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POSSIBLE PREVENTION OF REACTIVE OXYGEN SPECIES INDUCED HUMAN TRABECULAR MESHWORK CELL DAMAGE BY RESVERATROL AND ASCORBIC ACID.

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ABSTRACT… To analyze the antioxidant activity of Resveratrol and Ascorbic Acid against hydrogen peroxide (an oxidant) mediated cell injury of human trabecular meshwork cells. **Study Design:** Experimental study. **Setting:** Molecular Biology Laboratory at Medical Research Centre, Liaquat University of Medical & Health Sciences, Jamshoro. **Period:** Six months. (21-09-2016 to 20-03-2017). **Materials and Methods:** Human Trabecular Meshwork cells were purchased from ScienCell Research Laboratories, USA. TM cell metabolism, TM cell viability and Reactive oxygen species were detected by standard methods in co- and pre- treated TM cells. **Results:** A significant reduction in TM cell metabolism was observed approximating 61% at 1.0 mM H_2O_2 compared to Ascorbate – 99% and Resveratrol 99% ($p=0.0001$). Resveratrol was more effective than Ascorbate even at 4.0 mM H_2O_2, the TM cell activity was noted 76%. Compared to H_2O_2- treated TM cells, resveratrol improved mitochondrial function upto 4.0 mM H_2O_2 (76%). Compared to co-treatment, the pretreatment shows similar results except at 4.0 mM H_2O_2. At 4.0 mM H_2O_2 the pre-treat TM cell metabolic activity was found as 11%, 31% and 47% compared to co-treat as 9%, 31% and 76% in controls, ascorbate and resveratrol groups respectively ($p<0.05$). Resveratrol shows significant decrease in viability was seen in controls compared to Ascorbate and Resveratrol groups. Cell viability showed statistically significant differences at 2.0 and 4.0 mM H_2O_2 compared to controls ($P=0.0001$). For reactive oxygen species (ROS), cells were incubated and with Ascorbate and Resveratrol for 24 hours and TM cells were treated with 0.0mM, 0.5 mM, 1.0 mM, 2.0mM and 4.0mM H_2O_2. Significant decrease in ROS was noted by Resveratrol compared to Ascorbate. **Conclusions:** Resveratrol and Ascorbate may prove useful in preventing and delaying the glaucoma, and timely institution of these anti – oxidants may help maintain trabecular meshwork functions and prevent visual loss.

Key words: Ascorbate, H_2O_2, Reactive oxygen, Resveratrol, Trabecular Meshwork.

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INTRODUCTION

Pathogenesis of primary open angle glaucoma (POAG) is vague and not elucidated clearly. The previous research work shows the reactive oxygen species (ROS) play major role. The ROS mediated apoptosis of trabecular meshwork (TM) cells has been proposed as one of underlying mechanism in POAG pathogenesis.[1] Physiological impairment of TM cells retards the functioning of these cells, resulting in elevated intraocular pressure (IOP) and glaucoma. A progressive loss of TM cells occurs in patients of glaucoma that is caused by ROS over a long time period. ROS are capable of damaging the TM cells, reducing their physiological role in aqueous humor flow thereby altering the intraocular pressure.[2] Oxidative stress plays major role in the development of glaucoma. ROS damage the TM cells, decrease the nitric oxide, alter the vascular endothelial cell functions and nerve ganglion cell death.[3]

Previous research proved that a decrease in anti-oxidant mechanisms of TM cells progresses the POAG.[4] A previous study showed short term exposure of TM cells to sub-lethal doses of hydrogen peroxide (H_2O_2) damages the anatomical integrity of these cells. Adhesion of TM cells to growth matrix was decreased that compromises the tissue integrity and impairs the aqueous humor outflow.[5] While long term H_2O_2 exposure of cultured TM cells show increased expression of inflammatory markers.[6] ROS

enhances the oxidative damage of nuclear and mitochondrial DNA of cultured TM cells from glaucoma patients has been reported by previous studies.[6] This shows the role of oxidative injury in the pathogenesis of glaucoma. A previous study reported the resveratrol plays anti oxidative role against the ROS. Resveratrol is a phenolic compound which exerts anti oxidative, anti-angiogenic, anti-cancer, anti- inflammatory and vasodilator activities. Beneficial effects of resveratrol have been proved in diabetic retinopathy, macular degeneration, cataract and glaucoma.[6] Ascorbic acid is a proved anti-oxidant vitamin.[7]

National health survey 2003 reported prevalence of 2.7% blindness among Pakistanis which was surprisingly high in glaucoma patients.[8] The present study was conducted to determine anti-oxidant and anti-glaucoma activity of resveratrol and ascorbic acid against hydrogen peroxide induced oxidative injury in cultured trabecular meshwork cells.

