



INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND DEVELOPMENT (IJPRD)

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DIFFERENTIAL MORPHOLOGICAL CHANGES AND EXPRESSION STUDIES OF GFAP IN GLIOMA INDUCED IN RATS FOLLOWING SESAMOL TREATMENT

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ABSTRACT

Glioma is a highly invasive primary brain tumor that arises from glial cells. Malignant gliomas are invariably fatal and all efforts to develop new diagnostic and therapeutic modalities have failed to establish a curative regime. The present study was aimed to investigate the protective activity of sesamol in brain tissues of glioma induced rats on restoring the morphological changes and Glial fibrillary acidic protein (GFAP) expression. Experimentally glioma was induced in adult male Wistar rats by intracerebral injection of C6 cells. The effect of oral administration of sesamol (20mg/kg B.wt) on morphological changes and GFAP expression in the brain tissue of normal and experimental animals were analyzed. The brain tissue section of glioma induced rat showed large polygonal cells of fibrillary background with increased anaplasia and partly scattered cytoplasm. The brain tissue section of sesamol treated rat showed marked reduction in the neoplastic cells. The sum of the area occupied by GFAP reactive filamentous structures with astrocytic morphology were markedly increased in glioma induced rats when compared with control rats. The GFAP reactive filaments were suppressed by the administration of sesamol in glioma induced rats. These findings suggest that sesamol exhibits protective effect against glioma induced changes in the rat brain.

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Key Words

Glioma- astrocytes - sesamol -
GFAP- atypia – necrosis –
malignancy.

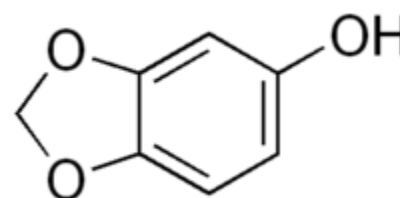
INTRODUCTION

Glioma the most common form of malignant brain tumor is relatively resistant to therapeutic strategies and has a median survival after first diagnosis of only around 12 months. This median survival has remained unchanged for decades despite multiple clinical trials designed to optimize radiation and/or chemotherapy regimens [1, 2]. Although systemic metastases of malignant gliomas are relatively rare, the highly infiltrative nature exhibited by these tumors is the main cause of treatment failure and high recurrence rates. Recent works suggest that malignant gliomas have a stem cell population, which is fundamental for tumor maintenance and growth [3].

The diffused astrocytomas are characterized by their infiltrating nature. Grossly, they tend to be bulky lesions that efface the normal anatomic boundaries, distorting, but not destroying, the structures being invaded. Extensive invasion into surrounding brain is often seen. Neoplastic astrocytes can range from cells closely resembling reactive astrocytes (in some grade II lesions) to showing bizarre anaplasia in which the cells are only recognizable as astrocytic by the presence of GFAP immunopositivity. Some tumor cells become so anaplastic that they even lose GFAP immunopositivity and can only be identified as being astrocytic within the context of the tumor.

Glial fibrillary acidic protein (GFAP) is an intermediate filament (IF) protein that is found in glial cells such as astrocytes. First described in 1971, GFAP is a type III IF protein that maps, in humans, to 17q21 [4, 5]. It is closely related to its non-epithelial family members, vimentin, desmin, and peripherin, which are all involved in the structure and function of the cell's cytoskeleton. GFAP helps to maintain astrocyte mechanical strength, as well as the shape of cells. The spontaneous tumor progression was confirmed by the presence of prominent vascularity, presence of pseudopalisading cells and increase of GFAP in a grade IV astrocytoma [6]. Many authors report the tumor progression phenotype as a result of expression of dedifferentiated characteristics of the cells. During the embryonic development of the CNS, astrocytes hypothetically are originated from progenitors that solely express vimentin

as a cytoskeleton filament [7,8]. These cells have a migratory pattern and before they migrate to the glia radial, they express vimentin and GFAP during cell maturation period [9]. By the end of this process, mature cells express mainly GFAP [10] as a cytoskeleton protein. Sesamol (3,4 methylene dioxy phenol , M.wt:138.12) is the major constituent of *Sesamum indicum* seed oil, which makes it more resistant to oxidative deterioration than other vegetable oils [11]. *Sesamum indicum* is a flowering plant in the genus *Sesamum* of Pedaliaceae family. The genus *Sesamum* consists of about 36 species of which 19 species are indigenous to Africa [12, 13]. It is widely naturalized in tropical regions around the world and is cultivated for its edible seeds. Sesamol is a powerful antioxidant and inhibits UV- and Fe³⁺/ascorbate-induced lipid peroxidation in rat brain [14]. It has been shown that sesamol inhibits several steps in the generation of neoplasia and mutagenesis [15].



