Effects of combined radiofrequency field exposure on amyloid-beta–induced cytotoxicity in HT22 mouse hippocampal neurones

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ABSTRACT

Alzheimer's disease (AD) is the most common progressive and irreversible neurodegenerative disease and it is caused by neuronal death in the brain. Recent studies have shown that non-ionizing radiofrequency (RF) radiation has some beneficial cognitive effects in animal models of AD. In this study, we examined the effect of combined RF radiation on amyloid-beta (Aβ)–induced cytotoxicity in HT22 rat hippocampal neurons. Treatment with Aβ suppressed HT22 cell proliferation in a concentration-dependent manner. RF exposure did not affect cell proliferation, and also had a marginal effect on Aβ-induced suppression of growth in HT22 cells. Cell cycle analysis showed that Aβ decreased the G1 fraction and increased the subG1 fraction, indicating increased apoptosis. Accordingly, Aβ increased the annexin V/propidium iodide (PI)–positive cell fraction and the degradation of poly (ADP ribose) polymerase and caspase-3 in HT22 cells. However, RF alone and the combination of Aβ and RF did not affect these events significantly. Aβ increased reactive oxygen species (ROS) generation, thereby suppressing cell proliferation. This was abrogated by N-acetylcysteine (NAC) treatment, indicating that Aβ-induced ROS generation is the main cause of suppression of proliferation. NAC also restored Aβ-induced annexin V/PI–positive cell populations. However, RF did not have a significant impact on these events. Finally, Aβ stimulated the ataxia telangiectasia and Rad3-related protein/checkpoint kinase 1 DNA single-strand breakage pathway, and enhanced beta-site amyloid precursor protein expression; RF had no effect on them. Taken together, our results demonstrate that RF exposure did not significantly affect the Aβ-induced decrease of cell proliferation, increase of ROS production, or induction of cell death in these cells.

KEYWORDS: radiofrequency fields, Alzheimer's disease, amyloid-beta, HT22 hippocampal neuronal cells

INTRODUCTION

The rapid increase in mobile phone usage worldwide has caused great concern about the potential health risk of electromagnetic radiation (EMR) exposure in the public [1]. Mobile phones emit EMR usually ranging from 800 to 2000 MHz, which is included in the radiofrequency (RF) spectrum. Tissues closest to where the phone is held, particularly the brain, can absorb the RF emitted from cell phones. Many studies have shown that RF–electromagnetic fields (EMFs) negatively affect the brain. After the International Agency for Research on Cancer (IARC) classified RF-EMFs as a group 2B human carcinogen, it was reported that RF-EMFs from mobile and cordless phones were associated with the initiation and progression of malignant brain

tumors [2, 3]. RF-EMFs may cause DNA damage in brain cells, which leads to altered gene expression, morphological change, and even cell death [4, 5]. In addition, RF exposure induced oxidative damage in mitochondrial DNA in primary cultured neurons [6].

In stark contrast, some studies insist that RF exposure may have beneficial effects on cognitive functions. Specifically, there have been controversies about the effect of RF on the neurodegenerative disease. Recent studies have shown that RF radiation may have some beneficial effect on neurodegenerative disorders like Alzheimer's disease (AD) [7–9]. Exposure to high-frequency EMFs provides cognitive benefits for both normal and AD mice [7, 8]. The serum transthyretin level is suggested as a marker for this effect [9]. However, EMF exposure was reported to increase amyloid-beta (Aβ) expression in rats [10].

Given these debates on the impact of RF in animal models of AD, it is important to define the effect of RF on the molecular mechanism(s) of AD *in vitro*. In the present study, we explored the effect of RF exposure on Aβ-induced cytotoxicity in HT22 hippocampal neurons. Our data indicate that Aβ suppressed cell proliferation and partially induced apoptosis via increasing reactive oxygen species (ROS). In addition, Aβ caused DNA single-strand breakage and induced the AD-related protein beta-site amyloid precursor protein (APP). However, RF did not have a significant impact on these events. To build on this work, further investigation of the consequences of long-term exposure to RF is required.

