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# Effect of Methylsulfonylmethane on Proliferation and Apoptosis of A549 Lung Cancer Cells Through $G_2$/M Cell-cycle Arrest and Intrinsic Cell Death Pathway

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**Abstract.** *Background/Aim: Methylsulfonylmethane (MSM) is a natural organic compound that displays anti-inflammatory as well as antioxidant properties. MSM reportedly has potential in inhibition of tumor cells. However, molecular mechanisms underlying the effects of MSM on lung cancer remain unclear. Materials and Methods: In this study, the effect of MSM on A549 cells was examined. We focused on the mode of apoptosis induced by MSM and investigated alterations in the integrity of the outer membrane of mitochondria. Results: Our results showed that MSM inhibited viability of A549 cells and changed the shape and permeability of nuclei. In addition, MSM induced $G_2$/M arrest. MSM reduced the mitochondrial membrane potential and contributed to release of cytochrome c from mitochondria to cytoplasm. Conclusion: MSM is a potential anticancer agent for the treatment of lung cancer.*

Lung cancer, which is a most common cancer, is associated with a high rate of mortality (1). It is classified as either small-cell lung cancer (SCLC) or non-small-cell lung cancer (NSCLC), according to the histopathological phenotype (2). NSCLC accounts for 80% of all lung cancer and is subdivided into large-cell carcinoma, squamous-cell carcinoma and adenocarcinoma (2). Patients with NSCLC are treated with surgery, radiation therapy, and chemotherapy, depending on the pathological progression and treatment response of the cancer. The facile nature of chemotherapy makes it efficient to use in a variety of lung cancer cases, such as when radiation therapy or surgery is difficult, or before and after other treatments (3, 4). Cisplatin and carboplatin are well-known lung cancer drugs. These agents suppress lung cancer cell proliferation by inhibiting cell division by directly binding DNA in cancer cells (5). However, these platinum-based drugs are associated with serious side-effects and lose efficacy due to cancer recurrence and drug resistance (6). Therefore, finding alternative chemical therapeutics that are safer and more effective as lung cancer treatments is urgently required. To this end, a variety of natural, synthetic, and biological chemicals have been proposed as chemopreventive agents suitable for treating multistep carcinogenesis (7).

Methylsulfonylmethane (MSM) is a simple, organic sulfur compound otherwise known as dimethylsulfone, methylsulfone, crystalline dimethyl sulfoxide, sulfonyl bis methane or organic sulfur (8). MSM occurs naturally in plants, animals, various natural products, and foods (9). MSM is considered as a 'Generally Recognized as Safe' grade compound by the US Food and Drug Administration. Thus, it is recognized as a safe substance (10). MSM was reported to exert numerous physiological functions and is particularly well-known for its antioxidant and anti-inflammatory properties (10, 11).

Reactive oxygen and chronic inflammation affect many stages of cancer and are important factors that promote cancer development and progression (12, 13). It is believed that diverse pharmacological effects of MSM, including anti-inflammatory and antioxidant, may regulate various genes that are either overexpressed or inhibited in carcinogenesis, thereby reducing the generation of malignant cancer cell phenotypes. Therefore, many studies have been conducted to

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examine the inhibitory effect of MSM on cancer cells. MSM was found to induce endoplasmic reticulum stress and subsequently cause apoptosis of HCT-116 (colon cancer) cells, thereby effectively suppressing their proliferative capacity (14). Another study reported that MSM inhibited proliferation, migration and invasion ability of prostate cancer cells (15). Moreover, MSM has been reported to effectively inhibit breast cancer cells through synergistic action with existing anticancer drugs (16). In a recent study, our team demonstrated that MSM treatment induced cell-cycle arrest and cell apoptosis in YD-38 gingival cancer cells (17). Furthermore, MSM reduced cell proliferation and cancer cell invasiveness by down-regulating signal transducer and activator of transcription 5B (STAT5B) and STAT3 signaling in breast cancer cells (18). However, the effects of MSM on malignant lung cancer cell phenotypes and mechanisms underlying such effects have not been completely elucidated.

The present study examined molecular mechanisms underlying the inhibitory effect of MSM on A549 cells, a typical NSCLC model. This study specifically focused on intrinsic apoptosis following MSM treatment and investigated variations in cytochrome *c* expression and mitochondrial membrane potential associated with mitochondrial damage.

## Materials and Methods

*Reagents*. MSM (Sigma-Aldrich, St. Louis, MO, USA) dissolved in RPMI-1640 medium (Gibco, Grand Island, NY, USA) was stored at 4˚C in dark. Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). NE-PER nuclear and cytoplasmic extraction reagents were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Antibodies specific to cleaved poly-ADP ribose polymerase (PARP), cleaved caspase 9, B-cell lymphoma 2 (BCL2), BCL2-like protein 4 (BAX), and cytochrome *c* were bought from Cell Signaling Technology (Danvers, MA, USA). The anti-mouse and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody were supplied by Merck (Merck, Darmstadt, Germany). 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$) was obtained from Enzo (NY, USA). Caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK), was obtained from R&D Systems (Minneapolis, MN, USA) and fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit I was purchased from BD Biosciences (San Jose, CA, USA). Mitochondria isolation kit was supplied by Thermo Fisher Scientific Inc.