METHODOLOGY

The present experimental study was conducted at Molecular Biology laboratory, Medical Research Centre, Liaquat University of Medical & Health Sciences, Jamshoro. Human Trabecular Meshwork cells (HTMC) isolated from the Juxta-canalicular and corneo-scleral regions of human eye along with culture media and reagents used in this study were purchased from The ScienCell Laboratories, USA . The vial purchased contained >5 x 10^5 cells in 1 ml volume and was delivered in dry ice, once received the cells were immediately transferred to liquid nitrogen and were kept their until required for experiments. Before culture of the cells, T-75 flasks were prepared with poly-L-lysine-coating by adding 10 ml of sterile water to T-75 flask and then adding 15 µl of poly-L-lysine stock solution (10 mg/ml). The flasks were left in 37°C incubator overnight. Complete medium was prepared under sterile conditions. The poly-L-lysine-coated vessels were rinsed twice with sterile water and then 15 ml of complete medium was added. Once flasks became ready, the frozen vial was placed in a 37°C water bath and then was rotated gently until the contents thawed completely. The vial was then wiped with 70% ethanol and transferred to sterile field. The cells were re-suspended in poly-L-lysine-coated culture vessel and the vessel was kept in 37°C incubator for 24 hours. After 24 hours the culture medium was replaced with fresh culture medium. Culture medium was changed every three days thereafter, until the culture was approximately 70% confluent. Once the culture reached 70% confluency, medium was changed every other day until the confluence reached approximately 90%.

Once cultures reached 90% confluence, subculture was started by rinsing the cells with DPBS (Ca++ - and Mg++ -free). Then 8 ml of DPBS and 2 ml of T/E solution was added into flask. The flask was gently rocked to ensure complete coverage of cells by T/E solution. The flask was then incubated in a 37oC incubator for 2 minutes or until cells completely round up. Change in cell morphology was monitored under light microscope. Use a microscope to monitor the change in cell morphology. During incubation, a 50 ml conical centrifuge tube was prepared with 5 ml of fetal bovine serum. The T/E solution was transferred from the flask to the 50 ml centrifuge tube, after making sure that all the residual cells were collected in 50 ml tube, the tube was centrifuged at 1000 rmp for 5 minutes and cells were resuspended in culture medium. Cells were plated in new poly-L-lysine coated culture flasks with 5x10^6 cells per ml.

Culture flasks were grouped and labeled as control, Ascorbate and resveratrol groups. The cells were treated with different concentrations of H_2O_2 (0mM, 0.5mM, 1.0mM, 2.0mM and 4.0mM). Ascorbate and resveratrol group cells were also treated with 1mM of Ascorbate and 1 mM resveratrol respectively in addition to different concentrations of hydrogen peroxide. Cells were observed under inverted microscope (made by Leica company) for morphological changes and TM cell metabolism was determined by MTT assay, TM cell viability by F528 and Reactive oxygen species were determined by ROS Elisa assay kit (Glory Science Co., Ltd. 2400 Veterans Blvd. Suite 16 - 101, Del Rio, TX 78840,

USA). Data was analyzed on SPSS 22.0 at 95% confidence interval (P-value ≤0.05) using Chi square test. Results were presented as frequency and percentage.

