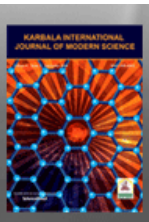

Karbala International Journal of Modern Science

Volume 8 | Issue 3 Article 19

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

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Abstract

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Keywords

Glioblastoma, C60 hydrofullerene, Hydrogen peroxide, Autophagy, GFAP

RESEARCH PAPER

C_{60} Hydrofullerene Induced Autophagy and Ameliorated GFAP in H_2O_2 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.

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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 anticancer 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].

Received 29 January 2022; revised 24 May 2022; accepted 28 May 2022.
Available online 1 August 2022

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https://doi.org/10.33640/2405-609X.3242

Among nanomaterials, C_{60} hydrofullerene is potent for its anticancer, cytoprotective, and cytotoxic effects [7]. It does not affect normal cells and tissue at low concentrations [6]. Nanocrystalline C_{60} particularly, has a cytotoxic effect against resistant chemotherapy glioma that is dependent and independent of oxidative stress [7]. The data about hydrated C_{60} hydrofullerene on glioblastoma is limited; nevertheless, some research has shown anti-proliferative effects of C_{60} hydrofullerene. The study observed that C_{60} derivatives significantly inhibited glioblastoma's growth [10]. In addition, researchers observed different anti-proliferative mechanisms of C_{60} hydrofullerene on cancer cell lines HeLa, A549, and MCF-7 [11]. Regarding anti-proliferation activity, recent studies demonstrated dual roles of C_{60} hydrofullerene on cancer cells and have cytotoxic effects [12]. On the other hand, C_{60} 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 C_{60} hydrofullerene nanoparticles and H_2O_2 on autophagy in human malignant glioblastoma cells. Because of, reviews demonstrated that Glioblastoma is resistant to apoptosis [25], C_{60} 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 C_{60} hydrofullerene can play as an anticancer agent? Does it recover the cells when treated with cytotoxic agent through intermediate filament protein protection? Does C_{60} 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 μg/ml penicillin, and 0.1 mg/ml streptomycin antibiotic solution. It was incubated in a humidified incubator (Esco, Singapore) at 5% CO_2 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 μM C_{60} 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 μl) was added and the cells were gestated in a humidified incubator for 4 hours in the dark. Then the solubilization reagent was added (100 μl/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 proteinase inhibitor, leupeptin and pepstatine 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 (ani-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. C_{60} hydrofullerene and H_2O_2 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 C_{60} hydrofullerene and H_2O_2 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 C_{60} hydrofullerene (**$p < 0.05$) and cell viability decreased approximately 15%. The 1 mM H_2O_2 inhibited cell proliferation (*$p < 0.05$) and cell viability decreased approximately 13%. The combined dose of C_{60} hydrofullerene and H_2O_2 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. C_{60} hydrofullerene and H_2O_2 induced autophagy

To elaborate on the effect of C_{60} hydrofullerene and H_2O_2 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. C_{60} 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 H_2O_2 for 24 hours (*$p < 0.05$) and the combined dose of (C_{60} hydrofullerene and H_2O_2) induced the expression and conversion of LC3 protein (*$p < 0.05$) as shown in Fig. 2.

3.3. C60 hydrofulleren 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 C_{60} hydrofullerene on the human malignant glioblastoma U-373 cell line. We found that C_{60} hydrofullerene at a low dose (0.5 μM) with H_2O_2 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 C_{60} hydrofullerene and H_2O_2 (***$p < 0.05$). The results in our study agree with other published studies. One study mentioned that H_2O_2 (125 μM) inhibited cell proliferation in C6 glioma cell line [30]. *Kim* et al. *2012,* said that 600–900 μM H_2O_2 decreased cell viability [31]. The high dose (400–1000) μm of H_2O_2

