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Resveratrol Pretreatment Protects Cardiomyocytes Against Catecholamine-Induced Beta Adrenergic Stimulation And Myocyte Injury During High Glucose Challenge.

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ABSTRACT

Resveratrol(3,5,4'-trihydroxystilbene) a polyphenolic compound produced in plants in response to stress is a phytoalexin, phytoestrogen reported to possess diverse biological activities both *in vitro* and *in vivo*. In the current study, the effect of resveratrol to protect rat cardiomyocytes H9c2 against beta adrenergic stimulation induced by the catecholamine isoproterenol during high glucose challenge was investigated *in vitro*. H9c2 cells were pretreated with different concentrations of resveratrol (10, 20, 30, 40 and 50µg/ml) for 30 minutes followed by high glucose (30 mmoles/L for 24 hours) and isoproterenol (100µmoles/L for 8 hours) challenge. Alternatively, a group of cells were also post treated with resveratrol at the specified concentrations for 30 minutes following exposure of the cells to high glucose/isoproterenol. Cell viability was assessed by NBT reduction test and sulphorhodamine B assay. Results of the study indicated that challenge with glucose/isoproterenol induced severe cytotoxic effects on the cardiomyocytes as observed by the decreased cell viability and survival. Pretreatment with resveratrol at a concentration of 30µg/ml for 30 minutes was able to confer considerable protective effects against the cytotoxic effects of high glucose/isoproterenol as revealed by statistically significant increase (P<0.001) in percentage survival determined by both NBT reduction test and sulphorhodamine B assay. Results implicate the cardioprotective effects of resveratrol against catecholamine induced beta adrenergic stimulation in cardiomyocytes during experimental hyperglycemic conditions *in vitro*.

Keywords: Resveratrol; cardiomyocytes; H9c2 cells, NBT reduction test, isoproterenol

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INTRODUCTION

Natural products have been the epicenter of drug discovery and development and the last three decades have seen tremendous research efforts to highlight the application of these natural products in the therapy of diseases. Polyphenols have gained considerable research attention because of their diverse biological actions owing to their immense antioxidant potential. Resveratrol is a naturally occurring polyphenolic compound found largely in the skin of the grapes (*Vitis vinifera*) and the products prepared from it such as redwine. Resveratrol has been reported to be present in several other plant species other than grapes such as peanuts, berries and legumes[1]. Its stilbene-based structure has two phenolic rings linked by a styrene double bond,which allows *cis* and *trans* orientation to generate 3,4,5-trihydroxystilbene[2].

Ischemia is a condition wherein there is an imbalance between oxygen supply and demand for the metabolizable substrates in the cardiac tissue rapidly leading to functional, metabolic, electrophysiological, and morphological alterations of the myocardium and may eventually cause cellular necrosis. Cardiomyocytes are extremely vulnerable to the detrimental effects of free radicals resulting in oxidative stress which ultimately culminates in damage to the myocardial tissue. Antioxidant supplementation is a promising approach in negating the oxidative damage induced in the cardiac tissue and resveratrol treatment has been reported to exert significant cardioprotective effects in both *in vivo* and *in vitro* models. [3]

Diabetes is the major risk factor for the development of cardiovascular disease and the risk conferred by diabetes in an individual is as detrimental as a previous cardiac event. Considerable research momentum has gained in the development of antidiabetic drugs which are capable of preventing the development of macrovascular changes and cardiovascular disease in the risk prone population at a later part of life[4]. The objective of the current study is to understand if resveratrol supplementation could be beneficial and prevent the injury inflicted in cardiomyocytes by the beta adrenergic agonist and catecholamine isoproterenol during experimental hyperglycemic conditions by high glucose challenge.Isoproterenol induced myocardial injury is a well standardized model to study the cardioprotective effect of test compounds and hence used for inducing myocyte injury in the present study also. Cardiomyocyte cells were challenged with high glucose to mimic diabetic conditions *in vitro*. Rat embryonic cardiomyocytes H9c2 cells were used as model systems in the study and basic cell growth and viability assays were carried out to investigate the specified objectives.