Structure of sesamol (C₇H₆O₃)

In this study we examined the protective activity of sesamol in brain tissues of glioma induced rats with a focus on above discussed GFAP reactive filaments and histological assessment.

MATERIALS AND METHODS

Drugs and chemicals

Tris- HCl, H₂O₂, bovine serum albumin (BSA) were purchased from Sisco research laboratory, India. Sesamol was purchased from Sigma Aldrich. GFAP antibody and FITC-labeled secondary antibody were purchased from Genei, Bangalore, India. C6 cell line was purchased from NCCS, Pune, India. Other chemicals of analytical reagent grade were purchased from Qualigens, India.

Animals

Male wistar rats, weighing 250-300g were used for the study. Rats were obtained from the King Institute of Preventive Medicine, Chennai, India. They were housed in polypropylene cages in air conditioned room and allowed free access to pellet diet and water *ad libitum*. Animals acclimatized under standard laboratory conditions were used for the experiment. This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (IAEC No.01/076/09).

Experimental protocol

The animals were divided into four groups (n=6). Group-1 rats were injected with 10 μ l of MEM supplemented with 10% FBS (Control). Group-2 animals were injected with cell suspension of C6 glioma cells (10 μ l of MEM supplemented with 10% FBS containing 10⁵ cells) under a controlled pressure. Group-3 rats were induced glioma as mentioned in group 2 and treated with sesamol orally (20mg/kg body weight) for 30 days. Group-4 animals served as drug control (20mg of sesamol administered orally /kg BW for 30 days)

Induction of Glioma

Rats were anesthetized by injection of xylazine (10 mg/kg, IM) and ketamine (100 mg/kg, IP). The animals were placed in a stereotaxic surgical frame (Instrument & Chemicals Pvt Ltd, Ambala city, India). A small burr hole was drilled into the right side at a location defined by the following stereotaxic coordinates: - 0.8 mm to the bregma; 4 mm medial to lateral; 5 mm dorso ventral from the skull surface for the injection of C6 glioma cell suspension with a Hamilton micro syringe. The craniotomy was sealed with bone wax and overlying skin incision was closed.

Tissue Preparation

At the end of the experimental period, animals were sacrificed by cervical dislocation. The brain tissue was excised immediately and rinsed in ice-cold normal saline. The brain tissue was then fixed in 4% formalin. The tissue sections were embedded in paraffin wax and 4 μ m thick serial sections were obtained and mounted on glass slides. For morphometric analysis, hematoxylin and eosin staining was performed according to standard

protocol. Unstained sections were used for Immunofluorescence studies.

Immunofluorescence Studies

The tissue sections were deparaffinized in two changes of xylene at 60°C for 20 min each and hydrated through a graded series of alcohol, the slides were incubated in a citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven for antigen retrieval. The sections were then allowed to cool to room temperature and then rinsed with Tris-buffered saline (TBS), and treated with 0.3% H₂O₂ in methanol for 10 min to block endogenous peroxidase activity. Nonspecific binding was blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h. The sections were then incubated with GFAP antibody (Genei, Bangalore, India) at 4°C overnight. The slides were washed with TBS and then incubated with anti-rabbit FITC-labeled secondary antibody (Genei, Bangalore, India) for 1 h in room temperature. The fluorochrome, fluorescein, will fluoresce by light closer the excitation wavelength of 495 nm and the emission wavelength of 520 nm.

