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RESEARCH ARTICLE

Antineoplastic Action of CAPE in *invitro* model of glioma

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Abstract

Glial tumors comprise the largest group of the central nervous system tumors. Genetic heterogeneity of tumor, redundant and overlapping signal transduction pathways and limited drug delivery to the tumor are among several mechanisms underlying therapeutic failure. While primary brain tumors account for only 2% of all adult cancers, these neoplasms cause a disproportionate burden of cancer-related disability and death. The lookout for novel treatment strategies for these tumors has led to heightened interest and optimism among oncologists. Research on alternative therapeutic means of targeting malignant gliomas is of great importance since current treatments have proven ineffective in guaranteeing patients long-term survival. Drugs from natural extracts are found to have potential for long-term treatment with minimal side effects, by targeting multiple pathways in the glioma cells. These drugs are seen as a potential adjunct to the treatment of brain cancer. The use of herbal medicines is on the rise in cancer patients and it is imperative, to allow for the development of safe and efficacious anticancer therapies. Among all the newly developed anti-tumor agents, natural extracts with effective anticancer activities have been researched. The present study was aimed at elucidating the potential of Caffeic acid phenethyl ester [CAPE] for treating glioma. In vitro results suggest that CAPE when used in appropriate proportions and durations presents an antitumor potential for glioma. Here, we show that the CAPE exerts a strong antitumor action on C6 glioma cells .CAPE treatment inhibits proliferation and induces apoptosis. Results suggest that CAPE therapy inhibits migratory property and invasiveness of C6 cells. The Nitric oxide [NO] levels were significantly reduced by CAPE treatment at all time points and effect was persistent over the time in comparison to control. The results in totality suggest that the CAPE therapy remarkably reduces the proliferation of glioma cells possibly through different mechanisms, targeting multiple pathways involved in tumor growth, proliferation and development implicating the relevance of using this drug for effective treatment of glioma. Invitro results suggest that CAPE treatment could be therapeutically exploited for the management of gliomas.

INTRODUCTION

Central nervous system [CNS] cancers account for approximately 5-9% of all cancers (Jemal, Siegel et al. 2009). Primary brain tumors account for 2.3 % of ten leading causes of cancer related deaths. The most common primary brain tumor is the glioma, accounting for 32 % of CNS tumors and 80 % of malignant CNS tumors (Soffietti, Ruda et al. 2002, Reifenberger and Collins 2004). The impracticability of current treatment modalities hampers patient survival and handicaps quality of life (Burton and Prados 2000, Castro, Cowen et al. 2003). Due to the limitations of

current treatment modalities, among all the newly developed anti-tumor agents, natural extracts with effective anticancer activities have been researched. The researchers believe that many drugs especially plant extracts could lower resistance of drugs in glioma, by reducing the expression of proteins that inhibit apoptosis of the glioma cells (Bemis, Capodice et al. 2006, HemaIswarya and Doble 2006). The herbal medicines provide safe and efficacious anticancer therapy hence their use is consistently increasing in cancer patients. Chemotherapeutic drugs work by targeting multiple mechanisms of tumorigenesis hence dwindling the possibility of drug resistance. CAPE is a constituent of honeybee propolis and has been found to have diverse biological activities like anti-proliferation and anti-inflammation. *In vitro* and *in vivo* studies validate the therapeutic potential of CAPE in neuroprotection (Hwang, Park et al. 2006, Onori, DeMorrow et al. 2009, Lin, Liang et al. 2010, Fontanilla, Wei et al. 2012).

1. MATERIALS AND METHODS

1.1. Reagents

Dulbecco's modified Eagle's medium [DMEM] with 4500 mg glucose/L,110mg sodium pyruvate and L-glutamine, fetal bovine serum[FBS] , trypsin-EDTA solution, penicillin-streptomycin [10,000 units penicillin and 10mg streptomycin/ml], dimethyl sulphoxide [DMSO],3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT],Wrights stain, phosphoric acid,N-(1-naphthyl) ethyl-enediamine [NED] and sulfanilamide were obtained from Sigma-Aldrich, Co. (St Louis, MO, USA).Lactate dehydrogenase [LDH] cytotoxicity assay kit was purchased from GBiosciences, 100% HPLC purified caffeic acid phenethyl ester was purchased from Calbiochem .All other chemicals were of ultrapure grade and obtained from Sigma-Aldrich, Co. (St Louis, MO, USA).

