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Original Article

Effect of *Trans*-resveratrol on Rotenone-induced Cytotoxicity in Human Breast Adenocarcinoma Cells

M. A. Siddiqui[1,3], Q. Saquib[1,3], M. Ahamed[2], J. Ahmad[1], A. A. Al-Khedhairy[1,3], F. M. Abou-Tarboush[3], J. Musarrat[1,3,4]

[1]A.R. Al-Jeraisy Chair for DNA Research, College of Science, King Saud University, Riyadh, [2]King Abdullah Institute for Nanotechnology, King Saud University, Riyadh, [3]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia, [4]Department of Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh, India

ABSTRACT

Rotenone, a botanical insecticide is known to cause apoptosis in various cell types. *Trans*-resveratrol, a natural phytophenol present in red grapes and wine, is also well documented for its antioxidant, anti-inflammatory, anti-mutagenic, and anticarcinogenic activities. Therefore, the present investigations were carried out to assess the protective effect of trans-resveratrol against rotenone-induced cell death in human breast adenocarcinoma (MCF-7) cells. MCF-7 cells were exposed with various concentrations of rotenone for 24 h, and the loss in percent cell viability was evaluated by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and neutral red uptake (NRU) assays. A significant decrease in percent cell viability in MCF-7 cells was observed at 50 μM and above concentrations of rotenone, as compared to untreated control. Furthermore, various concentrations (5, 10, and 25 μM) of trans-resveratrol were used to see its protective role on cell viability in rotenone-induced cell death in MCF-7 cells. Pre- or post- treatment of trans-resveratrol for 24 h was given to the cells. The data exhibited a significant dose dependent increase in the percent cell viability under pre- and post-treatment conditions. However, post-treatment of trans-resveratrol for 24 h after rotenone exposure to the cells was relatively less effective. Overall, the results suggest that trans-resveratrol significantly protects MCF-7 cells from rotenone-induced cell death. This model can be used as an effective and economical alternative to animal models for screening the antioxidant activity of a variety of natural compounds/drugs.

Key words: Cytotoxicity, MCF--7 cells, rotenone, *trans*-resveratrol

INTRODUCTION

Rotenone, a specific inhibitor of mitochondrial complex-I, is used as a botanical insecticide for at least 150 years to control crop pests.[1] Being a cell respiratory enzyme inhibitor, it acts as a stomach poison in insects.[2] It is well known to inhibit biochemical process at the cellular level, and can block electron transfer from complex to ubiquinone, resulting in a blockage of the oxidative phosphorylation, and an increase of the reactive oxygen species (ROS).[3,4] A number of studies have evaluated the effects of rotenone both *in vitro*[5] and *in vivo*.[3] Studies have shown that rotenone is capable to induce apoptosis in various cells derived from human B-cell lymphomas,[6] promyelocytic leukemias,[7] and neuroblastomas.[8] Recently, reports have also shown that rotenone induce oxidative stress mediated cytotoxicity in PC12 cells,[9] and in MCF-7 cells.[4]

Trans-resveratrol (*trans*-3,4′,5-tryhydroxystilbene), a natural phytophenol is reportedly present in high concentrations

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Address for correspondence: Prof. Javed Musarrat, Abdul Rahman Al-Jeraisy Chair for DNA Research, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia. E-mail: musarratj1@yahoo.com

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in red grapes and wine. Pharmacological activities of *trans*-resveratrol is well known through its antioxidant,[10,11] anti-inflammatory,[12] antimutagenic,[13] and anticarcinogenic[14] activities. Protective potential of *trans*-resveratrol has also been demonstrated by several investigators in different cell lines and primary cell cultures[15-18] including PC12 cells.[19] Moreover, the neuroprotective potential of *trans*-resveratrol has been extensively studied in both *in vitro* and *in vivo* models.[19-21] However, the protective potential of *trans*-resveratrol against rotenone-induced cytotoxicity in MCF-7 cells, a human breast cell line has not been reported. The MCF-7 cells have been chosen because this cell line has been extensively used for the toxicological and/or pharmacological studies.[22-24] Thus, the present studies were carried out to determine the antioxidant potential of *trans*-resveratrol against rotenone-induced cytotoxicity in MCF-7 cells.

