Research Article

The Inhibitory Effects of Hesperetin, a Natural Flavonoid, against Substance P-induced Inflammatory Responses in Glioblastoma Cells

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ABSTRACT

Objectives: As a key inflammatory neuropeptide, substance P (SP) is involved in Glioblastoma multiforme (GBM) carcinogenesis and tumor progression. Thus, the proinflammatory effects of SP must be strictly regulated in GBM patients. The purpose of the study is to examine whether Hesperetin, a natural flavonoid found in citrus fruits with strong anti-inflammatory and anticancer effects, can regulate SP-induced inflammation in GBM primary human cells.

Methods: The primary human cells were derived from fresh surgically resected tissue samples of GBM patients and characterized by immunocytochemical analysis of Ki-67. MTT assay was used to evaluate cell proliferation. Apoptosis was detected by an Annexin-V/propidium iodide assay kit using flow cytometry. The levels of the specific inflammatory mediators were measured by western blotting and enzyme-linked immunosorbent assay.

Results: We observed that Hesperetin effectively reduced GBM cell viability in a dose-dependent manner, which was associated with the induction of apoptosis. Obtained findings indicated SP increased the protein expression of phosphorylated-NF-κB, the main regulator of inflammatory processes, and the pro-inflammatory cytokines (IL-1β, TNF-α), while Hesperetin treatment reduced the effects of SP.

Conclusion: Taken together, our findings highlight the role of SP-induced inflammatory responses in GBM pathology and suggest that Hesperetin could be an effective therapeutic strategy in attenuating SP-associated inflammation.

Keywords: Substance p, Hesperetin, Glioblastoma, Inflammation, NF-κB

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Introduction

Glioblastoma multiforme (GBM), also known as grade IV astrocytoma, is the most aggressive subtype of glial tumors with high malignancy, remarkable annual incidence rate, and poor prognosis (1, 2). The current standard therapeutic modalities for GBM are surgical resection, chemotherapy, radiotherapy, and other innovative therapeutic approaches (e.g., gene or cell-based treatments) combined with conventional treatments (3, 4). However, these therapeutic interventions are highly insufficient, and the prognosis remains extremely poor, with an average survival of 14 months (5). Therefore, there is an imperative need to explore new therapeutic targets to optimize the GBM therapeutic approaches.

Inflammation has been recognized as an important factor in GBM tumorigenesis and development (6). As a member of the tachykinin neuropeptide family, substance P (SP) induces a series of neuroinflammatory responses and is, therefore, particularly important in cancer-associated inflammation (7). SP activation of neurokinin 1 receptor (NK1R), a class of neurokinin G protein-coupled receptors, initiates several pro-tumorigenic and inflammatory responses, including increased vascular permeability, vasodilatation, plasma extravasation, immune cell recruitment, overexpression of transcription factor nuclear factor-kappa B (NF-κB) as the main regulator of inflammatory processes, and subsequent overproduction of proinflammatory cytokines and chemokines (7-9). Accordingly, overexpression of NK1R has been reported in GBM, which is highly related to the degree of malignancy (10, 11). The SP/NK1R system has been reported to induce GBM cell proliferation by increasing pro-inflammatory cytokines involved in GBM growth and development (12). In view of these findings, modulation of the proinflammatory effects of SP could present an effective strategy to avoid inflammatory damage in patients with cancer, particularly GBM.

The idea of using naturally occurring compounds instead of chemical agents with unfavorable pharmaceutical activities is currently drawing considerable attention. Hesperetin, also known as 3′, 5, 7-trihydroxy-4′-methoxy flavanone, is the aglycone of hesperidin, the primary flavone glycoside in citrus fruits (13). This dihydroflavone has the chemical formula C16H14O6 and belongs to the flavanone group of flavonoids (13). It is commonly found in citrus fruits (e.g., oranges, lemons, and grapefruits) and has various pharmacological effects. Hesperetin exhibits a range of activities, such as anti-inflammatory, antibacterial, anticarcinogenic, and antioxidant properties (14, 15). In vivo, administration of hesperetin has been shown to be protective against LPS-induced neuroinflammation-affected mice by reducing the expression of inflammatory mediators, including phosphorylated NF-κB, tumor necrosis factor-α (TNF-α), and IL-1β (16). To date, our knowledge of the anti-inflammatory effects of hesperetin in GBM is very limited. Based on these considerations, we speculated that hesperetin could potentially target SP-associated inflammation. Thus, in this study, we first examined whether SP exerts inflammatory effects by evaluating NF-κB activation and the protein levels of inflammatory cytokines, TNF-α, and IL-1β. Additionally, we examined whether hesperetin can regulate SP-mediated inflammatory events in primary cells derived from patients with GBM.

