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Rheum emodi ameliorates hypoxia associated cytotoxicity in C6 glioma cells and thereby increases cell viability

Ajaz Ahmad Waza[1], Zeenat Hamid[2], Bashir A Ganai[3]

[1,3]Centre of Research for Development (CORD)
University of Kashmir, Srinagar, Jammu and Kashmir, (India)
[2]Department of Biotechnology,
University of Kashmir, Srinagar, Jammu and Kashmir, (India)

ABSTRACT

The aim of this work was to investigate the effect of methanolic extract of Rheum emodi (MER) on hypoxia induced cytotoxicity in human neuronal cells. Cells were subjected to chemically induced hypoxia conditions using 200mM CoCl2 alone or together with MER (150μM) for 24 hours. It was found that hypoxia is associated with different cytotoxicity processes like formation of oxygen radical formation (ROS), lactate dehydrogenase (LDH) leakage and lipid peroxidation as compared to untreated cells. Hypoxia induced ROS formation was significantly prevented by Rheum emodi treatment. Other beneficial effects associated with MER treatment in hypoxia challenged cells were reduction in LDH leakage and lipid peroxidation. Using MTT assay, it was further found that the hypoxia induced cell death was decreased by MER treatment. Our findings suggest that MER exert cytoprotective action against hypoxia induced cytotoxicity and cell death in C6 glioma cells.

Key words: Rheum emodi, Reactive Oxygen Species, hypoxia, LDH release, cytotoxicity, cell viability,

I.INTRODUCTION

A constant supply of oxygen is required for the proper development and survival of mammals. Among all organs of human body, the brain presents largest demand for the oxygen, which accounts for 20% of the body's oxygen. A continuous supply of oxygen is indispensable for proper functioning of brain [1]. Hypoxia is the deficiency either in the delivery or the utilization of oxygen at the cellular level [2], which can alter various physiological functions of the cells, with severe consequences to the organism. In humans, the hypoxic conditions occur during various pathophysiological conditions, like stroke, myocardial ischemia, tumorous growth, etc [3]. The brain is the most sensitive organ to hypoxia and initiates numerous adaptive responses at the molecular and cellular levels as well as at the whole organ level to counter the stress conditions [4] [5]. An insufficient supply of oxygen has been associated in the pathology of many central nervous system disorders, like head trauma, stroke, neurodegenerative diseases, neoplasia etc [6]. It should be noted here that astrocytes are considered to be the ultimate sensors of the brain environment; and these cells react immediately to any cellular change [7] [8]. The way astrocytes respond to hypoxia stress significantly affects the reaction of the brain to hypoxia and therefore to the extent of brain injury during hypoxia/ischemia stress [9]. During hypoxia

conditions, water is incompletely reduced at mitochondrial cytochrome oxidase and thus results in the accumulation of reduced equivalents in respiratory chain. The accumulation leads to formation of reactive oxygen species (ROS) like superoxide and hydroxyl radical [10]. It has been reported that other enzymes like nitric oxide synthase and xanthine oxidase may contribute to an increased ROS formation under hypoxia conditions [11] [12]. It should be noted that hypoxic cells are not only associated with biochemical alterations but also with morphological changes [13] [14] [15] [16] [17] [18]. Hypoxic has been found to increase lipid peroxidation and lactate dehydrogenase (LDH) release in brain cells [19] [20] and ultimately leads to neuronal cell death [21].

Herbal plants are playing an important role in health sector worldwide, and are considered to be the natural reservoirs for different medicines for treatment of various human diseases. *Rheum emodi* (*Himalayan rhubarb)* is one such herb used as tonic for several years in India. The herb has been traditionally used for treating different human ailments like fungal infections, bacterial infections, jaundice, ulcers, fevers and liver disorders [22] [23]. *Rheum emodi* (locally known as *Pam chalan*) is an effective anticholesterolemic, antispasmodic, antitumor, aperients, stomachic, astringent, antiseptic, cholagogue, diuretic and has been found to manages primary dysmenorrhoea and ameliorates ethanol induced cytotoxicity in liver cells [24] [25]. Aqueous extract of *Rheum emodi* protects the proximal tubule segments of kidneys against nephrotoxicity in rats induced by mercuric chloride, potassium dichromate, cadmium chloride and gentamicin [26]. It has been reported that the anthraquinone derivatives isolated from *Rheum emodi* like emodin, aloeemodin and rhein exhibit anti-proliferative activity against cancer cells obtained from different cancerous tissues like colorectal, breast, lungs, cervical and prostate [27] [28] [29] [30]. *Rheum emodi* extracts has been reported to exhibit hepatoprotective role against carbon tetrachloride (CCl4) and paracetamol induced liver damage in rats [31] [32]. It has been reported that the *Rheum emodi* wall possess protective effect in many oxidative stress-related injuries and inflammatory diseases [33]. The present work was carried out to look for the protective role of *Rheum emodi* against hypoxia stress in neuronal cells.

