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Caffeine Induces Apoptosis in Human Neuroblastoma Cell Line SK-N-MC

Caffeine is one of the most widely consumed neuroactive drugs, coming mostly from everyday beverages such as coffee and tea. To investigate whether caffeine induces apoptosis in the central nervous system, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, flow cytometric analysis, DNA fragmentation assay, and caspase-3 enzyme assay were performed on SK-N-MC human neuroblastoma cells. Cells treated with caffeine at concentrations as high as 10 mM exhibited several characteristics of apoptosis. In addition, caffeine was shown to increase the caspase-3 activity. These results suggest that high-dose of caffeine induces apoptosis in human neuroblastoma cells, probably by increasing the caspase-3 enzyme activity.

Key Words : *Caffeine; Apoptosis; Neuroblastomas; Caspases*

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INTRODUCTION

Caffeine is one of the most widely consumed neuroactive drugs, coming mostly from everyday beverages such as coffee and tea; it exhibits a variety of central effects including stimulation of locomotor behavior (1). Caffeine exerts multiple effects at the cellular level on the central nervous system (CNS); for instance, antagonizes adenosine receptor (2), inhibits GABA receptor-mediated effect (3), and inhibits phosphodiesterase (4). In electrophysiological studies, caffeine at high concentrations triggered Ca^{2+} release via ryanodine receptors (5, 6), and it is known as a classic pharmacological agonist for activating Ca^{2+}-induced Ca^{2+} release. It was also reported that high-concentration caffeine administration has some deleterious effects, induces various morphological anomalies on cultured rat embryonic cells, and modifies the rate of neural cell proliferation in certain regions of the brain (7, 8).

Apoptosis, also known as programmed cell death, is a form of cell death that occurs in several pathological situations in multicellular organisms and constitutes part of a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells (9). Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of "apoptotic bodies" (10). The caspase family of aspartate-specific cysteine proteases is emerging as the central executioner of apoptosis. Of particular interest is caspase-3, which is activated in a variety of cell types during apoptosis (11).

In the present study, to investigate whether caffeine induces apoptosis in the CNS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, flow cytometric analysis, DNA fragmentation assay, and caspase-3 enzyme assay were performed on SK-N-MC human neuroblastoma cells.

MATERIALS AND METHODS

Drugs and reagents

Caffeine and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The MTT assay kit and the TUNEL assay kit were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Japan) and the caspase-3 assay kit was from CLONTECH (Palo Alto, CA, U.S.A.).

Cell culture

SK-N-MC human neuroblastoma cells were purchased from

the Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured according to the previously reported method (12). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY, U.S.A.) at 37℃ in 5% CO_2 and 95% O_2 in a humidified cell incubator, and the medium was changed every 2 days.

MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit as per the manufacturer's protocol. In order to determine the cytotoxicity of caffeine, cells were treated with caffeine at concentrations of 0.1 mM, 1 mM, and 10 mM for durations of 12 hr, 24 hr, and 36 hr. Cultures of the control group were left untreated. Ten microliters of the MTT labeling reagent was added to each well, and the plates were incubated for 4 hr. One hundred microliters of the solubilization solution was then added to each well, and the cells were incubated for another 12 hr. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, U.S.A.) at a test wavelength of 595 nm and a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as "(O.D. of drug-treated sample/control O.D.)×100".

DAPI staining

DAPI staining was performed according to the previously described protocol (13). Cells were first cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.). After treatment with caffeine, cells were collected and fixed by incubation in 4% PFA for 30 min. Following washing in PBS, the cells were incubated in 1 μg/mL DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope (Zeiss, Oberköchen, Germany).

TUNEL staining

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag® peroxidase in situ apoptosis detection kit. SK-N-MC cells were cultured on 4-chamber slides at a density of 2×10^4 cells/chamber. After treatment with caffeine, the cells were washed with PBS and fixed by incubating in 4% paraformaldehyde (PFA) for 10 min at 4℃. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37℃ in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using 3,3′-diaminobenzidine (Sigma Chemical Co.) as the substrate for the peroxidase.