MATERIALS AND METHODS
Materials
The Aβ$_{25-35}$ fragment and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO, USA), and the Annexin V-FITC Apoptosis Detection kit was obtained from BD Biosciences (San Jose, CA, USA). Antibodies for p-ATM, p-ATR, p-Chk1, p-Chk2, cleaved poly (ADP ribose) polymerase (PARP), and Caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against beta-site APP-cleaving enzyme 1 (BACE), and CD10 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for APP and γ-H2AX, were purchased from Millipore (Bedford, MA, USA), and low density lipoprotein receptor–related protein 1 (LRP1) and A disintegrin and metalloproteinase 10 (ADAM10) were purchased from Abcam (Cambridge, MA, USA).

Cell culture and treatments
HT22 mouse hippocampal neuronal cells were grown in DMEM supplemented with 10% FBS at 37°C under a humidified atmosphere of 5% CO$_2$ and 95% air. Cells were subcultured once every 2 days. For the experiments, Aβ$_{25-35}$ was firstly dissolved in dimethylsulfoxide (DMSO) to obtain a stock 50 mM solution.

Exposure to RF radiation
A radial transmission line (RTL) exposure system was used as an *in vitro* multifrequency radiation exposure system for this study. A typical Code-Division Multiple Access (CDMA) signal at 837 MHz and a W-CDMA (Wideband Code-Division Multiple Access) signal at 1950 MHz were applied to the RTL after amplification. The

signals for *in vitro* exposure were modulated by real CDMA and W-CDMA signals. The specific absorption rate (SAR) distribution inside the exposed medium was characterized by numerical simulations using the finite-difference time-domain (FDTD) method (XFDTD 6.5, Remcom, State College, PA). The mean value and standard deviation of the SAR measurements for 1 W input power in the entire sample was 0.105 ± 0.019 W/kg for the CDMA frequency and 0.262 ± 0.055 W/kg for the W-CDMA frequency. The exposure system was warmed up for 30 min to equilibrate it prior to the RF exposure. The 100-mm Petri dishes were placed at 13.6 cm from the conical antenna, which was located in the center of the exposure chamber, and cells were then exposed to RF radiation in the Petri dishes. RF radiation exposure to multiple signals (CDMA at 2 W/kg plus W-CDMA at 2 W/kg) was performed for 2 h. During the exposure period, the temperature in the chamber was maintained within a range of 37 ± 0.3 °C by circulating water within the cavity, and a 5% CO$_2$ concentration was also maintained. After exposure, the cells were immediately transferred to a cell culture incubator. For sham exposure the cells were kept in the RF radiation exposure device but were not exposed to RF radiation.

Cell viability assays
Cell viability was assessed by a microculture 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–based colorimetric assay. Briefly, HT22 cells were seeded in 96-well plates at a density of 3 × 10^3 cells per well. Once the treatments were completed, 20 μl MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C under 5% CO$_2$. The supernatant was then discarded and the formazan crystals produced in the viable cells were solubilized with 100 μl of DMSO, after which the absorbance of each well at 570 nm was read using a microplate reader. Relative cell density was expressed as a percentage of the control, which was not treated with Aβ.

Western blot analysis
HT22 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM ethylenediaminetetra acetic acid, 0.5% Nonidet P-40, and protease inhibitor cocktail tablet). The protein content of the cell lysates was then determined using Bradford reagent (Bio-Rad, Hercules, CA). The proteins in each sample were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h at room temperature, membranes were incubated with the appropriate antibodies. After incubation with the secondary antibodies, membranes were developed with enhanced chemiluminiscence reagent.

Analysis of cellular ROS and glutathione level
To evaluate the levels of intracellular ROS, cells were treated with 10 μM of CM-H$_2$DCFDA (general oxidative stress indicator) for 30 min at 37 °C under 5% CO$_2$. The cells were then harvested and washed three times with PBS. Cells were trypsinized and analyzed using a flow cytometer.

