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Research Article

EFFECT OF BUTYLATED HYDROXYANISOLE ON HYDROGEN PEROXIDE INDUCED OXIDATIVE STRESS ON CEREBRAL GLIOMA CELL LINE

RIHAN ANSARI[1], QUAISARPERWEEN[2], GAURAV KUMAR*[3], JAYANAND[4], DURG VIJAY RAI[5]

[1,2,4]**Faculty of Biological Engineering, Shobhit University, Meerut, UP** [3,5]**School of Agriculture and Environmental Sciences, ShobhitUniversity, Gangoh, Saharanpur, Email:** gk.biotechnology@gmail.com

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ABSTRACT

Objective: Butylated hydroxyl anisole (BHA) has potential protective effects on hydrogen peroxide (H_2O_2) induced oxidative stress on cerebral glioma cell (BMG-1).The study was carried out to investigate the neuro-protective effects of BHA againstBMG-1 cerebral glioma cell in vitro.
Methods:In vitro cytotoxicity assay, Oxidative Stress Mediated Apoptosis, Determination of Oxidative Stress Indices by DCFH-DA, Effect of BHAon Extent of Lipid Peroxidation and Total GSH Levels Measurement was carried out on BMG-1 cerebral glioma cell using standard procedures.
Results: BHA was showing very less toxicity, it reached IC_{50} value approximately equal to 200µM in 24hr treatment, decreases the distorted morphologyof the BMG-1 cells, Significant reduction of apoptotic bodies, Oxidative Stress, Lipid Peroxidation Levels and significantly increases the levels of glutathione.
Conclusion: The present study indicates the BHA may be effective in both prevention and treatmentof brain and neurodegenerative disorders in which free radical processes give a modulatory effect.

Keywords: Neuro-protective effects, BHA, Cytotoxicity assay, Oxidative stress, Cerebral glioma cell, Lipid peroxidation

INTRODUCTION

Oxidative stress has been linked to a number ofneuro-pathological conditions, such as metal-induced neurotoxicity and neurodegenerative diseases [1].Butylatedhydroxylanisole (BHA) has been widely used as an antioxidant to stabilize fatty foods since 1947 [2]. BHA is primarily as an antioxidant and preservative in animal feed, food, food packaging, petroleum, cosmetics, and in rubber products. BHA in commercial use consists of a mixture of 3-*tert*-butyl-4-hydroxyanisole (3-BHA) and 2-*tert*-butyl-4-hydroxyanisole (2-BHA). BHA is insoluble in water, but is soluble in fats, oils, propylene glycol, petroleum ether, chloroform, and 50% alcohol. BHA is considered by the U.S. Food and Drug Administration (FDA) to be generally recognized as safe when the antioxidant content does not exceed 0.02% by weight of the food's total fat or oil content. BHA was listed in the Sixth Annual Report on Carcinogens; an additional study in experimental animals has been recognized. Dietary administration of BHA to fish (hermaphroditic *Rivulusmarmoratus*) as larvae caused liver cancer (hepatocellular carcinoma) in the adult fish [3]. Butylatedhydroxyanisole (BHA) is reasonably anticipated to be a human carcinogen and first listed in the Sixth Annual Report on Carcinogens (1991). On the other hand, BHA has been found to protect laboratory animals from chemically induced neoplasia under various experimental conditions [4].Additionally, BHA inhibits carcinogenesis in numerous tissues, including the breast, liver, colon and lung [5]. These protective properties of BHA are recognized, in part, to its ability to induce phase II detoxifying enzymes such asUDP- lucuronosyltransferases, epoxide hydrolases, glutathione S-transferaseand quinine reductases that results in the metabolic detoxification of carcinogens [6,7]. In addition, BHA inhibits the growth of tumor cells and fibroblasts and protects against the development of bronchioloalveolar hyperplastic lesions during pulmonary fibrosis [8]. Oxidative stress is an important contributor to the pathophysiology of various pathological conditions including atherosclerosis, cardiovascular dysfunction, drug toxicity, inflammation, carcinogenesis, neuro-degenerative diseases and reperfusion injury [9]. Reactive oxygen species (ROS), including free radicals such as superoxide anion radical (O^{2-}), hydroxyl radical (OH^{-}) and the 1,1-diphenyl-2-picrylhydrazyl free radical, are active oxygen compounds that are often produced by biological oxidation reactions with exogenous factors[10]. ROS are known to cause aging and cancer and to have many other toxic effects in the human body. There are many antioxidants that reduce the actions of ROS[11]. Antioxidants have been defined as substances that prevent the formation of ROS or other oxidants, repair the damage they cause or scavenge them [12]. Oxidative stress in neurons arises because of an imbalance between free radical production and antioxidant control. This process leads to cell damage and, when severe, can trigger apoptosis or necrosis. Indeed, such oxidative stress may initiate certain neuro-pathologies such as Alzheimer's and Parkinson's disease whereby oxidative damage to biomolecules causes cellular dysfunction and neuronal death[13].