RESULTS

Effects of Resveratrol and Ascorbate on co-incubated and pre-treated TM Cell metabolism against H_2O_2-Induced Injury showed significant differences for Resveratrol at 1.0 mM H_2O_2 concentration (p=0.0001). A significant reduction in TM cell metabolism was observed approximating 61% at 1.0 mM H_2O_2 compared to Ascorbate – 99% and Resveratrol 99% (p=0.0001). Resveratrol was more effective than Ascorbate even at 4.0 mM H_2O_2, the TM cell activity was noted 76%. Under co-treatment conditions, the 1mM Resveratrol retained TM cell metabolism. Compared to H_2O_2- treated TM cells, resveratrol improved mitochondrial function upto 4.0 mM H_2O_2 (76%) (Table-I and Figure-1). Compared to co-treatment, the pre treatment shows similar results except at 4.0 mM H_2O_2. At 4.0 mM H_2O_2 the pre-treat TM cell metabolic activity was found as 11%, 31% and 47% compared to co-treat as 9%, 31% and 76% in controls, ascorbate and resveratrol groups respectively (p<0.05) (Table-II and Figure-2).

Resveratrol shows significant decrease in viability was seen in controls compared to Ascorbate and Resveratrol groups. Cell viability showed statistically significant differences at 2.0 and 4.0 mM H_2O_2 compared to controls (P=0.0001) (Table-III and Figure-3).

For reactive oxygen species (ROS), cells were incubated and with Ascorbate and Resveratrol for 24 hours and TM cells were treated with 0.0mM, 0.5 mM, 1.0 mM, 2.0mM and 4.0mM H_2O_2. Significant decrease in ROS was noted by Resveratrol compared to Ascorbate (Table-IV and Figure-4).

	0.0 mM H_2O_2	0.5 mM H_2O_2	1.0 mM H_2O_2	2.0 mM H_2O_2	4.0 mM H_2O_2
Controls	100.0	98.0	61.0	32	9
Ascorbate (1mM)	100.0	100.0	99.0	65.0	31
Resveratrol (1mM)	100.0	100.0	99.0	90.0	76

Table-I. Co-Incubation of TM cells with ascorbate and resveratrol against H_2O_2-Induced metabolic injury
P value = 0.0001 is statistically significant calculated by ANOVA "t" test

	0.0 mM H_2O_2	0.5 mM H_2O_2	1.0 mM H_2O_2	2.0 mM H_2O_2	4.0 mM H_2O_2
Controls	100.0	98.0	63.0	53.0	11.0
Ascorbate (1mM)	100.0	100.0	98.0	76.0	31.0
Resveratrol (1mM)	100.0	100.0	99.0	89.0	47.0

Table-II. Pretreatment of TM cells with ascorbate and resveratrol against H_2O_2-induced metabolic injury
P value = 0.0001 is statistically significant calculated by ANOVA "t" test

Cell Viability	0 mM H_2O_2	0.5 mM H_2O_2	1.0 mM H_2O_2	2.0 mM H_2O_2	4.0 mM H_2O_2
Controls	100.0	98.0	61.0	32.0	9.0
Ascorbate (1mM)	100.0	100.0	99.0	65.0	31.0
Resveratrol (1mM)	100.0	100.0	99.0	90.0	76.0

Table-III. Co-Incubation of TM cells with antioxidants also protects against H_2O_2-induced cell death
P value = 0.0001 is statistically significant calculated by ANOVA "t" test

ROS	0 mM H_2O_2	0.5 mM H_2O_2	1.0 mM H_2O_2	2.0 mM H_2O_2	4.0 mM H_2O_2
Controls	21.0	115.0	321.0	657.0	789.0
Ascorbate (1mM)	20.0	98.0	295.0	534.0	711.0
Resveratrol (1mM)	23.0	75.0	280.0	489.0	669.0

Table-IV. Reactive oxygen species (ROS) levels (IU/ml)
P value = 0.01 is statistically significant calculated by ANOVA "t" test

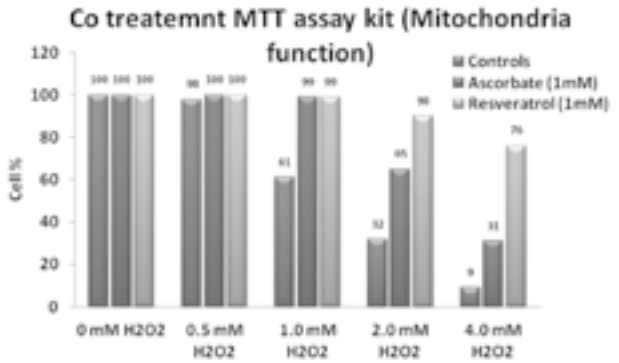

Figure-1. Co-Incubation of TM cells with antioxidants protects against peroxide-induced metabolic injury using MTT assay kit (Mitochondria function)