*Cell culture*. Human lung adenocarcinoma cell line A549 was purchased from the American Type Culture Collection (Manassas, MD, USA). Cells were cultured and sub-passaged in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin (ABAM; Invitrogen). Cells were cultured in a humidified incubator at 37˚C with 5% $CO_2$. Media were changed three times a week.

*Cell viability assay*. MTT assay was conducted to measure the cell viability. Lung cancer cells, A549, were seeded in 96-well culture plates at a density of $1\times10^4$ cells per 100 μl medium. After incubating overnight, the cells were treated with 100, 200 and 300 mM of MSM for 24 h. Then 5 mg/ml MTT was added to every well and plates were incubated at 37˚C for 4 h. The formazan product formed was dissolved by adding 100 μl of dimethyl sulfoxide (DMSO), and the wavelength absorbance was measured at 570 nm using an Ultra Multifunctional Microplate Reader (TECAN, Durham, NC, USA). All measurements were performed in triplicates and repeated at least three times.

*Morphological analysis*. Morphology of apoptotic nuclei and permeability to DAPI was observed using DAPI staining. A549 Lung cancer cells were plated at a density of $1.5\times10^5$ cells/plate on 6 cm culture dishes, and incubated at 37˚C for 24 h. MSM was then added at different concentrations (100, 200 and 300 mM) for 24 h. Cells were treated with 500 μl of 300 nM DAPI staining solution, then cells were washed twice with phosphate-buffered saline (PBS) and mounted on microscope slides using mounting solution. Cell images were acquired using an inverted IX71 microscope (Olympus, Tokyo, japan).

*Cell-cycle analysis*. Cell-cycle analysis was performed by PI staining with the help of flow cytometry. A549 cells ($1.5\times10^5$ cells/ml) were seeded into 6 cm culture dishes and treated with different concentrations (100, 200 and 300 mM) of MSM for 24 h. Cells were isolated with trypsin-EDTA and fixed with 70% ethanol. The cells were then washed twice with cold PBS and the supernatant from centrifugation discarded. The pellet was re-suspended with PBS and stained using 50 μg/ml of PI and 100 μg/ml of RNase A for 20 min in the dark. DNA contents were analyzed by flow cytometry using a FACS Calibur instrument and software (BD Biosciences, San Jose, CA, USA).

*Annexin V and PI staining*. A549 Lung cancer cells ($1.5\times10^5$ cells/ml) were seeded in 6 cm culture dishes and incubated for 24 h. Cells were treated with MSM (100, 200 and 300 mM) for 24 h and then harvested with trypsin-EDTA and washed thrice using PBS. Annexin V and PI staining were carried out using FITC-annexin V apoptosis detection kit I (BD Biosciences). Data were analyzed using FACSCalibur software (BD Biosciences).

*Western blotting*. A549 Cells were treated with different concentrations (100, 200 and 300 mM) of MSM for 24 h and isolated with PBS. The resulting cell pellets were re-suspended in RIPA lysis buffer containing phosphatase inhibitor and protease inhibitor cocktail. The cell lysates were incubated on ice for 1 h and purified by centrifugation at 17,000 × *g* at 4˚C for 10 min. Protein content was quantified by Bradford assay (Bio-Rad, Hercules, CA, USA) using a spectrophotometer. The cell lysates were separated by 10% SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane, which were blocked in 5% non-fat dried milk dissolved in Tris buffered saline with Tween 20 buffer for 1 h at room temperature. The membrane was incubated overnight at 4˚C with specific primary antibodies against PARP, cleaved PARP, cleaved caspase 9, actin, BAX, BCL2 and cytochrome *c*. After washing, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies for 1 h at room temperature. After washing, the blots were analyzed using West-Zol Plus and western blot detection