Very few reports are available on the potential protective effects of Butylatedhydroxylanisole (BHA) on hydrogen peroxide (H_2O_2) induced oxidative stress on BMG-1 cerebral glioma cell.

MATERIAL AND METHOD

Cell Culture

The cerebral glioma cell line (BMG-1; wild-type p53) were obtained from the American Type Culture Collection (ATCC, USA) and NCCS (PUNE). Stock cultures were maintained in the exponentially growing state by passaging twice weekly in DMEM containing 10mM HEPES and antibiotics supplemented with 10% fetal bovine serum [19]. Viability of cells used throughout theexperiment was always >95% as determined by trypan blue exclusion.

In vitro cytotoxicity assay:

The cells were cultured in DMEM F-12 (Dulbecco's minimum essential medium) in the presence of 10% fetal bovine serum, with 100units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO2 at 37oC. Proliferation of BMG-1 cells was assessed by MTT assay [14, 18]. Following different treatments and time points, sub-confluent monolayer culture of cerebral glioma cell line were trypsinized and cells counted with the help of hemocytometer. Cells were plated in 96-well plate 100µL in each well with the help of multichannel pipette. After 24h of cells incubation, the medium was replaced with 100µL medium containing different concentration of cytotoxic drug (BHA) ranging from 2nM to 20mM and incubated for 24 h. At the end

of treatment, media from control and extract -treated cells was discarded and 20µL of MTT (0.5 mg/ml Phosphate Buffered Saline) was added to each well. Cells were then incubated for 2 h at 37°C in CO2 incubator. MTT was then discarded and the colored crystals of produced formazan were dissolved by adding 100µL of DMSO. Absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570 nm. Optical density of each sample was compared with control optical density and graphs were plotted. Similarly, observation at different time interval i.e. after 24hrs., 48 hrs. and 72 hrs.were monitored.

Oxidative Stress Mediated Apoptosis

For Fluorescence based quantification of apoptosis, fresh media were plated in 6-well plate 2ml in each well with the help of pipette and Add 70µL cell suspension (seed 5000 cells) in each well. After overnight incubation the old media were removed from each well and then washed with PBS buffer and again incubated for 10 minutes. After incubation buffer is replaced with 2 mL fresh media for the control and 1990 mL media + 10µL H_2O_2 (500µM) for control plus H_2O_2 well. Similarly, 1000µL BHA (100µM) + 10µL H_2O_2 (500µM) + 990µl media is added to next well and 100µL BHA (10µM) + 10µL H_2O_2 (500µM) + 1890µl media were also added to another well and incubated for 4 hrs. After incubation the media were removed and washed 2 times by PBS buffer for detection of morphological changes of cells induced by H_2O_2. For fluorescence based quantification of apoptosis the cells werefixed by addition of 4 % (500µL) formaldehyde and kept at room temperature. After 30 minutes formaldehyde was removed and 500µL Hoechst strain is added in each wells and then washed 2 times by PBS buffer followed by 10 minutes of incubation, then micrographs were taken using fluorescent microscope.

