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Abstract

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Keywords

Glioblastoma, C60 hydrofullerene, Hydrogen peroxide, Autophagy, GFAP

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RESEARCH PAPER

C₆₀ Hydrofullerene Induced Autophagy and Ameliorated GFAP in H₂O₂ Treated Human Malignant Glioblastoma U-373 Cell Line

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Abstract

Glioblastoma is one of the most combative astrocytoma that is resistant to chemotherapy and radiotherapy. This resistance makes it very difficult to treat. However, researches have shown that nanoparticles especially C60 hydrofullerene have antioxidant and anticancer activity. The effect of C60 hydrofullerene in cancer has been extensively studied; however, the potential regulation of autophagy and modulation of the Glial Fibrillary Acidic Protein (GFAP) gene has not been addressed in glioblastomas. Glioblastoma U-373 cell was treated with 0.5 μM of C60 hydrofullerene and/or 1 mM of hydrogen peroxide (H₂O₂) for 24 hours. This study demonstrated that C60 hydrofullerene and H₂O₂ significantly decreased cell proliferation. The gene expression and conversion of LC3-II/LC3-I were significantly induced in the separate and combined treatments. The immunofluorescent results observe that H₂O₂ dramatically inhibits the expression of GFAP. However, when the cells were treated with both H₂O₂ and C60 hydrofullerene, GFAP expression was significantly restored. The anti-proliferative effects of C60 hydrofullerene and H₂O₂ are shown in the results. Both treatments induced LC3-based autophagy and H₂O₂ was down regulated GFAP.

Keywords: Glioblastoma, C60 hydrofullerene, Hydrogen peroxide, Autophagy, GFAP

1. Introduction

Glioblastoma was discovered by Burns in 1800 and Abernethy in 1804 [1]. It is the most aggressive grade IV form of glioma [2,3], which arises from glial cells [2]. It is characterized by its fast-growing, rapid invading ability into the adjacent tissue [2], it is more common among men and approximately 57% of all astrocytic tumors are glioblastoma [3,4]. However, glioblastoma is resistant to chemotherapy and radiotherapy [5]. The treatment of glioblastoma is the removal of the tumor followed by radiotherapy and chemotherapy [2,4]. Researchers are continuing to find out more ways to treat patients. These studies have observed

nanoparticles having anti-cancer effects on glioblastoma [6,7], but there is not enough data to fully understand about the ways that nanoparticles affect glioblastoma. Further research may aid in making carbon nanoparticles as potential factors for anti-cancer therapy [8].

Nanomaterials have large applications in scientific and medical research because of their external dimensions, high chemical properties, and physical activities [9]. Nanomaterials have various characteristics that make them beneficial. They do not aggregate in tissues at a high rate and have a high shelf-life rate. When consumed by non-healthy cells compared to the normal cells, they can induce abnormal apoptosis, pro-inflammatory effect, and augment oxidative stress [7].

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Among nanomaterials, C₆₀ hydrofullerene is potent for its anticancer, cytoprotective, and cytotoxic effects [7]. It does not affect normal cells and tissue at low concentrations [6]. Nanocrystalline C₆₀ particularly, has a cytotoxic effect against resistant chemotherapy glioma that is dependent and independent of oxidative stress [7]. The data about hydrated C₆₀ hydrofullerene on glioblastoma is limited; nevertheless, some research has shown anti-proliferative effects of C₆₀ hydrofullerene. The study observed that C₆₀ derivatives significantly inhibited glioblastoma's growth [10]. In addition, researchers observed different anti-proliferative mechanisms of C₆₀ hydrofullerene on cancer cell lines HeLa, A549, and MCF-7 [11]. Regarding anti-proliferation activity, recent studies demonstrated dual roles of C₆₀ hydrofullerene on cancer cells and have cytotoxic effects [12]. On the other hand, C₆₀ hydrofullerene has protective properties such as diminishing cytotoxicity. For example, studies have shown the protective effect of fullerene in rat brains exposed to alcohol by maintaining the expression of Glial Fibrillary Acidic Protein (GFAP) [13,14].

GFAP is the essential member of intermediate filament proteins type III that participates in the formation of the cytoskeleton building of astrocytes [15,16]. GFAP is expressed in glial cells and in the glioblastoma cell. It is necessary for cell survival [17], mitosis [15,17], normal cell functions, cell apoptosis, cell prognosis [17], cell migration [15,17], cell motility and attaching to the glutamate transporter in the cell membrane [15], and astrocytic reactive response [16].

