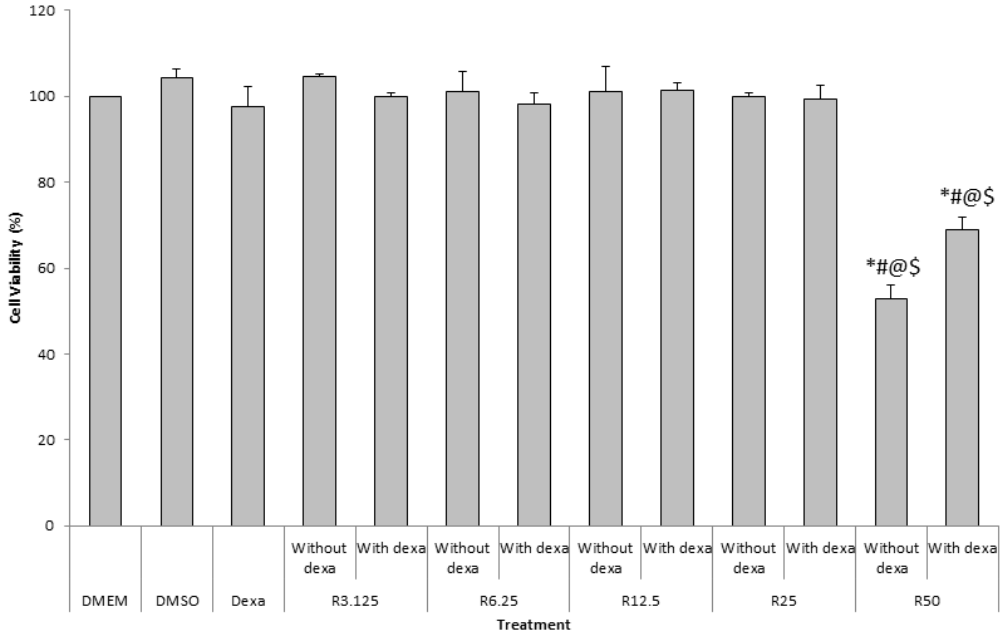


Figure 2c. Effect of trans-resveratrol in the presence and absence of dexamethasone on the viability of HTMC after 7 days of incubation. *p<0.001 vs DMEM; #p<0.001 vs DMSO; @p<0.001 vs Dexa; $p<0.0001 vs corresponding R 3.125, 6.25, 12.5 and 25. R: *trans*-resveratrol; Dexa: dexamethasone



Determination of CC_{50} by linear regression showed that *trans*-resveratrol produces cytotoxicity at relatively lower concentration when incubation was done in the absence of dexamethasone. The CC_{50} of *trans*-resveratrol in the presence of dexamethasone was 1.47, 1.60 and 1.47 folds higher compared to that in the absence of dexamethasone (Table 1).

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Table 1. The CC_{50} values (µM) for trans-resveratrol on HTM cells in the presence and absence of dexamethasone

Duration of treatment (days)	Treatment	
	Trans-resveratrol (µM)	Trans-resveratrol (µM) + Dexamethasone (100 nM)
2	57.76	85.19
5	58.20	93.23
7	59.07	86.78

DISCUSSION

This study evaluated the effect of various concentration of *trans*-resveratrol on the morphology and viability of HTMCs after 2, 5 and 7 days of incubation in the presence and absence of dexamethasone using MTS assay. We observed morphological changes in HTMCs incubated with 100 nM dexamethasone as early as 2 days of incubation. These morphological changes persisted up to 7 days with continued treatment with dexamethasone. Despite the morphological changes, the HTMCs viability remained unchanged. Similar effects of 100 nM dexamethasone have been observed on other cells such as KYSE150 esophageal squamous cell carcinoma cells [12]. Treatment with dexamethasone induces stress fiber rearrangement in HTMC [13] and Raghunathan et al. have reported that treatment with DEX for 3 days results in a 2-fold increase in HTM cell stiffness [14]. Treatment of HTMCs with dexamethasone has also been shown to cause reorganization of actin filaments at the cellular periphery [15]. These observations in previous studies correlate with the elongated cell shape and thickened margins observed in our study.

For the first time, this study demonstrated the effect of *trans*-resveratrol on the viability of HTMCs in the absence and presence of dexamethasone. Resveratrol at concentrations of 25 µM or below did not produce any significant decrease in cell viability when incubated up to 7 days, which is in accordance with previous studies [16]. However, at 50 µM concentration, we observed significantly reduced cell viability both in the presence and absence of dexamethasone compared to corresponding controls. This is in agreement with another study, which showed that 50 µM resveratrol significantly reduces the viability of cultured primary orbital fibroblasts after 24 hours of treatment [2]. In activated hepatic stellate cell model, treatment with resveratrol 50 µM significantly reduced cell survival as early as 24 hours and this was associated with increased lipid peroxidation [17]. Resveratrol 50 µM has been shown to induce apoptosis and reduce cell viability of cancer cells as well, by inhibiting NFκB-STAT3 signaling pathways [18], inducing release of Ca^{+2} [19] and inhibiting glucose metabolism [20]. In contrast to these observations, one of the previous studies demonstrated absence of cytotoxic effect of resveratrol on HTMCs at or below 100 µM concentration [21]. The differences may be attributed to the difference in assay method used. In our study, MTS assay was used that measures cell viability by measuring the metabolic activity of live cells whereas the assay used by Luna et al. measured the lactate dehydrogenase released by lysed cells [21]. It is likely that at 50 µM concentration the metabolic activity of HTMC is inhibited by resveratrol but the extent of inhibition remains insufficient to cause significant cell lysis. .Effects of resveratrol on cellular morphology observed in this study may be attributed to its effects on metabolic and other cellular pathways.

One of the important observations made in this study was that CC_{50} for resveratrol was higher in the presence of dexamethasone, compared to that in the absence of dexamethasone. Previous studies showed that dexamethasone could reduce the sensitivity of cancer cells to cytotoxic drug therapy, such as cisplatin [22]. It prevents cell death in response to cytotoxic drug, by enhancing the cellular adhesion to extracellular matrix [23]. In another study, treatment of primary cultures of human and rat hepatocytes with dexamethasone increased cell viability and inhibited apoptosis by reducing caspase-3 activity and increasing expression of anti-apoptotic Bcl-2 and Bcl-xL proteins [24]. In the current study, it is likely that at 50 µM concentration, cellular effects of *trans*-resveratrol overwhelm the apoptosis preventing effect of dexamethasone, hence resulting in reduced HTMCs viability. Further mechanistic studies, however, are needed to fully understand the mechanisms of these effects of *trans*-resveratrol.

In conclusion, this study demonstrated that incubation of HTMCs with *trans*-resveratrol up to a concentration of 25 µM does not affect the viability but at 50 µM, it significantly reduces viability both in the presence or absence of dexamethasone. This effect of *trans*-resveratrol on the viability of HTM cells is

independent of the duration of treatment. The findings of this study will provide a guide for future studies investigating the mechanisms of antiglaucoma effects of *trans*-resveratrol using normal and steroid-treated HTMCs.

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