1.2. Cell Culture

The studies were performed on C6 cell line ,cells whose protein profile has been found to be most similar to those reported in human brain tumors(Benda, Lightbody et al. 1968, Kleihues, Zulch et al. 1970). The cells were cultured in DMEM supplemented with 10% FBS and 100 units penicillin-streptomycin. The cells were continually maintained in a humidified incubator with 5% CO_2 at 37°C.

1.3. Statistical analysis

All the assays were repeated independently three times and values were expressed as Mean ± SD of three independent experiments .All data was analyzed using One Way ANNOVA by Dunnett's multiple comparisons test.

1.4. MTT Cell Proliferation Assay

Measurement of cell proliferation forms the basis for screening invitro cellular effect of drugs. MTT-tetrazolium is reduced by mitochondrial dehydrogenase enzymes of metabolically active cells, to generate reducing equivalents and intracellular purple formazan crystals. Water insoluble MTT –formazan crystals formed are dissolved in an acidified solvent, once solubilized it is quantified spectrophotometrically. The effect of CAPE on proliferation of C6 cells over a concentration range was quantified using the MTT colorimetric assay. 2×10^4 cells/well were plated out in 24-well plate in triplicates for each condition and allowed to grow. After 24 h the medium was changed and cells were treated with drug in increasing concentrations. Plates were incubated at 37°C and 5% CO_2 for 24hrs with media and cell controls. MTT was dissolved in PBS at 5 mg/ml. At the end of experiment, culture media was removed carefully and 300µl MTT solution was added to all wells, and plates were incubated at 37°C for 3 h. The reaction was stopped after incubation by discarding the supernatants, and then MTT solvent (0.1% NP-40 and 4mM HCI in isopropanol) was added to each well and mixed thoroughly to dissolve the blue formazan crystals trapped inside cells. Plates were incubated at 37°C for 20 minutes in dark, after ensuring that all crystals were dissolved, the plates were read on ELISA plate reader (BioTek instruments inc.) within 10 minutes at a test wavelength of 560 nm and reference wavelength of 650nm.

1.5. LDH Cell Death Assay

LDH assay is an enzymatic cell death assay, performed by assessing cytosolic LDH soluble enzyme released into the media due to damage of plasma membrane as a marker of dead cells. CytoScan™ LDH Cytotoxicity Assay kit (G Biosciences) was used to examine the effect of drugs on cell viability. LDH catalyses the reduction of NAD^+ to NADH in the presence of L-lactate, while the formation of NADH can be measured in a coupled reaction in which the tetrazolium salt is reduced to a red formazan product. The amount of the highly colored and soluble formazan is quantitated at 490 nm spectrophotometrically. Briefly, 2×10^4 C6 cells/ml were seeded in 24-well plates and besides test samples, positive and negative controls were included. After 24 h, medium was changed and cells were treated with IC-25 dose of CAPE. During experiments 100 µl of culture medium was removed from plates at three different time points; 6 h, 24 h, 48 h and transferred to 96 well plates.100 µl of LDH substrate prepared according to the instructions of manufacturer was added to each well and incubated for 20 minutes at room temperature; the enzymatic reaction was arrested by adding 50 µl of CytoScan™ stop solution. LDH activity was measured from the

samples spectrophotometrically at 490 nm in a micro plate reader (BioTek instruments Inc.). LDH concentrations were calculated by setting standards along with experimental samples. CytoScan^{TM} LDH that was provided with the kit was taken as positive control. The LDH levels corresponding to maximum cell death were measured by treating cells with CytoScan^{TM} lysis buffer .Basal LDH levels ,were determined from control cells (without any treatment).

1.6. Morphological Analysis by Wrights Staining

Wright's stain, a polychromatic stain consisting of eosin and methylene blue is used to differentiate cells in various pathologic conditions. When applied to cells, the dye produces multiple colors based on the ionic charge of the stain and various components of the cell. For morphological analysis, C6 cells were seeded in 60 mm dishes at 2×10^4 cells/ml in triplicates for each condition and allowed to grow. After 24 h the medium was changed and cells were treated with IC 25 - CAPE. Plates were incubated at 37°C and 5% CO_2 for 24hrs with media and cell controls. At the end of experiment culture media was carefully discarded and plates were washed with ice cold phosphate buffer saline [PBS] pH 7.4, thrice. Wrights stain was added to each plate as per manufacturer's instructions. Excessive stain was pipetted out and cells were washed with PBS. Cellular morphology was examined by optical microscopy to assess apoptosis. Six randomly chosen fields of view were observed; morphological changes were examined and images were captured using a phase-contrast inverted microscope (Olympus CKX41) using a 20X objective lens.