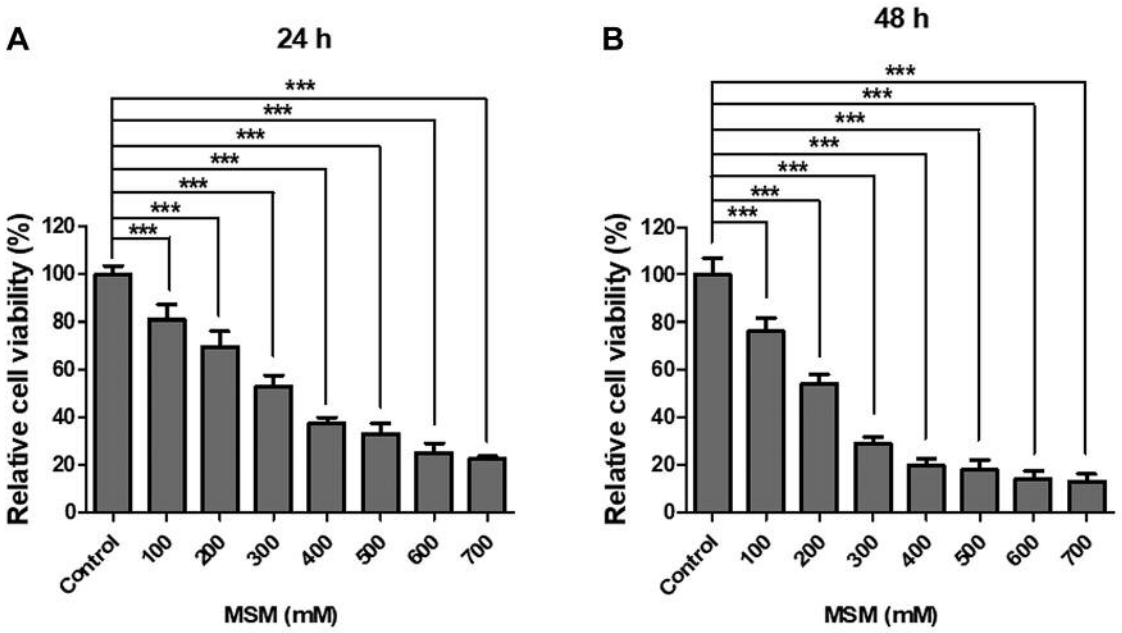


Figure 1. *Antiproliferative effect of methylsulfonylmethane (MSM) on A549 cells. Cytotoxic effects of MSM on A549 cell line were found by MTT assay. Cells were treated with MSM at increasing concentrations for 24 (A) and 48 (B) h, respectively. Results represent the mean±standard deviation (SD) of at least three independent experiments. ***Significantly different at p<0.001 (one-way ANOVA).*

system (iNtRON Biotechnology, SungNam, Republic of Korea). Relative densitometric quantification of western blot data were performed using Image J software from National Institutes of Health (Bethesda, MD, USA) and normalized to data for actin.

*Mitochondrial membrane potential analysis*. Mitochondrial membrane potential was determined by DiOC6 staining using flow cytometry. In brief, A549 cells ($2.5\times10^5$ cells/well) were cultured in 6 cm culture dishes for 24 h and treated with 100, 200 and 300 mM MSM for 24 h. Cells were isolated with trypsin-EDTA and 0.1 M DiOC6 was added to the cells, after which the cells were incubated for 15 min at 37˚C. The cells were then centrifuged (300 × *g* for 5 min at 4˚C), washed twice with PBS, and re-suspended in 200 μl of PBS. The samples were analyzed using FACSCalibur instrument (BD Biosciences). The whole protocol was performed under low light.

*Isolation of mitochondria fraction*. Mitochondria from MSM-treated and non-treated A549 cells were extracted by mitochondria isolation kit (Thermo Fisher Scientific Inc.). Mitochondria isolation reagent was added to the cells ($2\times10^6$) and incubated on ice. After incubation, Reagents B and C from the kit were applied and the preparation was incubated on ice in between each treatment. The mixture was then centrifuged at 700 × *g* for 15 min to collect the supernatant. The collected mitochondria pellet was washed with Reagent C and used for western blotting applications.

*Statistical analysis*. All data were expressed as the mean±standard deviation from three independent experiments. Graph Pad Prism software (Graph Pad Software, La Jolla, CA) was used to analyze the data. The statistical significance ($p<0.05$, $p<0.01$, or $p<0.001$) was assessed by paired one-way analysis of variance (ANOVA) or Student's *t*-test followed by *post-hoc* comparison (Tukey's HSD).

## Results

*MSM reduced the viability of A549 lung cancer cells*. To evaluate the effect of MSM on the A549 cell viability, we conducted MTT assays. Based on our previous study (17), we conducted an MSM dose-dependent study for 24 and 48 h. MSM displayed time- and concentration-dependent inhibition of cell proliferation in A549 cells (Figure 1). The half maxima- inhibitory concentration ($IC_{50}$) value at 24 h was approximately 300 mM in A549 cells (Figure 1A). At 48 h, the $IC_{50}$ value was 200 mM, indicating 48-h treatment also followed a similar trend with a more severe effect (Figure 1B). Based on these results, further experiments were conducted.

*MSM induced changes in morphological characteristics of A549 cells*. Morphology is an important clue that reflects changes in the state of cancer cells as well as in the expression of their genes (19, 20). In particular, the shape of cells changes considerably in the process of cell division and during the course of differentiation and death (20). Apoptosis is known to cause shrinkage and blebbing of the cell membrane (21). In addition, not only the shape of nuclei, but also their permeability to DAPI is known to increase during cell death (22). In this study, we monitored morphological changes in A549 cells for 24 h following MSM treatment by inverted phase-contrast microscopy. The shape of A549 cells changed considerably and the number of floating cells at the 24 h time