Determination of Oxidative Stress Indices by DCFH-DA

The cellular ROS (reactive oxygen species) accumulation was measured with the help of fluorescent probe DCFH-DA (2,7-dichlorodihydrofluoresceindiacetate)[15]. DCFH-DA is a non-fluorescent compound, and it can be enzymatically converted to highly fluorescent compound, DCF, in the presence of ROS. After exposure to 500µM hydrogen peroxide, with or without BHA, the cells (10^6/ml) were incubated in 2ml of 140mMNaCl, 5mMKCl, 1mM $MgCl_2$, 5.6mM glucose, 1.5mM $CaCl_2$, and 20mM HEPES-Na, pH 7.4, and allowed to take up 5µM DCFH-DA at 37°C for 20 min, in an atmosphere of 95% air and 5% CO_2. Then the cells from well were re-suspended in cell lysis buffer and after loading, the fluorescenceintensity (relative fluorescence unit) was measured at 485 nm excitationand 530 nm emission every 30 min for 3hrs. using the MicroplateFluorometer (Model 7620).

Effect of BHAon Extent of Lipid Peroxidation

Endogenous lipid peroxidation was determined by the measurement of thiobarbituric acid reactive substances (TBARS).The extent of lipid peroxidation wasdetermined by measuring thiobarbituric acid reactive substances(TBARS) as described by Ide and Lau, 1997 [16]. At the end of the treatment of 500µM hydrogen peroxide the cells were washed with twice with PBS and removed by scrapping in PBS, followed by low speed centrifugation. The cell pellets were re-suspended in PBS. The cell suspension was mixed with twice of its volume of TBARS reagent (15% Trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25N HCl), and heated for 15 minutes at 100°C. After centrifugation, the absorbance of the supernatant was measured at 532nm and concentration of TBARS is calculated.

Total GSH Levels Measurement

Reduced glutathione was estimated according to the method described by Ellman et al,1959 [17]. One milliliter of supernatant was precipitated with 1 ml of 4% sulphosalicylic acid and cold-digested for 1 hr at 4°C. The samples were then centrifuged at1200xg for 15mins at 4°C. To 1 ml of the supernatant obtained, 2.7 ml of phosphate buffer (0.1mmol/l, pH 8) and 0.2 ml of 5, 5' dithio-bis (2-nitrobenzoic acid) was added. The yellow colour the developed was measured at 412 nm using Perkin Elmer Lambda 20 spectrophotometer. Results were calculated using the molar extinction coefficient of the chromophore (1.36 X 104(mol/l)$^{-1}$ cm^{-1})

RESULTS AND DISCUSSION

In vitro Cytotoxicity Assay:-It is observed in the studies that the treatment of BHA is showing very less toxicity, it reached IC_{50} value approximately equal to 200µM in 24hrs treatment routine. The drug is showing same kind of trend while cells were continuously treated for longer duration of time (fig. 1). It is not even showing cytotoxicity to 100% even at concentrations as high as 20mM.

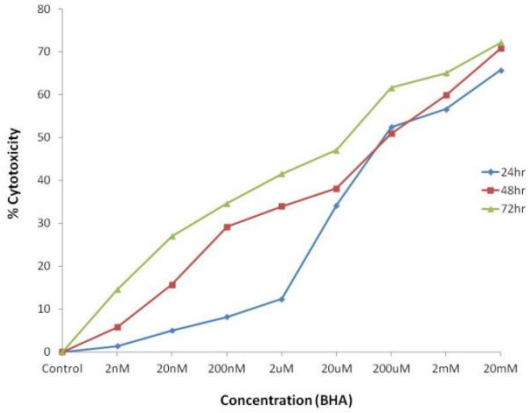
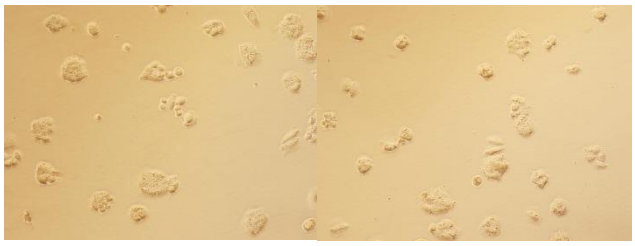
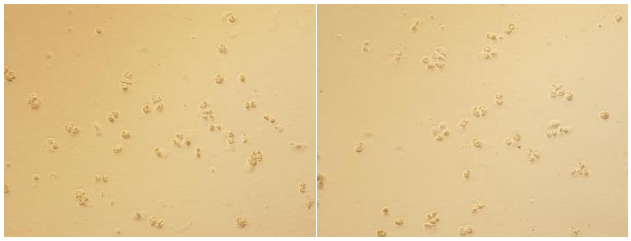


Fig 1: Cytotoxicity Assay.Concentration verses Cytotoxicity graph of 24 hr, 48 hr and 72 hr reading for IC_{50} Value.