Flow cytometric analysis

For flow cytometric analysis, after treatment with caffeine, cells were collected and fixed by incubation with 75% ethanol in PBS at -20℃ for 1 hr. Afterwards, the cells were incubated with 100 μg/mL RNase and 20 μg/mL propidium iodide (Sigma Chemical CO.) in PBS for 30 min at 37℃ and were analyzed using FACScan (Becton Dickinson, San Jose, CA, U.S.A.).

DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using ApopLadder EX™ DNA fragmentation assay kit (TaKaRa, Shiga, Japan). Cells were first treated with caffeine as mentioned earlier and were collected in Eppendorf tubes. The cells were then centrifuged and were lysed with 100 μL of lysis buffer per tube. The lysate was incubated with 10 μL of 10% SDS solution and 10 μL of Enzyme A at 56℃ for 1 hr and then at 37℃ for 1 hr following the addition of 10 μL of Enzyme B. Afterwards, 70 μL of precipitant was added, and the resultant pellet was resuspended in TE buffer. DNA fragmentation was visualized by electrophoresis on a 2% agarose gel containing ethidium bromide.

Caspase-3 enzyme activity assay

Caspase enzyme activity was measured using ApoAlert® caspase-3 assay kit according to the manufacturer's protocol. First, after treatment with caffeine, cells were lysed in 50 μL of chilled Cell Lysis Buffer. Fifty microliters of 2× reaction buffer (containing DTT) and 5 μL of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37℃ for 1 hr, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

Statistical analyses

Results are expressed as mean±standard error mean (SEM). The data were analyzed by one-way ANOVA followed by Scheffe's post-hoc test using SPSS. Differences were considered statistically significant at $p<0.05$.

RESULTS

Effect of caffeine on cell viability

As shown in Fig. 1, the viabilities of cells incubated with caffeine at a concentration of 0.1 mM for durations of 12 hr, 24 hr, and 36 hr were 96.06±1.85%, 101.56±2.93%, and 96.35±1.02% of the control value, respectively. The viabili-

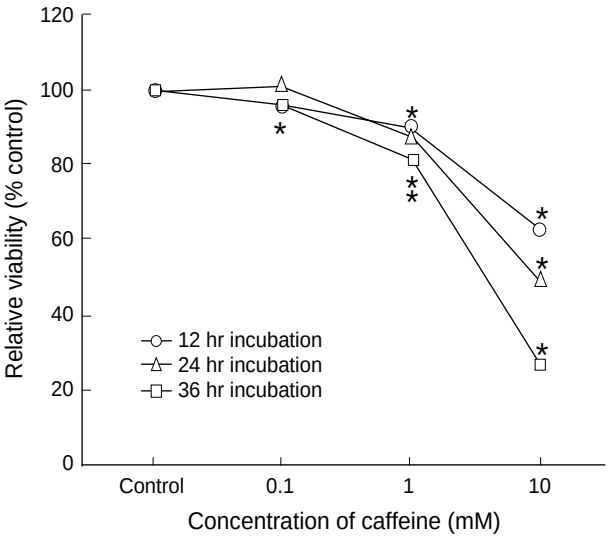

Fig. 1. Cytotoxic effects of caffeine. Human neuroblastoma cell line SK-N-MC cells were incubated with caffeine at various concentrations and duration prior to the determination of cellular viability through MTT assay. Results are represented as mean± standard error for two independent experiments, each with a minimum of three cultures. *represents $p<0.05$ compared to the control.

ties of cells incubated with caffeine at a concentration of 1 mM for durations of 12 hr, 24 hr, and 36 hr were 89.70±2.26%, 88.16±2.46%, and 81.14±1.76% of the control value, respectively. The viabilities of cells incubated with caffeine at a concentration of 10 mM for durations of 12 hr, 24 hr, and 36 hr were 63.01±0.10%, 50.11±0.80%, and 26.19±0.30% of the control value, respectively. These results show that caffeine at concentrations as high as 10 mM has a cytotoxic effect on SK-N-MC cells and this cytotoxic effect was increased in a time-dependent manner.

