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CYTOTOXICITY EFFECTS OF ASPARTAME ON THE HUMAN CERVICAL CARCINOMA CELLS

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Running title: Aspartame and HeLa cells

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Abstract

Aspartame used as artificial sweeteners in more than 6000 food varieties. The present study was aimed to determine the effects of aspartame at various concentrations on the cell viability, morphology, ROS level and DNA of human cervical carcinoma cells over two time periods of exposure. The effects of aspartame on HeLa cell viability were investigated by the sulphorhodamine-B assay (SRB assay) and flow cytometry. Alkaline comet assay determined the possible DNA damage induced by aspartame. Mitochondria-derived reactive oxygen species (ROS) was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The fluorescence microscope determined the presence of apoptotic and necrotic cells following aspartame treatment. Cell viability was significantly altered following the higher concentration of aspartame exposure. Mitochondria-derived ROS increased at higher concentration of aspartame exposure. Exposure of 10 mM and 20 mM of aspartame-induced DNA fragmentation. The apoptotic and necrotic bodies have found in the range of 1-20 mM aspartame exposure. The exposure of high concentration of aspartame may alter cell viability and morphology, and it may induce ROS generation and DNA damage in cervical carcinoma cells.

Keywords: Aspartame; HeLa cells; flow cytometry; DNA fragmentation; cell viability.

Introduction

Aspartame is one of the widely used as artificial sweeteners in several foods (1) and found in low-calorie beverages and desserts (2). It is a dipeptide of aspartyl--phenylalanine methyl ester facilitates its intestinal hydrolysis and absorption of amino acids together with methanol (3). Aspartic acid and phenylalanine have commonly present in natural proteins (4). Consumption of aspartame has been associated with several behavioral and neurological disturbances (5).

Consumption of small amounts of aspartame can increase methanol levels in the animals (6). Severe metabolic acidosis and clinical disturbances, including blindness, neurologic sequelae and death may occur upon accidental and suicidal uptake of aspartame (7). Methanol is a potential substance that can damage, liver and kidney cells. Methanol converted into formaldehyde and formate (8). This process accompanies elevation of NADH level and formation of superoxide anion and which may participate in the process of lipid peroxidation (9). Mitochondrial damage, increased microsomal proliferation and production of oxygen radicals are commonly associated with methanol intoxication (10). Oxidative stress was known as a state in which oxidative force exceeds the cellular antioxidant systems due to loss of the balance. The possibility of increased oxidative stress when the production of reactive oxygen species (ROS) level exceeds the animal body's ability to neutralize and eliminate them. Thus, the creation of an imbalance and overabundance of free radicals is reported during these conditions (11). Therefore, these factors with the excess amount of formaldehyde, formed during acute methanol intoxication may involve cell viability and membrane damage due to increased lipid peroxidation (9).

The several studies have evidenced the possible risk of cancer with use of aspartame. The use of high concentration of artificial sweeteners indicated the potential association of bladder cancer with rodents (12). However, these findings not correlate with findings in humans (13). There were no neoplasms observed in the saccharin treated rats (14). Andreatta et al., (15) have reported that the prolonged use of artificial sweeteners in combined form results in the formation of urinary track tumor in Argentina. Also, Sturgeon et al., (16) have reported that the heavy artificial sweeteners are known to contain an elevated risk of high-grade tumors. The association of brain and breast cancer has been associated with the positive occurrence of tumors at different sites of rats (13). The higher concentration of artificial sweeteners has been known to induce DNA damage in rats (14). A selection of HeLa cells for this study based on the being monocytic lineage could mimic local immune responses, and cervical carcinoma is one of the most common neoplastic diseases affecting women worldwide. Therefore, elucidating the mechanism of aspartame with tumor development in humans could provide the possible effect on mammalian cells.

The objective of the present study was to determine the effects of aspartame at various concentrations on the cell viability, morphology, ROS level and DNA of human cervical carcinoma cells over two time periods of exposure**.** SRB assay and flow cytometry analysis investigated the effect of aspartame on cell viability. The Comet assay determined the possible DNA fragmentation. ROS level was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The fluorescence microscopy was used to confirm the presence of apoptotic and necrotic cells.

Materials and Methods

Materials

Aspartame, dimethyl sulphoxide (DMSO), Sulforhodamine B (SRB) have purchased from Sigma. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA have obtained from Welgene (Daegu, South Korea). Fluorescein diacetate (FDA, propidium iodide (PI) and DCFH-DA have purchased from Santa Cruz Biotechnology, Inc. (Delaware Avenue, California, USA).

Cell Culture

Cervical carcinoma cells (HeLa) have purchased from the Korean Cell Line Bank (South Korea). Cells have maintained in growth medium supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin). The cells have grown in a CO_2 incubator at 37°C and 5% CO_2.