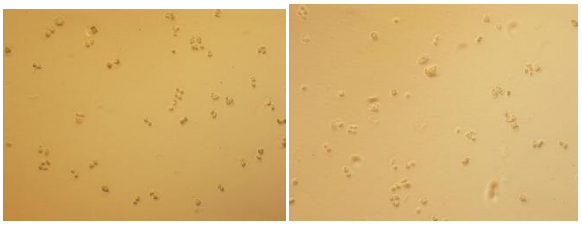
Morphological changes Induced by H_2O_2:- It is evident from the scanning micrograph (fig. 2) of the cells treated with hydrogen peroxide with BHA, that BHA decreases the distorted morphology of the BMG-1 cells. In control the cells were growing in groups with normal cell size, while in case of control with hydrogen peroxide the cells are smaller in size and showing bubbly outgrowth on the surface of the cells. The same is reduced in 100µM and 10µM in concentration dependent manner.



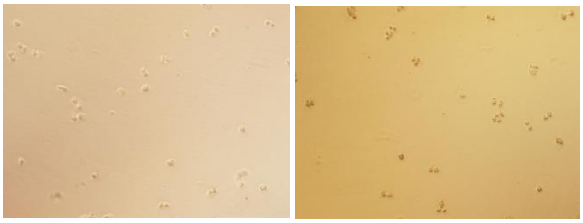
Control



Control + H_2O_2 (500 µM)



BHA (10 µM) + H_2O_2 (500 µM)

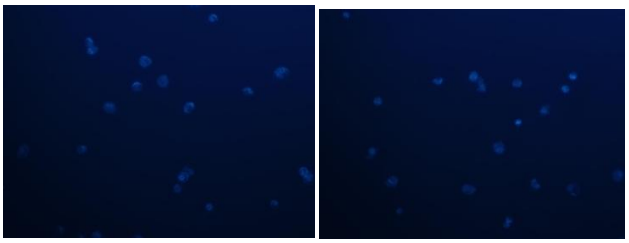


BHA (100 μM) + H_2O_2 (500 μM)

Fig. 2: Morphological changes Induced by H_2O_2. Images of cells survival and effect of drugs on different concentration

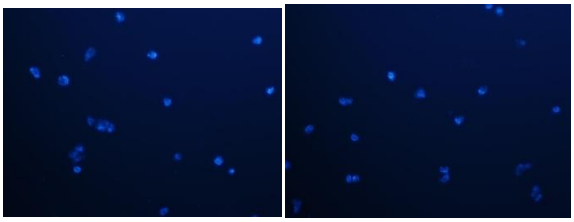
Fluorescence based Quantification of Apoptosis

Immunofluorescence micrograph of the BMG-1 cells (Fig. 3) with treatment of hydrogen peroxide and drug is shown here. In control cells the nuclei of cells is showing intact DNA which shows normal fluorescence. While in case of control cells with hydrogen peroxide shows nuclei fluorescence much brighter as compared to the normal suggestive of nuclei breakage and formation of apoptotic bodies shown by arrows in fluorescence micrograph. This formation of apoptotic bodies is significantly reduced in 100μM and 10μM BHA respectively.



Control

Control + H_2O_2 (500 μM)



BHA (10 μM) + H_2O_2 (500 μM)

BHA (100 μM) + H_2O_2 (500 μM)

Fig 3: Immunofluorescence micrograph of the BMG-1 cells with treatment of hydrogen peroxide and drug is shown here

DCFH-DA based measure of Oxidative Stress:-Fig. 4 representsthe quantitative bar graphs of cells with observed levels of oxidative stress in cells in various conditions of treatments. This shows that control cells is showing very less uptake or very less conversion of non-fluorescent probe into fluorescent one. While, highest reading in control with hydrogen peroxide indicative of highest level of oxidative stress produced in cells. While in BHA treated cells, its shows BHA significantly reduces the oxidative stress levels is a function of concentration.