RESULTS

Histochemical Analysis

Figure 1a shows H&E stained section of the brain tissue of control rat with normal architecture of the astrocytic cells in the brain tissue. Microscopical appearance of the brain tissue section of glioma induced rat shows the features of glioma (Fig. 1b). The tumor was quite well differentiated, richly cellular, typical nuclear changes with increased anaplasia were also found. Figure 1c shows the brain tissue section of sesamol pretreated in glioma induced rat with marked reduction in the neoplastic cells. Figure 1d shows the brain tissue section of sesamol only administered rat with normal architecture of the astrocytic cells. Figures 2, 3 and 4 show the morphological changes like nuclear atypia, increased cellular proliferation and necrosis in the brain tissue section of glioma induced rats.

Immunofluorescence studies

The immunostained section with the GFAP antibody of the brain tissue of control rat showed few GFAP antibody-reactive filaments (fig 5a). Fig.5b shows the immunostained section with the GFAP antibody of the

brain tissue of glioma induced rat. The sum of the area occupied by GFAP antibody-reactive filamentous structures with astrocytic morphology were markedly increased in glioma induced rats when compared with control rats. This GFAP reactive filaments were suppressed by the administration of sesamol in glioma

induced rats (fig 5c). The immunostained sections of the brain tissue of sesamol only treated rat with the GFAP antibody showed decreased GFAP immunoreactivity and filamentous structures (fig 5d).

Histopathological analysis in the brain tissue of control and experimental groups of rats

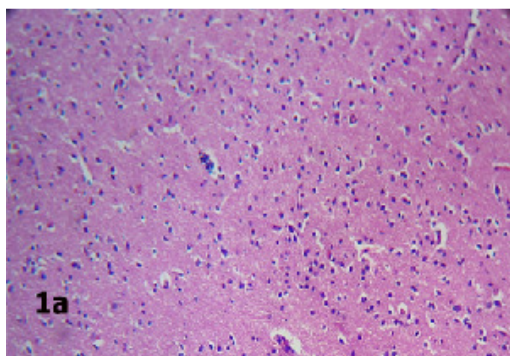


Fig 1a. Normal control rat with normal architecture

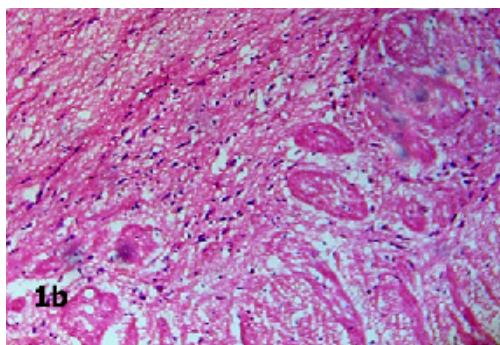


Fig 1b. Glioma induced rat with increased cell density

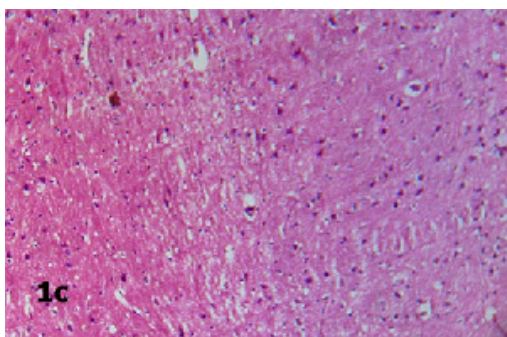


Fig1c. Sesamol treated with reduction in the neoplastic cells

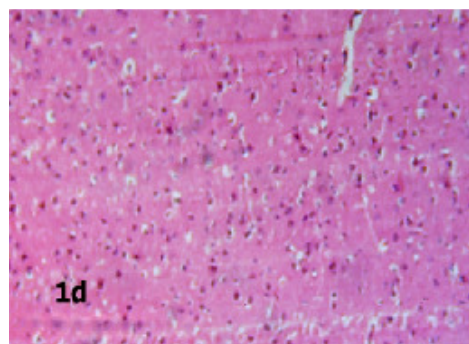


Fig1d. Drug control rat with normal architecture

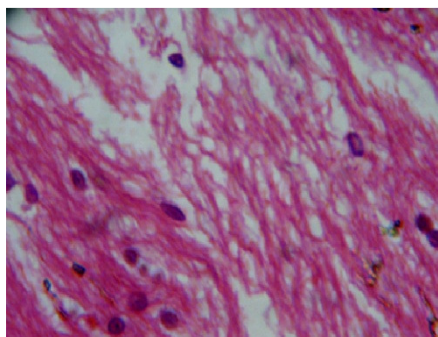


Fig 2. Nuclear atypia.