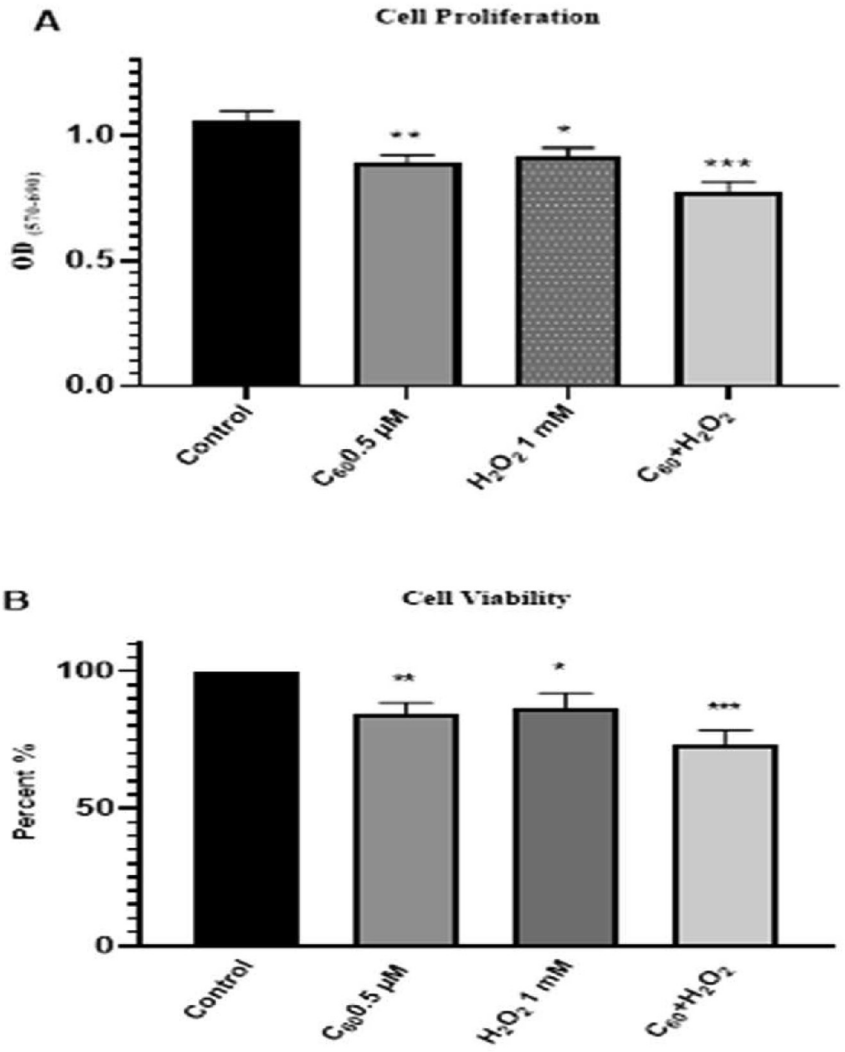

Fig. 1. The level of glioma U-373 cell proliferation (A) and Cell viability (B) of control and treated cells with C60 hydrofullerene 0.5 μM and H2O2 1 mM in vitro. n = 3.

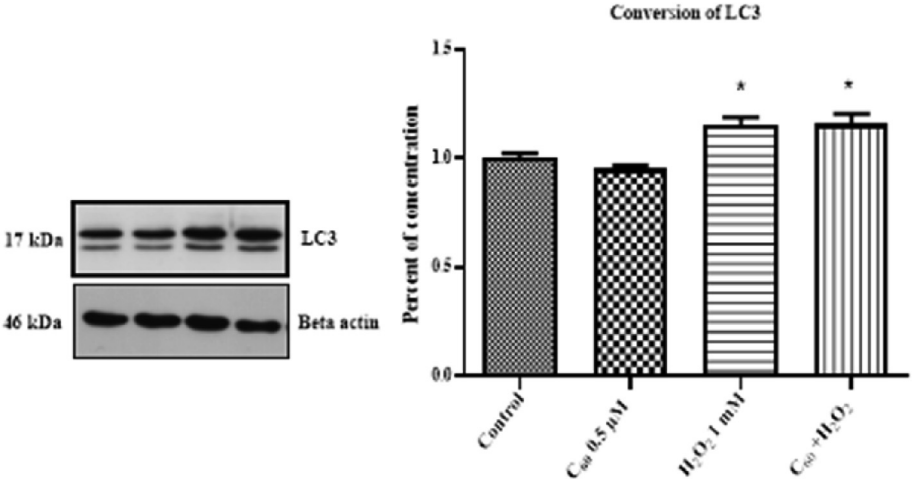

Fig. 2. The Western blot result for LC3 protein. n = 3.