MATERIALS AND METHODS

Cell culture

MCF-7 cells, were cultured in minimum essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate and antibiotic-antimycotic solution (100×; 1 ml/100 ml of medium, HiMedia, India) following the method described by Musarrat *et al*.[25] Culture were maintained at 37°C in 5% CO_2---95% atmosphere under high humid conditions. Medium was changed twice weekly and the culture was split at a ratio of 1:5 once a week. Prior to experiment, each batches of cells was assessed for cell viability by trypan blue dye exclusion test,[26] and batches showing more than 95% cell viability were used in the study. Cells of passage number between 8 and 10 were used in the study.

Experimental design

To identify the cytotoxic doses, the MCF-7 cells were exposed to various concentrations of rotenone (1--1000 μM) for 24 h. Under identical setup, biologically safe doses of *trans*-resveratrol were also detected in MCF-7 cells. Further, the experiments were conducted using rotenone (100 μM) and *trans*-resveratrol at 5, 10, and 25 μM for 24 h each. Responsiveness of MCF-7 cells to *trans*-resveratrol was determined by performing the following two treatment schedules; (a) cells treated with *trans*-resveratrol for 24 h prior to rotenone insult (pre-treatment group); (b) cells treated with *trans*-resveratrol for 24 h after 24 h of rotenone exposure (post-treatment group). Influence of *trans*-resveratrol was estimated by comparing the values of treatment groups with the non-treated cells exposed to rotenone alone.

Mitochondrial activity by MTT assay

MTT assay was performed following the protocols of Siddiqui *et al*.[27] In brief, cells (1×10^4) were seeded in 96-well culture plates and allowed to adhere properly for 24 h at 37 °C in a CO_2 incubator (5% CO_2---95% atmosphere at high humidity). After respective exposures, MTT (5 mg/ml of stock in PBS) was added (10 μl/well containing 100 μl of cell suspension) 4 h prior to the completion of exposure period. After the incubation period, the reaction mixture was carefully aspirated and 200 μl of dimethyl sulfoxide was added to each well with gentle mixing to ensure complete homogeneity. Subsequently, after 10 min, the color was read at 550 nm, using a multiwell microplate reader (Zynth 200, Germany).

Neutral red uptake assay

Neutral red uptake (NRU) assay was performed following the protocols of Siddiqui *et al*.[27] In brief, cells (1×10^4) were seeded in 96-well culture plates and allowed to adhere properly for 24 h at 37 °C in a CO_2 incubator (5% CO_2---95% atmosphere at high humidity). On the completion of respective incubation periods, the test solution was aspirated and cells were washed with PBS twice. Cells were then incubated for 3 h in medium supplemented with neutral red (50 μg/ml). Then medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. The cells were then subjected to further incubation further for 20 min at 37 °C, in a mixture of acetic acid (1%) and ethanol (50%), to extract the dye. The plates were then read at 540 nm using multiplate reader (Zynth 200, Germany).

Statistical analysis

Results are expressed as the mean ± standard error of three independent experiments with six replicates each. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Dunnett post-hoc test to compare the inter- and intra-group findings. The values depicting $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

The results of cytotoxicity assessment of rotenone are presented in Figures 1 and 2. Statistically significant decrease in percent cell viability (% control) was initiated following 24 h exposure of rotenone at 50 μM concentration. A concentration-dependent decrease in cell viability was observed at 50 μM and higher concentrations, whereas, 25 μM and lower concentrations did not exhibit any adverse effect in rotenone exposed MCF-7 cells [Figure 1]. Similar pattern of concentration dependent effects was also observed with NRU assay [Figure 2]. Under identical experimental setup, none of the doses (5, 10, and 25 μM) of *trans*-resveratrol was found to be cytotoxic in 24 h exposed MCF-7 cells by MTT [Figure 3] and NRU [Figure 4] assays.

