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In Vitro Antiproliferative and Apoptosis Inducing Activity of Bibimbap on HT-29 Human Colorectal Adenocarcinoma Cells

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Abstract

The present study was designed to investigate the antiproliferative activity and molecular mechanisms of Bibimbap in HT-29 human colorectal adenocarcinoma cells. Bibimbap extract inhibited the proliferation of HT-29 cells by 50% at a concentration of 10.1±0.17 mg/mL for 48 h. The population of live cells decreased slightly, and the morphology changed with a reduction in cell volume (pyknosis) with Bibimbap. Treatment with 5 mg/mL of Bibimbap resulted in slight cell shrinkage. Furthermore, as the Bibimbap dose increased to 10 mg/mL, these characteristics were more evident, and HT-29 cells exhibited partial detachment by staining with the DNA-binding dye Hoechst 33342. Flow cytometric analysis by Annexin V and PI double staining showed that Bibimbap increased the levels of apoptosis. Analysis of the mechanism of these events showed that Bibimbap-treated cells exhibited a mitochondria-dependent apoptotic pathway through the modulation of caspase-3, caspase-8, caspase-9, and poly-ADP ribose polymerase, as well as Bax and Bcl-2 expression in dose- and time-dependent manners. Consequently, Bibimbap exerts a significant antiproliferative effect on HT-29 human colorectal adenocarcinoma cells.

Key words: Bibimbap, antiproliferation, HT-29, human colorectal adenocarcinoma cells, mitochondrial apoptosis, Korean dish

Introduction

Bibimbap is well known Korean dish that has been watched as a traditional Korean food. Bibimbap has been developed on the basis of special district's own in Jinju, Jeonju, and Haeju (Kim et al. 2004). Bibimbap has been also appeared as a healthy food from people around the world (Ko et al. 2013), and it has been upgraded and listed in the menu of Korean restaurant and Air Lines as one-dish meal (Kim SH 2007). The main ingredients of Bibimbap are cooked rice, vegetables, ground meat, and hot pepper paste. As a staple food, rice constitutes the main source of carbohydrates and energy. For traditional Bibimbap rice, the "Beauty of Korean Food: With 100 Best-Loved Recipes" released by Institute of Traditional Korean Food suggests white rice, bean sprouts, spinach, bracken, Chwi-namul, carrot, beef, sesame oil, hot bean paste, green onions, salted sesame seeds, soy sauce (Lee et al. 2016).

Colorectal cancer is one of the most common cancers worldwide, accounting for 10% of all new cancer cases and responsible for 9.4% of all cancer deaths in 2020. A high incidence and burden of colorectal cancer occurs in eastern Asia (Sung et al. 2021). In Korea, colorectal cancer has become the second leading cancer in men and the third most frequent cancer in women (Jung et al. 2017). Dietary intake with processed or red meat is primary risk factor for colorectal cancer. It can be associated with an increased risk of colorectal cancer in a report from the World Cancer Research Fund (Kim & Park 2018). The increasing consumption of Western foods might be the one of risk factor for colorectal cancer in Korea. One promising approach involves the administration of dietary phytochemicals that possess biologically active components which inhibit the proliferation of cancer cells. It is recommended the more administration of phytochemicals to be reduced the rates of colorectal cancer (Jemal et al. 2010; Ryu & Chung 2015; Mahadevappa

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& Kwok 2017; Ranjan et al. 2019; Shim et al. 2022). Phytochemicals found in fruits, grains, and vegetables abundantly may protect against chronic diseases as cancer, cardiovascular disease, diabetic mellitus and neurodegenerative diseases. Human studies have assessed the chemoprotective effect of selected phytochemicals in colorectal cancer. For the ongoing study on the identification of cytotoxic phytochemicals from natural resources (Ryu et al. 2013; Guon & Chung 2016; Ryu & Chung 2016; Guon & Chung 2022; Kim & Chung 2022), potent apoptotic components have been isolated. In the present study, we examined the basics of hypothesis testing, and investigated proliferation of Bibimbap made with *Glycine max* L. *Merr*, *Brassica rapa* var. *glabra*, *Agaricus bisporos*, *Pleurotus eryngii*, *Lentinula edodes*, and *Brassica oleracea* var. *capitata* L. which showed high antiproliferative activities on HT-29 human colorectal adenocarcinoma cells, at various concentrations of 1-20 mg/mL. Through these experiments, we expected that the use of different and high bioactive ingredients in Bibimbap would have a significant healthy effect on colorectal cancer control.