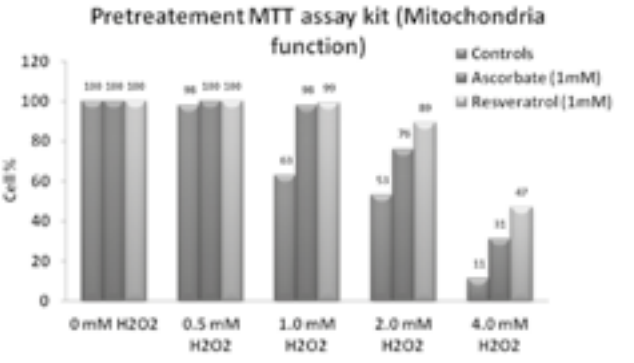

Figure-2. Pre-treatment of TM cells with ascorbate and resveratrol against H_2O_2-induced injury (MTT assay kit)

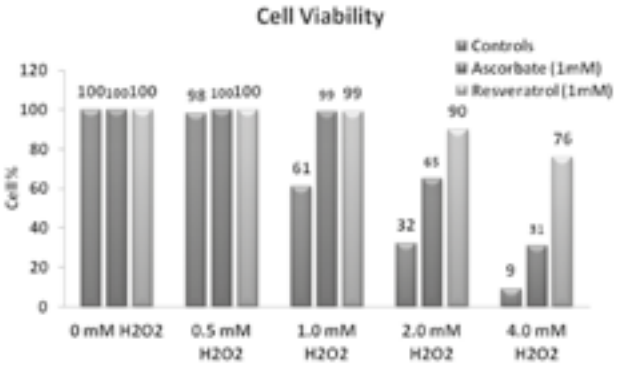

Figure-3. Co-Incubation of TM cells with antioxidants also protects against H_2O_2-induced cell death (Determined by F528)

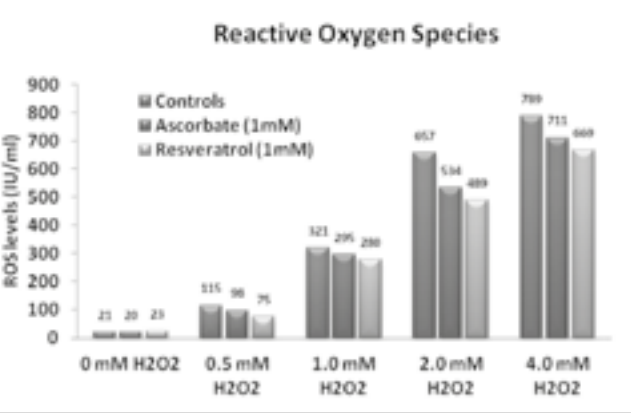

Figure-4. Reactive oxygen species (ROS) levels (IU/ml)

DISCUSSION

Previous research showed anti-oxidative and anti-inflammatory effects of phytochemicals in glaucoma.[9] The present study analyzed the anti-oxidant and ROS scavenging activity of Resveratrol and ascorbic acid in cultured TM cells.

Cardio-protective, neuroprotective, anti-aging effects and chemotherapeutic effects of Resveratrol have been reported. In present study, the Resveratrol showed more promising results as compared to ascorbic acid in determining the TM cell metabolism, TM cell viability and preventive effects on ROS. These findings corroborate with previous reports.[11] In controls, the TM cell metabolism was decreased by 61% compared to 99% Ascorbate and Resveratrol at 1.0 mM H_2O_2 concentration ($p=0.0001$). Resveratrol protected TM cell metabolism excellently at 1mM concentration on co-incubation. Mitochondrial function was retained by 76% at 4.0 mM H_2O_2 concentration by the co- treatment of Resveratrol (Table-I). Previous data also support the observations of this study related to protective effects of antioxidants in prevention of TM cell damage.[12,13]