MATERIALS AND METHODS

Chemicals

Isoproterenol (ISO), Streptozotocin (STZ/Strep), Resveratrol (RSV), Sulforhodamine-B and Nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Company. Sterile ready to use Dulbecco's Modified Essential Medium (DMEM), Foetal Bovine Serum, Trypsin-EDTA, Antibiotic antimycotic solution were procured from Himedia Laboratories Pvt Limited, Mumbai, India. All the other chemicals used were of analytical grade and were purchased from the local chemical companies.

Procurement and maintenance of H9c2 cell line

The cardiomyocyte cell line H9c2 was procured from the National Center for Cell Science, Pune, India and cultured in sterile ready to use Dulbecco's Modified Essential Medium (DMEM, AL007 , Himedia, India) supplemented with 10% Foetal Bovine Serum(FBS- RM1112 , Himedia, India) and 1x antibiotic antimycotic solution(A002, Himedia, India). Cells were grown under standard growth conditions (Temperature 37^0C, 95% humidity and 5% CO_2) in a CO_2 incubator (Forma Scientific, USA). When a confluent monolayer was formed, the cells were detached with 0.25% Trypsin-0.2% EDTA in Dulbecco's phosphate buffered saline (T-001, Himedia, India) and then subcultured at a split ratio of 1: 3 in a 12.5cm^2 tissue culture flask (TCG2, Himedia, India). The media was changed three times a week. The cells were grown in a growth medium containing 10% FBS or maintenance medium containing 5% FBS. On arriving at confluency, the cells were plated on to 96 well microtitre plates (TPP-96, Himedia, India) and were utilized for different *in vitro* assays.

***In vitro* assays for assessing the influence of resveratrol on cardiomyocyte viability and integrity.**

Induction of hyperglycemic conditions and stress on cardiomyocyte cells in vitro by challenge with glucose and isoproterenol- In vitro models to mimic myocardial stress in diabetic conditions.

On arriving at confluency, the cells (5 X 10^6 cells/ml) were plated on to 96 well microtiter plate. The cells were allowed to attach overnight and then pretreated with different concentrations of resveratrol (10, 20, 30, 40, and 50µg/ml for 30 minutes). Following this, experimental hyperglycemic conditions were induced in H9c2 cells by exposing them to high concentration of glucose (30 mMoles/L) [5] for 24 hours. After 24 hours, the cells were treated with isoproterenol (100 µmoles/L) for 8hrs [6]. A group of cells were challenged with glucose/isoproterenol at the same dose specified above and then treated with resveratrol (10, 20, 30, 40 and 50µg/ml for 30 minutes) to understand if resveratrol treatment could reverse the damage inflicted on the myocardium. Following the different treatment regimen the growth and viability of the cells were determined by NBT reduction test and sulforhodamine B assay and the results were expressed as percentage viability of the treated cells as compared to the untreated control cells.

NBT reduction Test

NBT reduction test was performed based on the method of Williams et al., 1977 [7]. Briefly, the untreated control cells, glucose/isoproterenol challenged cells and the resveratrol treated cells (both pre and post treatment) were allowed to proliferate for 72 h, 10 µl of nitro blue tetrazolium chloride (5mg/ml) was added and incubated in a CO_2 incubator at 37°C for 5 hours. The cells were then washed three times with isotonic phosphate buffered saline and the NBT reduced was solubilized in 100µl of Isopropanol. The optical density of each well was measured at 570nm using a micro plate reader.

Sulforhodamine B assay

Cell proliferation was assessed by Sulforhodamine-B (SRB) assay as previously reported [8]. Briefly, cell suspensions containing 1×10^4 viable cells/ml were plated onto 96-well plates and allowed to attach for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were then exposed to resveratrol for 24 h. Cells were washed with PBS and fixed with trichloroacetic acid at 4°C for 1 h. After washing with water, cells were stained with SRB. Protein bound stain was solubilized with unbuffered Tris base [tris(hydroxymethyl)aminomethane]. The absorbance was then measured at 540 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Results were expressed as relative absorbance of the treated cells compared with untreated controls.

Statistical Analysis

For *in vitro* assays all the experiments were carried out in triplicate on at least three different occasions and the mean of the replicate values were taken. Values were expressed as mean ± SD. Statistical analysis of the data was determined by Student's t-test and comparisons were made between the untreated control group and the treated groups.