Materials and Methods

1. Preparation of Bibimbap

The purple rice and vegetables were purchased at Nonghyup Hanaro mart at Yangjae, Seoul, Korea. Each of 30 g of *Glycine max* L. *Merr*, *Brassica rapa* var. *glabra*, *Agaricus bisporos*, *Pleurotus eryngii*, *Lentinula edodes*, and *Brassica oleracea* var. *capitata* L. were heated with 15 g of soybean oil in frying pan and cooled at room temperature. Mix all ingredients together with 300 g of cooked purple rice, 10 g of sesame oil and 25 g of hot bean paste, and then dried using an automatic food dryer (LD-528WG; L'equip Co. Ltd., Seoul, Korea) for 12 h. The dried Bibimbap was soaked in 70% ethanol and sonicated for 1 h at room temperature. The extracts were evaporated under reduced pressure using a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 40℃. The Bibimbap extracts were dried at 60℃ and stored at −20℃ (Operon Co. Ltd., Gimpo, Korea) until used *in vitro* assay.

2. Chemical reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 100 units/mL penicillin and 100 μg/mL streptomycin were purchased from Thermo Scientific Inc. (Waltham, MA, USA). Primary antibodies and rabbit monoclonal anti-Bcl-2 (sc-492) and anti-Bax (sc-493) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit monoclonal antibodies against cysteinyl aspartate-specific cleaved caspase-3, caspase-8, caspase-9 and poly-(ADP-ribose) polymerase (PARP; 5625) antibodies were purchased from Cell Signaling Technologies, Inc. (Danvers, MA, USA). All other chemicals and reagents were of the highest analytical grade.

3. Cell culture and viability assay

FHC human colorectal normal cells and HT-29 human colorectal adenocarcinoma cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in RPMI-1640, supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, and incubation was carried out at 37℃ in a humidified incubator in a 5% CO_2 atmosphere. Cell counts were performed using a hemocytometer from Hausser Scientific (Horsham, PA, USA). Cell viability of Bibimbap on HT-29 cells were estimated colorimetric using the MTT assay, based on the reduction of tetrazolium salt by mitochondrial dehydrogenase in viable cells (Carmichael et al. 1987; Ryu & Chung 2016). Briefly, cells were seeded (2×10^6 cells/mL) in a 96-well plate and then treated with Bibimbap at final concentrations of 1, 5, 10, 15, and 20 mg/mL. After 24, and 48 h incubation, MTT solution was added to each well at a final concentration of 0.2 mg/mL. After 2 h of incubation, the supernatants were aspirated and replaced with 150 μL of DMSO to dissolve the formazan product. The absorbance at 540 nm was then read using a spectrophotometric plate reader. Results were calculated as percentages of the unexposed control.

4. Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was observed using the DNA specific blue fluorescent dye Hoechst 33342. The viable cells were stained homogeneously, whereas apoptotic cells which had undergone chromatin condensation and/or nuclear fragmentation were not stained (Gschwind & Huber 1995; Lizard et al. 1995). The HT-29 cells were treated with Bibimbap at the concentrations of 1, 5, 10, 15, and 20 mg/mL. Cells were then fixed for 30 min in 100% MeOH, washed with PBS, and then stained with 2 μg/mL of Hoechst 33342. The cells were observed under

a fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

5. Apoptosis analysis

Annexin V/PI double staining assay was carried out for the determination between early and late apoptosis stages. It was determined using an ApoScanTM Annexin V-FITC apoptosis detection Kit (BioBud, Seoul, Korea). The amounts of early apoptosis and late apoptosis were determined as the percentage of Annexin V+/PI − or Annexin V+/PI+ cells. The cells were trypsinized, harvested, and then washed with PBS. The cells were resuspended in 500 μL of binding buffer and incubated with 1.25 μL of Annexin V-FITC (200 μg/mL) at room temperature, 15 min. After centrifugation, the supernatant was removed. The cells were resuspended in 500 μL of 1× binding buffer and cell suspensions were stained with 10 μL of PI (30 μg/mL) at 4℃ in dark. Fluorescence was quantified using FACSCalibur flowcytometry (Becton Dickinson, San Jose, CA, USA).

6. RT-PCR

RT-PCR was performed as previously described (Guon & Chung 2016). Cells were treated with dihydroxyflavone at 10 μg/mL for 48 h, and treated with Bibimbap of 0, 1, 5, and 10 mg/mL at the final concentrations. Total RNA (5 μg) was reverse transcribed into cDNA by incubating with Invitrogen SuperScript®™ RNase H reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PCR was conducted in a reaction composed of 40 μg of 1.25X reacting mix, 1 μL enzyme mix, 1 μL forward primer (200 nM), 1 μL reverse primer (200 nM) and RNA. cDNA was performed with following conditions: cDNA synthesis at 45℃ for 30 min, followed by denaturation at 94℃ for 2 min. A total of 40 cycles of PCR amplification were then performed with following conditions: 94℃ for 15 sec, 60℃ for 30 sec, and 68℃ for 1 min. The last cycle was followed by final extension step at 72℃ for 5 min. The primer pairs (Bionics, Seoul, Korea), forward and reverse were as follows: β-actin, 5'-CCTCTA TGCCAACACAGTGC-3' and 5'-ATACTCCTGCTTGCT GATCC-3'; Bcl-2, 5'-AGCTGCACCT GACGCCCTTCA-3' and 5'-AGCCAGGAGAAATCACAGAGG-3'; Bax, 5'-ATGGAC GGGTCCGGGGAGCAG-3' and 5'-CAGTTG AAGTTGCCG TCAGA-3'. PCR was performed for 40 cycles. Temperature cycling was initiated with each cycle, using the Takara PCR Thermal Cycler Dice (Takara Bio, Inc., Otsu, Japan), as follows: β-actin, 98℃ for 10 sec (denaturation), 55℃ for 30 sec (annealing), 72℃ for 1 min (extension); Bcl-2, 98℃ for 10 sec, 60℃ for 30 sec, 72℃ for 1 min; Bax, 98℃ for 10 sec, 60.5℃ for 30 sec and 72℃ for 1 min. The amplified products were resolved on 1% agarose gels, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and photographed under ultraviolet light using a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

7. Western blotting analysis

Western blotting analysis was performed as previously described (Ryu & Chung 2015). The cells were cultured, harvested, and lysed for 30 min on ice in an appropriate lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), and 0.1% NP 40) and were then centrifuged at 13,000×g for 15 min. Lysates were mixed with 5× sample buffer (0.375 M Tris-HCl, 5% SDS, 5% β-mercaptoethanol, 50% glycerol, 0.05% bromophenol blue, pH 6.8) and were heated to 95℃ for 5 min. Equal amounts of protein were separated by 12% SDS-PAGE and were transferred onto a nitrocellulose membrane. The membranes were then washed with Tris-buffered saline containing 0.05% TBST and were then blocked in TBST containing 5% nonfat dried milk. The membranes were then incubated with their respective specific primary anti-bodies overnight at 4℃. After three washes in TBST, membranes were incubated with the appropriate secondary antibodies coupled to HRP for 1 h at room temperature. The membranes were washed, and detection was carried out using an enhanced chemiluminescence Western blotting detection kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The values for the specific protein levels are presented as the fold-change relative to the control and densitometry was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

8. Statistical analysis

SPSS software version 22.0 (IBM SPSS, Armonk, NY, USA) has been used to analyze the experimental data. Significance was determined one-way ANOVA with Tukey's test. P-value < 0.05 was considered to indicate a statistically significant difference.

