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석 사 학 위 논 문

Mitigation of 3.5 GHz Electromagnetic Fields Radiation-induced Toxicity in BV2 Microglial Cells and Zebrafish Embryos by Biochanin A

계 명 대 학 교 대 학 원
의 학 과

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Mitigation of 3.5 GHz Electromagnetic Fields Radiation-induced Toxicity in BV2
Microglial Cells and Zebrafish Embryos by Biochanin A

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2024년 8월

Acknowledgment

First of all, I would like to express my highest gratitude to Allah for the blessings and mercies to finish my master degree. Next, I would like to devote my deepest gratitude and appreciation to my supervisor, Professor Byeong-Churl Jang, for his sincere and valuable guideline, advice, and never ending encouragement which made me better in what I do and improved.

I would also like to thank my thesis head committee chair, Professor Jong-Wook Park, and the committee member, Professor Ji-Hye Jang for the sincere guide and advise on my thesis.

Next, I would like to thank my parents for their continuous prayers, support and trust in me. I would not have sustained this far without their prayers and support. In addition, I would like to express my special thanks to my brothers who have supported me throughout this journey.

Last but not the least, I also particularly thank my fellow lab mates: Wang Saini, Shailashree Pachhapure, Nivethasri Lakshmana Perumal, Muhammad Amjad, and Muneer Hussain, for the willingness to help and share all the knowledges to me, for the valuable and memorable discussions, for the sleepless nights when we were working together, and all the fun we had during my study. It was always a pleasure working with them.

2024년 8월

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1. Introduction

1.1. Background of the Study:

High-frequencies of electromagnetic fields (EMF), ranging from 800 MHz to 300 GHz, are non-ionizing radiation emitted by various electronic devices and Wireless communication systems, such as smartphones, Wi-Fi routers, and microwave ovens. The widespread use of these devices and technologies has raised public concerns about potential health risks, particularly acute or chronic EMF exposure (1-3). Specifically, the brain is particularly susceptible to EMF exposure with its intricate electromagnetic and electrophysiological functions. Hence, this exposure can penetrate the skull and potentially cause neurotoxicity (4,5).

Studies have shown that the effects of EMF radiation at specific frequencies could affect brain tissue and neuronal function. For instance, exposure to EMF at 2.45 GHz for 2, 24, and 48 h leads to DNA damage and alterations in the central nervous system (CNS) (6). Furthermore, an epidemiological study revealed that long-term use of mobile phones at 1.9 GHz could increase the risk of brain tumor (7), and exposure to 1.8 GHz EMF for 24 h can induce oxidative damage in primary cultured neurons (8). Of further note, several in vitro and in vivo studies have indicated potential mechanisms and factors associated with EMF-induced neurotoxicity, including the phosphorylation (activation) of stress-activated protein kinases (SAPKs) and an elevation of reactive oxygen species (ROS) (9-11).

Microglial cells are the primary innate immune cells of the CNS,

acting as the first line of defense against infections and injuries (12). Their dysfunction has been linked to various neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (13-15). At present, EMF-induced microglial cell toxicity and its regulatory mechanisms as well as the natural substances that could inhibit it are remain poorly understood.

Biochanin A (4'-methoxy-5, 7-dihydroxy isoflavone) is a natural dietary isoflavone derived from edible plants such as soybean, red clover, peanuts, and other plants (16-17). Abundant pharmacological and biological properties of biochanin A, including as neuroprotective (18,19), antioxidant (20), anti-inflammation (21), and anti-cancer (22) have been well documented. However, the regulation of EMF-induced toxicity in BV2 cells and zebrafish by biochanin A remains unknown to date. In the present study, I investigated whether EMF exposure at 3.5 GHz, which 5th generation (5G) smartphones or microwave ovens can emit, has toxic effects on BV2 mouse microglial cells and zebrafish embryos and whether biochanin A can counteract these effects.

1.2. Aims of The Study:

1. To investigate whether exposure to 3.5 GHz EMF induces toxicity in BV2 microglial cells and zebrafish embryos.
2. To understand the molecular and signaling mechanisms underlying 3.5 GHz EMF-induced toxicity in BV2 microglial cells.
3. To evaluate whether biochanin A protects against 3.5 GHz EMF-induced toxicity in BV2 microglial cells and zebrafish

embryos.

4. To elucidate molecular and signaling mechanisms by which biochanin A mitigates 3.5 GHz EMF-induced toxicity of BV2microglial cells.

2. Materials and Methods

2.1. Chemicals and Antibodies:

Cell culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from Welgene company (Daegu, Korea). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was obtained from Promega company (Madison, WI, USA). Enzyme-linked chemiluminescence (ECL) western detection reagents were bought from ThermoScientific (Waltham, MA, USA). SP600125 and SB203580 were obtained from BiomolResearch Lab (Plymouth Meeting, PA, USA). Cell culture plastic wares were purchased from SPL Life Sciences (Gyeonggi-do, Korea). Primary anti- β -actin antibody (A5441) and dichlorodihydrofluoresceindiacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary anti-phosphorylated (p)-eukaryotic initiation factor (eIF)-2 α (S51) antibody was purchased from Abcam (Cambridge, UK). Primary antibodies of total (T)-eIF-2 α , p-JNK-1/2 (T183/Y185), T-JNK-1/2, p-p38 MAPK, T-p38 MAPK (T180/Y182), p-MK-2 (T334), and T-MK-2 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

2.2. Cell Culture:

BV2 murine microglial cells were cultured in DMEM-high glucose (Welgene, Daegu, Korea) and supplemented with 10% heat-inactivated

FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. BV2 cells were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂.