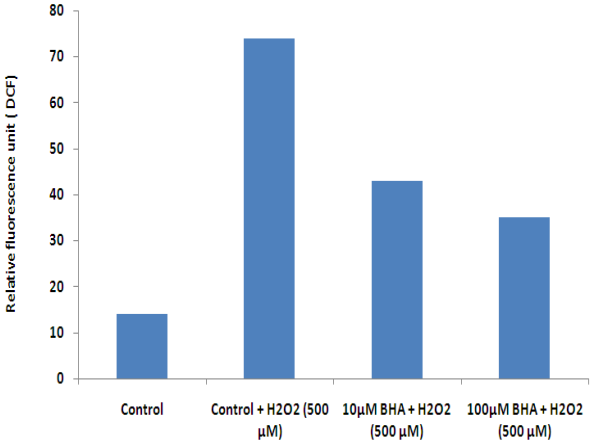


Fig 4: DCFH-DA based measure of Oxidative Stress. Quantitative bar graphs of cells with observed levels of oxidative stress in cells in various treatments

Quantification of Lipid Peroxidation Levels:-Fig.5 shows the quantitative bar graph showing lipid peroxidation in control and in treated cells. BHA decreases the lipid peroxidation levels in concentration dependent manner.

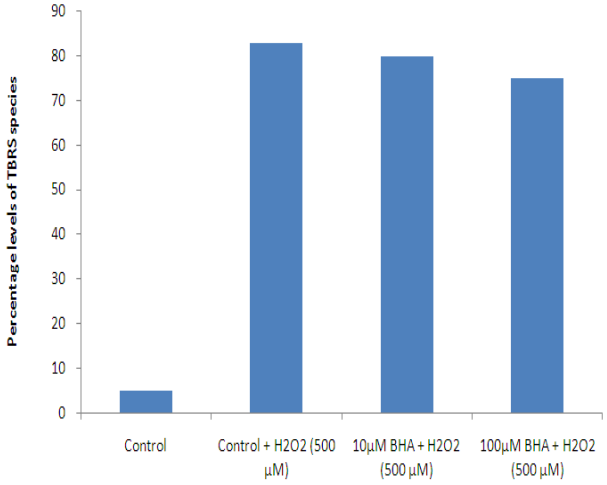


Fig 5:Quantification of Lipid Peroxidation Levels.Quantitative bar graph showing lipid peroxidation in control and in treated cells

Quantification of Total Reduced GSH Levels:-The quantitative bar graph of reduced glutathione levels in control and treated cells is illustrated by Fig 6. In control cells the levels are depleted when treated with hydrogen peroxide but after treatment of BHA significantly increases the levels of glutathione levels in concentration dependent manner.

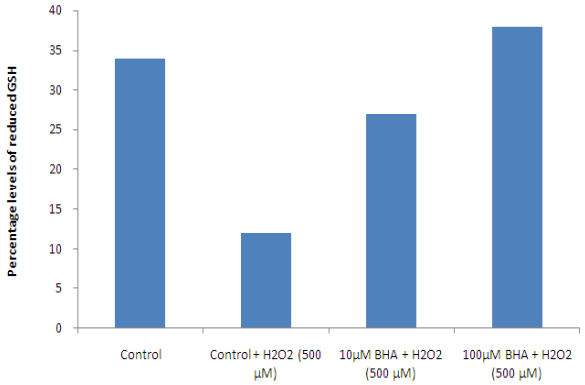


Fig 6: Quantification of Total Reduced GSH Levels. Quantitative bar graph of levels of reduced glutathione levels in control and treated cells

CONCLUSION

We have observed the antioxidant potential of the butylatedhydroxianisole. Administration of BHA has been shown to have remarkable effects on tissue thiol status, increasing intracellular GSH levels, probably by reducing extracellular cystine to cysteine, a natural metabolic antioxidant substance, has been shown in numerous types of neurological disorders to potentially be effective in both prevention and treatment of free radical mediated brain and neurodegenerativedisorders. Various analogues of this molecule could be formed and verified for their activity as antioxidant reducing drugs which could be very useful in therapies of these kinds of disorders.

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