1.7. Measurement of Nitric Oxide by Griess assay

NO, reactive nitrogen specie [RNS] serves an important role under physiological conditions in biological systems. NO production in the brain from glial cells has been correlated with the pathogenesis of neurological diseases. Due to its diverse role in neurological systems, effect of drugs on modulation of NO release in C6 cells was important. Nitric oxide was determined by measurement of nitrite [NO^{2-}] and nitrate [NO^{3-}], stable oxidation products of NO. The assay for quantification of NO relies on a diazotization reaction that was originally described by Griess. C6 cells were cultured in 24-well plates at a density of 2×10^4 cells/ml. After 24 h, medium was changed and cells were treated with IC-25 dose of CAPE. During experiments 100 µl of culture medium was collected from plates at three different time points ;6 h ,24 h, 48 h and transferred to 96 well plates. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. Equal volumes of cell supernatants and Griess reagent were added to individual wells of a 96-well plate and incubated at room temperature for 15 min. Absorbance was measured at 590 nm in an ELISA reader (BioTek instruments Inc.). Controls and blanks were run simultaneously. NO concentrations were calculated using a standard curve prepared with 0.1 M sodium nitrate dissolved in ddH_2O.The resulting equation was then used to calculate the unknown sample concentrations. A time dependent curve was formulated based on the NO levels corresponding to the drug treatment.

2. RESULTS

2.1 TREATMENT WITH CAPE SIGNIFICANTLY REDUCES PROLIFERATION OF C6 GLIOMA CELLS:

The MTT cell proliferation assay measures the cellular metabolic activity and conversely, when metabolic events lead to apoptosis or necrosis via NADPH-dependent cellular oxidoreductase enzymes. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. In order to evaluate effect of CAPE on proliferation of C6 cells MTT assay was performed as described in material and methods. C6 cells were treated with CAPE over a concentration range of 5µM to 200µM and incubated for 24 h along with untreated cell control and medium control wells to provide the blanks for absorbance readings. The proliferation of C6 cells decreased on treatment with CAPE in concentration dependent manner consistently, compared to control. After 24h of incubation, before density limitation of cell growth occurred in control wells MTT assay was performed. A significant proliferation decrement of C6 cells treated with increasing concentrations of CAPE was detected. There was about ~50% reduction in cell proliferation at 60µM of CAPE. Concentrations' at which inhibition in cell proliferation was ~25% (IC-25) was 40µM (Fig 1). Therefore, for further studies with CAPE we choose the most optional concentration of CAPE, IC-25 concentration; the semi inhibitory dose. Next we evaluated effect of IC-25 concentration of CAPE on proliferation of C6 cells at different time points. Proliferation of C6 cells was significantly decreased when treated with CAPE in comparison to control (Fig 2). We observed that CAPE showed a inhibitory effect which was persistent over time however there was no significant change reduction or inhibition in proliferation of C6 cells in group treated with PBS .Besides control and PBS treated group showed no significant differences in % proliferation with time.