Preparation of aspartame

Aspartame stock (1 M concentration) was initially prepared by dissolving in DMEM. The stock solution was filtered by using 0.22 µm filter prior to utilization in the experiment. The stock solution of aspartame was diluted with complete medium to prepare working concentrations and pH was adjusted to 7.4.

SRB assay for cell viability

Cells have seeded at a density of 2 x 10^4 cells/well and allowed to adhere. Cells have treated with aspartame at different concentrations (100 nM, 1µM, 10 µM, 100 µM, 1 mM, 10 mM & 20 mM) for 24 and 48 h. At the end of the treatments, the cytotoxic effect of aspartame on HeLa cells was measured by the SRB assay (17).

Flow cytometry assays for cell viability

HeLa cells have seeded at a density of 2.2 x 10^5 cells/well in 6-well plates. After 24 h adherence, the cells were treated with aspartame of different concentrations (10 µM, 100 µM, 1 mM, 10 mM & 20 mM) for 24 and 48 h. At the end of treatments, cells are washed once with PBS and re-suspended in PBS at an initial cell density of 1x10^5cells/ml. FDA was added to the each tube to attain a final concentration of 0.5µg/ml, and tubes have incubated in the dark place for 30 minutes. Then, PI was added each tube to achieve a final concentration of 50 µg/ml. All the tubes were kept on ice immediately up to flow cytometry analysis (18).

HeLa cell viability was assessed by determining the intracellular green and red fluorescence using a BD FACS Calibur Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ 07417). A 570-nm beam splitter has used for dual color fluorescence analysis, with a 625-nm filter used to collect red (PI) fluorescence, and a 530-nm filter used for green (FDA) fluorescence. Logarithmic amplification of green and red fluorescence signals is used. Viable cells have identified as those with high green fluorescence.

Alkaline comet assay

The possible DNA damage induced by aspartame at concentrations (100 µM, 1 mM, 10 mM & 20 mM) was studied after 24 and 48 h time periods by following standard protocols. This assay is very sensitive and rapid method for the quantization of DNA lesions in animal cells. The assay detects single strand breaks, DNA cross-linking, alkali labile sites, and incomplete excision repair sites (19). The presence of ‘‘comets’’ was observed by using a light microscope, and DNA damage have quantitated.

Determination of intracellular ROS level

HeLa cells were seeded in 96-well plates in growth medium at a cell density of 4500 cells/well. After adherence, cells were treated with aspartame of different concentrations (100 µM, 1 mM, 10 mM & 20 mM). At the end of treatments, the medium was removed, and the cells have incubated with 5 µM of DCFH-DA in the growth medium for 30 min at 37°C and 5% CO_2. Fluorescence was measured using a fluorescent plate reader at excitation/emission wavelengths of 490 and 525 nm, respectively, and images were taken using a fluorescence microscope (Axiovert 2000, Carl Zeiss, Germany). ROS level was determined using a fluorescent probe, DCFH-DA (20).

Fluorescence Microscope

Cells have seeded at a density of 2 x 10^4 cells/well in 6-well plates. After 24 h adherence, the cells were treated with aspartame of different concentrations (1 mM, 10 mM & 20 mM). After 24 and 48 h, cells were removed from the wells and centrifuged at 500xg for 3 minutes. The supernatant have withdrawn from the tubes and phosphate buffered saline (PBS) have added to the tubes. Cell volume was adjusted to 10^5-10^6 cells/ml. 95 µl of cell suspension have added to the Microtube, and then 5 µl of AO and EB dye mixture was added to the same tube. The tubes were incubated at 37°C for 15-30 minutes with protection from light. Ten µl of the cell staining solution have added to a glass slide and covered with a cover glass. Cells were examined with Fluorescence Microscope (Axiovert 2000, Carl Zeiss, Germany) (20).

Statistical analysis

Values have expressed as mean ± SD. The difference between control and aspartame treatment was evaluated using Student's *t*-test. A p value less than 0.05 was considered statistically significant.

Results

Effect of aspartame on cell viability measured by SRB assay

Aspartame showed a statistically insignificant cytotoxic effect on HeLa cells and slightly a concentration-dependent response relationship was observed. Exposure to high concentration of aspartame have induced cell death up to 12%, whereas at lower concentrations of 100 nM, 1µM, 10 µM, 100 µM of aspartame-induced less than 6% irrespective of exposure periods (Figure 1).

Effect of aspartame on cell viability measured by flow cytometry

We utilized FDA and PI to determine the amount of viable and nonviable cells. FDA is a non-polar and non-fluorescent compound. In viable cells, FDA can enter freely; there it is converted by intracellular esterase into a fluorescent compound (fluorescein). Fluorescein is an extremely polar compound, which have trapped within the viable cells that possess membrane integrity. On the other hand, fluorescein diffuses out of the cells, which lacks membrane integrity. Viable cells with the FDA have bright green fluorescence, whereas non-viable cells are non-fluorescent in flow cytometry analysis. PI is a highly polar and fluorescent substance that can enter freely the non-viable cells where membrane integrity is absent. Therefore, non-viable cells have bright red fluorescence during flow cytometry analysis.