2.3. Zebrafish Lines and Husbandry:

Zebrafish were maintained under standard conditions as previously described (23). A wild-type (WT) zebrafish line was provided by the Zebrafish Center for Disease Modeling (ZCDM), Korea. The change from dark to light incited zebrafish mating and spawning.

2.4. 3.5 GHz EMF Exposure System and Exposure to BV2

Cells:

A manufactured and specifically designed apparatus was used to expose BV2 cells to 3.5 GHz EMF with a power density of 5 mW/cm² (Figure 1A). The apparatus consists of an EMF generator, an EMF reader, and a chamber connected to a wire patch cell (WPC) antenna from the EMF generator. The EMF generator operated at 3.5 GHz, which the EMF reader monitored. The chamber is equipped with humidity and temperature systems and was set up to have 50% humidity and 37 °C during EMF exposure to BV2 cells.

2.5. 3.5 GHz EMF Exposure System and Exposure to Zebrafish

Embryos:

Zebrafish embryos at 3 or 4.5 hours post fertilization (hpf) were arrayed in a 6 well-plate in which each well contained 10 or 15 embryos and 2 mL egg water (0.6 g/L sea salt). The zebrafish embryos were placed and exposed to 3.5 GHz EMF in the specially designed apparatus and chamber. The chamber was set up to have 80% humidity and 28 °C during EMF exposure to embryos. Alternatively, considering that humans are daily exposed to EMF produced by using many electronic devices, including mobile phones, in an opening state and environment, 4.5 hpf zebrafish embryos were pretreated without or with biochanin A at the indicated doses for 1.5 h. They were then exposed to 3.5 GHz EMF produced by two mobile phones without or with biochanin A at the same concentrations for an additional 2 h, then measured by the number of survived or dead embryos and the change of their morphologies under a light microscope. After 22 h without 3.5 GHz EMF exposure, the number of survived or dead embryos and the change of their morphologies were also measured under a light microscope. During all exposure sessions, the lids of the plates containing cells or embryos were kept open to enhance EMF penetration.

2.6. Measurement of Intracellular ROS Levels:

Levels of cellular ROS were detected with DCFH-DA, a fluorogenic

dye, under exposure to 3.5 GHz EMF or 0.2 mM H₂O₂, a known ROS inducer, as a positive control. DCFH-DA is a nonfluorescent and nonpolar compound that permeates cell membranes. Once inside, deacetylation by cytosol esterase will form DCFH, rapidly oxidizing to the fluorescent DCF in the presence of ROS. For the experiment, BV2 cells were plated at a density of 3.0×10^5 cells per 1 mL in a 12-well plate overnight. The cells underwent pretreatment with or without 10 μ M of biochanin A for 1.5 h, followed by exposure to 3.5 GHz EMF or H₂O₂ for an additional 1 or 2 h. After pretreatment, cells were incubated with 10 μ M of DCFH-DA for 30 min, then washed twice with PBS. ROS levels were then observed using an inverted fluorescence microscope (Olympus Life Science, Shinjuku, Tokyo, Japan). Fluorescence intensity was quantified using Image J software, with results expressed as percentage intensity relative to controls.

2.7. Preparation of Whole-cell Lysates:

After treatments, BV2 cells were washed twice with PBS supplemented with 1 mM Na₃VO₄ and 1 mM NaF and subsequently exposed to cell lysis buffer [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium vanadate, 1 mg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride]. The cells were then harvested and centrifuged for 15 min at 4 °C and $13,000 \times g$. The supernatant was saved, and its protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Tempe, AZ, USA) at 560 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

2.8. Western Blot Analysis:

Proteins (40 μ g) were separated using 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were washed with TBS solution (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and then blocked in TBST containing 5% non-fat dried milk. They were incubated overnight at 4 °C with primary antibodies at a dilution of 1:2000 for p-JNK-1/2, T-JNK-1/2, p-p38 MAPK, T-p38 MAPK, p-MK-2, T-MK-2, p-eIF-2 α , T-eIF-2 α , and 1:10000 for actin. Afterward, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and washed again with TBST. Immunoreactivity was detected using enhanced chemiluminescence (ECL) reagents. The consistent expression of actin was used to confirm equal protein loading across samples.

2.9. Statistical Analysis:

Cell count analysis was measured in triplicate and repeated three times. The results were expressed as the mean \pm standard error (SE). One-way ANOVA was used to compare the significance of the difference. All significant testing was established on a p-value of <0.05.

3. Results

3.1. Exposure to 3.5 GHz EMF for Either 2 or 24 h Significantly Inhibited the Growth of BV2 Microglial Cells:

Using a specially designed apparatus for EMF exposure (Figure 1A), I initially examined whether exposure to 3.5 GHz EMF for 2 or 24 h affected the growth of BV2 cells using cell count assay. Results revealed that exposure to 3.5 GHz EMF for 2 or 24 h significantly inhibited the growth of BV2 cells compared with sham-exposed cells (Figure 1B). Microscopic observations also further confirmed the capability of 3.5 GHz EMF exposure for 2 or 24 h to markedly suppress the growth of BV2 cells (Figure 1C). Given the rapid and pronounced suppressive effects observed on BV2 cell growth, I selected the 2 h (and earlier than 2 h) exposure period of 3.5 GHz EMF for further investigations.