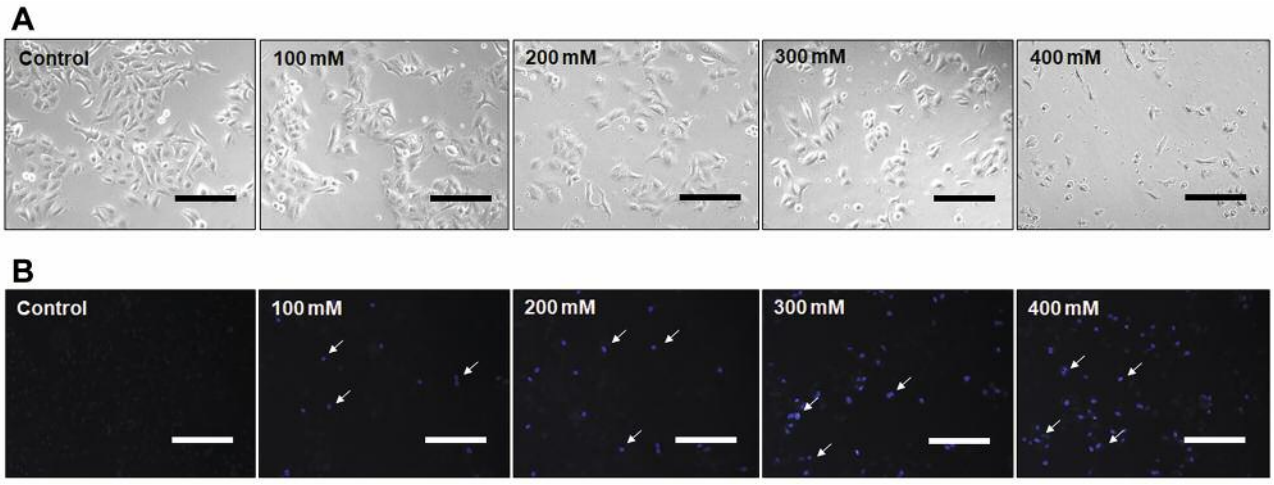


Figure 2. *Methylsulfonylmethane (MSM) induced changes on A549 cell morphology. Cells were treated with MSM at different concentrations for 24 h. A: Observation of A549 cell morphology using phase-contrast microscopy. B: 4',6-Diamidino-2-phenylindole staining was performed to analyze nuclear morphological changes using inverted phase-contrast microscopy. White arrows indicate abnormal nuclei. Representative photographs are presented. (Scale bar: 200 μm).*

point increased with increasing MSM concentration (Figure 2A). In addition, DAPI staining was used to observe nuclear abnormalities (23). Nuclear permeability of A549 cells to DAPI increased with MSM concentration. Additionally, fragmentation of nuclei associated with the process of apoptosis was elevated by MSM in a concentration-dependent manner (Figure 2B).

*MSM induced $G_2$/M cell-cycle arrest and increased the sub-$G_1$ population of A549 cells*. Abnormal cell cycles are key features of cancer cells (24). Cell-cycle changes provide important information to researchers, enabling the identification of pharmacological effects of anticancer agents, and are considered important targets in chemoprevention and cancer treatment (25). The sub-$G_1$ phase contains apoptotic cells with hypodiploid fragmented DNA. Therefore, changes in the cell cycle were examined using PI staining following MSM treatment. When A549 cells were treated with MSM for 24 h, the ratio of $G_2$/M and S phases increased, while that of $G_0/G_1$ decreased in a concentration-dependent manner. The sub-G1 population was also augmented by MSM (Figure 3A and B). These results clearly indicated that MSM induced $G_2$/M cell-cycle arrest and increased the sub-$G_1$ population of A549 cells.

*MSM treatment induces apoptosis of A549 cells*. Increased permeability of the nuclear membrane and surface exposure of phosphatidylserine is one of the key features of apoptosis (26). Therefore, annexin V-FITC and PI staining are commonly used as a method for detecting apoptosis (27). As a result, the ratio of annexin $V^+/PI^-$, and annexin $V^+/PI^+$ cells increased with MSM concentrations for 24 h (Figure 4A). In addition, early apoptosis (annexin $V^+/PI^-$, ANOVA, $p<0.01$) and the late apoptosis (annexin $V^+/PI^+$, ANOVA, $p<0.05$) cell populations increased significantly (Figure 4B). This result indicated that MSM induced apoptosis of A549 cells.

*MSM induced A549 cell death through the intrinsic pathway*. Apoptosis is activated through both intrinsic and extrinsic pathways *via* induction of caspase cascades. Intrinsic apoptosis is associated with mitochondria and accompanied by activation of caspase 9 (28, 29). In the extrinsic pathway, caspase 8 activation is induced by death receptors such as FAS, FAS-associated protein with death domain, and death receptor (30). These two pathways of cell death ultimately lead to cleavage of PARP through the caspase cascade. Western blot results indicated that cleaved caspase 9, a component of the intrinsic apoptosis pathway, increased significantly (one-way ANOVA, $p<0.001$) with MSM concentration in A549 cells. Expression of cleaved PARP also increased in the same manner (Figure 5A and B). Z-VAD-FMK, an irreversible general caspase inhibitor, was used to validate the effect of MSM on apoptosis. MTT assay showed that Z-VAD-FMK treatment successfully restored the viability of A549 cells (Figure 5C). Moreover, expression of cleaved caspase 9 and cleaved PARP were significantly reduced following application of Z-VAD-FMK to MSM treated cells (Figure 5D and E). These results clearly indicated that MSM induced apoptosis of A549 cells. Mitochondria constitute a major component of intrinsic apoptosis (31). The formation of a mitochondria outer membrane pore, is an important aspect of intrinsic apoptosis, which in turn is affected by variations in the expression of BCL2 family genes. Pore formation induces changes in the mitochondrial membrane