Material and methods

Cell Culture and reagents

The primary human glioblastoma cells were derived from fresh surgically resected tissue samples of 3 patients with WHO Grade 4 gliomas. Each sample was first soaked in phosphate-buffered saline (PBS) to remove blood. After complete blood removal, tissue samples were mechanically homogenized by a sterile scalpel and subsequently transferred to a falcon tube containing 0.05% Trypsin/EDTA (DNAbiotech Co., Tehran, Iran) and kept for 15 minutes in a 37°C water bath. Thereafter, the tissue suspension was centrifuged for five minutes at 1500 g, and the cell pellets were then resuspended in DMEM/F12 medium (DNAbiotech Co., Tehran, Iran) followed by filtration through a 70 µm cell strainer. The obtained cells were finally resuspended in a proliferation medium containing DMEM/F12 with 10% fetal bovine serum (FBS), 1% GlutaMAX, and 1% penicillin-streptomycin (Gibco) and cultured as a monolayer in a T25 cell culture flask. Cells were passaged when cell confluency reached approximately 80%. Hesperetin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and SP was purchased from Abcam. Hesperetin was dissolved in dimethyl sulfoxide (DMSO), and SP was dissolved in distilled water.

Immunocytochemistry

To characterize the primary human glioblastoma cells obtained from fresh surgically resected tissue samples, immunocytochemistry was employed. For this, primary cells were fixed with 4% paraformaldehyde as a fixation solution for 15 minutes at room temperature. Subsequently, cells were blocked with the blocking buffer (10% FBS and 0.1% Triton X-100 in PBS) for 30 minutes. Cells were incubated overnight with anti-Ki67 (1:100, rabbit polyclonal, Abcam) diluted in PBS with 5% FBS and 0.1% Triton X-100. After washing three times with PBS, cells were incubated with a secondary antibody (1:200, FITC-conjugated polyclonal goat anti-rabbit antibody) diluted in PBS with 5% FBS and 0.1% Triton X-100 for 1 hour at room temperature with

gentle agitation. Nuclei were counterstained with DAPI for 5 minutes, and images were finally acquired using fluorescence microscopy.

Cell viability assay

To evaluate cellular viability in Hesperetin-treated cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed. The human primary glioblastoma cells (5 x 10^3 cells) were cultured in 96-well plates and incubated for 24 hours to allow attachment. After reaching 80% confluence, cells were treated with varying concentrations of Hesperetin and incubated for 24 hours. After 24 hours of treatment, 20 µL MTT solution was added to each well, followed by a further 4 hours of incubation at 37°C. Thereafter, the remaining supernatant was removed, and each well received 150 µL DMSO (dimethyl sulfoxide) to dissolve the insoluble formazan crystals, followed by a further 10 minutes of incubation at 37°C. Absorbance was then measured at 570 nm with a microplate reader. Half-maximal inhibitory concentration (IC50) was calculated using GraphPad Prism software with a nonlinear regression curve.

Flow cytometric analysis of apoptosis

Abcam Annexin V-DY-634/PI assay kit was used to assess apoptosis following the manufacturer's protocol. The human primary glioblastoma cells were cultured in 6-well plates and treated with Hesperetin at the desired concentration. After 24-hour treatment, 1×10^5 cells were separated by trypsinization and washed twice with PBS. After washing, 100 µL of 1X binding buffer was added to the cells and subsequently, 5 µL of Annexin V-DY634 conjugate and 5 µL of Propidium Iodide staining solution were added, followed by further incubation in the dark at room temperature for 15 minutes. Ultimately, 400 µL of 1X binding buffer solution was added for each sample prior to undergoing a flow cytometry analysis. All data were analyzed using FlowJo cell analysis software.

Western blotting

The human primary glioma cells (1 x 10^6 cells) were cultured in 6-well plates and treated with SP and Hesperetin at the desired concentration. After treatment, total protein was extracted using RIPA buffer, and the protein concentration was measured by the Bradford method. An equal amount of protein (30 µg) from each sample was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto nitrocellulose membranes. The membranes were then incubated for two hours in 5% non-fat milk Tris-buffered saline (TBS) as a blocking solution. After blocking, the membranes were incubated with anti-phospho-NF-κB p65 (Cell Signaling Technology) and anti-β-actin (Abcam) overnight at 4°C. The membranes were then washed with TBS and incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology) for 1 hour. Protein bands were then developed using the DAB substrate solution. Band intensity was finally quantified by densitometry using ImageJ densitometry software.