Flow cytometry results have presented in dot plot on the log scale of green versus red fluorescence. Nonviable cells and debris events have separated from the viable cells in the dot plots, which confirms non-viable, and cell debris have not counted in the viable cell window. HeLa cells incubated with the FDA for 30 minutes and PI before the measurement. A large population of cells showed FDA fluorescence under controlled conditions (area R1 of Figure 2 & 3), indicating they were intact active living cells.

Exposure to different concentrations of aspartame at 24 h (10 µM, 100 µM, 1 mM, 10 mM & 20 mM) did not produce significant changes in the cell viability. Viable FDA fluorescence was more than 90% (area R1 of Figure 1), and non-viable PI fluorescence was less than 4% (area R2 & R3 of 20 mM, Figure 2). In 48 of aspartame exposure, FDA and PI fluorescence were significantly changed in the MDCK cells. The percentage of non-viable (PI) cells were 0.04±0.001, 0.07±0.001, 0.19±0.001, 3.25±0.01 and 3.65±0.01 following 10 µM, 100 µM, 1 mM, 10 mM & 20 mM of aspartame exposure respectively (area R2 + R3 of Figure 3). The percentage of viable (FDA) cells were 93.6±2.2, 83.20±1.1, 79.27±1.4, 93.80±1.7 and 45.87±1.5 following 10 µM, 100 µM, 1 mM, 10 mM & 20 mM of aspartame exposure respectively (area R1 of Figure 3).

Effect of aspartame on DNA damage

The results obtained for the alkaline comet assays for the HeLa cells were shown in figure 4. No comet tails were observed in control and a lower concentration of aspartame treatment, indicating margin or no damage to the DNA of the HeLa cells. However after 10 and 20 mM of aspartame treatment showed a long comet tail and a tiny head section could be observed, which indicates of severe DNA fragmentation. Cells treated with a low concentration of aspartame yielded no or little DNA fragmentation at 24 and 48 h. Elongated tail formation after cells have treated with aspartame indicated severe DNA fragmentation after 24 and 48 h of incubation (Figure 4).

Effect of aspartame on ROS level

Intracellular ROS levels were determined by using fluorescent probe DCFH-DA following exposure of aspartame at 24 and 48 h. Green fluorescence intensity of DCF has gradually increased in the aspartame-treated cells compared with the control cells. A clear dose

and time-dependent increase of ROS level have observed in the aspartame-treated cells (Figure 5-6).

Effect of aspartame on apoptosis

Fluorescence microscopy was carried out to determine whether the cytotoxic effect of apoptosis has related to the induction of apoptosis, morphological features of cell death. This method combines the dual uptake of fluorescent DNA binding dyes AO and EB. The chromatin condensation in the stained nucleus, allowing one to distinguish viable, apoptotic, and necrotic cells. Control cells have normal morphology. Viable cells possess a uniform bright green nucleus. Early apoptotic cells show bright green areas of fragmented chromatin in the nucleus, and necrotic cells show a uniform bright orange nucleus. However, HeLa cells exposed to aspartame for 24 and 48 h exhibited fragmented and condensed chromatin, fragmented nuclei and appearance of apoptotic bodies (Figure 7-8).

Discussion

The association of aspartame usage and the increased risk of developing cancer are still highly controversial. Recent studies have reported that there is an association between the usage of aspartame and the increased danger of developing tumors, especially when used at higher consumption levels for extended periods of time. The morphological alterations have observed in cervical carcinoma cells exposed to high concentrations of aspartame (10 and 20 mM). The cells were observed to be flattened, granular and enlarged with multiple nuclei as compared to untreated controls. Food and Drug Administration (FDA) approved aspartame use in food (21, 22). Visual impairments, high aspartate transaminase, ear buzzing, loss of equilibrium, muscle aches, pancreatitis, depression and hypertension have associated with intake of aspartame (4).

Aspartic acid, phenylalanine, and methanol have produced as metabolites of aspartame (23). Aspartic acid usually removed in the form of CO_2 through the lungs. Stegink (24) has reported that the plasma level of aspartic acid did not increase significantly following the oral administration of aspartame at a dose of 34 mg/kg in humans. Most of the phenylalanine transported into the amino acid pool for protein synthesis, whereas a small amount of phenylalanine removed in the form of CO_2 through the lungs (25). Parthasarathy et al., (9) have reported that the methanol have primarily metabolized into formaldehyde and formate by oxidation. Hydrogen peroxide (H_2O_2) and superoxide anion have produced during these oxidation processes.