Intracellular glutathione levels were measured using a glutathione assay kit (Sigma) according to the manufacture's protocol.
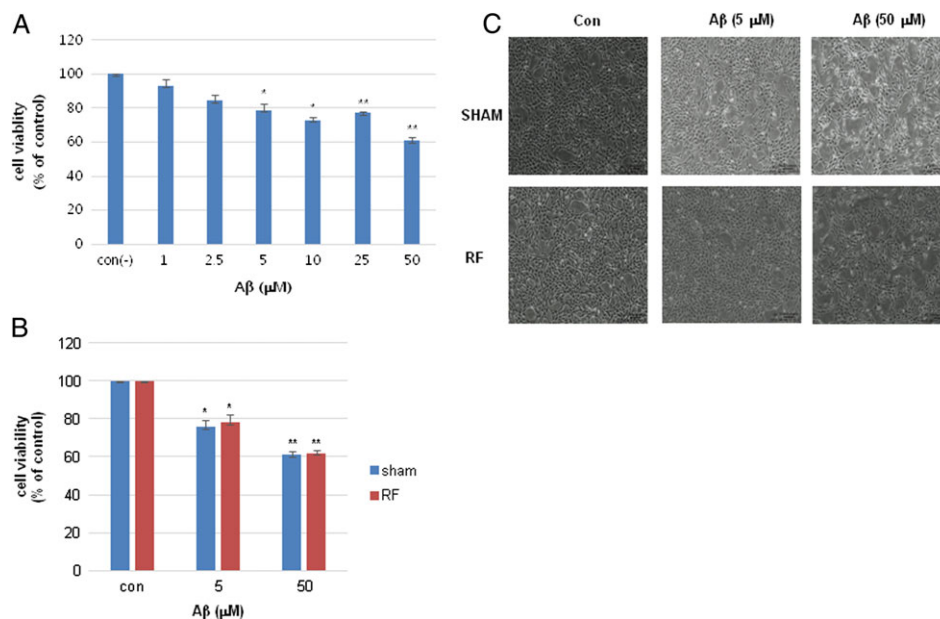
Fig. 1. Effect of Aβ and RF exposure on the growth of HT22 cells. (A, B) MTT assays. Cells were treated with Aβ at the concentrations indicated in the figure, followed by exposed to RF radiation (CDMA at 2 W/kg plus WCDMA 2 W/kg for 2 h). Results were presented as percentages of the untreated control. (C) Representative pictures of HT22 cells exposed to SHAM or RF in the presence or absence of Aβ (5 and 50 μM) after 24 h of culture. Magnification: ×100. *0.01 < P < 0.05; **0.005 < P < 0.01.

Annexin V/Propidium Iodide assay

To discern the molecular processes involved in the cytotoxic effect of Aβ$_{25-35}$, apoptosis was evaluated by Annexin V/Propidium Iodide (PI) staining. After treatments were completed, cells were trypsinized, washed with PBS, and centrifuged. The staining by Annexin V/PI was performed using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA).

Cell cycle analysis

Once treatments were concluded, cells were trypsinized, and washed with PBS. The pellet was resuspended in 75% EtOH and chilled over ice for 15 min. The cells were washed with PBS and stained with PI buffer containing PI (50 μg/ml) and ribonuclease A (100 μg/ml) for 30 min in the dark. After centrifugation, cells were resuspended in PBS. Flow cytometric analysis was performed on the FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Statistical analysis was performed using an independent samples t-test. Differences were considered statistically significant at P < 0.05.

RESULTS
Effect of RF exposure on the growth of HT22 cells

We first performed a MTT-based cell viability assay to investigate the effects of RF exposure on Aβ-induced cytotoxicity in HT22 cells. HT22 cells were treated with Aβ at concentrations between 0 and 50 μM for 30 min, followed by exposure to RF radiation (CDMA at 2 W/kg plus WCDMA at 2 W/kg for 2 h). As shown in Fig. 1A, treatment with Aβ suppressed proliferation of HT22 cells in a concentration-dependent manner up to 40% at 50 μM in MTT assay. RF exposure did not show any significant impact on cell proliferation. Also, RF exposure had a marginal effect on growth and Aβ-induced growth suppression in HT22 cells at both low (5 μM) and high (50 μM) concentrations (Fig. 1B). Examination of cell morphology using phase contrast microscopy did not show any significant morphological change in HT22 cells exposed to sham or RF in the presence or absence of Aβ (5 μM) after 24 h of culture (Fig. 1C).