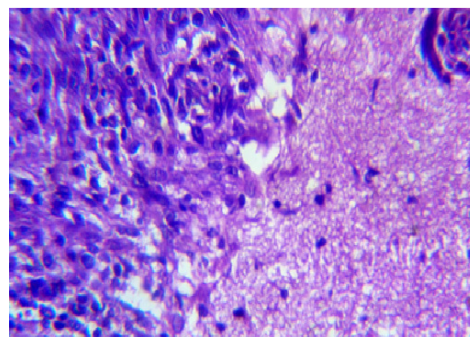


Fig 3. Increased cellular proliferation

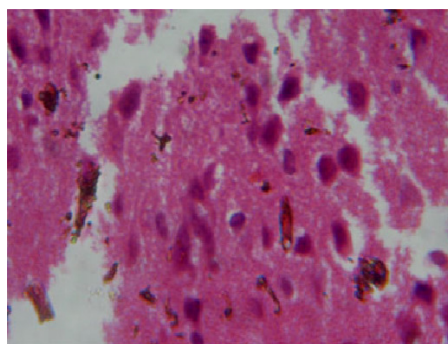


Fig 4. Necrosis

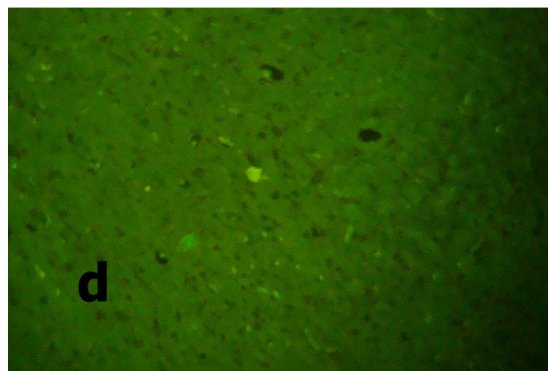
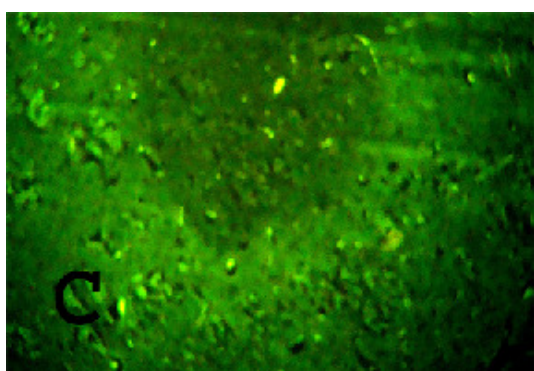
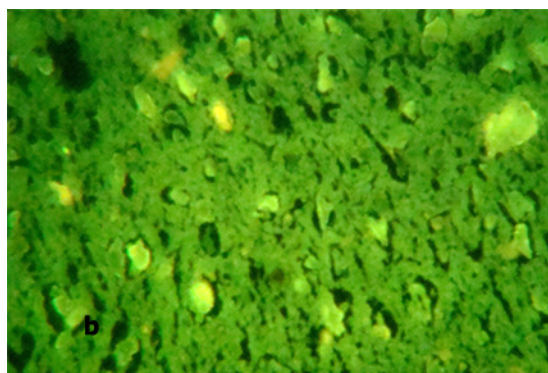
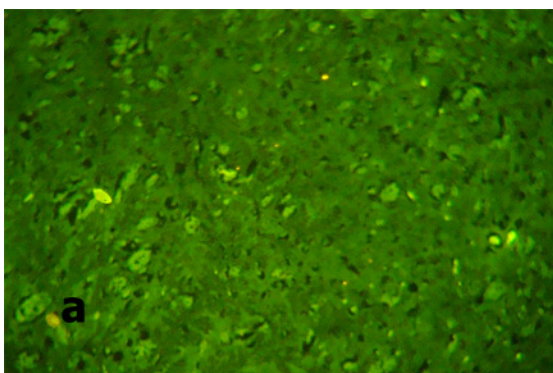