Flow cytometry provides a highly sensitive and accurate means of quantifying viable/non-viable MDCK cells following exposure of aspartame. Results obtained with the flow cytometry assay at 24 and 48 h after aspartame exposure approximated those obtained from the SRB assay. Moreover, we found a significant increase of non-viable (PI) estimates with the FDA/PI method following exposure of aspartame. Aspartame showed minor changes in the cell morphology in a dose and time-dependent manner. Rounding, loss of adherence and sporadic distribution of cells have observed at a higher concentration of aspartame exposure. Change cell morphology commonly associated with general cellular functions including locomotion, mitosis, cytokinesis, phagocytosis and secretion of macromolecules (26). Cell-to-cell and cell-to-substrate attachments have mediated via actin filaments (27). Aspartame may loosen this attachment as the actin filaments to move for cell contraction. Aspartame may have a greater effect on the periphery region of the culture, compared to the confluent region due to cell crowding and adhesion of the cells to the substrate. The peripheral cells are younger and adhere more loosely than confluent cells, which have greater adhesion to the substrate and cell-cell.

Oxidative stress is an imbalance between the elevated level of ROS and impaired function of the antioxidant. Overproduction of ROS can induce death of immature cultured cortical neurons (28) and DNA damage (29). Chandra et al., (30) have reported that increased ROS can trigger cell damage. The increase in ROS level may be due to methanol that have released during aspartame metabolism in the GI tract, as the pathway leading formaldehyde and formate by catalase enzyme. Cell death could occur in response to high oxidative stress (31). In agreement with this, ROS level was increased in our study due to oxidative stress in a time and dose-dependent manner.

There is a possibility for the larger and multinuclear cells during aspartame treatment, and therefore DNA content may increase in the cells. Therefore, the comet assay was carried out to understand the effect of various concentration of aspartame on the DNA content of cells at 24 and 48 h. The results of this study revealed that aspartame-induced DNA strand breaks observed as increased comet tail. The results indicated a time- and concentration-dependent effect on DNA within cells after exposure to aspartame. The effect of incubation time was, however, found to be not statistically significant while the effects of higher concentrations of aspartame were highly significant. The increased DNA fragmentation observed at the concentrations in excess of 1 mM of aspartame could be attributed to osmolarity as well as high concentration-induced apoptosis. Bandyopadhyay et al. (32) found that aspartame had the potential to induce DNA damage in mouse bone marrow cells. Aspartame did not have a time- or concentration-dependent effect on the DNA content. The results found in this study are in agreement with results obtained by Sasaki et al. (33). The metabolites of aspartame might have the potential to cause severe deleterious effects within cells at high concentrations (2).

AO/EB double staining, Fluorescence Microscopical study, showed distinct apoptotic morphological changes including cell shrinkage, chromatin condensation and formation of apoptotic bodies following 24 and 48 h of aspartame treatment. Our results cannot be directly extrapolated to in vivo situation since damage could observed at concentration levels in excess of 1 mM of aspartame that is possible in vivo after ingesting orally. The present study serves to provide further information on the potential effect of aspartame on cervical carcinoma cells.

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Declaration of Interest

The authors declare no conflict of interest

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Figure Legends

Figure 1: Cytotoxic effect of aspartame on HeLa cells by SRB assay at 24 and 48 h. Results were presented as a percentage of growth inhibition compared with the control. The percentage of growth inhibition was calculated as follows: Growth inhibition (%): (Control-Sample/Control)*100. Values have expressed as means ± SD.

Figure 2: Effect of aspartame on the HeLa cells discriminated by FDA and PI. Area R1 is FDA positive, R2 is PI positive, R3 is both FDA and PI positive, and R4 is both FDA and PI negative. The effect of aspartame (10 µM, 100 µM, 1 mM, 10 mM & 20 mM) on HeLa cell viability was examined at 24 h.

Figure 3: Effect of aspartame on the HeLa cells discriminated by FDA and PI. Area R1 is FDA positive, R2 is PI positive, R3 is both FDA and PI positive, and R4 is both FDA and PI negative. The effect of aspartame (10 µM, 100 µM, 1 mM, 10 mM & 20 mM) on HeLa cell viability was examined at 48 h.

Figure 4: Effect of aspartame on DNA fragmentation. HeLa cells were grown in 6 well plates and allowed for the adherence for 24 h. Cells were treated with aspartame (100 µM, 1 mM, 10 mM & 20 mM) for 24 and 48 h.

Figure 5: Effect of aspartame on ROS production. HeLa cells were grown in 6 well plates and allowed for the adherence for 24 h. Cells were treated with aspartame (100 µM, 1 mM, 10 mM & 20 mM) for 24 h. Scale bar is 200 µm. The representative images from three independent experiments (20x).

Figure 6: Effect of aspartame on ROS production. HeLa cells were grown in 6 well plates and allowed for the adherence for 24 h. Cells were treated with aspartame (100 µM, 1 mM,

10 mM & 20 mM) for 48 h. Scale bar is 200 μm. The representative images from three independent experiments (20x).