Enzyme-linked immunosorbent assay (ELISA) assay

The human primary glioblastoma cells were cultured in 96-well plates and treated with SP and Hesperetin at the desired concentration. After 24-hour treatment, the cell culture supernatant of each sample was collected and centrifuged at 12,000 rpm for 15 minutes at 4°C. The concentrations of IL-1β and TNF-α in the culture supernatant were measured by a commercial ELISA kit (R&D Systems) following the manufacturer's instructions. The experiments were carried out in triplicates

Statistics

The statistical software GraphPad Prism 8 was utilized to analyze variance (ANOVA), followed by post-hoc Tukey tests. A p-value less than 0.05 was deemed statistically significant. All data were indicated as mean ± standard deviation (SD).

Results

In vitro characterization of primary human glioblastoma cells

The primary human glioblastoma cells were extracted directly from resected tissue samples by a process based on mechanical disruption of tissue followed by enzymatic digestion with trypsin, cell centrifugation, and finally cell purification. **Figure 1A** indicates a representative image of the morphology of primary glioblastoma cells. For in vitro characterization, immunohistochemical expression of Ki67 was determined in primary cells. Ki-67 is a cell proliferation marker that is strongly associated with glioblastoma tumorigenesis(17). Immunocytochemistry results on early passage culture cells show that these cells were positive for ki67 as indicated by the green staining (**Figure 1B**).

Hesperetin reduced cell viability and induced apoptosis in primary glioblastoma Cells

The MTT assay was employed to determine the effects of Hesperetin (Figure 2A) on the viability of primary glioblastoma cells. Cells were exposed to various concentrations of Hesperetin (50, 100, 200, 400, 800,

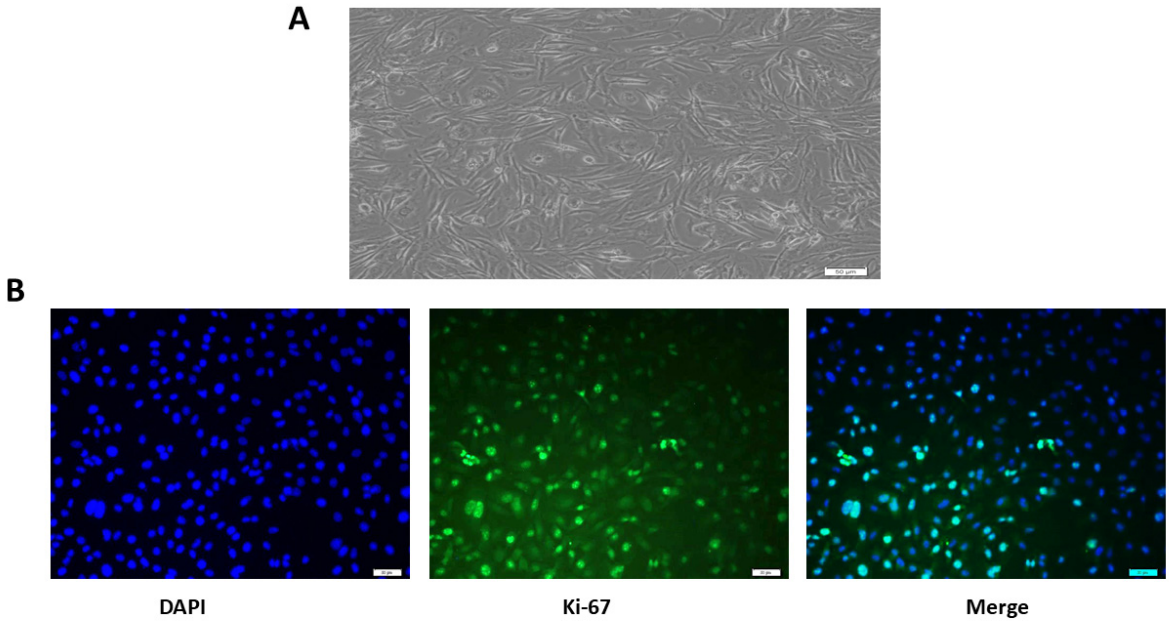

Figure 1. (A) A representative image of the morphology of human primary glioblastoma cells from passages between P3-P5. Scale=100 µm. **(B)** Immunohistochemical expression of Ki67 (green) in human primary glioblastoma cells. Nuclei were stained with DAPI (blue). The merged pictures are also indicated in the final column. Scale=50 µm.