RESULTS AND DISCUSSION

Effect of different doses of resveratrol pretreatment and post treatment on the growth and viability of cardiomyocytes as determined by NBT reduction test

NBT reduction test is an important diagnostic tool to study the influence of test compounds on growth and viability. The test is based on the principle that the dye nitroblue tetrazolium on reaction with viable cells produces a coloured complex, the intensity of which is directly proportional to the number of viable cells present in the culture [9]. The test is precise, accurate and could be an alternative to conventional MTT assay wherein the insoluble nature of the formazan crystals formed pose a problem with the accuracy and reproducibility of the results.

Figure 1a and Figure 1 b shows the effects of different doses of resveratrol pretreatment and post treatment respectively (10, 20, 30, 40 and 50 µg/ml) on the percentage viability of cardiomyocytes H9c2 during beta adrenergic stimulation and high glucose challenge. Results implicate that high glucose challenge

and consequent beta adrenergic stimulation resulted in a statistically significant decrease in percentage survival of cardiomyocytes as compared to control (p<0.001). Resveratrol pretreatment exhibited cardioprotective effects at low dosages with maximum protective effect observed at a dose of 30µg/ml for 30 minutes. No statistically significant increase in percentage viability was observed at doses above 30µg/ml - 50µg/ml. Doses above 50µg/ml resulted in decrease in growth and viability of cardiomyocytes implicating a toxic effect (data not shown). Post treatment with resveratrol following high glucose challenge and beta adrenergic stimulation failed to repair the damage inflicted in the cardiomyocytes. This also shows that resveratrol is more effective as a prophylactic agent rather than a curative agent.

Figure 1 a: Effect of resveratrol pretreatment on the viability of cardiomyocytes during high glucose /isoproterenol challenge- NBT reduction test

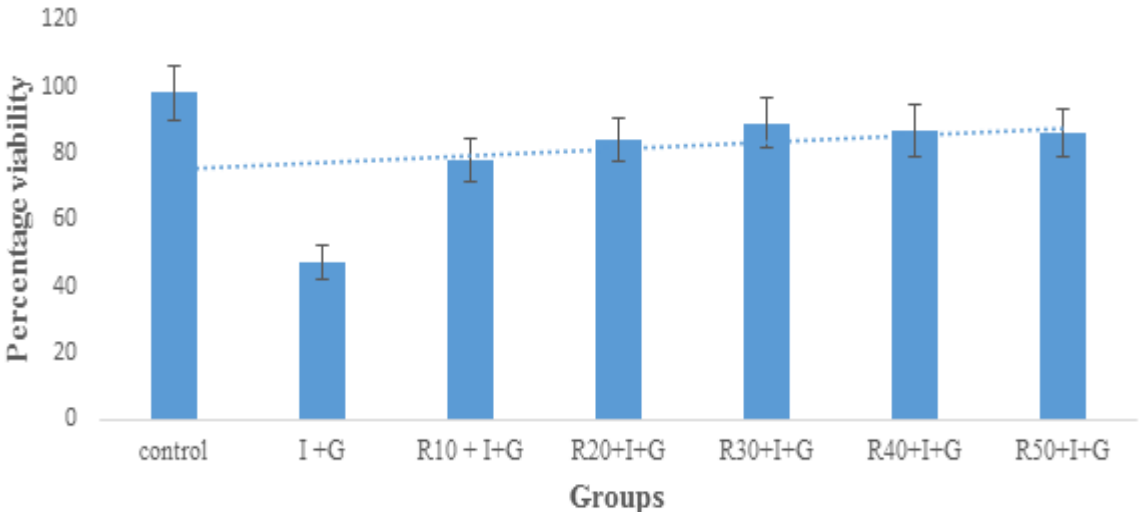

Figure 1 b: Effect of resveratrol post treatment on the viability of cardiomyocytes during high glucose/isoproterenol challenge- NBT reduction test