Effect of Aβ on cell cycle progression in HT22 cells

To determine if the cell growth inhibition observed in response to Aβ treatment was associated with a change in cell cycle progression, we examined cell cycle distribution using flow cytometry. Cell cycle analysis indicated that Aβ decreased the G1 fraction and increased the subG1 fraction slightly but significantly, but the G2 fraction was not significantly changed (Fig. 2A). Because the subG1 phase of the cell cycle indicates cell death, we performed fluorescence-activated cell sorting (FACS) analysis after staining the cells with annexin V and PI. As shown in Fig. 2B, Aβ increased the annexin V/PI–positive cell fraction, which indicated induction of apoptosis. Western blot analysis also showed that Aβ induced cleaved PARP and capase-3, markers of apoptosis (Fig. 2C). However, RF did not have significant effect on these events. Therefore, Aβ-induced suppression of cell growth is caused, at least in part, by partial cell death in these cells, and RF has a marginal effect on these events.
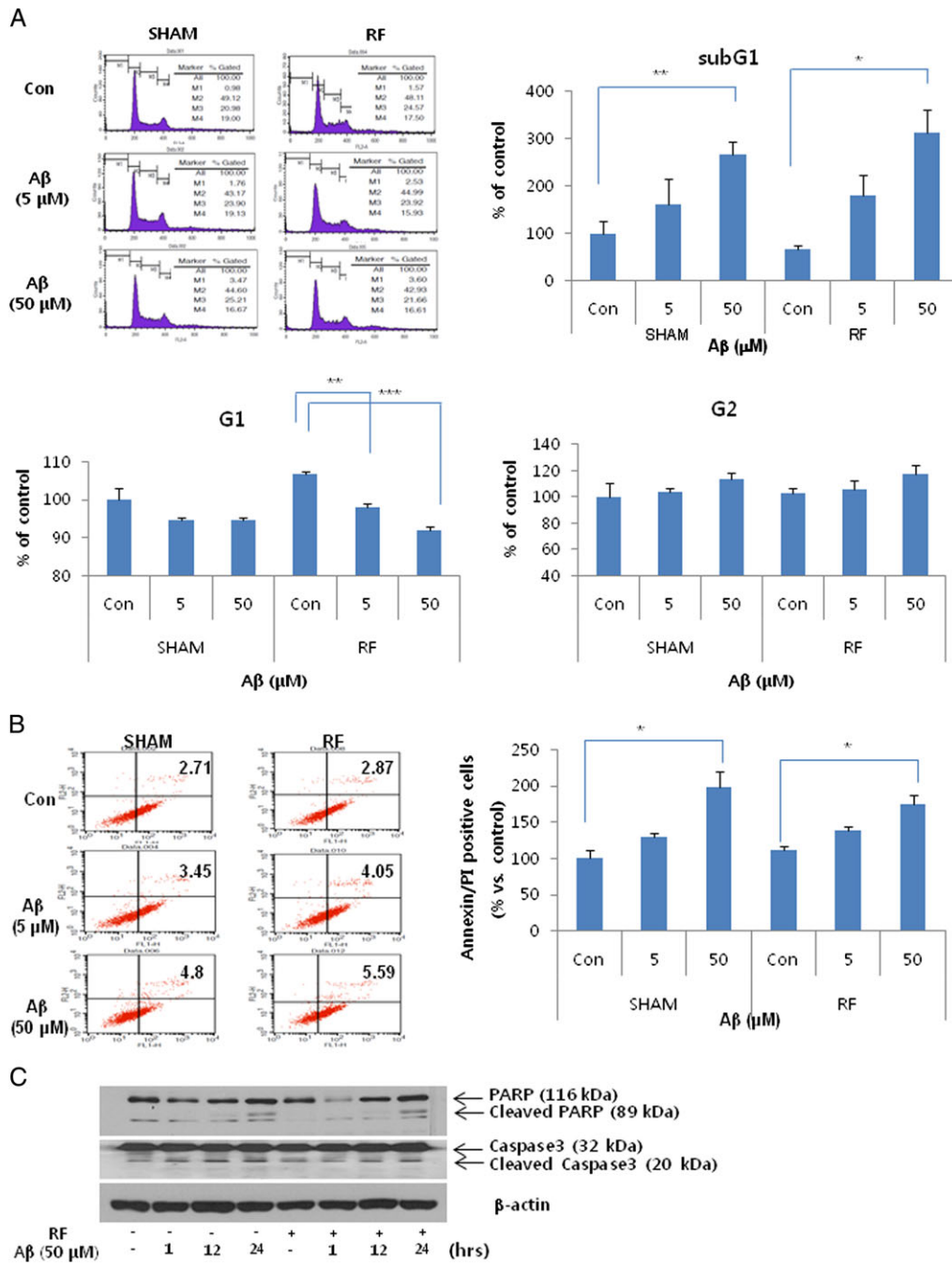
Fig. 2. Effect of Aβ in cell cycle progression and cell death in HT22 cells. (A) Cell cycle phase distribution study by flow cytometry in HT22 cells: representative histogram and quantification of the analysis. (B) FACS analysis of Annexin V/PI positive cells: representative histogram and quantification of the analysis. (C) Western blot analysis of the degradation of apoptosis-related proteins (PARP and Caspase-3). Cells were exposed to SHAM or RF radiation in the presence or absence of Aβ (5 and 50 μM) for 24 h. *0.01 < P < 0.05; **0.005 < P < 0.01; ***P < 0.005.