GFAP has been used to clinically diagnose glioma for many years [17–20]. A high level of GFAP protein was found in patients with glioblastoma grade II when compared to grades III and IV [21,22]. A low level of GFAP protein expression was found in glioblastoma grade IV [15,23] and the level of GFAP most likely does not reflect the differentiation state of glioblastoma [15]. On the other hand, a high level of GFAP- δ protein (the most abundant isoform of GFAP protein) was anticipated in glioma grade IV compared to low-grade glioma [19]. The study also demonstrated that glioma with a high level of GFAP is more invasive [24]. The progression and expression of GFAP positive glioblastoma is higher than GFAP negative glioblastoma [15].

This study was designed to elaborate on the effects of C₆₀ hydrofullerene nanoparticles and H₂O₂ on autophagy in human malignant glioblastoma cells. Because of, reviews demonstrated that Glioblastoma is resistant to apoptosis [25], C₆₀ induces autophagy [26] and autophagy is hypothesized to have dual role such as; anti-cancer activity and

cancer protective role [27]. The questions are, does C₆₀ hydrofullerene can play as an anticancer agent? Does it recover the cells when treated with cytotoxic agent through intermediate filament protein protection? Does C₆₀ hydrofullerene induce LC3-based autophagy whereas Glioblastoma is resistant to apoptosis?

2. Methods and materials

2.1. Cell culture

Human Glioblastoma U-373 Cell Line was acquired from ATCC organization (ATCC, USA). The U-373 cell was seeded in a 75 cm² flask (Sigma-Aldrich, Germany) with a DMEM medium containing 10% fetal bovine serum, 64 μ g/ml penicillin, and 0.1 mg/ml streptomycin antibiotic solution. It was incubated in a humidified incubator (Esco, Singapore) at 5% CO₂ at 37 °C. The cells were incubated under these conditions throughout the experiment unless stated otherwise. The medium was changed every 48 hours.

2.2. Treatment

This study is designed to treat the glioblastoma U-373 cell line with 0.5 μ M C₆₀ hydrofullerene and/or 1 mM hydrogen peroxide for 24 hours.

2.3. Cell proliferation assay (MTT)

To analyze cell proliferation, MTT cell proliferation set (Roche, Germany) was used [28]. The glioblastoma cell was seeded in 96 well-plates (Sigma-Aldrich, Germany) containing 6×10^3 cells/well. The cells were incubated overnight as mentioned before and then treated with its respective treatment. The first MTT reagent (10 μ l) was added and the cells were gestated in a humidified incubator for 4 hours in the dark. Then the solubilization reagent was added (100 μ l/well). The plate was allowed to stand overnight away from light. The absorbance of formazan crystals was measured by using an ELISA reader (Molecular devices LLC, USA). The ELISA reader took measurements at a wavelength of 550–600 nm along with a reference wavelength at more than 650 nm. Finally, the cell viability was extracted from cell proliferation MTT assay result [28].

2.4. Western blot assay

Fresh lysis buffer (RIPA) containing 1 mM protease inhibitor, leupeptin and pepstatin was used

to prepare the total protein [29]. Loading buffer was added to the sample and warmed up to 94 °C. Then, 20 µg from each sample was loaded to 12% SDS-polyacrylamide gel. The separated polypeptide bands were transferred to the PVDF membrane (Millipore, Germany) and blocked by using 5% skimmed milk. The membrane was bathed with primary antibodies (LC3 diluted 1:1000, β-actin 1:2500) (Santacruz, USA), 24 hours at 4° then washed out by PBS solution 5 times and placed in tubes containing different secondary antibodies (anti-mouse and anti-rabbit 1:5000) (Advansta, USA), for an hour at 25 °C. The signals were developed by the chemiluminescence X-ray method (Carestream, USA).

2.5. Immuno-fluorescent assay

5×10^5 cells/well were cultured in a 96-well plate, incubated overnight, and then treated. The treated cells were fixed by 4% paraformaldehyde then washed with PBS at room temperature. It incubated in FBS containing GFAP primary antibody (anti-rabbit 1:500, Abcam) overnight at 4 °C. After the primary antibody had been washed out with PBS, the cells were covered with the solution containing secondary antibody (anti-mouse 1:1000 diluted, Abcam) for 2 hours at room temperature. The cells were washed and incubated at room temperature with a blocking solution containing nuclear dye DAPI for 15 minutes. The last step was to wash the cells with PBS. The images were obtained using a Nikon microscope (Nikon, Japan) connected to a computer and armed with a fluorescent illuminator (Rockland, USA).