Figure 7: Morphological observation with AO/EB double staining by Fluorescence Microscope (100x). HeLa cells have seeded in 6-well plates and over 24 of adherence, cells were treated with aspartame (1 mM, 10 mM & 20 mM) for 24 h. Scale bar is 20 μm. The representative images from three independent experiments.

Figure 8: Morphological observation with AO/EB double staining by fluorescence microscope (100x). HeLa cells were seeded in 6-well plates and over 24 of adherence, cells were treated with aspartame (1 mM, 10 mM & 20 mM) for 48 h. Scale bar is 20 μm. The representative images from three independent experiments.

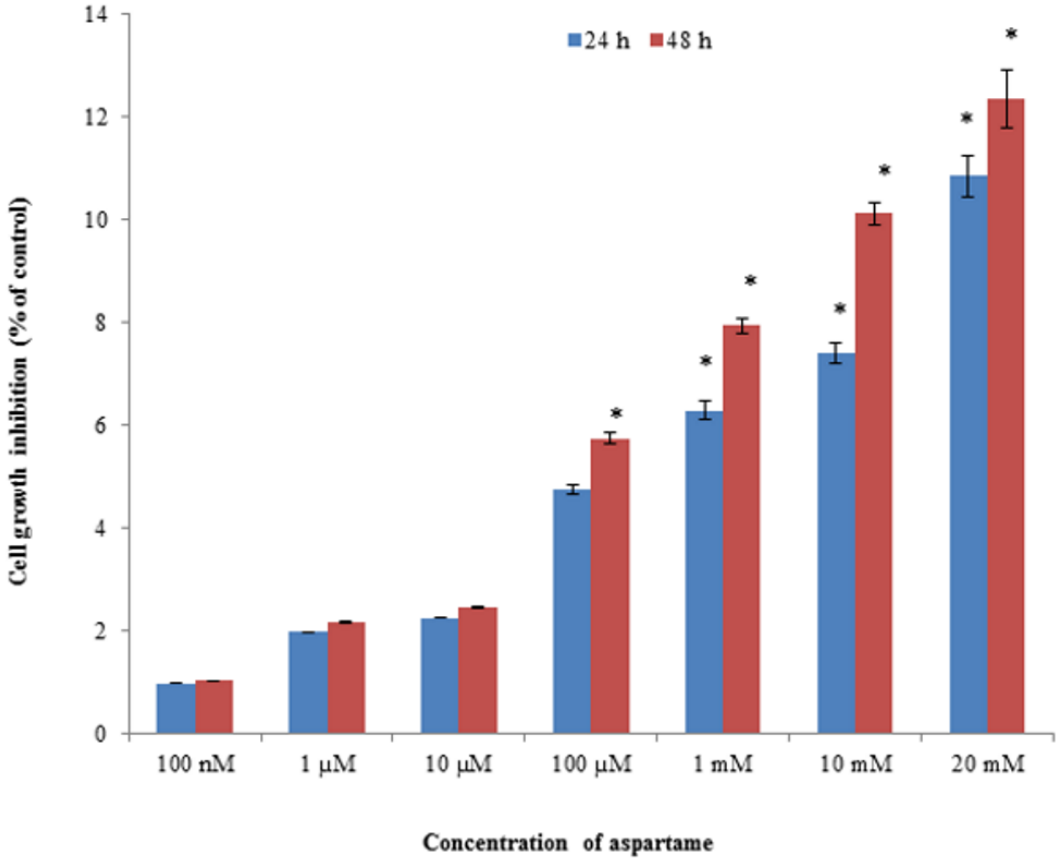
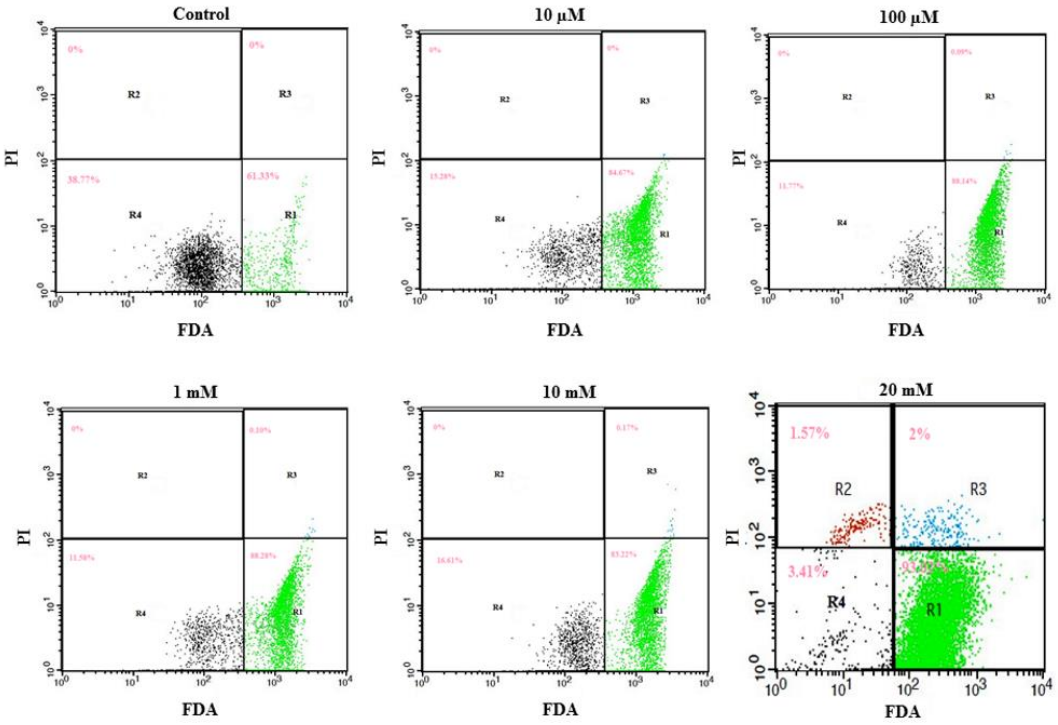


