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Mitochondrial dysfunction in H9c2 cells during ischemia and amelioration with *Tribulus terrestris* L.

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ABSTRACT

Aims: The present study investigates the protective effect of partially characterized *Tribulus terrestris* L. fruit methanol extract against mitochondrial dysfunction in cell based (H9c2) myocardial ischemia model.
Main methods: To induce ischemia, the cells were maintained in an ischemic buffer (composition in mM — 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 HEPES, 20 2-deoxy-D-glucose, pH — 6.2) at 37 °C with 0.1% O_2, 5% CO_2, and 95% N_2 in a hypoxia incubator for 1 h. Cells were pretreated with various concentrations of *T. terrestris* L. fruit methanol extract (10 and 25 μg/ml) and Cyclosporin A (1 μM) for 24 h prior to the induction of ischemia.
Key findings: Different parameters like lactate dehydrogenase release, total antioxidant capacity, glutathione content and antioxidant enzymes were investigated. Studies were conducted on mitochondria by analyzing alterations in mitochondrial membrane potential, integrity, and dynamics (fission and fusion proteins — Mfn1, Mfn2, OPA1, Drp1 and Fis1). Various biochemical processes in mitochondria like activity of electron transport chain (ETC) complexes, oxygen consumption and ATP production was measured. Ischemia for 1 h caused a significant ($p \leq 0.05$) increase in LDH leakage, decrease in antioxidant activity and caused mitochondrial dysfunction. *T. terrestris* L. fruit methanol extract pretreatment was found effective in safeguarding mitochondria via its antioxidant potential, mediated through various bioactives. HPLC of *T. terrestris* L. fruit methanol extract revealed the presence of ferulic acid, phloridzin and diosgenin.
Significance: T. terrestris L. fruit ameliorate ischemic insult in H9c2 cells by safeguarding mitochondrial function. This validates the use of *T. terrestris* L. against heart disorders.

1. Introduction

The prevalence of cardiovascular diseases is growing and it is the most common cause of death worldwide. Ischemic coronary heart disease, resulting in myocardial infarction is causing 7.2 million deaths worldwide [14]. Myocardial ischemia is caused by an imbalance in the demand and supply of oxygen and nutrients to the heart. The deprivation of oxygen disrupts oxidative phosphorylation, alteration of membrane potential, ATP depletion and the cell switches to adopt anaerobic glycolysis. The anaerobic glycolysis leads to accumulation of lactate and acidosis which ultimately leads to intracellular calcium overload, mitochondrial dysfunction and ultimately death of the myocyte [13]. The function of the heart cell is entirely dependent on the O_2 consumption by the mitochondria and electron transport chain with minimum electron leak. Any abnormality will result in high mitochondrial calcium and increased oxidative stress. Oxidative damage to mitochondrial membranes, enzymes, and electron transport chain (ETC) components culminate in impaired mitochondrial ATP production, which facilitates mitochondrial permeability transition pore (mPTP) opening [49] that leads to cellular apoptosis and necrosis.

The innate antioxidant system of the myocardium also prevents damage caused by oxidative stress. Myocardial antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase act in concert to protect the myocardium from ischemia induced oxidative insult [11]. Thus, focusing on the prevention of surplus ROS mediated mitochondrial dysfunction will be a promising therapeutic target. During ischemia, the change in transmembrane potential causes increased calcium uptake and impaired ATP synthesis that lead to a loss of ion homeostasis, stimulation of ROS generation, mPTP opening, matrix swelling, and outer mitochondrial membrane rupture and finally death of the myocyte [20]. Therefore, maintaining mitochondrial transmembrane potential and preventing mPTP opening is protective against ischemia. Mitochondrial dynamics and mitophagy have an important role in maintaining the quality of the mitochondria. During ischemia there is an increase in mitochondrial fission, making the mitochondria more susceptible to mitophagy and a decrease in mitochondrial fusion. Thereby, maintaining the integrity of the mitochondria prevents damage caused by ischemia. Thus, protecting the mitochondria by a therapeutic

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agent is an effective strategy to prevent the deleterious effects of myocardial ischemia.

Tribulus terrestris L. (Family: Zygophyllaceae) also known as *Gokshura* in Ayurveda and *Bai Ji Li* in traditional Chinese medicine is widely used in traditional practice against heart diseases [8]. It is also used as an aphrodisiac, anthelminthic, carminative, demulcent, diuretic, emmenagogue, galactogogue, pectoral and tonic [17,30]. It has been reported earlier that the aqueous extract of *T. terrestris* L. fruit has antihypertensive property [39,44]. Our previous studies revealed that the ethyl acetate fraction from the methanol extract of *T. terrestris* L. root protects the H9c2 cell from ischemic alterations by its potent antioxidant activity [41]. However, to the best of our knowledge there is no detailed study on the property of protecting the mitochondria during cardiac ischemic injury by *T. terrestris* L. fruits. So, considering the importance of mitochondria in ischemia mediated pathological lesions, we studied the effect of pretreatment of *T. terrestris* L. fruit methanol extract particularly on mitochondrial bioenergetics and redox signaling.