II. MATERIALS AND METHODS

2.1. Preparation of the methanolic extract of *Rheum emodi*

The dried rhizome of plant was powdered and extracted with methanol. The alcohol was distilled off at 45°C and aqueous part was dried to obtain the extract. The extract of *Rheum emodi* was prepared in dimethyl sulfoxide (DMSO) for cell line studies.

2.2. Cell culture and treatments

The rat C6 glioma cells were purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were grown in dulbecco's modified eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% pencillin-streptomycin at 37°C in a humidified incubator containing 5% CO_2. Cells were sub-cultured by trypsinization and cultured in plates according to the requirement of the experiment. After 24 hrs of seeding, cells were treated with treated with 200mM CoCl2 (cobalt chloride hexahydrate) as described [34] for 24hrs in presence/absence of MER (150µM).

2.3. Measurement of ROS

ROS level was measured by DCFDA - Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, USA). The kit contains 2', 7'–dichlorofluorescin diacetate (DCFDA) a fluorogenic dye that measures peroxyl, hydroxyl and other reactive oxygen species (ROS) activity. C6 cells were cultured in a 96-well plate for overnight. Next day, media was removed and followed by addition of 100 µl/well of 1X buffer. Buffer was removed and cells were stained with diluted DCFDA solution (100µl/well) for 45 minutes at 37°C. DCFDA solution was removed followed by treatment with CoCl2 (200mM) for 24hrs alone or together with MER (150µM) and later on fluorescence detection was done.

2.4. LDH leakage

Cells were cultured in a 24-well plate for 24hrs and subsequently treated with CoCl2 (200mM) alone or together with MER (150µM) for 24hrs. 150µl of medium was taken out for the extracellular LDH activity analysis. Total LDH activity was determined by using the LDH assay kit after cells were disrupted by sonication. The percentage of LDH released was calculated using the formula

$$\text{LDH release} = \frac{\text{Absorbance of the supernatant}}{\text{Absorbance of the supernatant and cell lysate}} \times 100$$

2.5. Lipid peroxidation

Cells were cultured in a 24-well plate for 24hrs and were treated with CoCl2 (200mM) alone or together with MER (150µM) for 24hrs. Cells were incubated with 1ml (0.5 M KCl in 10 mM Tris-HCl), mixed properly and then treated with 0.5 ml (30% trichloroacetic acid (TCA)) and 0.5 ml (52 mM thiobarbituric acid) and finally heated in water bath (90°C for 30 min). The mixture was cooled and later on centrifuged (5000 rpm for 7 minutes). Supernatant was collected and its absorbance was measured at 532 nm and the amount of Thiobarbituric acid reactive substances (TBARS) was used to measure lipid peroxidation [35].

2.6. Cell viability assay

Cell viability assay was carried out by using MTT assay. For this purpose, the C6 cell line was seeded at 10^4cells/well and allowed to adhere for 24 hours. Media was replaced with 150µl of fresh medium before treatment with CoCl2 (200mM) alone or together with MER (150µM). After 24 hrs of treatment, cell growth was evaluated by MTT assay. MTT solution of 50µl (5 mg/ml of PBS) was added to each well and the plates were incubated for 4 hrs at 37^{o}C in dark. The media was aspirated and 150µl of MTT solvent (DMSO) was added to each well to solubilize the formazan crystals. The absorbances of plates were measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. The sample was performed in triplicate, and the experiment was repeated thrice.

2.7. Statistical analysis

In the present study, results for each experiment are given as mean of triplicates ± SE. Statistically significant differences between sample groups were determined using Student's t-test. A p value of <0.05 was considered significant.

III. RESULTS AND DISCUSSION

3.1. Effect of MER on ROS levels

ROS production is a major factor in oxidative damage of cells and effect main biological molecules like nucleic acids, proteins and lipids. It has been earlier found that hypoxia stimulates ROS production in neuronal and therefore leads to cell injury [36]. As shown in figure 1, C6 cells when treated with CoCl2 alone increased the ROS production (Bar 2) as compared to control (Bar 1). However, incubation together with MER decline hypoxia associated ROS production (Bar 3) in C6 cells.

