Beneficial Effects of Salacia Oblonga on Mitochondrial Localization in Cells and NADPH oxidase Activity in Glucose Induced Cytotoxicity on rat Muscle Cell Line

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Abstract

Diabetes mellitus is fast acquiring the status of an epidemic and Type 2 diabetes has reached epidemic proportions throughout the world. The latter is associated with high blood glucose level due to inadequate insulin production and/or defective cellular uptake of glucose. Salacia oblonga is a widely used anti-diabetic herb in countries like India and Sri Lanka. Present study is an attempt to investigate the protective potential of Salacia oblonga extract in glucose induced cytotoxicity in L6 cell line in order to simulate the conditions of skeletal muscle in diabetics. The L6 cells were exposed to the toxic doses of glucose (with or without pretreatment with Salacia oblonga) and the effects were analyzed in terms of cell viability, NADPH oxidase enzyme activity, and mitochondrial localization and abundance. The results showed a dose dependent increase in cell death in glucose treated groups, which was prevented on pretreatment of cells with the plant extract. The NADPH oxidase enzyme activity was enhanced in glucose treated groups. In the pre-treated groups (with extract), the activity of NADPH oxidase enzyme was decreased in comparison to glucose treated groups. The fluorescent microscopic analysis (NAO) showed distinct changes in mitochondrial organization as they appear to get focused near perinuclear region with evident alteration in green fluorescent intensity in extract treated samples. This indicated a probable alteration in mitochondrial number/mass/activity. In summary, our results suggest that Salacia oblonga exerts protective role against high glucose-induced oxidative stress by reducing NADPH oxidase enzyme activity and changing energetics of the cell through
mitochondria. The plant seems to be promising in protecting the mitochondrial function by probably interrupting the vicious circle of reactive oxygen species production and maintaining mitochondrial integrity. The above results suggest that Salacia oblonga can further be analyzed to be developed as a drug to treat diabetes and its complications.

**Keywords:** Salacia, Diabetes, Oxidative stress, Mitochondria.

1. Introduction

Increased level of blood glucose in hyperglycemic conditions appears to be a key factor leading to reactive oxygen species (ROS) production. Since, diabetes is a metabolic disorder, there are several metabolic pathways that contribute to ROS formation and among these pathway intermediates, NADPH oxidase enzyme activation has drawn the attention of several researchers. Experimental evidences have shown that high glucose level stimulates ROS production through activation of NADPH oxidase in aortic smooth muscle cells and endothelial cells (Inoguchi et al, 2000).

In diabetic patient, mitochondrial dysfunction has been reported to be associated with skeletal muscle insulin resistance. Mitochondrion is the power house of a cell and has several enzymes and membrane proteins involved in the coupled electron transport chain (ETC) and oxidative phosphorylation pathways that lead to ATP production. Since, the components of ETC are located in the inner mitochondrial membrane, a well integrated mitochondrial membrane is of prime importance for biological function of a cell. This membrane is composed of several phospholipids and cardiolipin is one of them. Cardiolipin contains three glycerol backbones and four acyl chains, and forms contact sites between the outer and inner mitochondrial membranes. It is also required for optimal activity of several mitochondrial enzymes including the respiratory chain and the mitochondrial apoptotic pathway (Houtkooper and Vaz, 2008). In a hyperglycemic state, an imperfect coupling of ETC and oxidative phosphorylation occurs. The proton gradient generated due to electron transport through ETC intermediates leads to an increase in the overall electrochemical potential difference. All these events finally cause an increased half-life of superoxide-generating electron transport intermediates and ROS production, thereby causing oxidative stress and leading to development of diabetic complications (Rolo and Palmeira, 2006).

Since, the risk of developing long-term diabetic complications can be overcome by strict glycemic control, Ayurvedic antidiabetic herbs are becoming the choice of medication in a large section of the world’s population. Among several traditional antidiabetic plants, *Salacia oblonga* has been undertaken for scientific evaluation. In India, this plant is used in Ayurvedic antidiabetic formulations and marketed (Jarald et al, 2008). The plant possesses antioxidant, anti-hyperglycemic, anti-hypoinsulinemic effects (Mukherjee et al, 2006). But, the mechanism of action and effect of *Salacia*
Salacia Oblonga on mitochondria in a hyperglycemic state are still unknown. The aim of our work was to investigate the effects and mechanism of action of Salacia oblonga (root and stem) extract on high glucose-induced oxidative stress in L6 (rat skeletal muscle) cell line. This study gives an insight into the effects of superoxide radicals generated due to high glucose on mitochondria through change in fluorescent intensity on staining cells with nonyl-acridine orange dye.

2. Materials and Methods
2.1 Chemicals and Reagents
DMEM/nutrient mixture F-12 Ham, fetal bovine serum (FBS), and nonyl acridine orange (NAO) were purchased from Sigma Aldrich, India. Trypsin-EDTA, MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide], and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from HiMedia Laboratories Private Limited, India. Dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), ethylenediamine tetra acetic acid (EDTA), and Triton X-100 were purchased from Central Drug House Private Limited, India.

2.2 Plant Material
The Salacia oblonga powder (root and stem) was purchased from Natural Remedies Private Limited, Bangalore, India.

2.3 Cell Line and Culture Condition
The L6 (rat skeletal muscle) cell line was purchased from National Centre for Cell Science, Pune, India and was cultured by standard method. When required for the assay, confluent monolayers of L6 cells were trypsinized, centrifuged, and the cell suspension thus obtained was used for performing the assays.