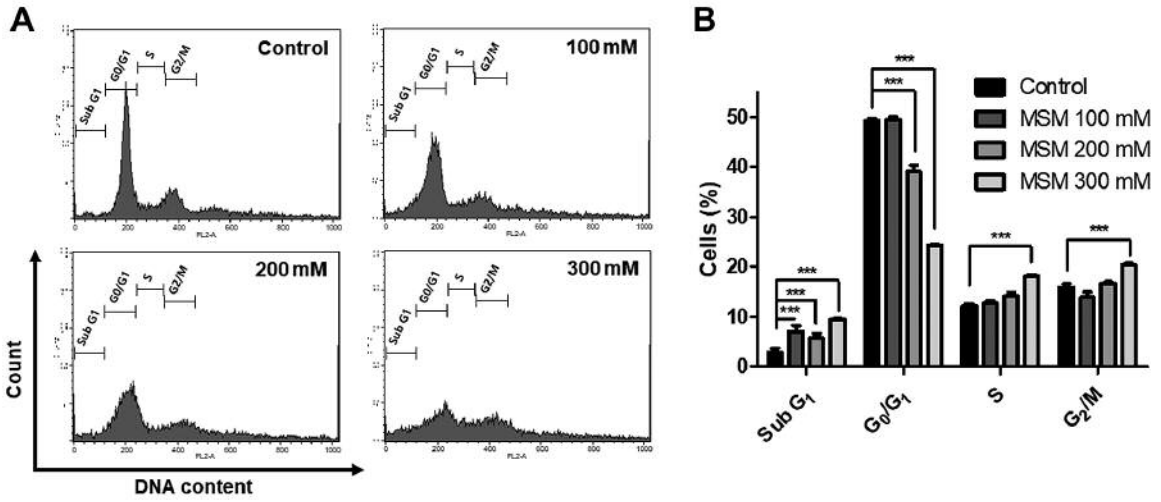


Figure 3. *Methylsulfonylmethane (MSM) induced $G_2$/M cell-cycle arrest and apoptosis of A549 cells. A: A549 Cells were treated with increasing concentrations of MSM for 24 h and then stained with propidium iodide for analysis by flow cytometry. B: The cell population in each cell-cycle phase was calculated as a percentage of the total number of cells. Data are expressed for three independent experiments ***Significantly different at p<0.001 (one-way ANOVA). AU: Arbitrary unit.*

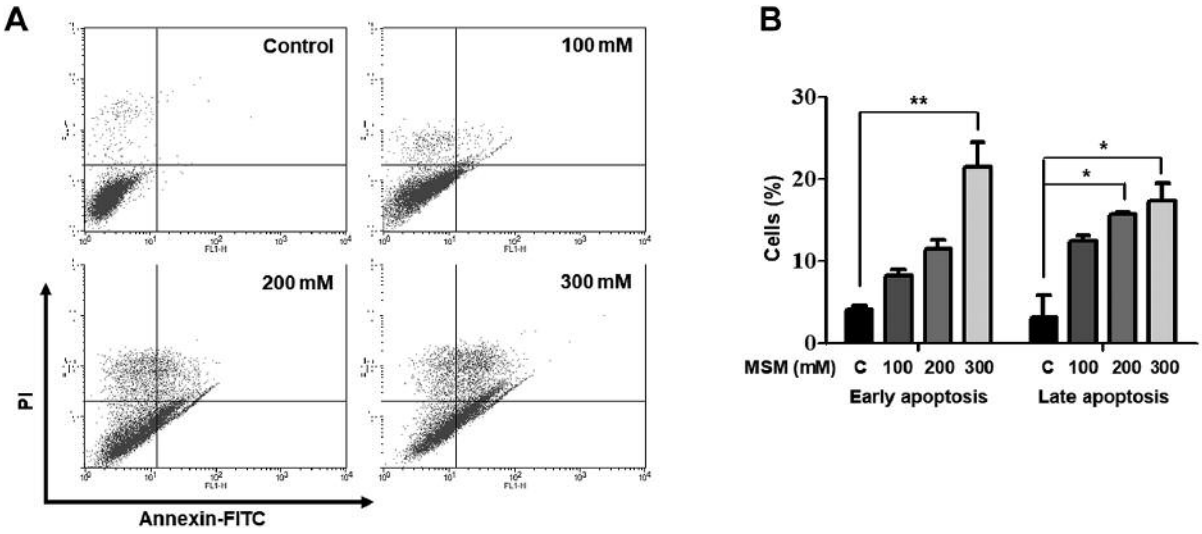


Figure 4. *Methylsulfonylmethane (MSM) induced early and late apoptosis of A549 cells. A: Flow cytometric analyses after annexin V and propidium iodide (PI) staining. A549 Cells were treated with MSM for 24 h. B: Graphical representation of the early- and late-apoptotic populations of A549 cells. Data are expressed as the mean±SD of three independent experiments. Significantly different at *p<0.05, and **p<0.01 (one-way ANOVA).*