3.2. Exposure to 3.5 GHz EMF Led to the Altered Phosphorylation and Expression Levels of JNK-1/2, p38 MAPK, eIF-2 α , and ROS Production in BV2 Microglial Cells:

Next, to scrutinize the molecular and signaling mechanisms or factors underlying the cytotoxic effects of 3.5 GHz EMF on BV2 cells, I investigated the effect of exposure to 3.5 GHz EMF on the

phosphorylation and expression levels of stress-related proteins, including JNK-1/2, p38 MAPK, and a translation-regulatory protein, eIF-2 α , in BV2 cells using Western blot analysis. I observed that exposure to 3.5 GHz EM for 1 or 2 h significantly increased the phosphorylation levels of JNK-1/2 and p38 MAPK in BV2 cells compared with sham-exposed cells (Figure 2A). Shorter exposures to 3.5 GHz EMF for 0.25 or 0.5 h did not affect the phosphorylation levels of JNK-1/2 and p38 MAPK in these cells. Additionally, compared with sham-exposed cells, there was a notable increase in levels of phosphorylated eIF-2 α in BV2 cells exposed to 3.5 GHz EMF for 2 h. Despite these changes in phosphorylation, the total expression levels of JNK-1/2, p38 MAPK, and eIF-2 α remained constant across these experimental conditions. The expression levels of actin, used as a control, remained stable under all tested conditions.

Next, using fluorescence microscopy, I investigated whether exposure to 3.5 GHz EMF elicits oxidative stress by measuring ROS levels in BV2 cells. This study used H₂O₂, an ROS inducer (24), as a positive control. As shown in Figure 2B, exposure to H₂O₂ at 0.2 mM for 1 or 2 h led to an elevation of ROS in BV2 cells. Distinctly, while exposure to 3.5 GHz EMF for 1 h did not elevate ROS levels in BV2 cells, the 2 h exposure increased ROS levels in these cells. Fluorescence microscopic observation further demonstrated the ability of 3.5 GHz EMF or H₂O₂ exposure at times tested to induce green fluorescence (ROS production) in BV2 cells (Figure 2C).

3.3. Activation of JNK-1/2 and p38 MAPK was Crucial for 3.5 GHz EMF-induced Growth Inhibition of BV2 Murine Microglial Cells:

Next, I tested the role of phosphorylated (activated) JNK-1/2 or p38 MAPK in 3.5 GHz EMF-induced growth inhibition of BV2 cells using a pharmacological inhibition study with SP600125, an inhibitor of JNK-1/2 or SB203580, an inhibitor of p38 MAPK. For this, BV2 cells were exposed to 3.5 GHz EMF in the absence or presence of SP600125 (25 μ M) or SB203580 (25 μ M) for 2 h, followed by measurement of the number of survived cells and the phosphorylation levels of JNK-1/2 or MK-2, a downstream effector of p38 MAPK, in these conditioned cells. As shown in Figure 3A, both treatments with SP600125 and SB203580 significantly blocked 3.5 GHz EMF-induced growth inhibition of BV2 cells. Microscopic observations further confirmed the capability of SP600125 and SB203580 to block 3.5 GHz EMF-induced growth inhibition of BV2 cells (Figure 3B). As shown in Figure 3C, treatment with SP600125 significantly suppressed 3.5 GHz EMF-induced phosphorylation of JNK-1/2 without affecting their total protein expression levels in BV2 cells. Moreover, SB203580 significantly blocked 3.5 GHz EMF-induced phosphorylation of MK-2 without affecting its total protein expression levels in BV2 cells.

3.4. Biochanin A Blocked 3.5 GHz EMF-induced Growth Inhibition in BV2 Murine Microglial Cells:

Next, I evaluated the effect of biochanin A at different concentrations (2.5, 10, and 40 μ M) on the growth inhibition of BV2 cells induced by 3.5 GHz exposure. BV2 cells were pretreated with biochanin A at the designated concentrations for 1.5 h, followed by exposure to 3.5 GHz EMF for an additional 2 h, with or without the same concentrations of biochanin A. As illustrated in Figure 4A, exposure to 3.5 GHz EMF for 2 h significantly reduced BV2 cell growth compared to sham-exposed cells. Conversely, biochanin A treatment at 2.5 and 10 μ M mitigated 3.5 GHz EMF-mediated growth inhibition of BV2 cells. Microscopic observations further demonstrated the ability of biochanin A to dose-dependently interfere with 3.5 GHz EMF-induced growth suppression of BV2 cells (Figure 4B).

3.5. Biochanin A Inhibited 3.5 GHz EMF-induced Phosphorylation of JNK-1/2 and ROS Production in BV2 Murine Microglial Cells:

Next, to understand how biochanin A at 10 μ M blocks 3.5 GHz EMF-induced growth inhibition in BV2 cells, I investigated whether biochanin A at 10 μ M modulates the altered phosphorylation and expression levels of JNK-1/2 and p38 MAPK in BV2 cells in response to 2 h exposure to 3.5 GHz EMF. As depicted in Figure 5A, biochanin A at the concentration tested substantially abolished the ability of 3.5 GHz EMF to induce the phosphorylation levels of JNK-1/2 without

changing respective total protein expression levels in BV2 cells. However, biochanin A did not affect the phosphorylation of p38 MAPK induced by 3.5 GHz EMF, nor did it influence the total protein expression levels of p38 MAPK in BV2 cells. Expression levels of control actin protein remained constant under these experimental conditions.

Next, I tested whether biochanin A at 10 μ M inhibits 3.5 GHz EMF-induced ROS production in BV2 cells. As shown in Figure 5B, biochanin A at 10 μ M significantly suppressed 3.5 GHz EMF-induced ROS production in BV2 cells. Fluorescence microscopic observation also exhibited the capability of biochanin A to inhibit ROS generation induced by 3.5 GHz EMF in BV2 cells (Figure 5C).