2.6. Statistical analyses

The data was evaluated in the Graph Pad Prism 9 soft program. The result was analyzed by one-way ANOVA Tukey nonparametric. The Densitometry analysis was performed in ImageJ software (USA) and normalized against its respective load control. Each treatment was replicated three times.

3. Results

3.1. C₆₀ hydrofullerene and H₂O₂ inhibited cell proliferation

Cell proliferation of normal and damaged cells were measured by MTT assay, and cell viability was extracted from MTT assay result [28]. The results show that nanoparticle C₆₀ hydrofullerene and H₂O₂ have an anti-proliferation effect against the U-

373 cell line. The obtained results and the MTT assay were observed that Malignant glioblastoma cell proliferation was inhibited when treated with 0.5 µM C₆₀ hydrofullerene (**p < 0.05) and cell viability decreased approximately 15%. The 1 mM H₂O₂ inhibited cell proliferation (*p < 0.05) and cell viability decreased approximately 13%. The combined dose of C₆₀ hydrofullerene and H₂O₂ greatly inhibited U-373 cell proliferation (***p < 0.05) and cell viability decreased by mean approximately 26%. The results are shown in Fig. 1.

3.2. C₆₀ hydrofullerene and H₂O₂ induced autophagy

To elaborate on the effect of C₆₀ hydrofullerene and H₂O₂ on autophagy, the level of LC3 was measured by the Western blot technique. The results of our study observed the induction effect of treatments on LC3 protein in the Malignant Glioblastoma U-373 cell line. C₆₀ hydrofullerene (0.5 µM) does not have a significant effect on LC3 expression. On the other hand, the LC3 protein was induced when exposed to 1 mM H₂O₂ for 24 hours (*p < 0.05) and the combined dose of (C₆₀ hydrofullerene and H₂O₂) induced the expression and conversion of LC3 protein (*p < 0.05) as shown in Fig. 2.

3.3. C₆₀ hydrofullerene prevents decline of GFAP

The GFAP in the U-373 malignant glioma cell did not change significantly when treated with C₆₀ hydrofullerene. On the other hand, the pictures presented show that C₆₀ hydrofullerene prevents decline of GFAP caused by H₂O₂ exposure. Therefore, C₆₀ hydrofullerene ameliorates the disturbance in glial cytoskeleton as shown in Fig. 3.

4. Discussion

In our study, a unique discovery was observed about the effect of C₆₀ hydrofullerene on the human malignant glioblastoma U-373 cell line. We found that C₆₀ hydrofullerene at a low dose (0.5 µM) with H₂O₂ separately and combined inhibited malignant glioma cell proliferation in a dose-dependent manner. The highest inhibition among these treatments was noticed in the combined treatment of C₆₀ hydrofullerene and H₂O₂ (***p < 0.05). The results in our study agree with other published studies. One study mentioned that H₂O₂ (125 µM) inhibited cell proliferation in C6 glioma cell line [30]. Kim et al. 2012, said that 600–900 µM H₂O₂ decreased cell viability [31]. The high dose (400–1000) µM of H₂O₂