potential and triggers a caspase cascade by increasing the cytosolic fraction of cytochrome *c* (32). The current study measured changes occurring in the mitochondrial membrane potential following MSM treatment, using DiOC6 staining (33). The mitochondrial membrane potential of A549 cells decreased on treatment with MSM in a concentration-dependent manner (Figure 6A and B). To quantify these data, the A549 cell population was divided into M1 and M2 based on the value of fluorescence intensity (FL1-H=102). The cells in M1 and M2 populations changed significantly (ANOVA, $p<0.001$) with MSM treatment (Figure 6C). Furthermore, protein expression of BAX increased (ANOVA, $p<0.05$), while that of BCL2 down-regulated (ANOVA, $p<0.01$) significantly (Figure 6D and E). Figure 6F shows the

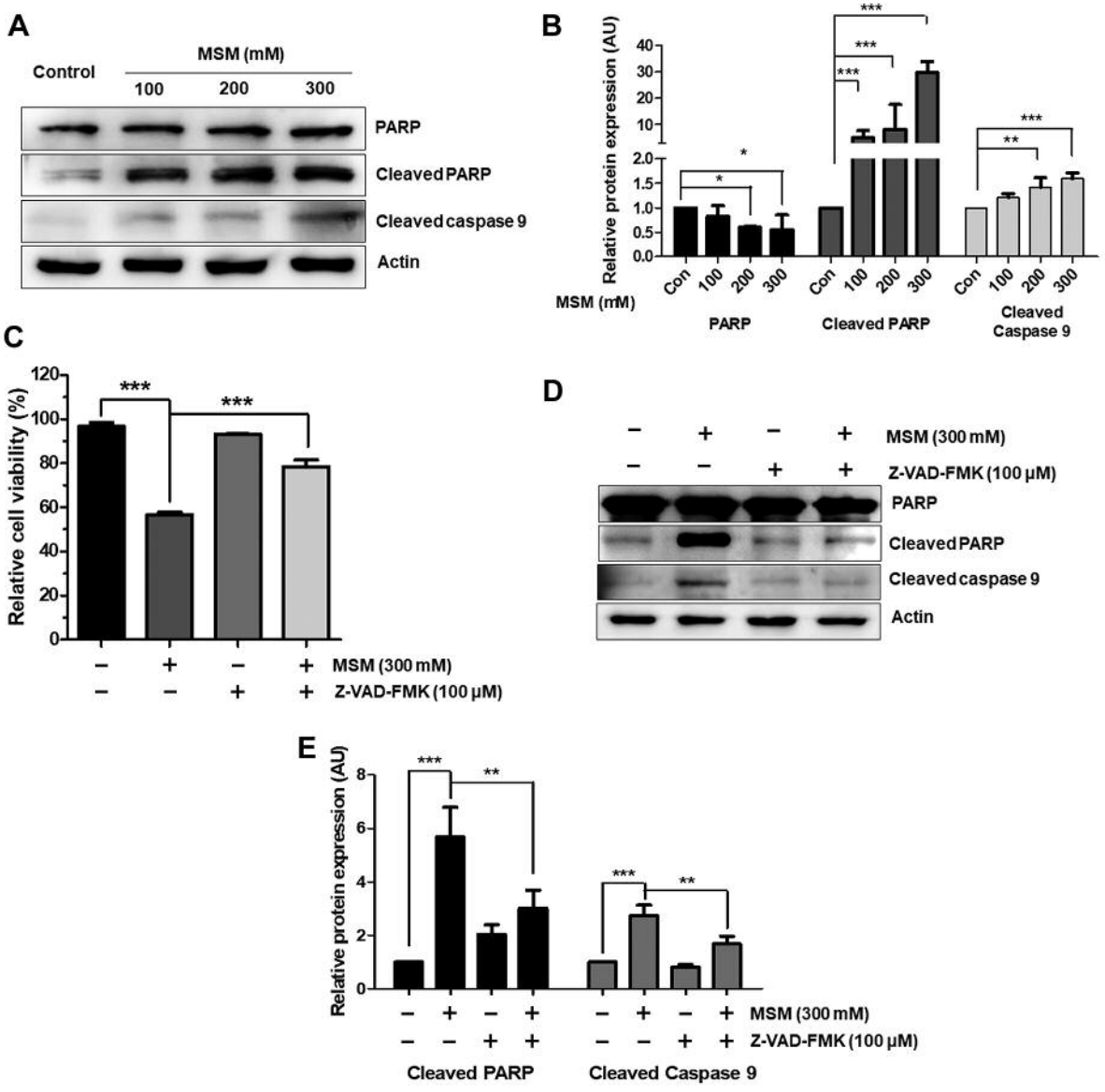


Figure 5. *Methylsulfonylmethane (MSM) induced intrinsic apoptosis pathway in A549 cells. A: Expression of cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase 9 were measured by western blotting. A549 Cells were treated with different concentration of MSM for 24 h. Actin was used as a reference. B: Graphical representation of the expression level of cleaved PARP and caspase 9 after MSM treatment for 24 h. C: Effect of pan-caspase inhibitor Z-VAD-FMK on A549 cells treated with 300 mM MSM was assessed by MTT assay. A549 cells were treated with or without MSM and Z-VAD-FMK for 24 h. Z-VAD-FMK was administered 6 h before MSM treatment. D: Western blot analysis of, cleaved PARP and caspase 9 after treatment with and without 300 mM MSM and 100 μM Z-VAD-FMK for 24 h. E: Relative protein expression depicted in graphical representation. Data are expressed from three independent experiments. Significantly different at *p<0.05, **p<0.01, and ***p<0.001 (one-way ANOVA). AU: Arbitrary unit.*

localization of cytochrome *c* to be mostly in the cytosol in control cells, whilst at 300 mM MSM, it was found in mitochondria, although the amount of cytochrome *c* in the total protein extract was not changed markedly. These results demonstrated that MSM-induced intrinsic apoptosis was triggered by cytochrome *c* that was released due to the breakdown of mitochondrial membrane potential.

## Discussion

Development of cost-effective anticancer drugs using naturally-derived chemical compounds which induce cell cycle and apoptosis in cancer cells is challenging (34). Disruption of the normal cell cycle leads to the development of most tumors, and inhibition of the cell cycle is a strategy used to inhibit cancer