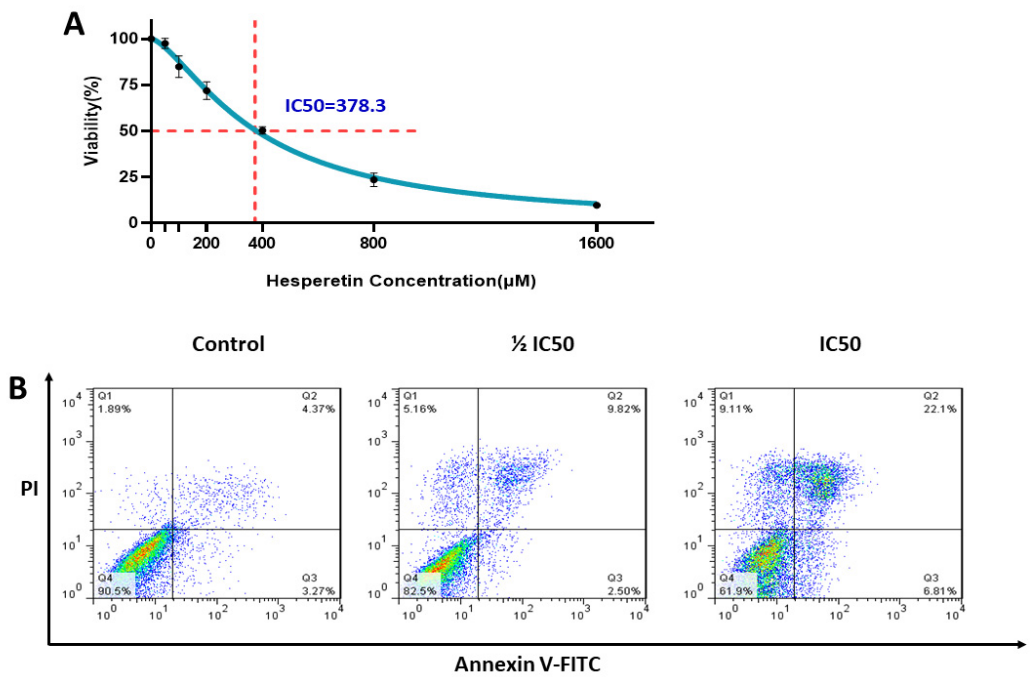

Figure 2. (A) The growth inhibitory effect of Hesperetin in primary glioblastoma cells. Cells were exposed to various concentrations of Hesperetin (50,100,200,400, 800, and 1600µM), and cell viability was determined by MTT assay after 24 hours. **(B)** The effect of Hesperetin ($^{1/2}$ IC50 and IC50) on primary glioblastoma cell apoptosis. Flow cytometry histogram showing the changes in the percentage of cells undergoing early apoptosis [(the lower right quadrant (Q3)] and late apoptosis [(upper right quadrant (Q2)] in each group.

and 1600 µM) for 24 hours. The MTT results showed that Hesperetin reduced cell viability in a dose-dependent manner, and the IC50 concentration of Hesperetin after a 24-hour incubation time was about 378.3 µM (Figure 2A). To investigate whether apoptosis is involved in the growth inhibitory effect of Hesperetin, flow cytometry was applied to examine the rate of cell apoptosis. As indicated in Figure 2B, the total percentages of cells