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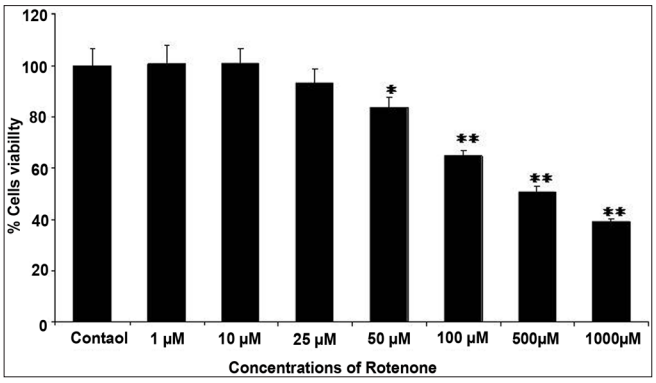


Figure 1: Cytotoxicity assessment by MTT assay in MCF-7 cells following the exposure of various concentrations (1--1000 µM) of rotenone for 24 h. Values are mean ± SE of three independent experiments. **P* < 0.05, ***P* < 0.01 vs control.

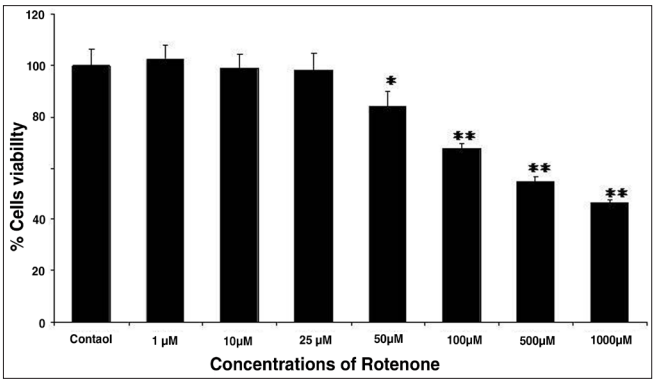


Figure 2: Cytotoxicity assessment by NRU assay in MCF-7 cells following the exposure of various concentrations (1--1000 µM) of rotenone for 24 h. Values are mean ± SE of three independent experiments. **P* < 0.05, ***P* < 0.01 vs control.

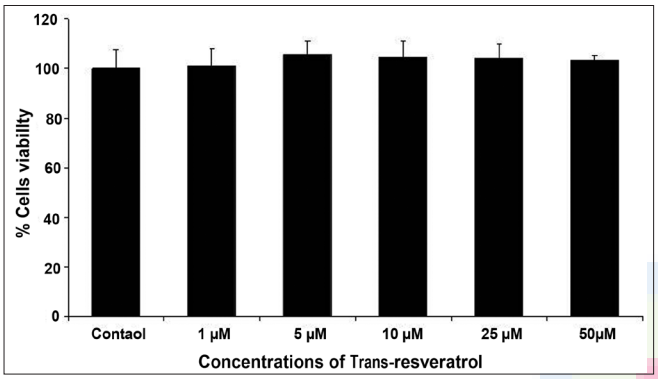


Figure 3: Cytotoxicity assessment by MTT assay in MCF-7 cells following the exposure of various concentrations (1--50 µM) of *trans*-resveratrol for 24 h. Values are mean ± SE of three independent experiments.