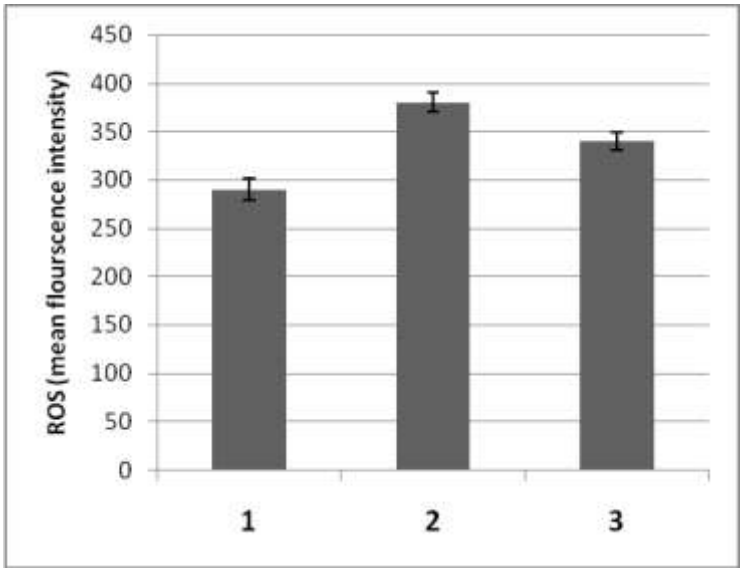

Figure 1: Showing effect of MER on ROS level: ROS level was determined by measuring DCFH fluorescent dye. Bar 1 represents ROS level of untreated C6 cells. Bar 2 represents ROS level of hypoxia-treated C6 cells. Bar 3 represents ROS level of C6 cells treated with MER in presence of hypoxia stress.

3.2. Protective role of MER on LDH release

LDH is an enzyme responsible for cellular respiration and is found within the cells. Disruption of cell membrane by any stress results in release of LDH to the external medium. Presence of this enzyme in the culture medium is considered to be a death call. Treatment of C6 cells with 200mM CoCl2 resulted in membrane damage as shown by LDH release. Prevention of LDH leakage by MER treatment reflects its role in protecting C6 cells against the hypoxia-induced toxicity.

As shown in figure 2, hypoxia-challenged C6 cells showed significant increase in the LDH leakage (Bar 2) as compared to control untreated cells (Bar 1)**.** This enhanced LDH leakage may partly explain the basis of stress. However, treatment with MER significantly decreased the hypoxia associated LDH leakage (Bar 3) in C6 cells.

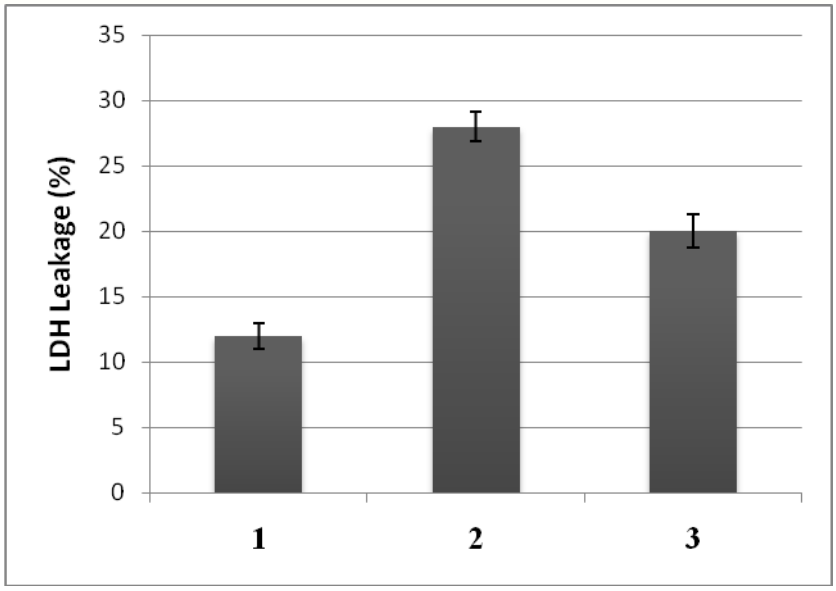

Figure 2: Showing effect of MER on LDH leakage: LDH leakage was determined using LDH assay kit. Bar 1 represents LDH leakage from untreated C6 cells. Bar 2 represents LDH leakage level from hypoxia-treated C6 cells. Bar 3 represents LDH leakage level from the C6 cells treated with MER in presence of hypoxia stress.

3.3. Effect of MER on lipid peroxidation

Lipid peroxidation is associated with oxidation of unsaturated fatty acids (FA) within the cell membrane and therefore leading to cell damage. TBARS are the byproducts of the lipid peroxidation and their detection is used to measure the cell damage. As shown in figure 3, treatment with 200mM CoCl2 resulted in almost three times increase in TBARS levels (Bar 2) as compared to control (Bar 1) in C6 cells. However incubation together with MER decline hypoxia associated lipid peroxidation (Bar 3).