Co- and pre- treatment with Resveratrol showed equivalent TM cell metabolism at 4.0 mM H_2O_2. Co- and pre- treated TM cells metabolism was noted as 9%, 31% and 76% & 11%, 31% and 47% in controls, Ascorbate and Resveratrol respectively ($p<0.05$). Our findings of resveratrol are consistent with previous studies. In a study conducted on cancer lines, showed the antioxidant properties of resveratrol when the cells were cultured in high glucose and oxygen, which shows the ability of this drug to prevent the ROS induced cell injury[14], also its positive effects on mitochondrial function

were observed in literature.[15]

TM cells showed significant viability at 2.0 and 4.0 mM H_2O_2 concentration compared to controls (P=0.0001). These findings are also in keeping with previous studies. In present study, TM cells co- incubated with 0.0mM, 0.5 mM, 1.0 mM, 2.0mM and 4.0mM H_2O_2 concentrations along with Ascorbate and Resveratrol for 24 hours revealed significant anti- ROS activity by these drugs compared to controls. Similar findings were observed by Boumaza S, and his colleagues, in a study conducted to see protective role of resveratrol on fibroblast cells induced oxidation state.[16], Similar findings were also noted in other studies[17,18] These results prove the efficacy of Resveratrol and Ascorbate as antioxidants. As ROS play pivotal role in POAG, and anti ROS activity of Resveratrol and Ascorbate may be exploited for its prevention and treatment.[19,20]

A previous study reported decreased adhesion of TM cells to matrix when exposed to sub lethal H_2O_2 (1 mM). Hence it is postulated that the anti-ROS potential of Resveratrol and Ascorbate may help in prevention of glaucoma. The findings of present study are supported by another previous study that treated the cultured TM cells with sub- lethal H_2O_2 (0.2 mM) doses. This previous study reported the inflammatory markers were increased in the trabecular meshwork.[21,22]

CONCLUSION

From the evidence based findings of present study supported by literature review, it is concluded that the Resveratrol and Ascorbate may prove useful in preventing and delaying the process of glaucoma, and timely institution of these anti-oxidants may help maintain trabecular meshwork functions and prevent permanent visual loss .

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REFERENCES

1. Evangelho K, Mogilevskaya M, Losada-Barragan M, Vargas-Sanchez JK. **Pathophysiology of primary open-angle glaucoma from a neuroinflammatory and neurotoxicity perspective: A review of the literature.** International ophthalmology. 2019; 39(1):259-71.

2. Babizhayev MA. **Generation of reactive oxygen species in the anterior eye segment. Synergistic codrugs of N-acetylcarnosine lubricant eye drops and mitochondria-targeted antioxidant act as a powerful therapeutic platform for the treatment of cataracts and primary open-angle glaucoma.** BBA Clin. 2016; 6:49-68.

3. Kaur C, Rathnasamy G, Foulds WS, Ling EA. 2015. **Cellular and molecular mechanisms of retinal ganglion cell death in hypoxic-ischemic injuries.** J Neurol Exp Neurosci 1(1): 10-19.

4. Abu-Amero K, A Kondkar A, Mousa A, Osman E, A Al-Obeidan S. **Decreased Total Antioxidants in Patients with Primary Open Angle Glaucoma.** Curr Eye Res. 2013 Sep; 38(9):959-64.

5. Ammar DA, Hamweyah KM, Kahook MY. **Antioxidants protect trabecular meshwork cells from hydrogen peroxide-induced cell death.** Transl Vis Sci Technol. 2012; 1(1):4.

6. Nita M, Grzybowski A. **The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults.** Oxid Med Cell Longev. 2016; 2016:3164734.

7. Lancon A, Frazzi R, Latruffe N. Anti-Oxidant, **Anti-Inflammatory and Anti-Angiogenic Properties of Resveratrol in Ocular Diseases.** Molecules. 2016; 21(3):304.

8. Dineen B, Bourne RR, Jadoon Z, Shah SP, Khan MA, Foster A, et al. **Causes of blindness and visual impairment in Pakistan. The Pakistan national blindness and visual impairment survey.** Br J Ophthalmol. 2007; 91(8):1005-10.