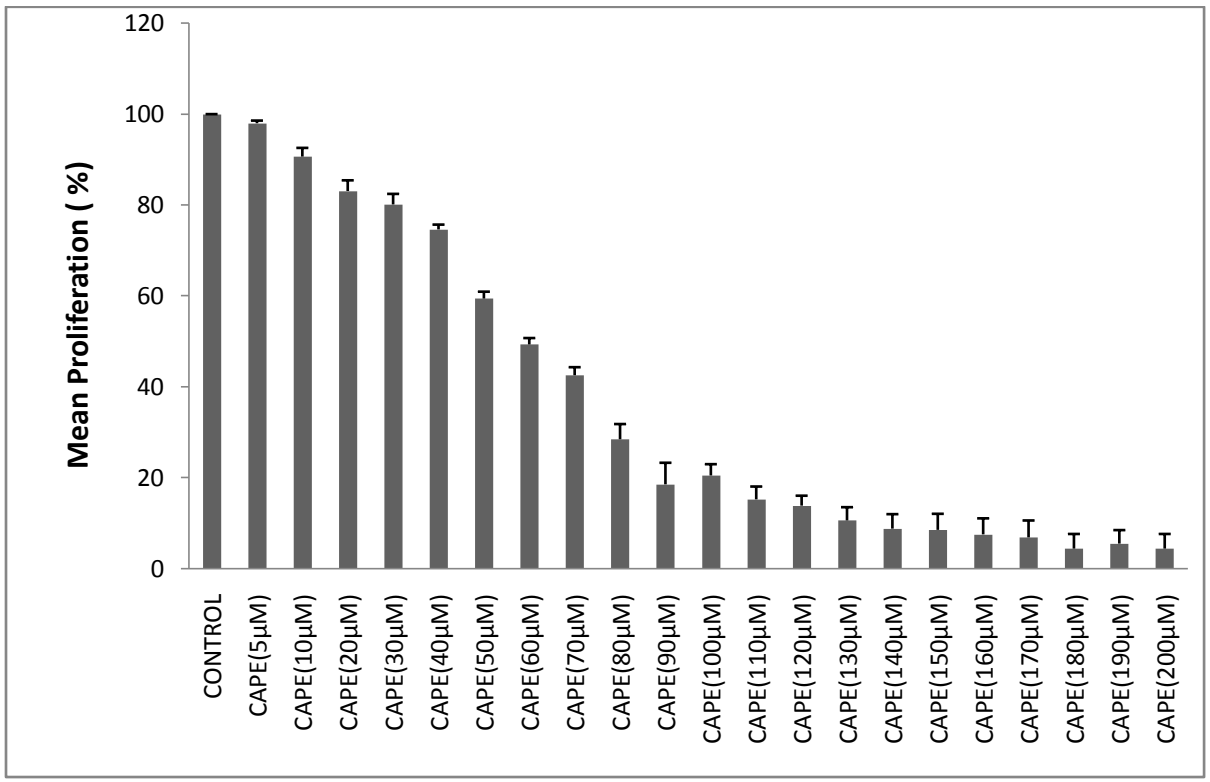


Fig 1: Dose responsiveness of C6 glioma cells treated with the indicated concentrations of CAPE as assessed by MTT assay; CAPE inhibited cell proliferation of C6 cells in a dose -dependent manner , IC-25 being 40µM .