Morphological changes induced by caffeine

The morphological changes induced by caffeine were examined by phase-contrast microscopy. Cells treated with caffeine at a concentration of 10 mM for 24 hr were seen to have detached from the dish, and cell rounding, cytoplasmic blebbing, and irregularities in shape were observed. In the DAPI assay, cells were observed via fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. The assay revealed the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon caffeine treatment at a concentration of 10 mM for 24 hr. To further confirm the induction of apoptosis by caffeine in SK-N-MC cells, 10 mM caffeine-treated cells were analyzed via TUNEL assay. As shown in Fig. 2, TUNEL-positive cells were shown to be stained dark brown under the light microscope, and nuclear condensation was observed.

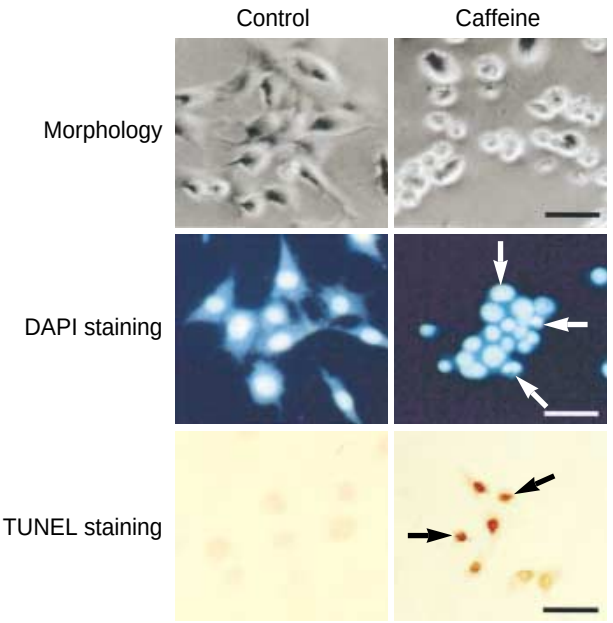

Fig. 2. Characterization of caffeine-induced cell death in SK-N-MC cells. Cells were cultured without caffeine (control) or with 10 mM caffeine for 24 hr in each case. Top: photomicrograph of phase-contrast microscope. Cell shrinkage, irregularity in cellular shape, and cellular detachment are seen in the nicotine-treated cultures. Middle: SK-N-MC cells stained with DAPI. White arrows indicate condensed nuclei. Bottom: SK-N-MC cells stained via TUNEL assay. Black arrows indicate where condensed and marginated chromatin have been labeled. All scale bars represent 100 μm.

Cell cycle distribution change

Flow cytometric analysis revealed an increase in the sub-G1 phase fraction, from 38.26% to 42.07% and 52.83% and a decrease in the G1 phase fraction, from 43.39% to 38.26% and 32.10%, in cells treated with caffeine at concentrations of 1 mM and 10 mM for 24 hr, respectively (Fig. 3).

Characterization of apoptosis via examination of DNA fragmentation

In order to ascertain the induction of apoptosis by caffeine, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was assessed. Caffeine treatment resulted in the formation of definite fragments, which could be seen via electrophoresis as a characteristic ladder pattern (Fig. 4).

Caspase-3 enzyme activity analysis

Caspase-3 enzyme activity was measured using DEVD peptide-nitroanilide (pNA). After 24 hr of exposure to caffeine at concentrations of 1 mM, and 10 mM, the concentration of DEVD-pNA cleavage product at the end of an 18-hr reaction was increased from 11.46±0.54 pM to 15.34±0.48