2. Materials and methods

2.1. Chemicals, drugs and reagents

Ascorbic acid, butylated hydroxyl toluene (BHT), ferric chloride, thiobarbituric acid (TBA), trichloroacetic acid (TCA), Folin-Ciocalteu reagent, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and Cyclosporin A were from Sisco Research Laboratories (Mumbai, India) and fetal bovine sera (FBS) was from Gibco (Langley, OK, USA). The cell culture flasks and plates were from BD Biosciences (USA). HPLC grade solvents were from Merck (NJ, USA). Kits for the enzyme assays were from Cayman Chemical Company (MI, USA), and all other chemicals were purchased from Sigma (St. Louis, MO, USA). Anti-Mfn2 (sc-50331), anti-OPA1 (sc-367890), anti-Drp1 (sc-21804), anti-Fis1 (sc-48865), anti-HIF-1α (sc-13,515) and anti-β actin antibodies (sc-4778) were obtained from Santa Cruz Biotechnology (CA, USA). Primers for q-RTPCR were obtained from Sigma (USA).

2.2. Plant material

T. terrestris L. an accepted name in "the plant list" (Sp. Pl. 1: 387. 1753) was collected during the month of April from Virudhunagar district (Latitude: N 9° 35′ 1.0032″ Longitude: E 77° 57′ 30.0384″) of Tamil Nadu, India, as per advice from a traditional healer and identified by Dr. Biju Haridas, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, India. *T. terrestris* L. specimen has been kept at the Herbarium at CSIR —NIIST (AC-4/2011). The fruits were washed, dried in an oven at room temperature and pulverized using a blender. The dried powdered fruits (250 g) were extracted with methanol at room temperature by maceration. The solution was filtered, evaporated under reduced pressure and further lyophilized to yield 17.5 g of methanol extract of *T. terrestris* L. fruit (TFM).

2.3. Cell culture, cytotoxicity studies and induction of ischemia

H9c2 cells were purchased from National Centre for Cell Sciences, Pune, India and maintained in DMEM, containing 10% FBS, 1× antibiotic solution (Sigma-Aldrich, MO, USA) and grown to subconfluence prior to the experiments. The cells were maintained in a 37 °C incubator (Sanyo, Japan) in an atmosphere of 5% CO_2. Cyclosporin A (1 μM) was used as the positive control (PC) and cells were treated with TFM extract or PC for 24 h before any experiment.

2.3.1. Measurement of cell viability

MTT assay was performed to determine the cytotoxicity of the various concentrations of the extract (10, 25, 50, 75, 100, 150 and 200 μg/ml) according to the method of Mosmann [31]). Different concentrations were tried to select suitable concentrations for experiments.

2.3.2. Induction of ischemia

Ischemia was induced by maintaining cells in a buffer containing (composition in mM: 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 HEPES, 20 2-deoxy-D-glucose, pH −6.2) according to the reported method [28] and maintained at 37 °C, 0.1% O_2, 5% CO_2, and 95% N_2 in a hypoxia incubator (New Brunswick Scientific, USA) for 1 h. The cells were grouped as control, treatment control, ischemia, positive control and TFM [cells pretreated with TFM (10, 25 μg/ml) 1 h prior to ischemia]. Schematic diagram of treatment regime is shown in Fig. 1.

2.3.3. Determination of lactate dehydrogenase (LDH) release

H9c2 cells were seeded in T25 flasks at a density of 5×10^5 cells per well. The culture medium was collected after the experiment and LDH release to the medium from different experimental groups was determined by the method of Haslam [12].

2.4. Cell-based antioxidant studies

For this, H9c2 cells were seeded in 6 well plates at a density of 5×10^4 cells per well. At sub-confluence, cells were pretreated with extract and positive control for 24 h before the induction of ischemia for 1 h. The cells were harvested and homogenized with 20 mM Tris/HCl buffer (pH 7.5) containing 0.2% Triton X-100 and 0.5 mM PMSF and sonicated for 30 s on ice. Total cell lysates were centrifuged at 3000 rpm at 4 °C for 15 min and aliquots of the supernatant were utilized for subsequent antioxidant assays.

2.4.1. Total antioxidant capacity determination

The antioxidant capacity exhibited by TFM was measured in a cell-based system [21]. The assay depends on the ability of antioxidants in the sample to inhibit the oxidation of ABTS by metmyoglobin and absorbance was measured at 750 nm. This is compared with standard trolox.

2.4.2. Estimation of reduced glutathione (GSH)

The cellular GSH content in control and treated cells was measured according to the method of Hissin and Hilf [15]. Cells after respective treatment were pelleted by centrifugation and were washed with PBS. To the cell pellet, 50 μl of 25% HPO_3 and 188 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA (pH 8.0) were added, and then the cells were homogenized on ice. After homogenization, the samples were centrifuged at 13,000 × *g* for 5 min at 4 °C. The supernatant was diluted with the above phosphate buffer; 0.1 ml of diluted sample was incubated with 0.1 ml of o-phthalaldehyde solution (0.1% in methanol) and 1.8 ml of phosphate buffer for 15 min at room temperature. Fluorescence was then read with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cellular GSH content was expressed as nanomoles of GSH per milligram of cellular protein.

2.4.3. Superoxide dismutase (SOD) activity

SOD was measured by the method of Sandstrom [42]. Cell lysate (250 μl) was added to 0.5 ml of water, 1.25 ml of ethanol, and 0.75 ml of chloroform and centrifuged at 3000 rpm for 5 min. To the supernatant, sodium pyrophosphate (0.025 M, pH 8.3), phenazine methosulphate (PMS) (186 μM), nitroblue tetrazolium (NBT) (300 μM) and NADH (780 μM) were added and incubated at 30 °C for 90 s. After incubation, glacial acetic acid and butanol were added to the reaction mixture and the absorbance was read at 560 nm. One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in 1 min under the assay conditions and expressed as specific activity in units/min/mg protein.