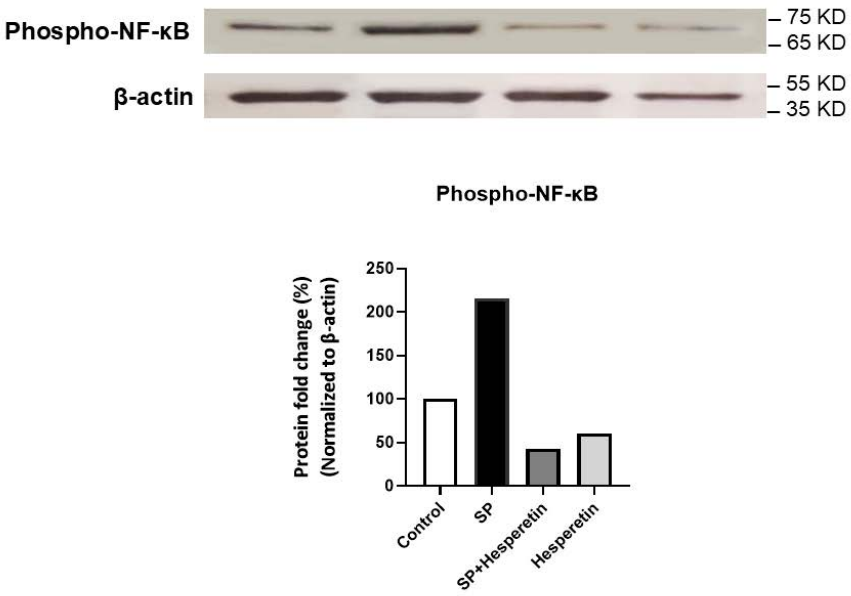

Figure 3. Hesperetin regulates SP-induced NF-κB activation. Primary glioblastoma cells were treated with the SP (100 nM) with/without Hesperetin, for 24 h, and then, the protein expression level of phosphor-NF-κB was measured by western blotting. Western blot was conducted two times, and a representative picture is indicated. Western blot bands were analyzed by Image J software.

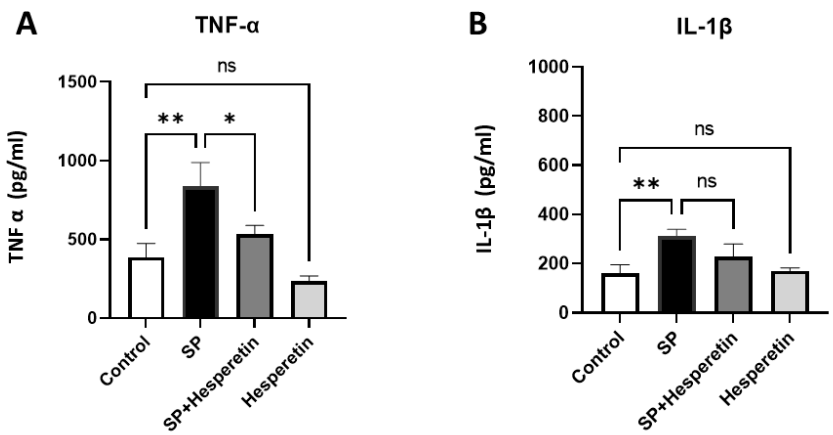

Figure 4. Hesperetin inhibits SP-induced production of TNF-α and IL-1β. Primary glioblastoma cells were treated with the SP (100 nM) with/without Hesperetin, for 24 h, and subsequently, the culture supernatant was separated to measure the protein levels of TNF-α **(A)** and IL-1β **(B)** by an ELISA kit. Statistical significance was determined at (*P<0.05, **P ≤ 0.01).

undergoing apoptosis were increased in Hesperetin-treated cells compared to the control group. Hesperetin increased the apoptotic rate (early + late apoptosis) from 7.64% in the control group to 12.32% in the ½ IC50 group and 28.82% in the IC50 group. Collectively, our results confirmed that Hesperetin induces anti-proliferative activity in primary glioblastoma cells. The sublethal doses of Hesperetin, about half of the IC50, were selected for the rest of the experimental tests

Hesperetin reduced SP-induced NF-κB activation and cytokine production

NF-κB, as a key transcriptional regulator of inflammatory genes, is involved in the pathogenesis of glioblastoma (18). We therefore investigated the inhibitory effect of Hesperetin on SP-induced NF-κB activation. In this case, primary glioblastoma cells were treated with SP (100 nM) with/without Hesperetin for 24 hours, and then the protein expression level of phospho-NF-κB was measured by western blotting. As shown in Figure 3, SP ncreases the protein expression of phospho-NF-κB *and Hesperetin effectively reversed this effect* . We further evaluated the NF-κB downstream inflammatory cytokines, including TNF-α and IL-1β, in response to SP and Hesperetin by ELISA. As shown in Figure 4, SP significantly increases the protein levels of TNF-α and IL-1β(**P ≤ 0.01). Hesperetin reduced SP-induced production of TNF-α (significant, *P < 0.05) and IL-1β (not meaningful)

Discussion

This study was undertaken to evaluate the SP-induced pro-inflammatory effects and the therapeutic effects of its inhibition by Hesperetin in GBM primary cells. Obtained findings indicated that SP enhances the pro-inflammatory responses in GBM as evidenced by increased protein expression of phosphorylated-NF-κB and the pro-inflammatory cytokines (IL-1β, TNF-α), while Hesperetin treatment reduced the effects of SP.