Fig 5.a. the immunostained section with the GFAP antibody of the brain tissue of control rat showing GFAP antibody-reactive filaments; 5b. Immunostained section of the brain tissue of glioma induced rat showing relatively enhanced GFAP immunoreactivity 5c. GFAP reactive filaments were suppressed by the administration of sesamol in glioma induced rats; 5d. The immunostained section of the brain tissue of drug control exhibiting similar picture to that of group 1. (Excitation wavelength of 495 nm and the emission wavelength of 520 nm)

DISCUSSION

Histopathologically, tumor tissue of glioma is composed of two distinct components. Cells with abundant eosinophilic cytoplasm and atypical pleomorphic nuclei were dispersed in fine fibrillary stroma. High mitotic activity, microvascular proliferation and geographic necrosis with obvious nuclear palisading were observed in those areas. Tumor tissue consisted of small undifferentiated cells with scant cytoplasm and ovalround hyperchromatic nuclei in those areas which coincide with the tumor histology of glioma^[16].

Low-grade diffuse astrocytomas show an increase in cellularity and an irregularity of spacing between tumor cells. Although the differences between reactive astrogliosis and infiltrating grade II astrocytoma may be subtle, particularly at the infiltrating edge, the central region of most grade II astrocytomas has sufficient increases in cell density, irregular spacing, and nuclear/cytoplasmic atypia to identify it as a tumor.

As astrocytomas become more malignant, a range of histological features could be observed as noted in the current results of the study indicating with increasing malignancy: increased cell density/ proliferation with the presence of nuclear and cytoplasmic atypia (Fig. 2) and increased mitotic activity (Fig 3). Increased malignancy is associated with increasing nuclear and cytoplasmic atypia. This is manifest by increasing nuclear size, frequently with irregularity of nuclear shape (Fig. 2).

Necrosis is another histological hallmark of malignancy in astrocytomas.. Necrosis can consist of either of two types: pseudopalisading necrosis and geographic necrosis. Pseudopalisading necrosis is characterized by a serpiginous pattern of ribboning of tumor cells around necrotic centers. Geographic necrosis consists of zones of coagulative necrosis in which cell outlines can still be identified, but cell staining and detail are lost (Fig. 4). Such morphological changes were not prominent in sesamol treated brain tissue of glioma induced rat suggesting the protective effect of sesamol on glioma induced rats.

Glial fibrillary acidic protein (GFAP) is considered to be relatively astrocyte-specific marker proteins [17, 18, and [19]. To understand the effect of sesamol on glioma induced rats, we investigated the distribution and quantity of GFAP in brain using immunohistochemistry. We analyzed the area occupied by filamentous structures in control, glioma induced and sesamol treated rats.

In this study, the most specific antibody GFAP was used for detecting the GFAP protein in the brain tissues of normal, glioma induced, sesamol treated and sesamol only treated rats. GFAP labeling, which showed spider-like thick filaments, was greatly increased in the glioma induced rats compared with control rats (Fig. 5). Since GFAP is a gliofilament produced by reactive astrocytes,

GFAP-labeled structures should have a typical spider-like appearance [20]. Reyaz N et al [21] had reported that the comparison of the histological grade with GFAP score was significantly higher in high grade tumour when compared with tumour of grade I. The prominent expression of GFAP in astrocytoma was reported by Kimura T, et al. [21]. In astrocytic neoplasms the number of GFAP positive cells and the intensity of the stain were directly proportional to the degree of malignancy [22].

In our study astrocytes labeled with the GFAP antibody were significantly reduced in the sesamol treated glioma induced rats when compared with glioma induced rats. It has been shown that sesamol neuroprotective [23] and chemo-preventive [24] properties in rats. Very few GFAP reactive structures and filamentous structures were found in the control and sesamol only treated rats. Our results are in agreement with the results of Dittmann et al., which showed that the GFAP concentration in astrocytomas is 1.2-6 times the GFAP concentration found in normal brain tissue [25]. The presence of the GFAP in astrocytomas has previously been demonstrated by a report showing enhanced GFAP immuno-reactivity of tumour cells getting in contact with collagenous tissue in the majority of astroglial tumours [26].

Sesamol induced reduction in the expression of GFAP reactive structures and the restoration of morphological characteristics in sesamol treated glioma models could be arguably, a the sign of protective effect of sesamol on glioma induced rat brain paving the way for related investigations to strengthen and unravel the potential of sesamol.

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