3.6. Biochanin A Blocked 3.5 GHz EMF-induced Zebrafish Embryo Toxicity:

To next see whether 3.5 GHz EMF exposure elicits *in vivo* toxicity and biochanin A defends it, I investigated whether exposure to 3.5 GHz EMF in the specially designed apparatus and chamber affects the development of zebrafish embryos over time. Compared with sham-exposed zebrafish embryos, the 2 h exposure to 3.5 GHz EMF caused severe toxicities in zebrafish embryos (data is not shown). Alternatively, I further challenged whether exposure to 3.5 GHz EMF produced by two mobile phones influences the development of zebrafish embryos in the open state and whether biochanin A regulates it. As shown in Figure 6A, the sham-exposed zebrafish embryos at 2 h showed normal embryo development. At the same time, exposure to 3.5 GHz EMF

produced by two mobile phones elicited severe impairment of zebrafish embryo development in the open state. Notably, biochanin A at 10 μ M vastly attenuated two mobile phones derived 3.5 GHz EMF-induced zebrafish embryotoxicity at times tested. As further shown in Figure 6B, microscopic observations demonstrated that while the sham-exposed zebrafish embryos at 2 or 24 h showed normal embryo development, at the same time, exposure to 3.5 GHz EMF produced by two mobile phones elicited severe impairment of embryo development in the open state. Biochanin A at 10 μ M vastly abolished the ability of two mobile phones derived 3.5 GHz EMF to cause zebrafish embryotoxicity at times tested.

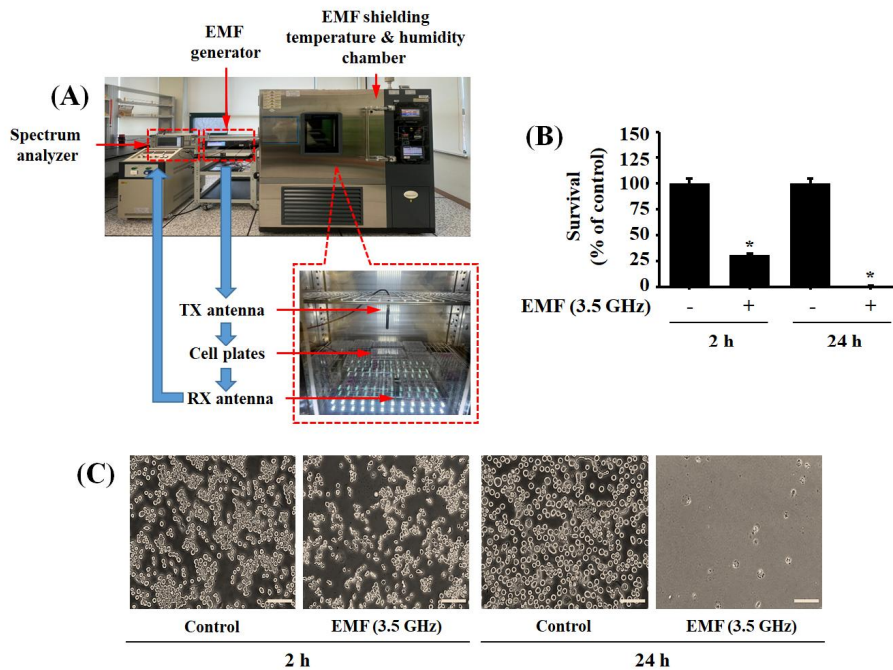


Figure 1. The EMF exposure system and the effect of 3.5 GHz EMF on the growth of BV2 cells. (A) The specifically designed 3.5 GHz EMF exposure system and apparatus. (B) BV2 cells were exposed to either sham or 3.5 GHz EMF for 2 or 24 h. The number of surviving cells was analyzed using cell count analysis. Data are presented as the mean \pm standard errors (SE) from three independent experiments. *: $p < 0.05$ indicates a significant difference from the control values (no 3.5 GHz EMF exposure). (C) A representative image showing morphological changes in BV2 cells exposed to sham or 3.5 GHz EMF as described in (B).

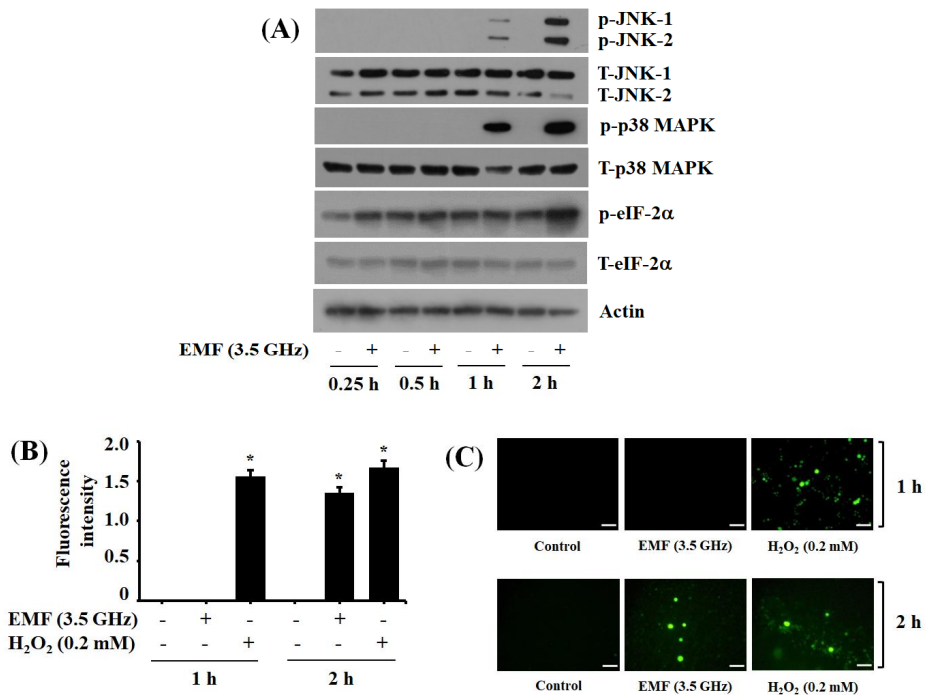


Figure 2. Effect of 3.5 GHz EMF exposure on the phosphorylation and expression of stress-related and translation regulatory proteins, as well as ROS production in BV2 cells. (A) BV2 cells were exposed to 3.5 GHz EMF for specified durations. At each time point, whole-cell lysates were prepared and analyzed by Western blotting using antibodies specific to JNK-1/2, p38 MAPK, and eIF-2 α . (B) BV2 cells were exposed to 3.5 GHz EMF or H₂O₂ for 1 or 2 h. At each time point, cells were loaded with DCFH-DA, and DCF fluorescence intensity (ROS generation) in the conditioned cells was measured using fluorescence microscopy. Quantification was conducted using % intensity for each picture using image J. The DCF fluorescence is presented as the mean \pm