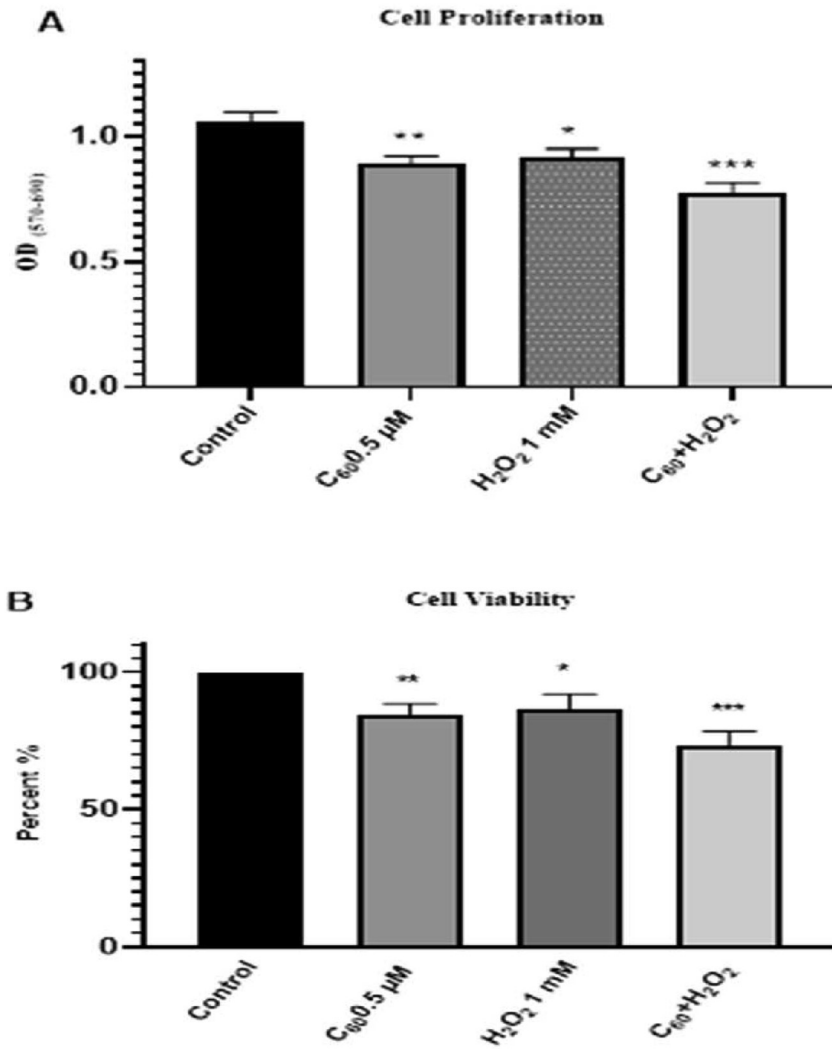


Fig. 1. The level of glioma U-373 cell proliferation (A) and Cell viability (B) of control and treated cells with C60 hydrofullerene 0.5 μM and H2O2 1 mM in vitro. n = 3.

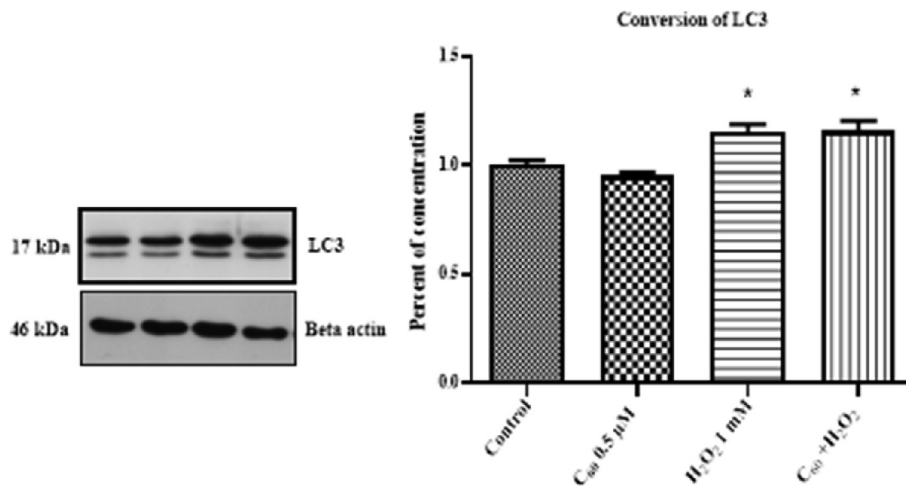


Fig. 2. The Western blot result for LC3 protein. n = 3.