Effect of RF exposure on the production of Aβ-induced ROS and glutathione in HT22 cells

Several studies have demonstrated the involvement of ROS in Aβ-induced neurotoxicity, which has been shown to induce neuronal apoptosis. Therefore, we examined whether RF exposure affects Aβ-induced ROS production in HT22 cells. As shown in Fig. 3A, treatment of HT22 cells with Aβ significantly increased ROS generation. However, RF exposure did not affect the ROS generation
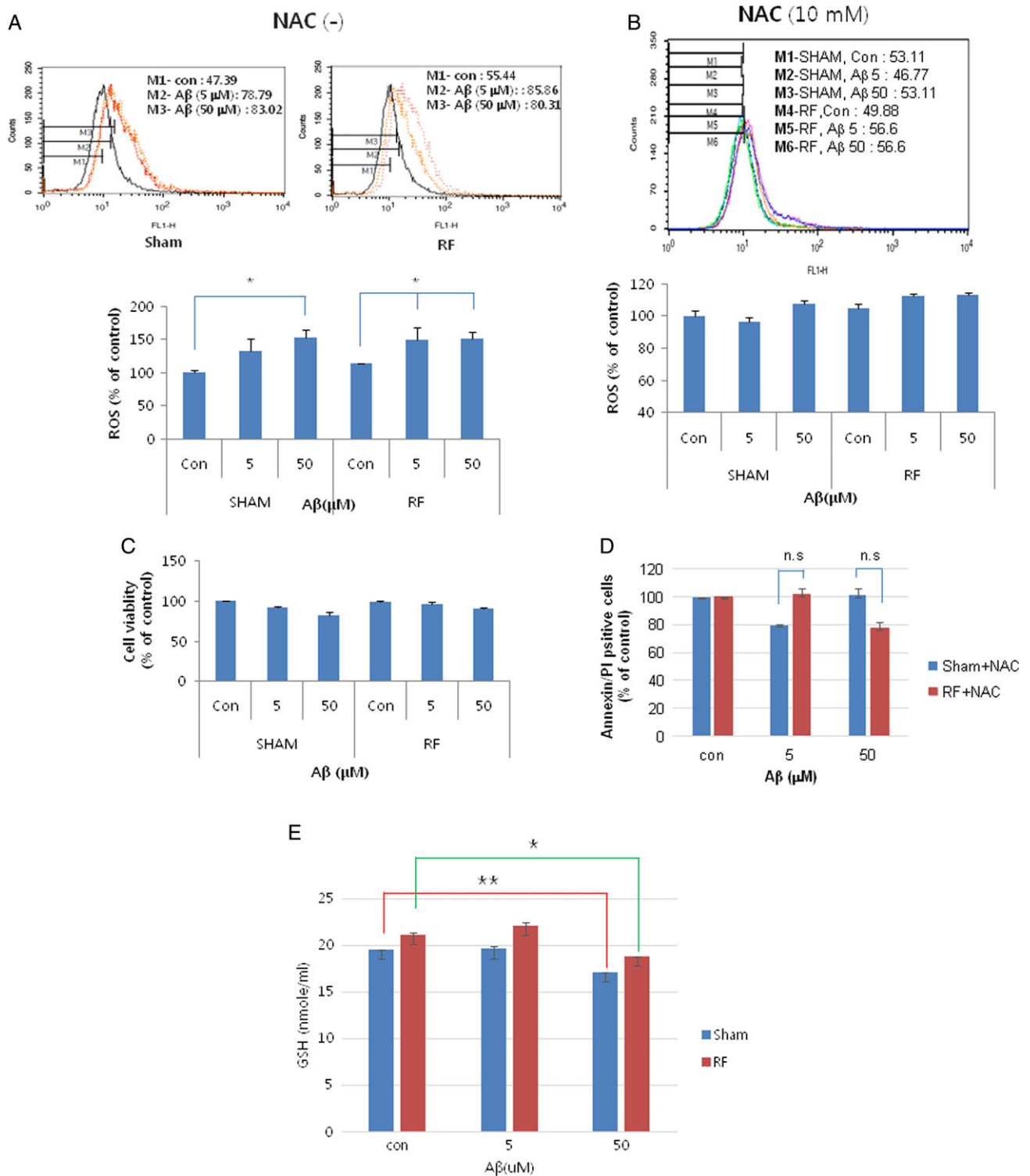
Fig. 3. Effect of RF exposure on Aβ-induced ROS production and cell death in HT22 cells. (A, B) FACS analysis of ROS production. Cells were exposed to SHAM or RF radiation in the presence or absence of Aβ (5 and 50 µM) for 12 h, pretreated without (A) or with (B) NAC (10 mM), followed by incubation with DCFDA (10 µM) for an additional 30 min. The intracellular levels of ROS were determined using flow cytometry: representative histogram (upper panel) and quantification of the analysis (lower panel). MTT assays (C), flow cytometric analysis for measuring Annexin and PI double-positive cell populations (D) and glutathione assays (E) in HT22 cells using the same experimental conditions as in (B).