standard deviation (SD) (n=3) from the histogram statistics. *: $p < 0.05$ vs. control (0 min). (C) Intracellular ROS levels of the conditioned cells in (B) were measured by fluorescence microscope.

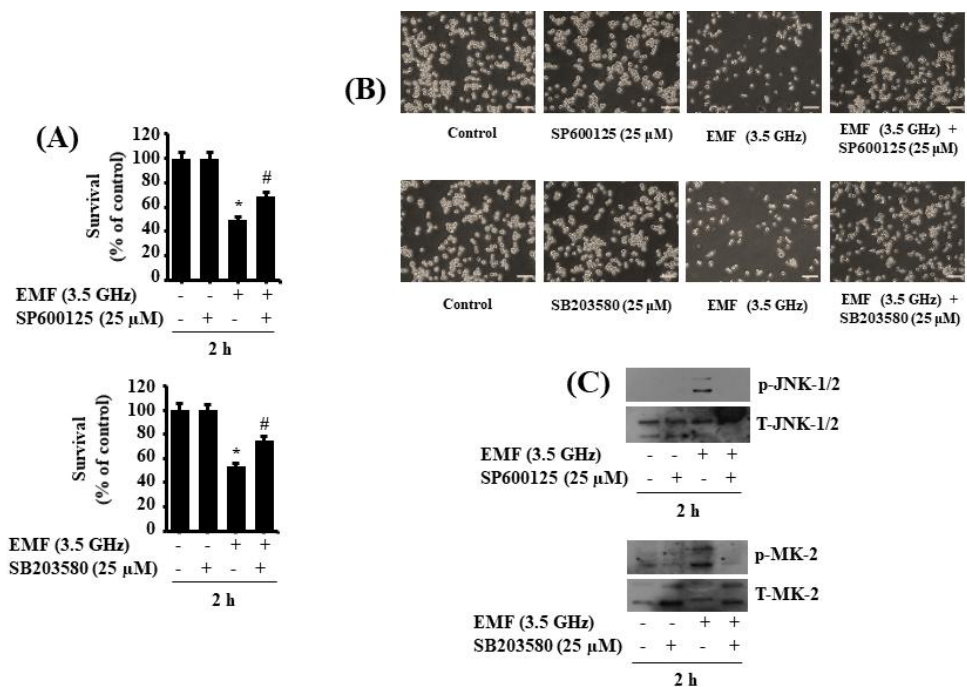


Figure 3. Effect of SP600125 or SB203580 on 3.5 GHz EMF-induced growth suppression and phosphorylation of JNK-1/2 and MK-2 in BV2 cells. (A) BV2 cells were exposed to 3.5 GHz EMF without or with SP600125 (25 μM), a JNK-1/2 inhibitor, or SB203580 (25 μM), a p38 MAPK inhibitor, for 2 h. The number of surviving cells was analyzed using cell count analysis. Data represent the means \pm SE of three independent experiments. *: $p < 0.05$ compared with the control values (no 3.5 GHz EMF exposure). #: $p < 0.05$ compared with the values of 3.5 GHz EMF exposure (no drug). (B) A representative image of morphological changes in the conditioned cells in (A). (C) BV2 cells were exposed to 3.5 GHz EMF without or with SP600125 (25 μM) or SB203580 (25 μM) for 2 h. Whole-cell lysates from the conditioned cells were prepared and analyzed using Western blotting with antibodies.