Figure 1



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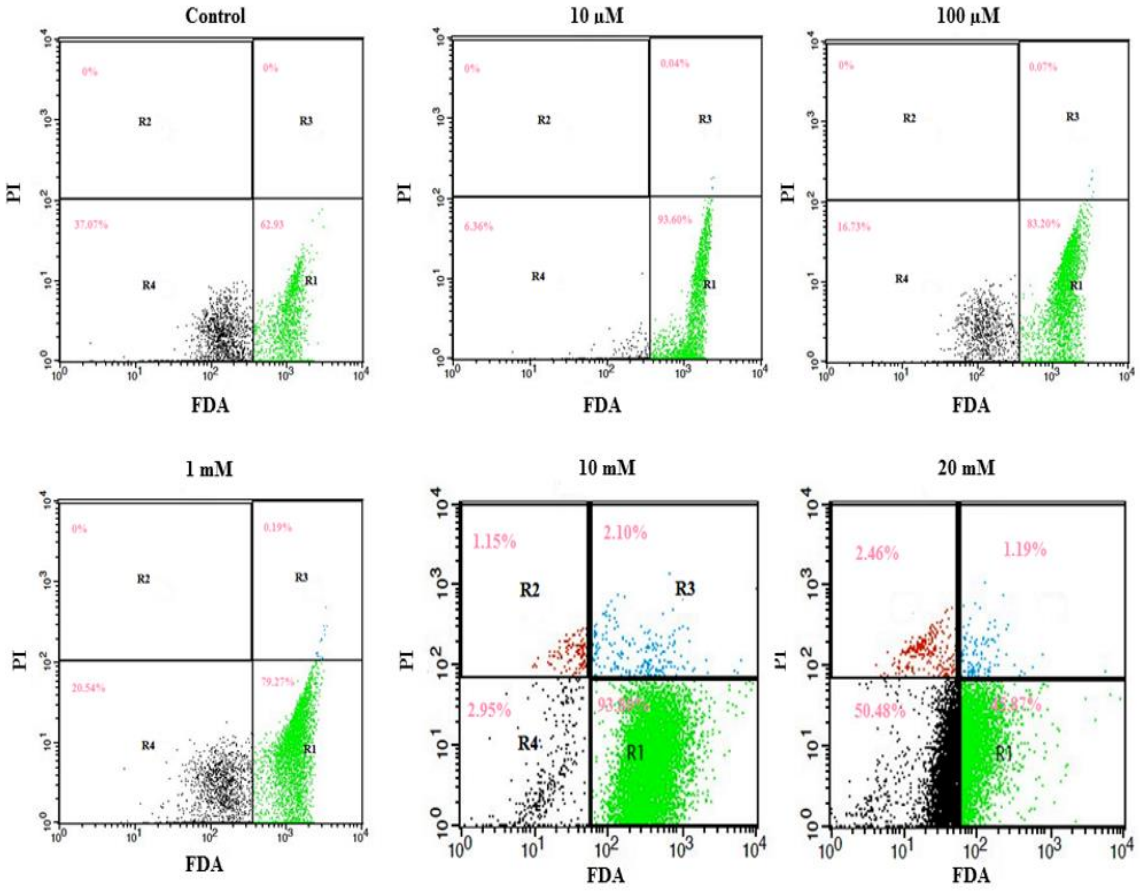