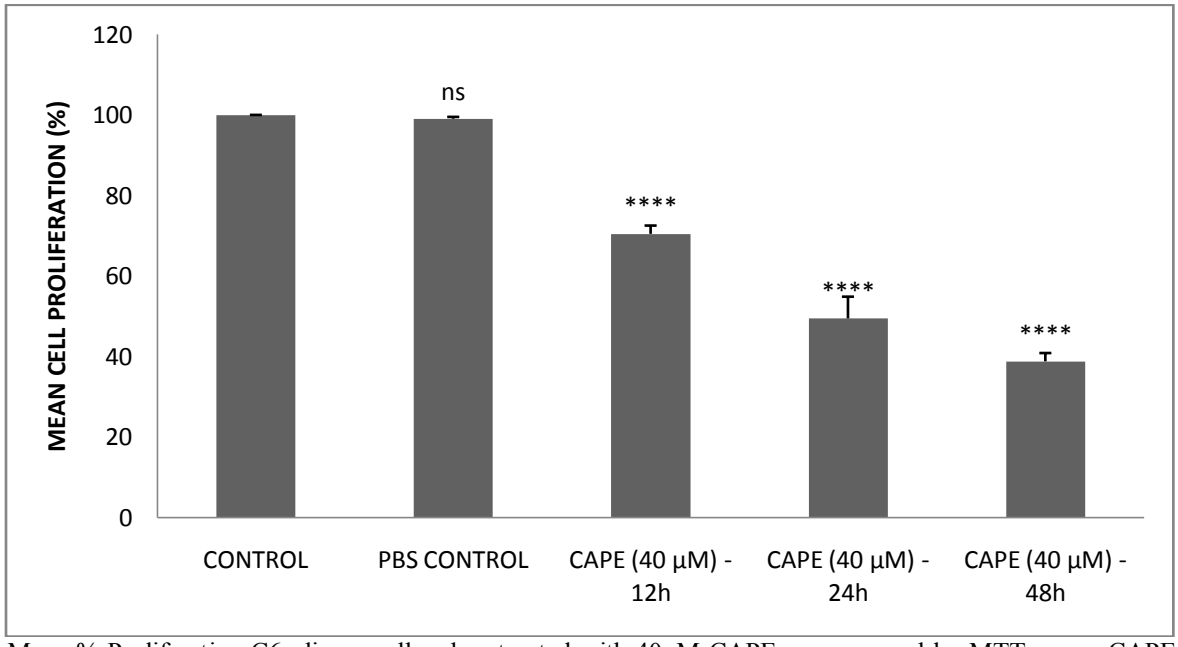


Fig 2: Mean % Proliferation C6 glioma cells when treated with 40µM CAPE as assessed by MTT assay; CAPE inhibited cell proliferation of C6 cells in comparison to control and inhibitory effect was persistent over time.

2.2 CAPE Enhances C6 Glioma Cell Death:

We examined the effect of CAPE on programmed cell death, one of the hallmarks of any anticancer therapy. The increase in LDH activity in culture medium is proportional to loss of membrane integrity and number of dead cells. Our results show a time-dependent decrement in viability of C6 cells when treated with IC-25 dose of CAPE with

respect to control cells. Treatment with CAPE at concentration of 40µM caused significant decrease in cell viability than control, after 6 h (3%), 24h (23.13%) and 48h (26.27%). From the above result, it becomes evident that CAPE effectively leads to cell death (Fig 3)

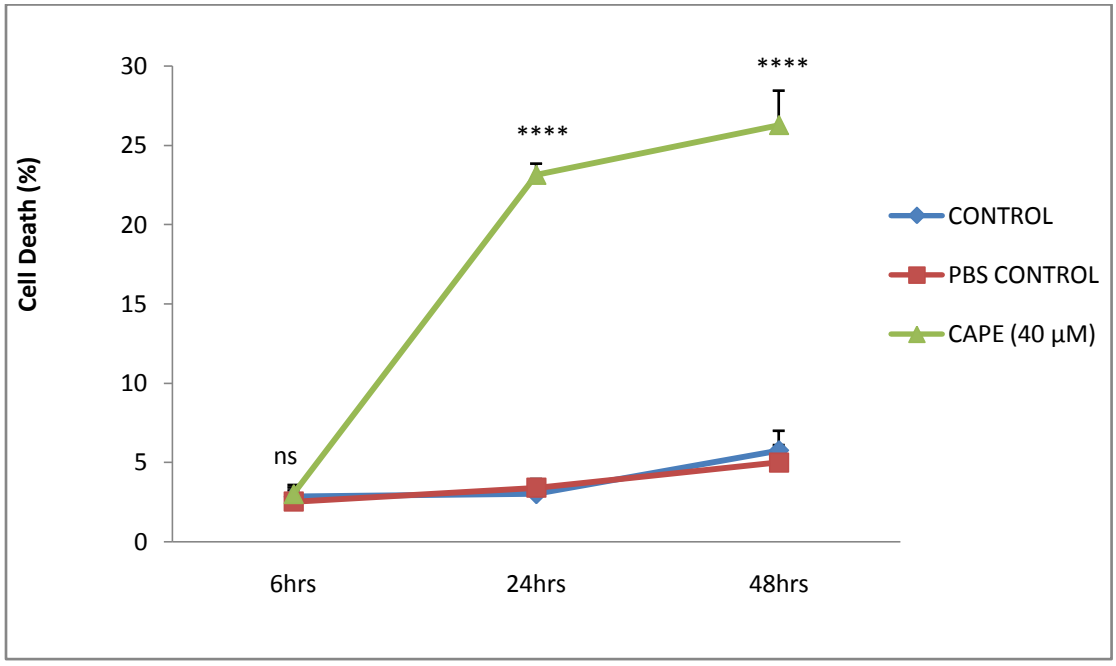
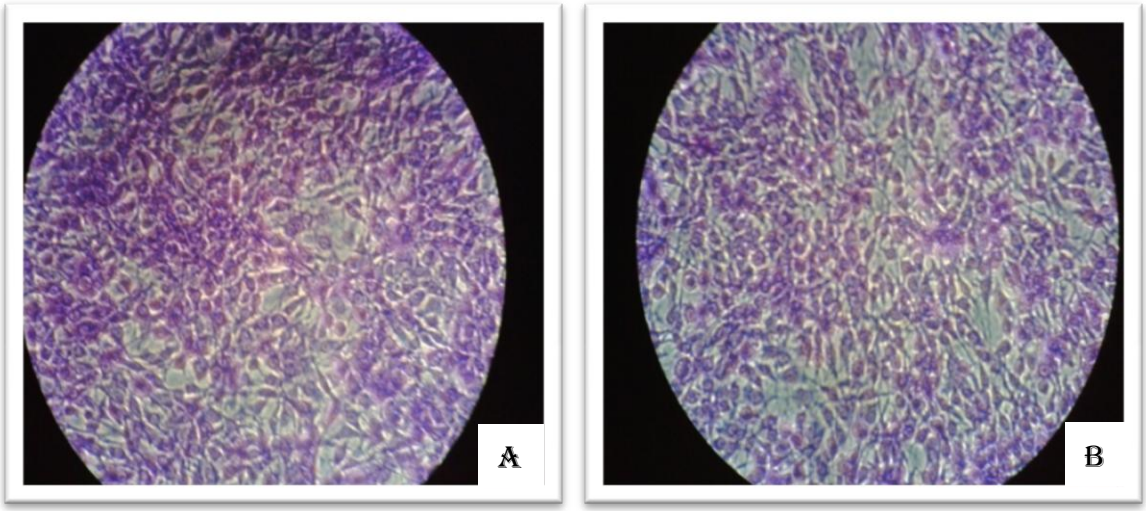