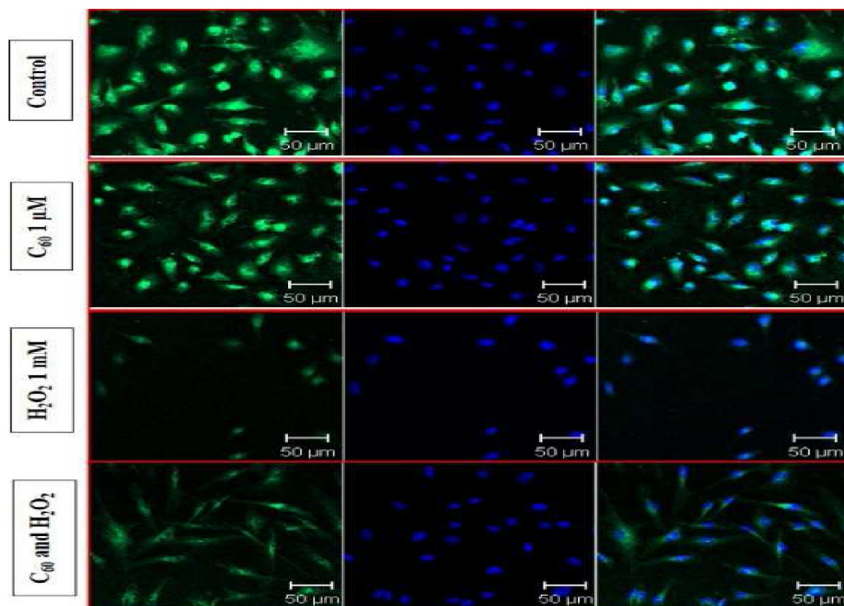


Fig. 3. The immunofluorescent pictures of GFAP protein in Human Malignant Glioblastoma U-373 cell line treated with C₆₀ hydrofullerene and H₂O₂. n = 3.

significantly reduced cell viability in the HepG2 cell line [27]. As we illustrated in our results, C₆₀ hydrofullerene has an anti-proliferative effect against the U-373 cell line [32]. In addition, the photoexcited C₆₀ and H₂O₂ (both about 50 μm) inhibited 50% cell viability in leukemic cells after 24 hours [33]. In Ershova et al. 2016, a high dose of C₆₀ hydrofullerene significantly decreased the number of human embryonic lung fibroblasts cells [34]. Prylutska et al., 2011 and 2014, demonstrated the C₆₀ hydrofullerene effect against tumor growth in mice transplanted lung cancer [6].

In this study, a high level of LC3 expression was observed in the U-373 cell line when treated with H₂O₂ and C₆₀ hydrofullerene. To examine the effect of H₂O₂ and C₆₀ hydrofullerene, the Western blot experiment was performed. The result demonstrated that the H₂O₂ dose and combined dose of H₂O₂ and C₆₀ hydrofullerene significantly induced autophagy through LC3 protein when compared to the control group (*p < 0.05, **p < 0.05). However, the C₆₀ hydrofullerene dose alone, does not significantly regulate the expression and conversion of LC3 protein as shown in Fig. 2. Recent studies supported our results. The ADS-I increased the autophagy in the U-373 glioblastoma cell line through LC3-II protein [35]. In addition, the conversion of LC3-I to LC3-II induced autophagy in the U-373 glioblastoma cell line [36]. One study demonstrated autophagic cell death in the U-251 malignant glioma cell line by up-regulating LC3-II [37]. Another published study demonstrated that

H₂O₂ after 12 hours induced autophagy and up-regulated LC3-II protein [38]. Considerable evidence from the references demonstrate that H₂O₂ treatment induced autophagy and increased LC3-II and GFP-LC3 puncta in human malignant glioma [39]. Finally, the results from our study coincides with the study published by Kong et al., 2011 that 1mM of H₂O₂ induced autophagy in human malignant glioma U-251 cell line [40] and autophagic cell death was seen in U-373 cell line based on up-regulation of LC3 protein when treated with H₂O₂ and C₆₀ hydrofullerene [41].

In our study, the U-373 cell line was used to determine the effect of H₂O₂ and C₆₀ hydrofullerene. Glioblastoma cells express the GFAP protein [15] and might play a vigorous role in glioblastoma malignancy [21,42], elaborate adhesion, and proliferation [42]. In our previous findings, C₆₀ hydrofullerene inhibited GFAP expression while C₆₀ hydrofullerene improves GFAP reduction [32]. One study demonstrated that C₆₀ hydrofullerene inhibited loss of cytoskeleton GFAP in rat brain astrocytic cells against alcoholic impact Prischepa et al., 2015 discussed that C₆₀ hydrofullerene restored GFAP expression in STZ-Diabetic rat retina [14,43]. Huang et al., 2018 said that GFAP is restored in H₂O₂ degenerated rat retina when treated with C₆₀ nanoparticle [44]. There were similarities in our results; H₂O₂ dramatically inhibited GFAP expression when compared to the control group and even more so when compared to the C₆₀ hydrofullerene group. On the other hand, C₆₀ hydrofullerene slightly

inhibited GFAP expression, but ameliorates GFAP expression when the cells were treated with H₂O₂ and C₆₀ hydrofullerene for 24 hours.

5. Conclusion

C₆₀ hydrofullerene and H₂O₂ have anti-proliferative effects separately and in combined dose. C₆₀ hydrofullerene can act as an anti-cytotoxic against the hydrogen peroxide effects, which partially ameliorates GFAP protein. In addition, it could be used in the future as an anti-cancer treatment and a cytoprotective drug for normal cells against various diseases but needs further investigation.

Declaration of competing interest

None.

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