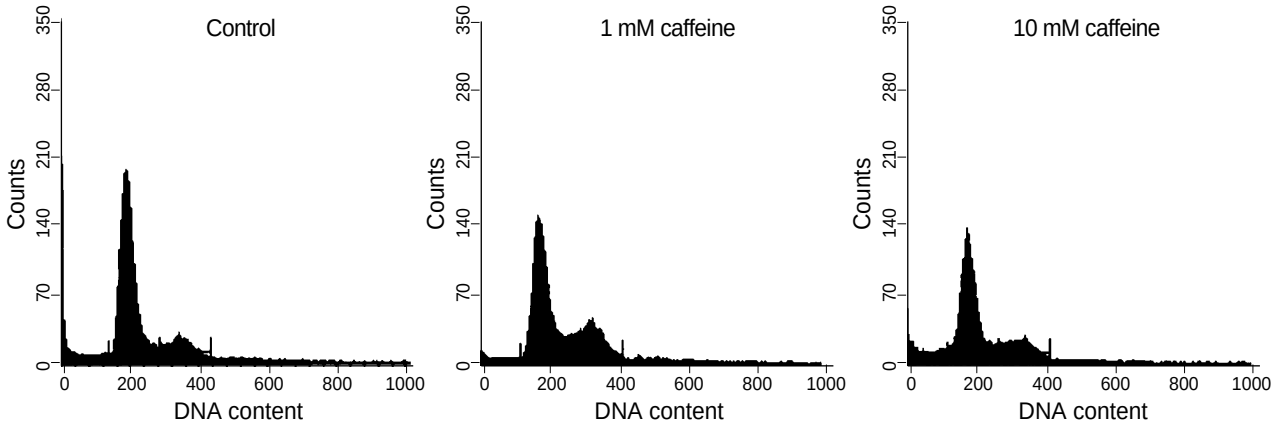

Fig. 3. Flow cytometric analyses. An increase in the sub-G1 phase fraction and a decrease in the G1 phase fraction were observed following caffeine treatment.

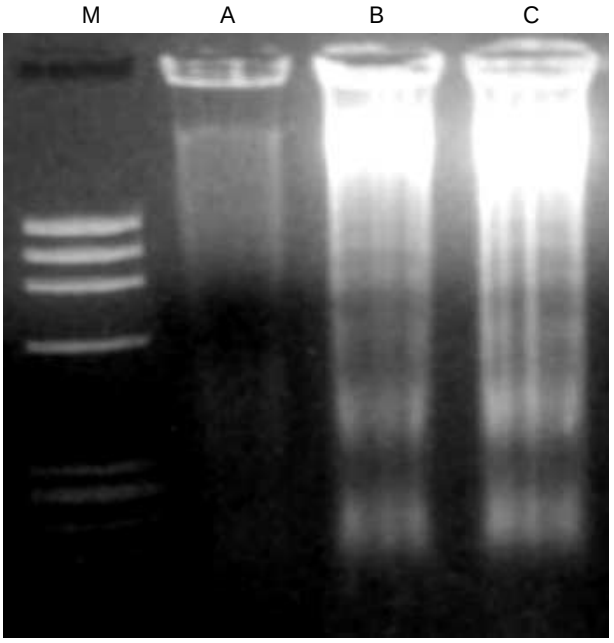

Fig. 4. Electrophoretic examination of the genomic DNA of SK-N-MC cells. Genomic DNA was extracted and analyzed via electrophoresis on 2% agarose gels containing ethidium bromide. A, control group; B, 1 mM caffeine-treated group; C, 10 mM caffeine-treated group.