Fig 3: Effect of CAPE (40µM) on viability of C6 cells at selected dose at different time point's in comparison to control cells.

2.3 CAPE Induces Morphological Alterations in C6 Glioma Cells

The morphology of the apoptotic cells as detected by light microscopy included characteristic features as chromatin condensation, cell-volume shrinkage, and membrane-bound apoptotic bodies. Effect of drugs after 24hrs of treatment on morphological features of C6 cells was assessed by phase contrast microscopy (20X) after wrights staining as described in material and methods. C6 cells without treatment showed a branchy and polygonal astrocytic shape, considered as normal cell growth effect. When the cells were treated with CAPE there was change in morphology from astrocytic to elongated form and decrease in cell density (Fig 4).



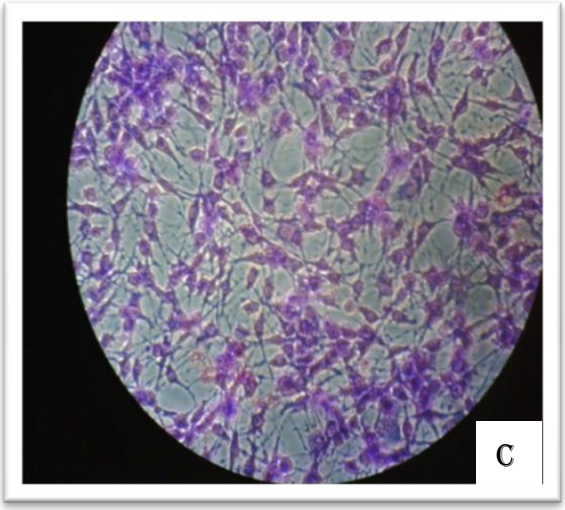


Fig 4: Morphological changes in C6 cells after 24hrs of treatment with (A) control,(B) PBS treated and (C) CAPE treated (40µM) as assessed under phase contrast microscope (20X) after wrights staining; (→) indicates cell volume shrinkage, apoptotic bodies and change in morphology.

2.4 Treatment Inhibits NO Production in C6 Glioma Cells

To determine whether CAPE has an impact on NO levels, we treated C6 cells with CAPE assayed the NO levels by Griess assay. In comparison to basal levels at different time points (6h: 48 µM/L, 24h:26 µM/L, 48h: 23 µM/L) NO levels at different time points were reduced by CAPE (6h: 31 µM/L, 24h: 17 µM/L, 48h: 11 µM/L).There was no significant change in NO levels on treatment with PBS. The NO levels were significantly reduced by CAPE treatment at all time points and effect was persistent over the time (Fig 5).