The SP/NK1R complex plays a crucial role in the inflammatory microenvironment of tumors (8). Overexpression of SP/NK1R has been observed in various inflammatory conditions (e.g., ulcerative colitis), thereby providing a supportive microenvironment for tumor development (19-21). Consistently, overexpression of SP/NK1R has been reported to be increased in colonic epithelial cells from patients with colitis-associated cancer (22). In our study, we investigated the effect of SP on cytokine production. We observed that SP increased the protein levels of pro-inflammatory cytokines (IL-1β, TNF-α). Excessive production of pro-inflammatory cytokines can result in the spread of inflammation, leading to tumor progression (23). IL-1β and TNF-α are critical glioma-derived factors triggering the inflammatory cycle in GBM and thus promoting GBM tumorigenesis (24). Therefore, SP-mediated production of pro-inflammatory cytokines is a possible oncogenic mechanism in GBM.

We also evaluated the effect of SP on NF-κB, a crucial transcriptional regulator of inflammatory mediators. NF-κB is known to be overactivated in GBM patients, contributing to tumor progression and treatment resistance (25). We observed that SP significantly enhanced NF-κB expression. Similar inflammatory responses mediated by SP have been observed in other inflammatory conditions as well. In head and neck cancers, SP has been shown to create an inflammatory tumor microenvironment and promote tumor progression by increasing the expression of chemotactic factors, pro-inflammatory cytokines, and activating the NF-κB signaling pathway (26). Consistent with this, SP has exerted potent inflammatory effects in U373 MG astrocytoma cells by activating NF-κB and its controlled target genes (27). Given that SP exacerbates inflammatory processes within the tumor microenvironment, identifying novel anti-inflammatory agents able to interfere with SP-associated inflammation is of great clinical significance.

Natural plant-derived compounds have been shown to provide significant health benefits in various pathological disorders, with lower toxicity and improved patient compliance compared to chemical agents (28). Several studies have highlighted the effectiveness of natural products in controlling SP-mediated inflammatory responses. In this line, Tsai et al. indicated that a green tea component, L-theanine, suppresses overactive bladder by inhibiting SP-induced oxidative stress and inflammation (29). Catechins, another component found in green tea, have been shown to reduce respiratory tract hyperactivity, oxidative stress, and proinflammatory response caused by SP released by thoracic vagus nerve stimulation in rats (30). The therapeutic potential of catechins in alleviating SP-induced neurogenic inflammation and oxidative injury following oil smoke exposure has also been reported (31).

Hesperetin is a natural flavonoid found in citrus fruits with strong anti-inflammatory and anticancer effects. The anti-glioblastoma effects of Hesperetin have been previously reported. According to Li et al., Hesperetin significantly reduced GBM U-251 and U-87 cell viability and induced GBM cell apoptosis via p38 MAPK activation (32). Consistently, Cheng et al. indicated that Hesperetin reduced GBM cell line GL261 proliferation and induced apoptosis through the PI3K/AKT and NF-κB pathways (33). In our study, we observed similar anti-proliferative effects of Hesperetin against GBM cells. However, in this study, human primary cells extracted directly from tissues were utilized instead of cell lines. This choice was made to improve the physiological relevance of the obtained in vitro data, as primary cells are more representative of tissues compared to cell lines. We further examined the therapeutic effect of Hesperetin against SP-induced inflammation in GBM primary human cells. Hesperetin attenuated SP-induced inflammation by suppressing NF-κB activation and production of inflammatory cytokines. Taken together, our findings highlight the role of SP-induced inflammatory responses in GBM pathology and provide insight into utilizing Hesperetin as an anti-inflammatory agent. Hesperetin is a natural flavonoid with significant benefits, including safety, accessibility, low production costs, and the ability to easily diffuse across the blood-brain barrier. Therefore, Hesperetin can be developed as a new potential therapeutic approach to better manage SP-associated inflammation in cancer patients as well as GBM

Conflict of Interests

The authors have nothing to declare.

Authors Contributions

Conceptualization: SE and SIH. Methodology: SE and AA. Formal analysis: SE, and AA. Writing—original draft preparation: SE; writing—review, and editing: All authors.; Supervision: SE. All authors have read and agreed to the published version of the manuscript.

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