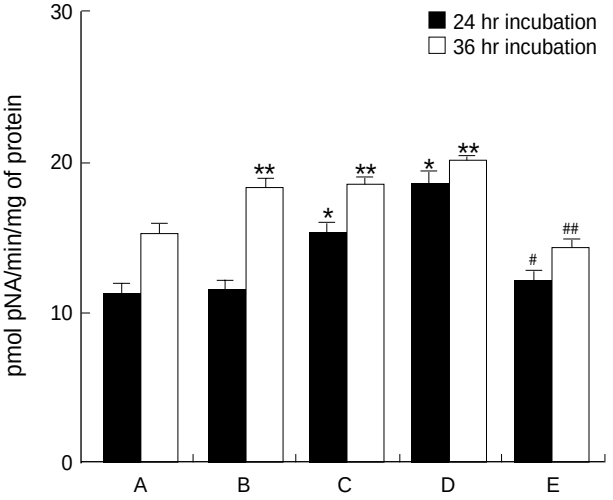

Fig. 5. Result of caspase-3 enzyme assay. Caffeine increases the caspase-3 enzyme activity. Results are presented as mean± standard error mean. *represents $p<0.05$ compared to the 24 hr incubated control group. **represents $p<0.05$ compared to the 36 hr incubated control group. #represents $p<0.05$ compared to the 24 hr incubated 10 mM caffeine-treated group. ##represents $p<0.05$ compared to the 36 hr incubated 10 mM caffeine-treated group. As the positive control, 1 μM stausporine was treated. DEVD-fmk is a caspase inhibitor. The rate of DEVD-*p*NA cleavage was measured at 405 nm. A, Control; B, 1 μM stausporine-treated group; C, 1 mM caffeine-treated group; D, 10 mM caffeine-treated group; E, 10 mM caffeine- and DEVD-fmk-treated group.

pM and to 18.56±0.66 pM, respectively. After 36 hr of exposure to caffeine at concentrations of 1 mM, and 10 mM, the concentration of DEVD-pNA cleavage product at the end of an 18-hr reaction was increased from 15.41±0.34 pM to 18.45±0.52 pM and to 20.14±0.26 pM, respectively (Fig. 5).

DISCUSSION

At the molecular level, caffeine interferes with cell division (14) and induces disturbances in early neurogenesis in mouse embryo cultures (8). In electrophysiological studies, caffeine at high concentrations (e.g. 1-10 mM) was shown to be necessary in order to initiate calcium release from intracellular stores via a caffeine-sensitive ryanodine receptor (5, 6), and increased intracellular calcium is known to induce cell death in the CNS (15). In addition, caffeine has been shown to attenuate G2 delay produced by DNA-damaging agents and to augment the cytotoxicity of these agents in a number of cell lines in vitro (16). In embryonic cell culture system, caffeine

induced various morphological anomalies (7). However, it has not yet been reported whether caffeine actually induces apoptotic cell death in the CNS. In the present study, the effect of caffeine on cells of the neuroblastoma cell line SK-N-MC was investigated.

Assessment of cell viability in the present study via MTT assay confirmed that caffeine exerts cytotoxic effects on SK-N-MC cells at high concentrations. In addition, caffeine at concentration as high as 10 mM was shown to cause characteristic changes in the morphology of SK-N-MC cells. Apoptotic bodies, the presence of which is a stringent morphological criterion for apoptosis, were seen in cultures treated with caffeine at high concentration upon DAPI staining. It has been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape, and retraction of processes (11). In addition, DNA strand breaks are known to occur during the process of apoptosis, and such breaks in the DNA molecules can be detected via TUNEL assay (17). In the present study, TUNEL-positive cells, indicative of the occurrence of apoptosis, were observed among caffeine-treated cells. It is also known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments which appear as a characteristic ladder pattern upon agarose gel electrophoresis (18). To provide further evidence supporting the involvement of apoptosis in caffeine-induced cytotoxicity, DNA fragmentation assay was performed. Caffeine-treated cells presented with the distinctive ladder pattern characteristic of apoptosis. Furthermore from flow cytometric analysis, increased apoptosis and decreased DNA synthesis were observed. In addition, caspases, a family of cysteine proteases, are known to form integral parts of the apoptotic pathway; in particular, caspase-3, when activated, has many cellular targets that, when severed and/or activated, produces the morphologic features of apoptosis (11). In the present study, an increase in the caspase-3 enzyme activity was observed in cells exposed to caffeine.

Based on these results, it is possible that caffeine at high concentrations induces apoptotic death in neuroblastoma cells by increasing the caspase-3 enzyme activity.

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