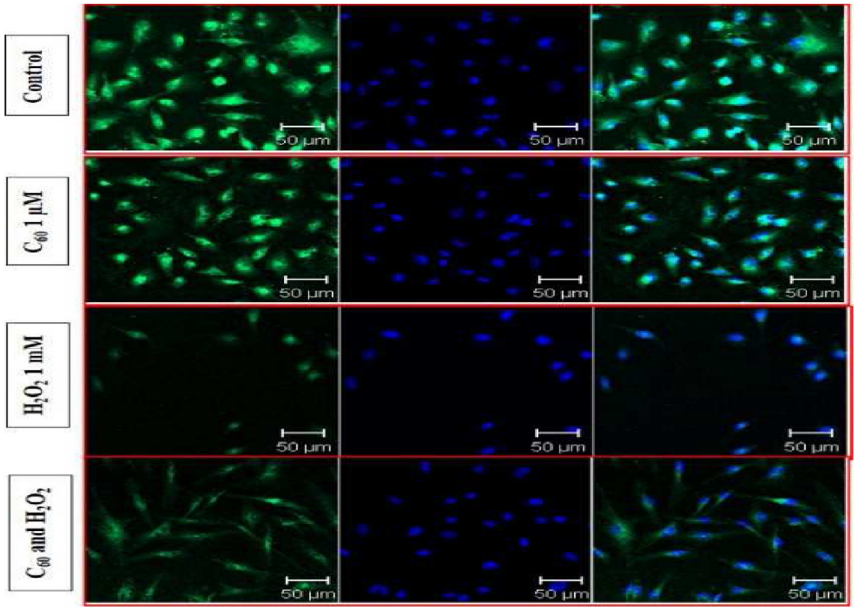

Fig. 3. The immunofluorescent pictures of GFAP protein in Human Malignant Glioblastoma U-373 cell line treated with C60 hydrofullerene and H2O2. n = 3.

significantly reduced cell viability in the HepG2 cell line [27]. As we illustrated in our results, C_{60} hydrofullerene has an anti-proliferative effect against the U-373 cell line [32]. In addition, the photoexcited C_{60} and H_2O_2 (both about 50 μm) inhibited 50% cell viability in leukemic cells after 24 hours [33]. In *Ershova* et al. *2016*, a high dose of C_{60} hydrofullerene significantly decreased the number of human embryonic lung fibroblasts cells [34]. *Prylutska* et al., 2011 and 2014, demonstrated the C_{60} 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 H_2O_2 and C_{60} hydrofullerene. To examine the effect of H_2O_2 and C_{60} hydrofullerene, the Western blot experiment was performed. The result demonstrated that the H_2O_2 dose and combined dose of H_2O_2 and C_{60} hydrofullerene significantly induced autophagy through LC3 protein when compared to the control group (*p < 0.05, **p < 0.05). However, the C_{60} 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 H_2O_2 after 12 hours induced autophagy and up-regulated LC3-II protein [38]. Considerable evidence from the references demonstrate that H_2O_2 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 H_2O_2 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 H_2O_2 and C_{60} hydrofullerene [41].

In our study, the U-373 cell line was used to determine the effect of H_2O_2 and C_{60} 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, C_{60} hydrofullerene inhibited GFAP expression while C_{60} hydrofullerene improves GFAP reduction [32]. One study demonstrated that C_{60} hydrofullerene inhibited loss of cytoskeleton GFAP in rat brain astrocytic cells against alcoholic impact *Prischepa* et al., 2015 discussed that C_{60} hydrofullerene restored GFAP expression in STZ-Diabetic rat retina [14,43]. *Huang* et al., 2018 said that GFAP is restored in H_2O_2 degenerated rat retina when treated with C_{60} nanoparticle [44]. There were similarities in our results; H_2O_2 dramatically inhibited GFAP expression when compared to the control group and even more so when compared to the C_{60} hydrofullerene group. On the other hand, C_{60} hydrofullerene slightly

inhibited GFAP expression, but ameliorates GFAP expression when the cells were treated with H_2O_2 and C_{60} hydrofullerene for 24 hours.

5. Conclusion

C_{60} hydrofullerene and H_2O_2 have anti-proliferative effects separately and in combined dose. C_{60} 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.

Acknowledgement

This article was revised linguistically by American English native speaker Ms. Aveen Saeed.

This work was partially supported by grant 215S192 from the Scientific and Technological Research Council of Turkey, Turkey (TUBITAK).

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