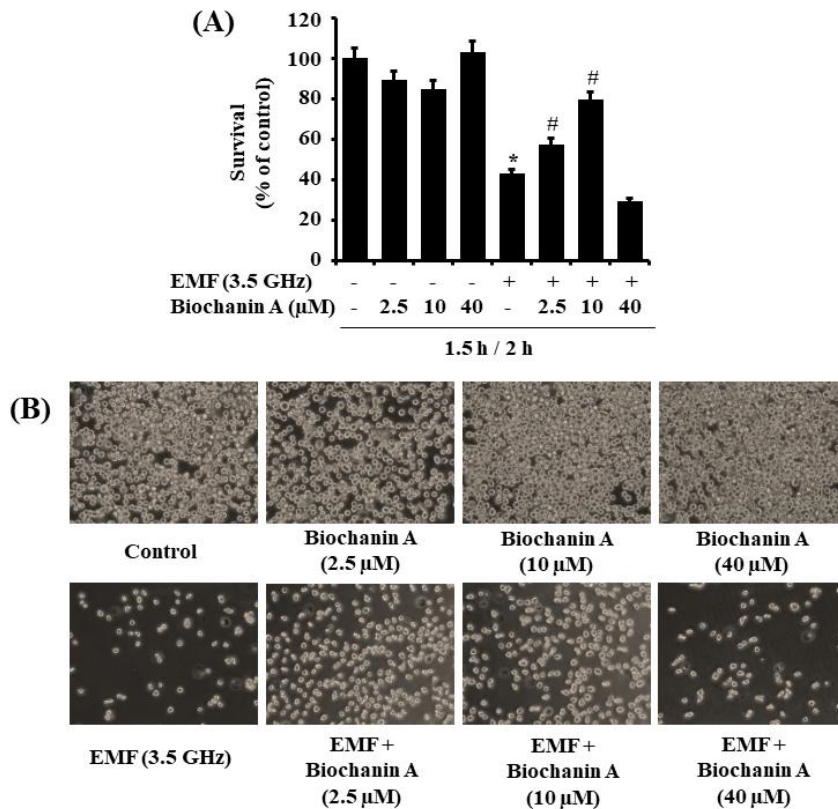


Figure 4. Effect of 3.5 GHz EMF and biochanin A on the growth of BV2 cells. (A) BV2 cells were pretreated without or with biochanin A at the designated concentrations for 1.5 h and exposed to 3.5 GHz EMF in the absence or presence of biochanin A at the same doses for an additional 2 h. The number of surviving cells was analyzed using cell count analysis. Data represent the means \pm SE of three independent experiments. *: $p < 0.05$ compared with the control values (no 3.5 GHz EMF exposure). #: $p < 0.05$ compared with the values of 3.5 GHz EMF exposure (with EMF and biochanin A). (B) A representative image of morphological changes in sham or 3.5 GHz EMF-exposed BV2 cells in (A).

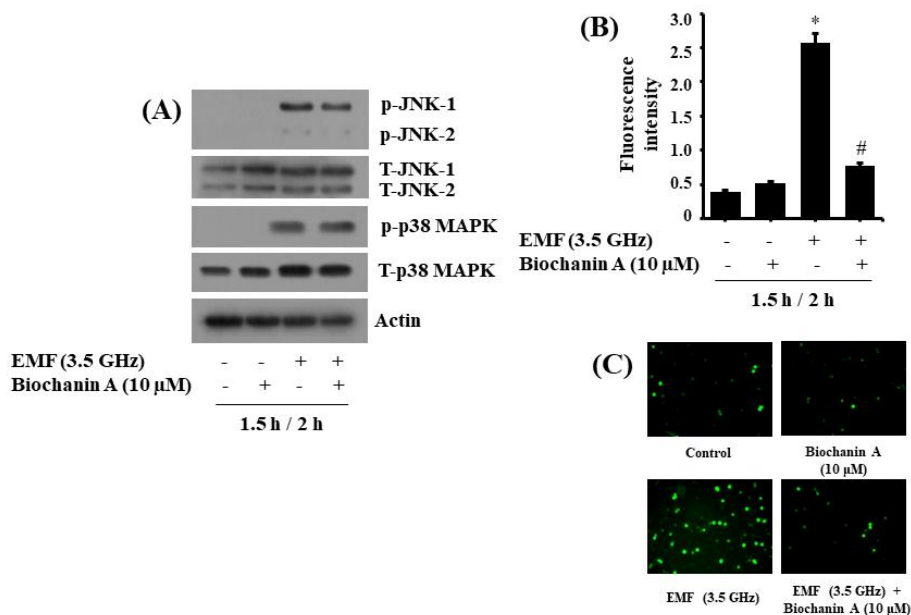


Figure 5. Effect of biochanin A on 3.5 GHz-induced phosphorylation and expression of JNK-1/2 and p38 MAPK, as well as ROS production in BV2 cells. (A) BV2 cells were pretreated without or with biochanin A at 10 μ M for 1.5 h and exposed to 3.5 GHz EMF in the absence or presence of biochanin A at the same concentrations for an additional 2 h. Whole-cell lysates from the conditioned cells were prepared and analyzed using Western blotting with antibodies. (B) BV2 cells were pretreated without or with biochanin A for 1.5 h and exposed to 3.5 GHz EMF without or with biochanin A at 10 μ M for an additional 2 h. The conditioned cells' DCF fluorescence intensity (ROS generation) and their quantification were measured using fluorescence microscopy and image J, respectively. The DCF fluorescence is presented as mean \pm standard deviation (SD) (n=3) from the histogram statistics. *: p < 0.05 compared with the values of control (no 3.5 GHz

EMF exposure). #: $p < 0.05$ compared with the values of 3.5 GHz EMF exposure (with EMF and biochanin A). (C) Intracellular ROS levels of the conditioned cells in (B) were measured by fluorescence microscope.