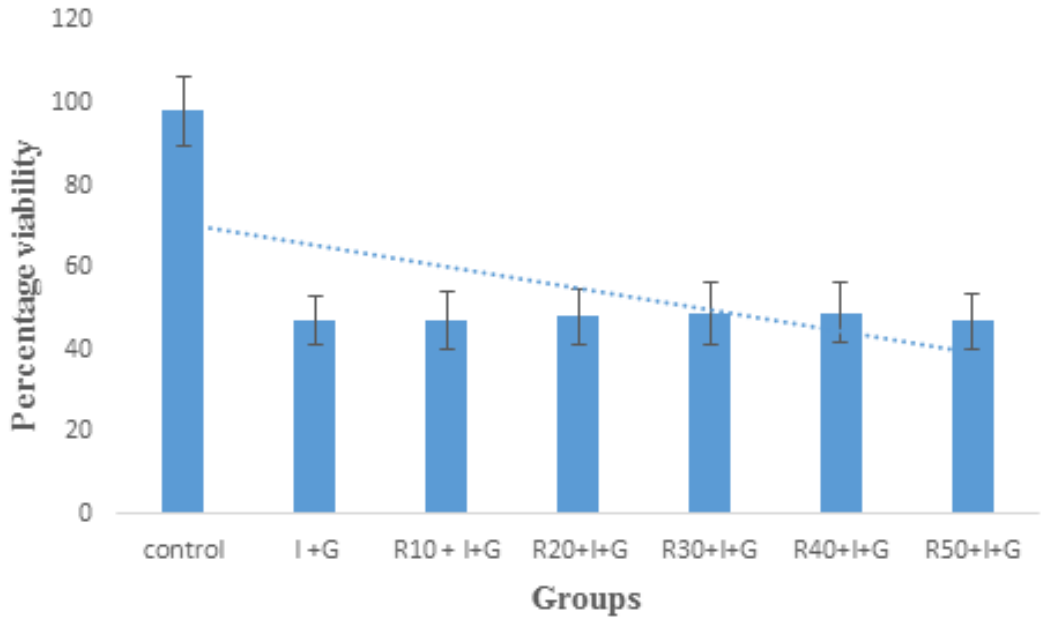

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Figure 2a: Effect of resveratrol pretreatment on the viability of cardiomyocytes during high glucose/isoproterenol challenge- Sulforhodamine B assay

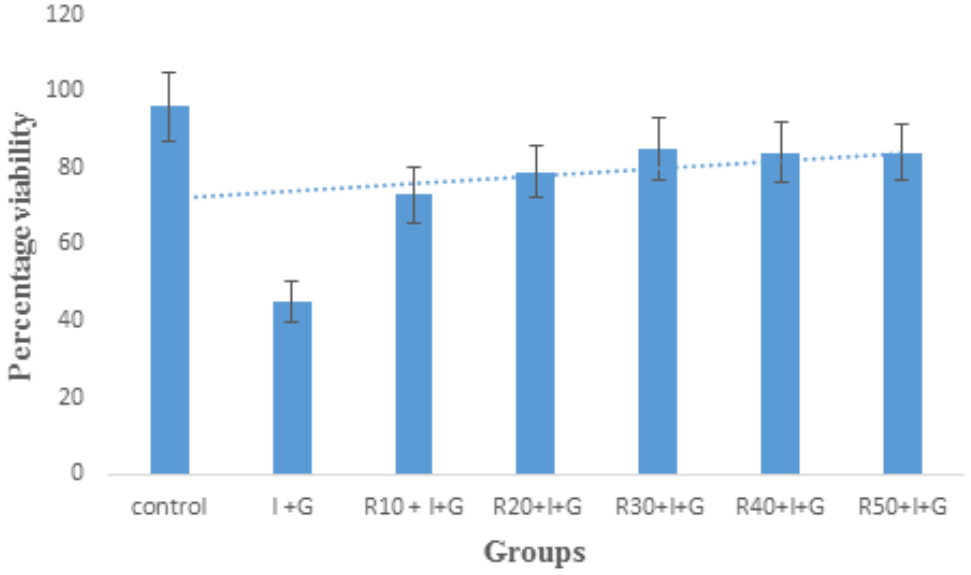
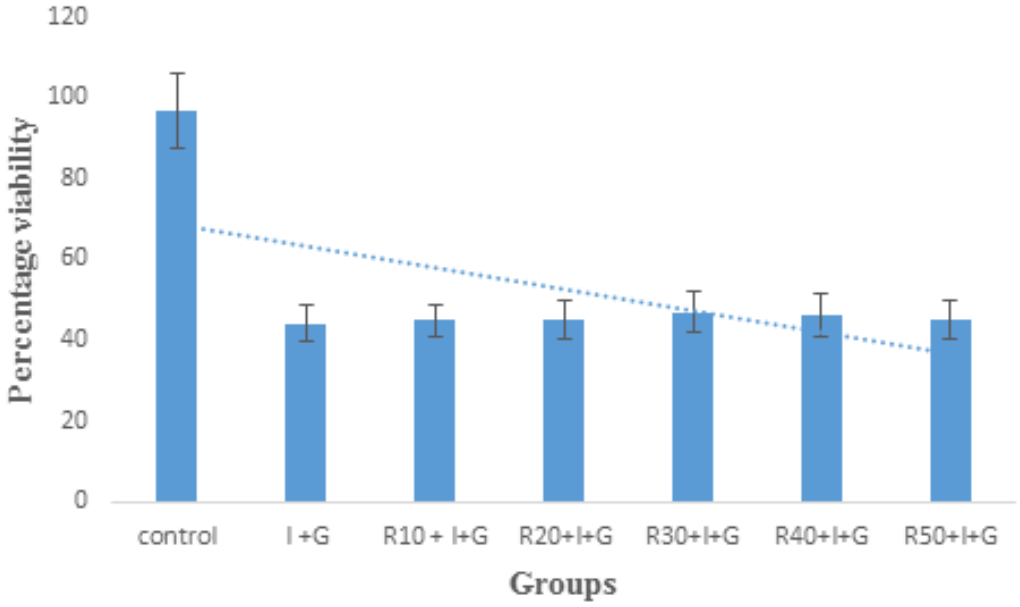