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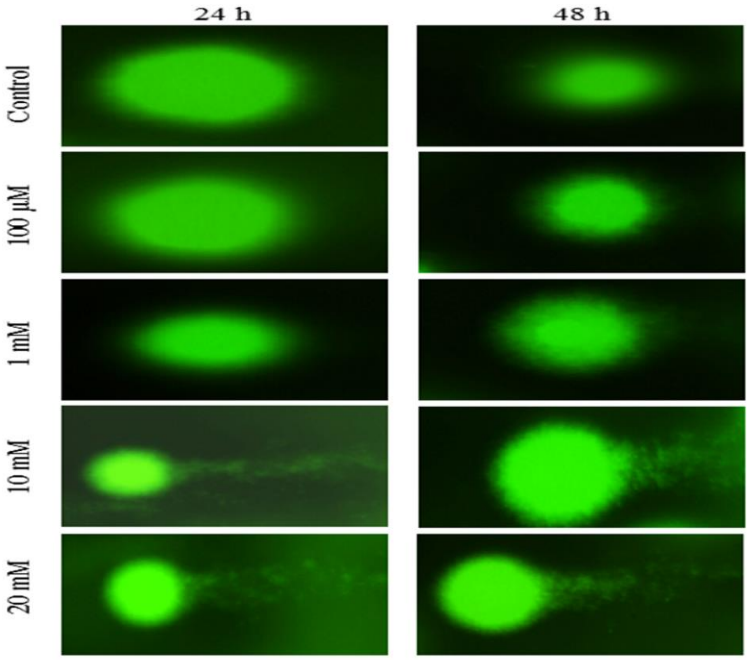

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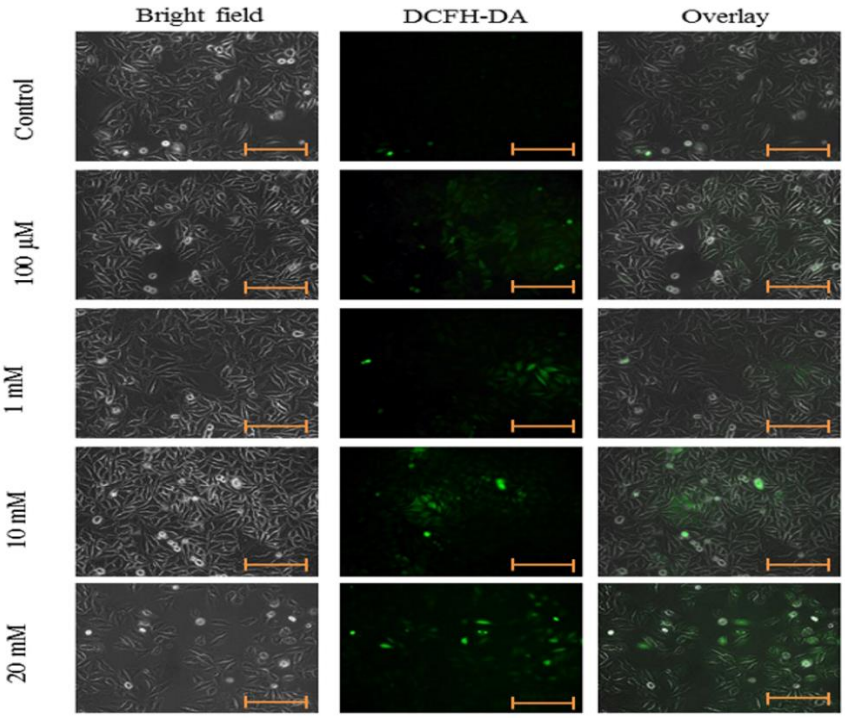

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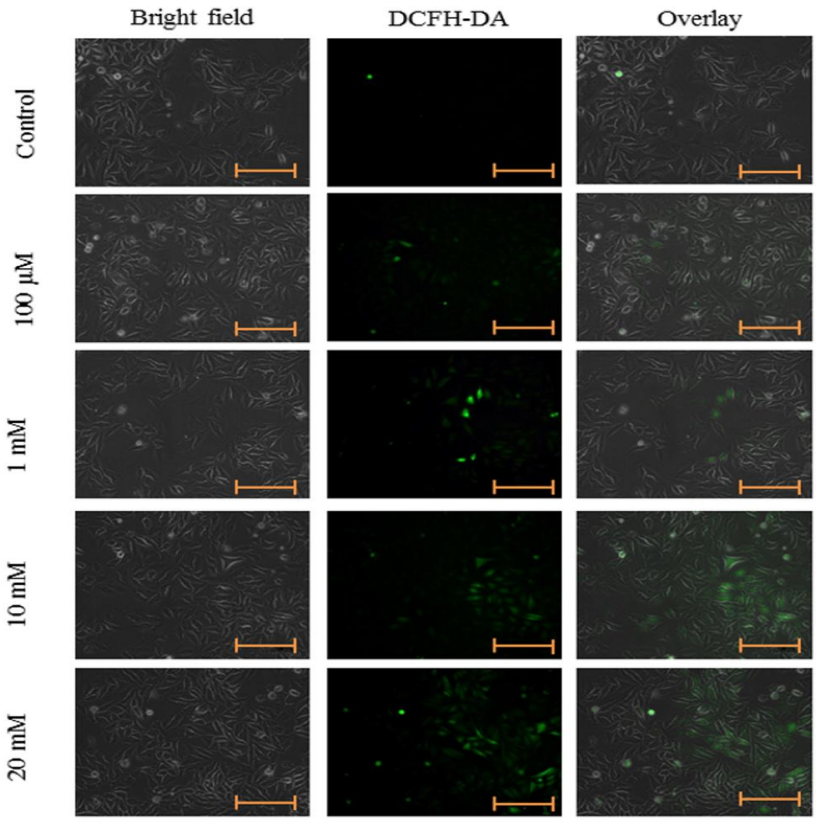