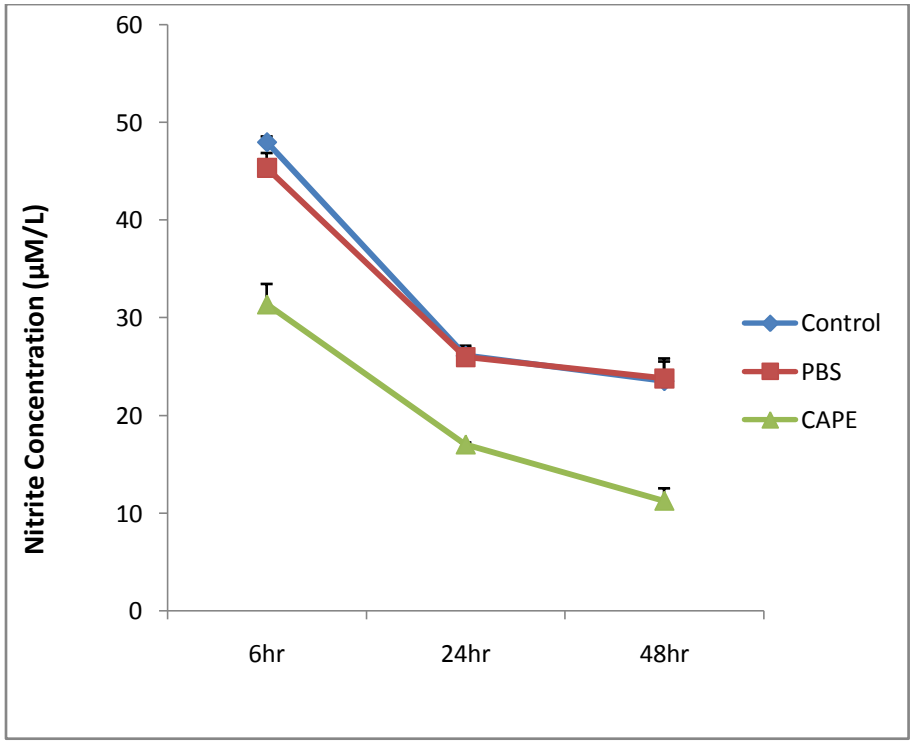


Fig 5: Time dependent curves of nitrite released by C6 cells when treated with CAPE 40Mm at different time points compared to control and PBS treated.

3 DISCUSSION

The standard therapy for the management of gliomas includes surgical resection, radiotherapy, and treatment with the alkylating agents such as Temozolomide (Stupp, Hegi et al. 2009) .Unfortunately this therapeutic approach increases only modestly the survival of patients(Wen and Kesari 2008). Due to the limitations of current treatment modalities, among all the newly developed anti-tumor agents, natural extracts with effective anticancer activities are seen as a potential adjuvants to the treatment of glioma (Bemis, Capodice et al. 2006, HemaIswarya and Doble 2006). CAPE targets cell growth, proliferation and development implicating the relevance of using this drug for effective treatment of glioma (Lee, Kuo et al. 2003, Lin, Liang et al. 2010). CAPE being a natural product is devoid of the strong side effects associated with other chemotherapeutic agents and since CAPE is used at very low concentrations overt toxic effects were observed in studies. Treatment with CAPE inhibited cell proliferation, induced cell death in C6 cells that are resistant to contact inhibition. Furthermore CAPE resulted in an induction of morphological changes typical of cells undergoing apoptosis. The untreated cells were well spread with flattened fibroblastic morphology while as CAPE cells were elongated. It has been reported that cells divide while maintaining their differentiated morphological features, such as elaborated multiple, branched processes protruding from the cell body(Lee, Kuo et al. 2003).Suggesting a high therapeutic potential of the therapy in glioma. Future perspective resides on molecular insights into the course of action of this drug in glioma that would provide better understanding into mechanism of action of this drug.

4 CONCLUSION

CAPE works by targeting multiple mechanisms of tumorigenesis hence dwindling the possibility of drug resistance. CAPE can be used at its optimal dose, without unendurable side effects and be an effective strategy for treating resistant gliomas. *In vitro* studies validate the therapeutic potential of CAPE for glioma and suggest anti-glioma effects for slowing disease progression implicating the relevance of using this drug for effective treatment of glioma.

5 CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest in the publication of the paper.

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7 AUTHOR CONTRIBUTIONS

HMB drafted the manuscript and performed the lab work. EH designed the study, edited the manuscript, and coordinated the group.TG & MZB provided helpful insights during the work and while drafting the manuscript. All authors have read and approved the manuscript.

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