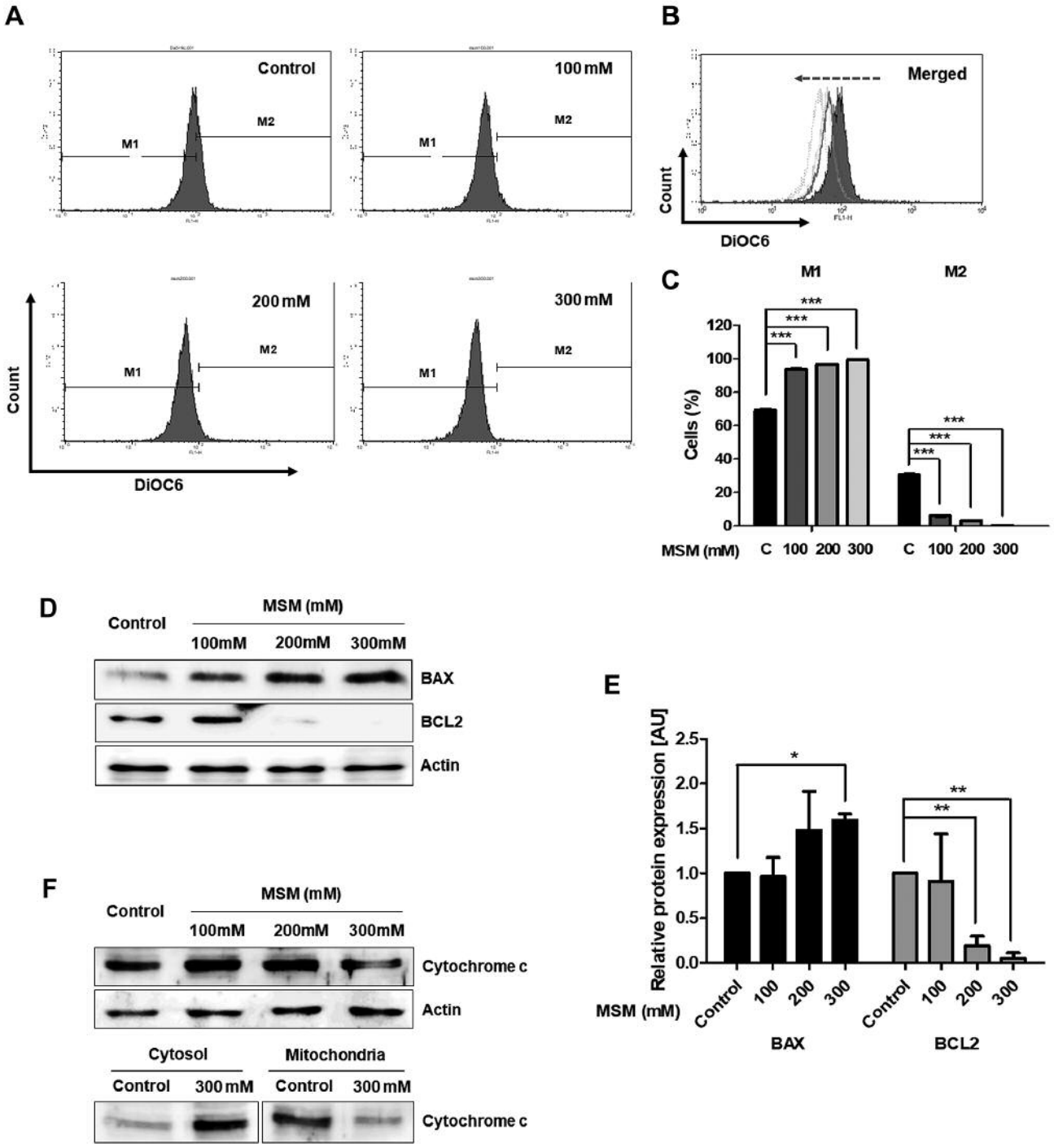


Figure 6. *Involvement of mitochondrial apoptosis pathway in the effects of methylsulfonylmethane (MSM) on A549 cells. A: MSM-treated A549 cells were stained by DiOC6 for measuring mitochondrial membrane potential. A549 cells were treated with increasing concentration of MSM for 24 h. Data were acquired using FACS. The point for separation of the cell population was located at FL1-H 102 which divided the A549 cell population into M1 and M2. B: Merged image of data from each analysis of part (A). Dotted red arrow indicates the shift of cell population from control cells to 300 mM MSM-treated cells. C: Graphical representation of percentage of M1 and M2 cell population. D: Patterns of expression of BCL2 apoptosis regulator (BCL2) and BCL2-associated X, apoptosis regulator (BAX) were measured by western blotting. E: Relative protein expression depicted in graphical representation. F: Western blot analysis of cytosolic and mitochondrial cytochrome c expression. Total cytochrome c expression was investigated using total cell extract, while cytosolic and mitochondrial cytochrome c were accessed by lysates of each fraction. Data are expressed from three independent experiments. Significantly different at *p<0.05, **p<0.01, and ***p<0.001 (one-way ANOVA). AU: Arbitrary unit.*

cell growth (35). Therefore, we investigated whether MSM suppresses A549 cell proliferation along with cell-cycle arrest. The results of MTT and FACS analysis demonstrated that MSM induced $G_2$/M phase and inhibited cell proliferation in A549 cells (Figure 1B-D and Figure 3A and B), and these results were parallel to those of a previous study (36). However, the results of a previous study, which used same time exposure to MSM, on gingival and prostate cancer cells showed that MSM induced $G_0/G_1$ phase arrest (17), indicating that the effect of MSM on cell-cycle arrest may differ depending on the human cancer cell type. Collectively, these findings suggested that treatment with MSM inhibited cell viability by inducing $G_2$/M cell-cycle arrest in A549 cells.

Notably, MSM caused an increase in the sub-$G_1$ population (Figure 3C), suggesting that reduced cell viability may be related to DNA damage or an increase in the number of dead cells (24). Results of FACS analysis using annexin V and PI staining showed that MSM-induced cell death was associated with both early and late apoptosis (Figure 4). There are two key pathways in apoptosis, intrinsic and extrinsic. These apoptotic pathways can either coexist or each pathway can be activated separately (37). The