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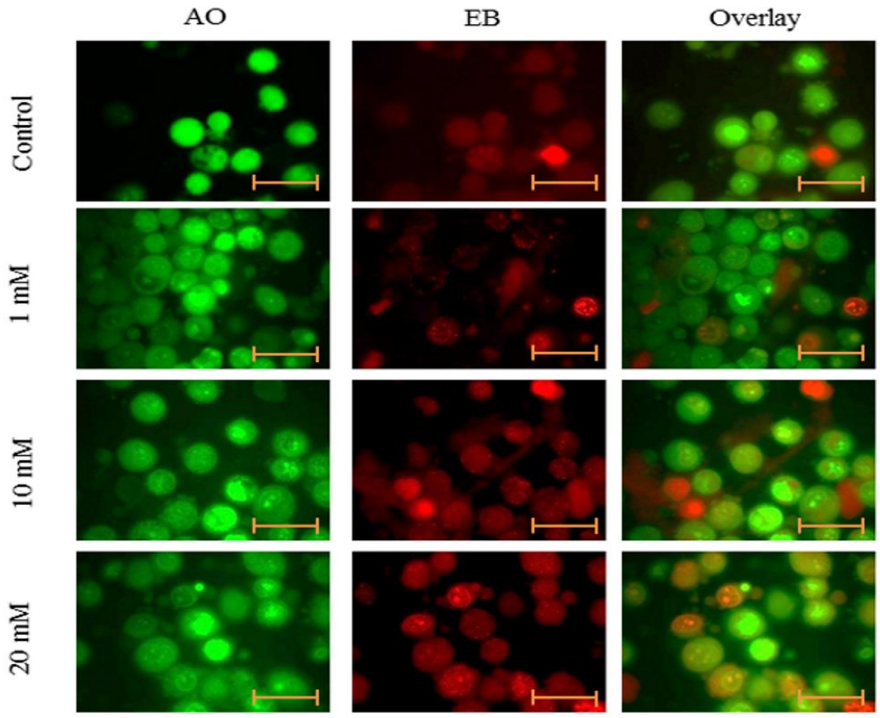

Figure 7

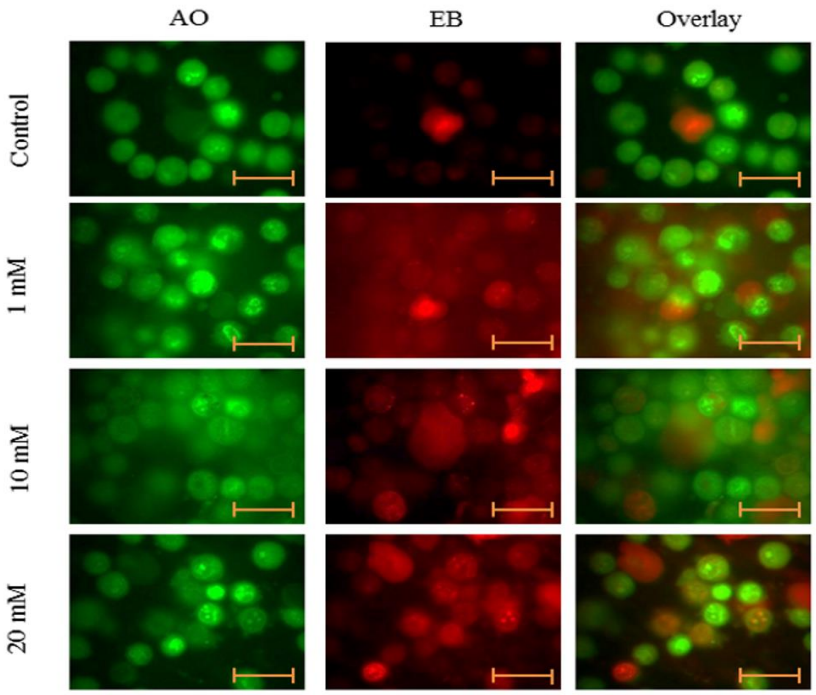

Figure 8