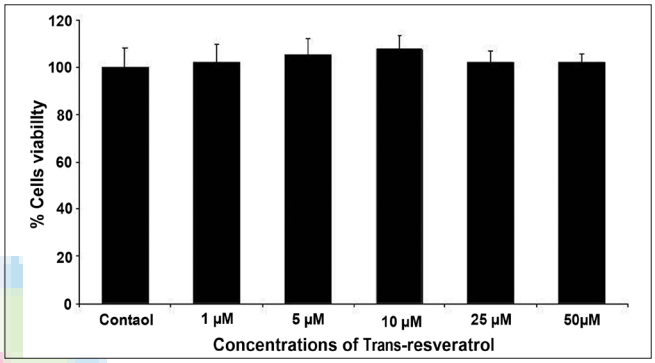


Figure 4: Cytotoxicity assessment by NRU assay in MCF-7 cells following the exposure of various concentrations (1--50 µM) of *trans*-resveratrol for 24 h. Values are mean ± SE of three independent experiments.

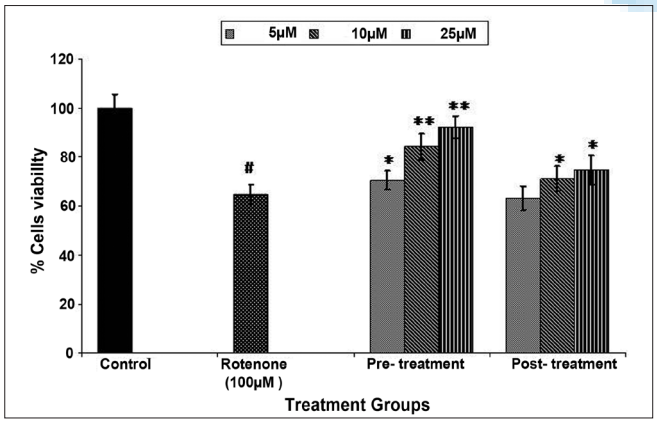


Figure 5: Protective potential of trans-resveratrol in MCF-7 cells exposed to 100 µM of rotenone for 24 h by MTT assay. Values are mean ± SE of three independent experiments. #*P* < 0.01 vs control and **P* < 0.05, ***P* < 0.01 vs rotenone exposure.

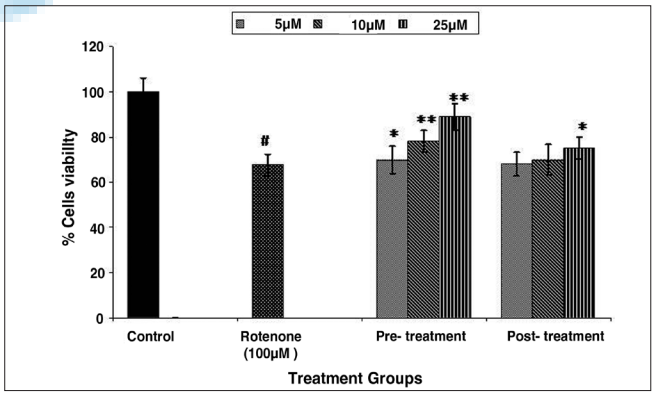


Figure 6: Protective potential of trans-resveratrol in MCF-7 cells exposed to 100 µM of rotenone for 24 h by NRU assay. Values are mean ± SE of three independent experiments. #*P* < 0.01 vs control and **P* < 0.05, ***P* < 0.01 vs rotenone exposure.

Result highlights of protective potential of *trans*-resveratrol against toxic insult of rotenone (100 μM) in MCF-7 cells are depicted in Figures 5 and 6. Statistically significant (6%, 20%, and 28% of rotenone control) increase in percent cell viability was recorded following 5, 10, and 25 μM concentrations of *trans*-resveratrol, respectively, in pre-treatment schedule for 24 h in MCF-7 cells. In post-treatment group, a similar trend of *trans*-resveratrol mediated protection was observed. However, the magnitude of protection was comparatively lower at all the doses used, i.e., 7% and 10% of rotenone control at 10 and 25 μM *trans*-resveratrol respectively, whereas, *trans*-resveratrol at 5 μM could not protect MCF-7 cells against rotenone-