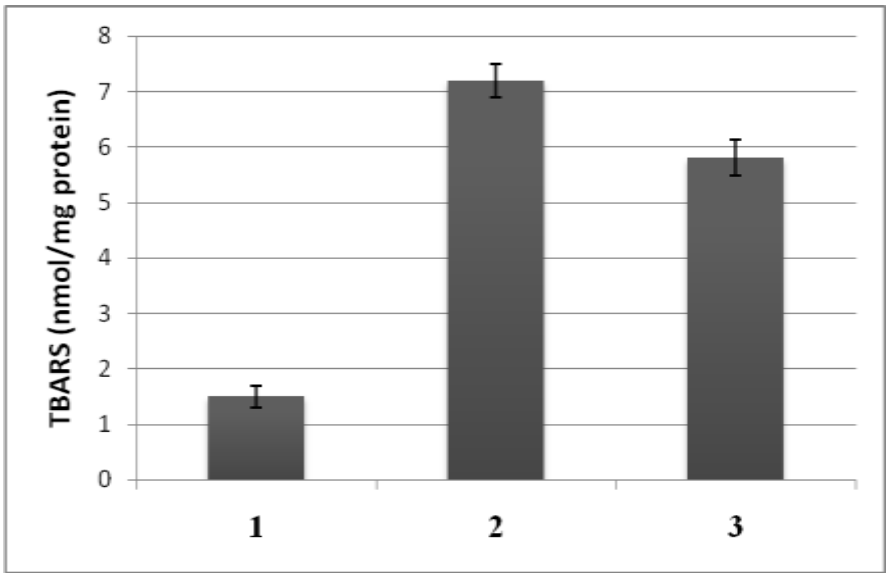

Figure 3: Showing effect of MER on lipid peroxidation: Lipid peroxidation was determined by measuring TBARS release. Bar 1 (control) represents lipid peroxidation from untreated C6 cells. Bar 2 represents lipid peroxidation level from hypoxia-treated C6 cells. Bar 3 represents lipid peroxidation level from the C6 cells treated with MER in presence of hypoxia stress.

3.4. Effect of MER on cell viability

Cell viability activity of the MER treatment on the hypoxia challenged cells was determined by using MTTT assay. Results of the MTT activity showed maximum growth inhibition in C6 cells by hypoxia treatment as shown in figure 4. However, treatment along with MER significantly increases cell viability in C6 cells.

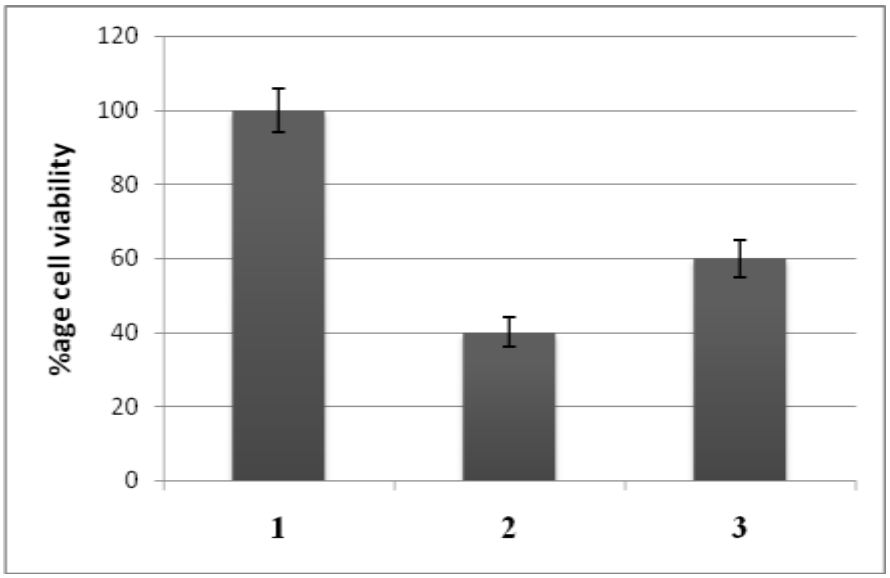

Figure 4: Showing effect of MER on cell viability: Bar 1 (control) represents cell viability of untreated C6 cells. Bar 2 represents cell viability percentage in hypoxia-treated C6 cells. Bar 3 represents cell viability level in the C6 cells treated with MER in presence of hypoxia stress.

IV. CONCLUSION

In conclusion, methanolic extract of *Rheum emodi* (MER) was found to successfully ameliorate the cytotoxicity induced by chemical hypoxia in C6 cells. It was further found that hypoxia associated cell death can be somehow controlled by *Rheum emodi* treatment.

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