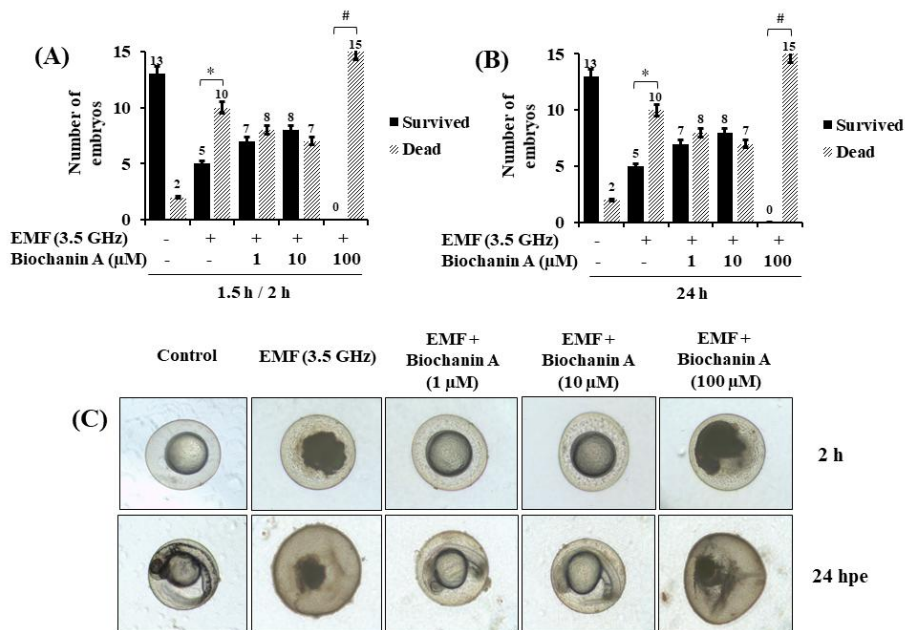


Figure 6. Effects of 3.5 GHz EMF and biochanin A on the development of zebrafish embryos. 4.5 hours post-fertilization (hpf) embryos were arranged in a 6 well-plate, with each well containing 15 embryos (n=15) and 2 mL egg water (0.6 g/L sea salt). Embryos were pretreated without or with biochanin A at designated concentrations for 1.5 h and exposed to 3.5 GHz EMF in the absence or presence of biochanin A at the same doses for an additional 2 h. The number of surviving or dead embryos (A,B) and changes in their morphologies (C) were assessed under a light microscope. Subsequently, the number of surviving or dead embryos and alterations in their morphologies were further evaluated under a light microscope at 22 h post-exposure (hpe) in the absence of 3.5 GHz EMF exposure. Data represent the means \pm SE (n=15 embryos

per group). *: $p < 0.05$ compared with the control values (no 3.5 GHz EMF exposure). #: $p < 0.05$ compared with the values of 3.5 GHz EMF exposure (with EMF and biochanin A).

4. Discussion

EMF exposure to the human brain from different sources such as cell phones, microwaves, Wi-Fi, and other wireless devices has grown scientific interest in recent decades. The advancement of 5G networks, which utilize a 3.5 GHz band, has raised concerns regarding its potential impacts on human health. However, research specifically addressing the toxic effects of acute 3.5 GHz EMF on brain cells, particularly microglial cells, is limited. This study explores the harmful effects of 3.5 GHz EMF acute exposure on BV2 microglial cells and zebrafish embryos and how biochanin A regulates these effects. Here, I report that acute exposure to 3.5 GHz EMF inhibits the growth of BV2 cells and causes severe toxicity in zebrafish embryos, and biochanin A blocks it. The present study further shows that biochanin A's blockage effect on 3.5 GHz EMF-induced BV2 cytotoxicity is mediated by regulating JNK-1/2 phosphorylation and ROS production.

Through initial experiments, I have shown that the 2 h exposure to 3.5 GHz EMF elicits BV2 cytotoxicity, characterized by its growth-inhibition effects on BV2 cells. Research indicates that exposure to EMF can alter neuronal activity, including increased the production of ROS leading to oxidative stress (25,26). It is important to note that exposure to 3.5 GHz EMF causes cellular stress and high ROS production in live fibroblasts and keratinocytes (27). Interestingly, results from this previous study parallel with my

current study, which shows that the 2 h exposure to 3.5 GHz EMF leads to an elevation of ROS in BV2 cells, but NAC, an antioxidant, significantly attenuates 3.5 GHz EMF-induced ROS production and growth inhibition of BV2 cells herein, it is likely that production of ROS is essential for the cytotoxic effects induced by 3.5 GHz EMF in BV2 cells. It is remarkable that several studies consistently demonstrated that biochanin A possesses antioxidant properties (28,29). Biochanin A regulation of 3.5 GHz EMF-induced ROS production and BV2 cytotoxicity is not fully elucidated. Of interest, the present study illustrates that biochanin A at 10 μ M significantly inhibits 3.5 GHz EMF-induced ROS production and BV2 cytotoxicity, emphasizing that biochanin A's protective effect against 3.5 GHz EMF-induced cytotoxicity in BV2 cells is partly attributed to its antioxidant properties.

SAPKs, including JNK-1/2 and p38 MAPK, are a group of serine/threonine protein kinases that play a critical role in cellular responses to various stress stimuli, including EMF exposure (30,31). Exposure to 2.45 GHz EMF has been reported to activate SAPKs in the brain, triggering stress response pathways that may lead to neuronal damage and cell death (9). Evidence suggests that JNK-1/2 and p38 MAPK are activated by EMF exposure, mediating the toxic effects of 918 MHz EMF on astrocytes (32). Supporting this, I also confirmed the ability of 3.5 GHz EMF exposure to induce the phosphorylation (activation) of JNK-1/2 and p38 MAPK in BV2 cells. Considering the present findings that SP600125, a JNK-1/2 inhibitor, or SB203580, a p38 MAPK inhibitor, significantly abrogates 3.5 GHz

EMF-induced BV2 cytotoxicity, it is evident that activating JNK-1/2 and p38 MAPK is crucial for the toxic effects of 3.5 GHz EMF in BV2 cells. However, biochanin A regulation of 3.5 GHz EMF-induced activation of SAPKs in BV2 cells remains to be clarified. In the present study, biochanin A at 10 μ M substantially abolished 3.5 GHz EMF-induced activation of JNK-1/2 but not p38 MAPK in BV2 cells. Thus, biochanin A's protective effect on 3.5 GHz EMF-induced BV2 cytotoxicity is further likely due to inhibition of JNK-1/2.

eIF-2 α is crucial in regulating translation initiation (33). The phosphorylation of eIF-2 α , an inactive form of the protein (34), can result in the global inhibition of protein synthesis in cells (35), and its sustained phosphorylation often leads to cellular dysfunction and apoptosis (36). In the current study, exposure to 3.5 GHz EMF for 2 h leads to increased phosphorylation of eIF-2 α in BV2 cells.