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induced cell death. Although, the *trans*-resveratrol mediated increase in percent cell viability was statistically significant ($P < 0.05$ and $P < 0.01$), when compared with rotenone (100 μM) exposed group, but the recovery response of pre-treatment of *trans*-resveratrol even at highest dose, i.e., 25 μM was significantly ($P < 0.01$) lower than unexposed control cells [Figure 5]. In case of NRU assay, the loss of percent cell viability was significantly increased by 10% and 12% at 10 μM and 25 μM of *trans*-resveratrol, respectively, compared with the cells exposed to 100 μM concentration of rotenone. The trend of recovery at all the concentrations of *trans*-resveratrol was almost similar as in case of MTT assay. However, *trans*-resveratrol treated MCF-7 cells after rotenone exposure could not get significantly increase, except 25 μM ($P < 0.05$) of *trans*-resveratrol when compared with rotenone (100 μM) exposed cells [Figure 6].

An essential task in pharmacology is the search and development of new drugs for the prevention and treatment of diseases.[28,29] Natural compounds could be the suitable candidates in this respect.[30,31] Moreover, in recent years it has been shown that *trans*-resveratrol offers protection against different toxins.[32,33]

The present study shows that rotenone induce significant decrease in cell viability in a concentration dependent manner in MCF-7 cells following 24 h exposure based on MTT and NRU assays, as the endpoints for cytotoxicity assessment. Similar trend in the decrease in cell viability was obtained by MTT and NRU assays. A possible explanation for this is that the cytotoxicity of rotenone is mediated through the both mitochondrial and lysosomal damage. Both the parameters have been used as direct measurements of cell viability by different investigators.[9,34]

Our results are in accordance with the previous findings that the rotenone-induce cytoxicity in various types of cells, including the MCF-7 cells.[4,9,35] Rotenone-induced oxidative stress and death of dopaminergic neurons in chronic midbrain slice model is documented.[36] Evidences suggest that complex-I may be a source of ROS generation, since, there is a site of electron leakage in complex-I upstream of the rotenone-binding site, which partially inhibits the complex I following rotenone exposure.[9,37]

The protective potential of *trans*-resveratrol studied with pre- and post-treatment schedules ascertained the therapeutic applicability of *trans*-resveratrol in MCF-7 cells, under experimental exposure of rotenone. A dose dependent synergistic protective potential could be observed under all treatment groups i.e. pre, and post. In general, pre-treatment group has shown higher restoration than post-treatment group by MTT and NRU assays. This might be due to pre-treatment of *trans*-resveratrol induced increase in the level of antioxidant and anti-inflammatory enzymes in MCF-7 cells. These kinds of pre-treatment of *trans*-resveratrol have already been studied by different investigators showing better protection than post-treatment.[19,27] The pre-treatment of many other antioxidants and anti-inflammatory drugs have also been demonstrated showing better protection than therapeutics.[27,38]

Increase in cell viability was concentration dependent but the recovery response of *trans*-resveratrol at higher concentrations, i.e., 10 and 25 μM was more protective that 5 μM of *trans*-resveratrol. *Trans*-resveratrol is well known for its anti-inflammatory and antioxidant activities, therefore, our data on the protective effects of *trans*-resveratrol are well corelated with previous *in vitro* studies demonstrating the antioxidative effects of this drug on toxicant-induced apoptosis in PC12 cells,[39,40] β-amyloid neurotoxicity,[41] and in oxygen-glucose deprivation.[42-44] Studies performed in rodents also suggested that this drug has a good protective role against toxicants.[45,46]

It is concluded that the study has significance in understanding the cytotoxicity of rotenone and also the protection against the cytotoxic activity by *trans*-resveratrol at cellular level, using MCF-7 cells as a model for simple, easy and inexpensive screening the antioxidant potential of a variety of natural compounds/pharmaceuticals.

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