Figure 2 b: Effect of resveratrol post treatment on the viability of cardiomyocytes during high glucose/isoproterenol challenge- Sulforhodamine B assay



Effect of different doses of resveratrol pretreatment and post treatment on the growth and viability of cardiomyocytes as determined by Sulforhodamine B assay

The sulforhodamine B (SRB) assay is a widely used technique for large scale drug screening. Its principle is based mainly on the ability of the protein dye sulforhodamine B to bind electrostatically and pH dependent on protein basic amino acid residues of trichloroacetic acid–fixed cells. It can be quantitatively extracted from cells and solubilized for optical density (OD) measurement by weak bases such as Tris base. During the mild acidic conditions it binds to and under mild basic conditions it can be extracted from cells and solubilized for measurement [10].Figure 2a and Figure 2 b shows the effect of different doses of resveratrol pretreatment and post treatment respectively (10, 20, 30, 40 and 50 µg/ml) on the percentage viability of cardiomyocytes H9c2 during beta adrenergic stimulation and high glucose challenge as determined by sulforhodamine B assay. Results of this assay also implicated that high glucose challenge and consequent beta adrenergic stimulation resulted in a statistically significant decrease in percentage survival of cardiomyocytes as compared to control (p<0.001). This was in line with the observations of NBT reduction test. Resveratrol

pretreatment exhibited maximum protective effects on the cardiomyocytes at a dosage of 30µg/ml for 30 minutes. Doses less than 30µg/ml also exhibited protective effects but the effects were found to be less pronounced as compared to the dose of 30µg/ml. There was no significant increase in the percentage viability of the cardiomyocytes at dosages 40 and 50µg/ml as compared to 30µg/ml thereby implicating that increasing the dose above 30µg/ml did not exert any appreciable protective effects on the cardiomyocytes. Doses above 50µg/ml were found to decrease the percentage viability of cardiomyocytes (data not shown) thereby indicating the toxic effects of resveratrol above the dose of 50µg/ml. All these were in line with the results obtained with the NBTreduction test. Also, as with NBT reduction test it was observed that post treatment with resveratrol following high glucose challenge and beta adrenergic stimulation failed to repair the damage inflicted in the cardiomyocytes. This again confirms that the protection offered by resveratrol against myocyte injury induced by beta adrenergic stimulation during experimental high glucose challenge is preventive rather than curative.

CONCLUSION

The results of the current study indicate the protective effects of the phytoestrogen resveratrol against cardiomyocyte injury induced by beta adrenergic agonist and catecholamine isoproterenol during high glucose challenge. Resveratrol supplementation could be a promising strategy to prevent macrovascular cardiac complications in risk prone diabetic population.

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