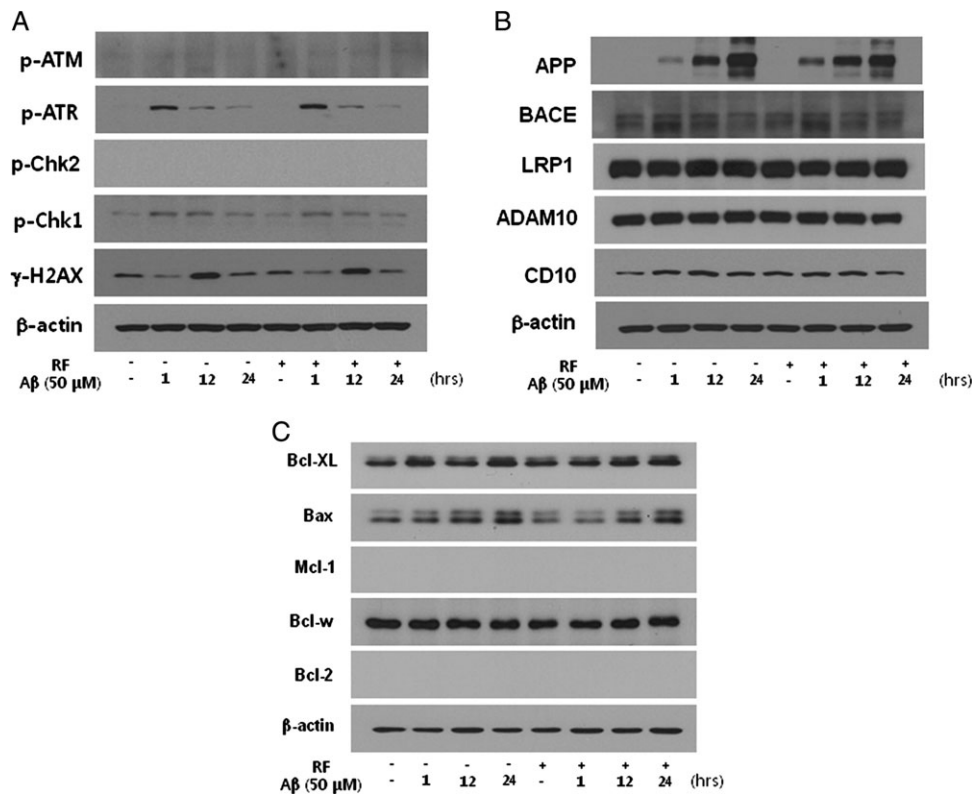*$0.01 < P < 0.05$; **$0.005 < P < 0.01$. n.s, = not significant.

Fig. 4. Effect of RF exposure on the Aβ-induced expression of DNA damage–associated proteins (A), AD-associated proteins (B), and Bcl2 family proteins (C). Cells were exposed to RF radiation followed by incubation with 50 μM Aβ for the indicated time points.

induced by Aβ. Pretreatment of the cells with N-acetylcysteine (NAC), an antioxidant that reduces free radicals, inhibited Aβ-induced ROS generation (Fig. 3B) and almost completely restored the cell proliferation rate suppressed by Aβ (Fig. 3C). Also, NAC treatment reduced the annexin V/PI double-positive cell fraction induced by Aβ (Fig. 3D). Cellular glutathione levels were slightly but significantly reduced by Aβ at 50 μM concentration; however, RF had no effect on this (Fig. 3E). These data indicate that ROS is the major cause of suppression of cell growth and induction of apoptosis by Aβ.