9. Panchal SS, Patidar RK, Jha AB, Allam AA, Ajarem J, Butani SB. **Anti-Inflammatory and antioxidative stress effects of oryzanol in glaucomatous rabbits.** J Ophthalmol. 2017;2017:1468716.

10. Gerszon J, A R, Puchała M. **Antioxidant properties of resveratrol and its protective effects in neurodegenerative diseases.** 2014; 4(2):97.

11. Orihuela Campos R, Tamaki N, Mukai R, Fukui M, Miki K, Terao J, et al. **Biological impacts of resveratrol, quercetin, and N-acetylcysteine on oxidative stress in human gingival fibroblasts.** J Clin Biochem Nutr. 2015 May; 56(3): 220–27.

12. Ungvari Z, Sonntag WE, de Cabo R, Baur JA, Csiszar A. **Mitochondrial protection by resveratrol.** Exerc Sport Sci Rev. 2011;39(3):128-32.

13. Kim SK, Joe Y, Zheng M, Kim HJ, Yu JK, Cho GJ, et al. **Resveratrol induces hepatic mitochondrial biogenesis through the sequential activation of nitric oxide and carbon monoxide production.** Antioxid Redox Signal. 2014;20(16):2589-605.

14. Fonseca J, Moradi F, Valente AJF, Stuart JA. **Oxygen and glucose levels in cell culture media determine resveratrol's effects on growth, hydrogen peroxide production, and mitochondrial dynamics.** Antioxidants. 2018;7(11):157.

15. Roberto de Oliveira M, Nabavi S, Manayi A, Daglia M, Hajheydari Z. **Resveratrol and the mitochondria: From triggering the intrinsic apoptotic pathway to inducing mitochondrial biogenesis, a mechanistic view.** Biochim Biophys Acta. 2016 Apr;1860(4):727-45.

16. Boumaza S, Belkebir A, Neggazi S, Sahraoui H, Sihem B, Smail L, et al. **Therapeutic role of resveratrol and quercetin on aortic fibroblasts of psammomys obesus after oxidative stress by hydrogen peroxide.** Am J Ther. 2018 May/Jun;25(3):e299-e313.

17. Dib B, Lin H, Maidana DE, Tian B, Miller JB, Bouzika P, et al. **Mitochondrial DNA has a pro-inflammatory role in AMD.** Biochim Biophys Acta. 2015; 1853(11 Pt A):2897-906.

18. Wu J, Hsieh T-C. **Resveratrol: A cardioprotective substance** 2011 Jan; 1215:16-21

19. Subramanian M, Goswami M, Chakraborty S, Jawali N. **Resveratrol induced inhibition of Escherichia coli proceeds via membrane oxidation and independent of diffusible reactive oxygen species generation.** Redox Biol. 2014; 2:865-72.

20. Heo JR, Kim SM, Hwang KA, Kang JH, Choi KC. **Resveratrol induced reactive oxygen species and endoplasmic reticulum stressmediated apoptosis, and cell cycle arrest in the A375SM malignant melanoma cell line.** Int J Mol Med. 2018; 42(3):1427-35.

21. Luna C, Li G, Liton PB, Qiu J, Epstein DL, Challa P, et al. **Resveratrol prevents the expression of glaucoma markers induced by chronic oxidative stress in trabecular meshwork cells.** Food Chem Toxicol. 2009; 47(1):198-204.

22. Nasir N, Agarwal R, Krasilnikova A, Hamimah Abdul Kadir S, Iezhitsa I, Md Radzi AB, et al. **The effect of trans-resveratrol on the viability of human trabecular meshwork cells.** RJPBCS 2017; 8(3)1-88.

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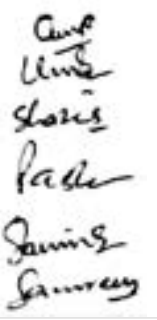
1	M. Yaqoob Shahani	Principal investigator and Corresponding author.	
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3	Shazia Begum Shahani	Writing correstion, data interpretation.	
4	Pashmina Shaikh	Statistical analysis, and data interpretation.	
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