2.4 Effect of High Glucose on the Viability of L6 Cells
The L6 cells were seeded at a density of 5x10⁴ cells/well (Dypbuky et al, 1994) in a 96-well cell culture plate and cultured overnight at 37°C under a humidified, 5% CO₂ atmosphere prior to treatment. The cells were exposed to glucose at a dose range of 10 - 75mM for 24 hrs in order to determine its toxic dose for attaining almost 50% cell death. The viability of the cells was determined by measuring spectrophotometrically the amount of formazan formed due to reduction of yellow MTT by succinate dehydrogenase inside the cells.

2.5 Effect of Salacia oblonga Extract (SOE) in Glucose-Induced Cytotoxicity in L6 Cells
In order to determine the safe dose range of SOE, the L6 cells were treated with SOE (10 - 75μg/ml, 3hrs) and the cell viability was determined through MTT assay. Further,
the cytoprotective role of SOE (in pre-treated cells) was determined in glucose treated samples.

2.6 Preparation of Cell Lysate for Enzyme Assay
The L6 cell pellets from the control and treated groups were lysed according to standard protocol (Janknegt et al, 2007) and used for enzyme assay.

2.7 Measurement of NADPH Oxidase Activity
The L6 cells were treated with high glucose in the absence or presence of SOE and the NADPH oxidase enzyme activity was determined in cell lysates of control and treated groups according to standard protocol (Whaley-Connell 2007).

2.8 Analysis of Mitochondrial Distribution
In order to analyze the distribution of intramitochondrial cardiolipin in control and treated L6 cells, the cells were stained with fluorescent dye nonyl acridine orange (NAO) according to standard protocol (Fernandez et al, 2002). Two molecules of NAO bind with high affinity to one molecule of cardiolipin, thereby forming NAO dimers. In this experiment, 10^6 cells were seeded onto a cover slip and incubated in a 6-well plate inside a CO2 incubator. Then the cells were exposed to various treatment combinations with glucose and SOE. The cells were fixed with 1% formaldehyde and stained with NAO. Finally, the cover slip was mounted on a glass slide and observed under a fluorescent microscope using 490 nm excitation and 520 nm emission wavelength, respectively.

3. Results
3.1 Effect of High Glucose on the Viability of L6 Cell Line
On exposure of the rat skeletal muscle cell line to high glucose dose range from 10mM to 75mM for 24 hrs, it was observed that 10mM glucose and 30mM glucose brought about almost more than 50% cell death (Figure 1A) in comparison to control.
Beneficial Effects of Salacia Oblonga on Mitochondrial Localization in Cells

3.2 Preventive Effect of SOE in High Glucose-Induced Toxicity
As shown in Figure 1B, on exposure of the cells to increasing concentrations of SOE, it was observed that SOE at a concentration of 10µg/ml, 15µg/ml and 20µg/ml showed an increase in cell viability to almost near or more than control. Thus, 10µg/ml SOE and 10mM glucose were taken up for further experiments. Further, in the SOE pre-treated cells that were exposed to high glucose (Figure 1C), the cells showed resistance to the toxic effects of glucose through enhancement in cell viability by SOE to almost 80%.

3.3 Measurement of Oxidative Stress in L6 Cell Line
NADPH oxidase enzyme activity was measured in terms of percentage NADPH consumed in comparison to control. As shown in Figure 4, treatment with 10mM glucose lead to increased utilization of NADPH by the enzyme, in comparison to control, thus indicating increased enzyme activity. The plant extract showed a minimal enzyme activity almost similar to control. In the treatment combination group, the enzyme activity was balanced by SOE almost near to control.

Figure 1: Effect of treatment with various concentrations of glucose (A), SOE (B), and their combinations (C) on viability of L6 cell line.

Figure 5: NADPH oxidase activity in L6 cell line treated with high glucose, SOE, and a combination of both.
3.4 Distribution of Mitochondrial Cardiolipin in Oxidatively Stressed L6 Cell Line

While investigating into the localization of mitochondria in various treated cells, it was observed that high glucose treatment resulted in a significant alteration in the skeletal muscle cellular morphology (Figure 6C) and green fluorescent intensity in comparison to control, wherein mitochondria appears to be centered around the nucleus evenly (Figure 6A). The high glucose-stressed cells appear elongated, having condensed and brightly fluorescent nuclear region, thereby indicating an increased mitochondrial functionality. The SOE treated cells (Figure 6B) showed mitochondrial localization, abundance and fluorescent intensity almost similar to control. In the SOE pre-treated cells (Figure 6D), there appears a clear nuclear zone (almost near to control) with bright fluorescent intensity that has spread radially across in the cytoplasm of all the cells. This indicates a possible re-location of mitochondria for carrying out highly functional activities in order to combat the high glucose-induced oxidative stress.

Figure 6: Effect of high glucose and SOE pre-treatment on mitochondrial distribution in L6 cell line.

4. Conclusion

The data from this investigation suggests that high glucose causes increased oxidative stress in rat skeletal muscle cell line through production of increasing amount of superoxide radicals by NADPH oxidase enzyme. This increase in enzyme activity was balanced on pre-treatment of cells with Salacia oblonga extract. In continuation with this observation, this data also suggests that increased ROS production during high
glucose-induced oxidatively stressed condition could modify the mitochondrial distribution and hence, the organelle number/mass as well as its functional ability. As a whole, our study proves the antioxidant potential of SOE and indicates possible involvement of skeletal muscle mitochondria in helping cells survive the stress. Further investigations are required to understand at the molecular level into the role of NADPH oxidase enzyme towards channelizing the oxidative stress pathway from the cell membrane towards and inside the mitochondria.

References