Effect of RF exposure on the expression of Aβ-induced DNA damage signaling proteins, AD-associated proteins and Bcl2 family proteins in HT22 cells

Because ROS frequently damages cellular DNA, we investigated DNA damage signaling pathways. As shown in Fig. 4A, Aβ induced the DNA single-strand breakage pathway (p-ATR/p-Chk1), but not the DNA double-strand breakage pathway (p-ATM/p-Chk2). Phosphorylation of H2AX (γ-H2AX) indicated the DNA breakage induced by Aβ treatment (Fig. 4A).

Dysfunction of Aβ metabolism is involved in the pathogenesis of AD. Several proteins, including APP, ADAM10, CD10, BACE and LRP1, are crucially involved in Aβ metabolism [10]. To investigate the effects of RF exposure on these proteins, cells were exposed to RF radiation and incubated with Aβ (50 μM) for 24 h; protein

levels were then assayed by western blotting. As shown in Fig. 4, Aβ enhanced APP significantly. Other protein levels were marginally changed by Aβ treatment in HT22 cells. However, RF had no effect on the expression pattern of these proteins in HT22 cells.

Finally, we examined the effect of RF and Aβ on the expression of Bcl2 family member proteins. As shown in Fig. 4C, expression of the pro-survival factors Bcl-xL and Bcl-w was not changed after RF and Aβ exposure. Expression of the pro-death factor Bax was elevated by Aβ treatment, but RF did not affect this elevation. Pro-survival factors Mcl-1 and Bcl-2 were not detected in the western blot analysis.

DISCUSSION

The exponential increase in mobile phone usage worldwide has caused great concern for public health because of the RF emitted from mobile phones. Since mobile phones are usually positioned near the ear for use, much attention has been focused on the impact of RF on the brain, including any effects on cognitive function. Specifically, several studies have shown that RF may impact AD by improving cognition in mice or, conversely, increasing Aβ deposition in rats [7–9, 11]. Determining the effect of RF in an experimental AD model *in vitro* will be beneficial to understanding its impact *in vivo*. In the present study, we used HT22 rat hippocampal neurons to investigate the effect of RF on Aβ-induced cytotoxicity.

Our results indicate that Aβ suppressed cell proliferation and partially induced apoptosis in HT22 cells. Inhibition of proliferation and induction of apoptosis were caused by the increase in ROS induced by Aβ in these cells. RF exposure did not significantly affect Aβ-induced cytotoxicity in HT22 cells. However, long-term exposure to RF for these cells would be needed to confirm this result.

There are fierce debates about the effect of RF-EMFs on public health, specifically on the brain. Some studies have shown that long-term use of mobile phones could affect the occurrence of brain tumors [2, 3, 12]. However, other studies indicate that RF-EMF exposure is not related to the incidence of brain tumors and does not have any impact on the biological systems in brain [13–15]. Recently, the impact of RF-EMFs on neurodegenerative disease, especially on AD, has received much attention. RF exposure reduced brain AD deposition by preventing Aβ aggregation, which had a beneficial effect on cognitive impairment in AD mice [7]. This effect might be explained by the elevation of transthyretin via RF, because transthyretin sequesters Aβ and prevents the formation of Aβ plaques in the brain [8]. In addition, Banaceru et al. reported that RF exposure improved the cognitive behavior of 3xTg-AD mice [9]. However, Jiang et al. insist that RF exposure has a negative impact on AD by inducing Aβ overexpression in rats [11]. In this study, we tried to define the effect of RF-EMFs on Aβ-induced toxicity in HT22 rat hippocampal neurons. Our data indicate that Aβ treatment inhibited cell proliferation and induced partial apoptosis by increasing intracellular ROS levels along with partial induction of apoptosis in HT22 cells. However, short-term exposure to RF-EMFs did not have any significant effect on the impact of Aβ in these cells. Studies on the impact of long-term exposure to these events are needed to reach a final conclusion.

FUNDING

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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