results of western blot analysis for cleaved caspase 9 and cleaved PARP indicated that MSM-induced cell death was associated with the intrinsic apoptosis pathway (Figure 5). Interestingly, Kim et al. reported that MSM induced the extrinsic pathway in hepatic tumor cell lines, such as HepG2 and Huh7 (36). Therefore, it is assumed that involvement of the apoptotic pathway may differ depending on the cancer cell type.

Mitochondria play a vital role in intrinsic apoptosis. Among the apoptosis-related proteins, the ratio of BCL2 and BAX is important for the progression of apoptosis. BCL2 functions as an anti-apoptotic factor that inhibits the release of cytochrome *c* from mitochondria into the cytosol, thus inhibiting apoptosis. BAX is a pro-apoptotic protein that acts as an apoptotic factor in mitochondria. These proteins act as regulators of mitochondrial membrane pore formation, and down-regulate mitochondrial membrane potential. The caspase cascade is activated subsequent to the release of cytochrome *c* from mitochondria to the cytosol (32). To confirm whether mitochondrial cytochrome *c* release is takes part in the intrinsic apoptosis pathway, we performed western blot with mitochondrial and cytosolic extracts (Figure 6). Our results clearly showed that the MSM-induced intrinsic apoptosis pathway was coupled with a decrease in mitochondrial membrane potential. However, MSM reportedly induces the extrinsic apoptosis pathway and down-regulates BCL2 expression simultaneously in HepG2 cells (36). Therefore, elucidation of molecular mechanisms underlying cross-talk between extrinsic and intrinsic apoptosis may be needed prior to applying MSM.

A549 cells used as a NSCLC model exhibited mutations in *KRAS* and in various other genes that are involved with various malignant characteristics of cancer cells, including abnormal proliferation and resistance to apoptosis (38, 39). The current study observed that MSM treatment induced cell-cycle arrest and intrinsic apoptosis of A549 cells. These findings also suggest that MSM can be used as a potential chemopreventive agent for the treatment of lung cancer.

## Conflicts of Interest

The Authors declare no conflict of interest.

## Authors' Contributions

Y.M.Y. and K-J.J. planned the experiments. D.H.K., N.S. and D.Y.K. conducted most of the experiments. E.S.J. and A.R. helped in some tests. Y.M.Y., K-J.J., D.H.K., N.S. and D.Y.K. evaluated the data, and D.H.K. wrote the article. All Authors helped to revise the article and accepted the final version for publication.

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