The potential biological risks of EMF exposure at various frequencies on numerous organisms, including zebrafish, have been suggested (37). For instance, cell phone-generated EMF affects the zebrafish's locomotor activity, behavior, development, and physiology (38). There is limited information on the toxicity of zebrafish embryos exposed to EMF at 3.5 GHz. In the present study, I have demonstrated that two mobile phones-derived 3.5 GHz EMF exposure for 2 or 24 h causes severe toxicity of zebrafish embryos while biochanin A at 1 and 10 μ M markedly attenuates it. These results emphasize that biochanin A's protective effect on 3.5 GHz EMF-induced toxicity in zebrafish embryos extends beyond its impact in BV2 cells, highlighting its potential as a broad-spectrum protective agent

against EMF-induced damage.

5. Summary

These results demonstrate that acute exposure to 3.5 GHz EMF causes BV2 cytotoxicity and zebrafish embryotoxicity. In contrast, biochanin A blocks it, and biochanin A's blockage effect on BV2 cytotoxicity is mediated by regulating JNK-1/2 phosphorylation and ROS production. The present study advocates that biochanin A can be utilized as a promising novel therapeutic agent against neurotoxic pathologies or diseases where microglial cell injury or damage is caused by acute exposure to 3.5 GHz EMF.

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Mitigation of 3.5 GHz Electromagnetic Fields Radiation-induced Toxicity in BV2 Microglial Cells and Zebrafish Embryos by Biochanin A

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(Abstract)

Emerging evidence highlights the biological risks associated with electromagnetic fields (EMF) generated by electronic devices. Biochanin A is a naturally occurring isoflavone compound in plants like red clover. The toxic effects and mechanisms induced by exposure to EMF on microglial cells and natural substances that inhibit it are limited. This study explored the toxicological effects of exposure to 3.5 GHz EMF radiation, commonly emitted by smartphones working in 5G communication or cooking using microwave oven, on BV2 mouse brain microglial cells and examined the protective effect of biochanin A in it.

Of note, acute (2 h) exposure to 3.5 GHz EMF significantly inhibited the growth of BV2 cells, along with elevating levels of reactive oxygen species (ROS) and phosphorylation of JNK-1/2, p38 MAPK, and eIF-2 α . Remarkably, biochanin A at 2.5 or 10 μ M blocked 3.5 GHz EMF-induced growth inhibition of BV2 cells. Biochanin A at 10 μ M further suppressed 3.5 GHz EMF-induced JNK-1/2 phosphorylation and ROS production in BV2 cells. In contrast, biochanin A at 10 μ M did not affect 3.5 GHz EMF-induced p38 MAPK phosphorylation in BV2 cells. Of importance, acute exposure to 3.5 GHz EMF induced severe toxicity in zebrafish embryos, and biochanin A at 10 μ M effectively blocked it. In conclusion, these results demonstrate that acute exposure to 3.5 GHz EMF causes strong BV2 cytotoxicity and zebrafish embryotoxicity and biochanin A mitigates them, mediated through control of JNK-1/2 phosphorylation and ROS production.

바이오차닌 A에 의한 3.5 GHz 전자파 유도 BV2 뇌미세아교세포 및 제브라피쉬 배아 독성 완화

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(초록)

최근 연구에서 다양한 전자기기로부터 일상생활 중 방출되는 전자파의 생물학적 위해성이 강조되고 있다. 천연물 바이오차닌 A는 레드 클로버와 같은 식물에서 발견되는 이소플라본 화합물이다. 현재 전자파의 뇌미세아교세포 독성 효과 및 기전이 불분명하고, 또한 전자파 유발 뇌미세아교세포 독성을 억제(감소)할 수 있는 천연물질 역시 제한적이다. 이에 본 연구에서 5G 통신 스마트폰 또는 전자레인지에서 방출되는 3.5 GHz 주파수의 EMF 노출에 따른 BV2 마우스 뇌미세아교세포 독성과 제브라피쉬 배아 독성을 분석하고 이에 대한 바이오차닌 A의 보호 효과를 조사하였다.

흥미롭게도 3.5 GHz EMF를 2 시간 노출 시 BV2 세포의 수적 감소와 함께 이들 세포내 활성산소종 증가 및 JNK-1/2, p38 MAPK, eIF-2 α 의 인산화 증가가 나타났다. 놀랍게도, 바이오차닌 A를 2.5 또는 10 μ M 투여 시 전자파에 의한 BV2 세포의 수적 감소가 크게 완화되었다. 또한 바이오차닌 A를

10 μ M 투여 시 전자파 노출시킨 BV2 세포내 JNK-1/2 인산화 및 활성산소종 증가를 크게 억제하였다. 이와는 달리 바이오차닌 A를 10 μ M 투여 시 전자파 노출시킨 BV2 세포내 p38 MAPK 인산화 증가는 변화를 보이지 않았다. 중요하게도 3.5 GHz EMF를 2 시간 노출 시 제브라피쉬 배아 독성이 크게 나타났으나, 바이오차닌 A를 10 μ M 투여 시 전자파 유발 제브라피쉬 배아 독성이 크게 완화되었다. 결론적으로, 단시간 전자파 (3.5 GHz) 조사가 BV2 뇌미세아교세포 및 제브라피쉬 배아에서 강한 독성을 유발하였고, 바이오차닌 A는 이러한 전자파 유발 BV2 뇌미세아교세포 및 제브라피쉬 배아 독성에 대해 강한 보호효과를 나타냈다. 바이오차닌 A는 (JNK-1/2 인산화 및 활성산소종 감소 통해) 급성 전자파 유발 뇌미세아교세포 독성을 완화함으로써 단시간 (또는 장기간) 전자파 유발 뇌(세포)손상 또는 질환의 예방/치료제로 사용될 수 있음을 제안한다.