Results and Discussion

1. Inhibitory effects of Bibimbap on FHC and HT-29 cells

For the preparation of Bibimbap, antiproliferative activity on HT-29 cells of 24 kinds of vegetables were investigated.

Bibimbap was made with *Glycine max* L. *Merr*, *Brassica rapa* var. *glabra*, *Agaricus bispoantiproliferaros*, *Pleurotus eryngii*, *Lentinula edodes*, and *Brassica oleracea* var. *capitata* L. which showed strong antiproliferative activity, and purple rice. The effects of Bibimbap on the growth of FHC and HT-29 cells (FB) were examined using the MTT assay. The cells were exposed to various concentrations (0~20 mg/mL) of the Bibimbap for 24 and 48 h, and the cytotoxicity was determined as a percentage of the viable treated cells in comparison with the number of viable untreated control cells. As shown in Fig. 1, Bibimbap did not affect the proliferation on FHC colon normal cells in dose-, and time-dependent manners. However, Bibimbap significantly inhibited the proliferation of HT-29 cells (FB) in dose-, and time-dependent manners. Subsequent to 48 h of exposure, Bibimbap (FB) induced 50% growth inhibition at a concentration of 10.1±0.17 mg/mL (Fig. 2). Therefore, the concentrations of 1, 5, and 10 mg/mL of Bibimbap were used for the further experiments.

2. Induction of apoptosis on HT-29 cells

The apoptosis can be characterized by a series of morphological changes and chromatin condensation of cellular nucleus by distinct mechanisms. Nuclear Hoechst 33342 staining was performed in order to investigate whether the anti- proliferative effect of Bibimbap was due to apoptosis or not. Apoptosis is the primary means for eliminating unwanted cells in cellular organisms in order to preserve tissue homeostasis. It is characterized by distinct changes in the morphology and chromatin condensations of the dying cell can be preserve cellular biochemical changes. As shown in Fig. 3, HT-29 cells which were treated with Bibimbap showed a number of morphological changes, including cell shrinkage and a higher density of apoptotic bodies compared with the untreated control cells. At the concentrations of 5 and 10 mg/mL of Bibimbap, crescent shaped nuclei, which are one of the typical characteristics of apoptotic cells, were also found. These results suggest that Bibimbap showed anti-proliferative activities in a dose-dependent fashion for 48 h, and is consistent with MTT results. A high consumption of fruits, vegetables, aspirin, non-steroidal anti-inflammatory drugs, magnesium, folate, and dairy products brings a decreased incidence of colorectal cancer (Chapelle et al. 2020). These results indicate that Bibimbap made with strong antiproliferative active vegetables may induce apoptosis on HT-29 cells.

3. Effects of Bibimbap on apoptosis on HT-29 cells

To quantify the extent of apoptotic cells, flowcytometry analysis was performed using double staining with Annexin V and PI. The Annexin V－/PI－ population was considered represent unaffected cells, Annexin V+/PI－ as early apoptosis, Annexin V+/PI+ as late apoptosis, and Annexin V－/PI+ as necrosis. As shown in Fig. 4, Bibimbap-treated HT-29 cells showed that early apoptotic cell populations were increased 12.7% at 10 mg/mL of Bibimbap, compared with 1.7% for the control. The late apoptotic cells were increased 25.1% at 10 mg/mL, compared with 6.8% for the control. These results suggest that Bibimbap can induce apoptosis on HT-29 cells. Annexin V and PI staining is widely used for determining the cellular death through apoptosis. In the presence of Ca^{2+} ions, annexin V has a strong binding affinity for phosphatidylserine (PS), a membrane phospholipid that during apoptosis is translocated from the inner

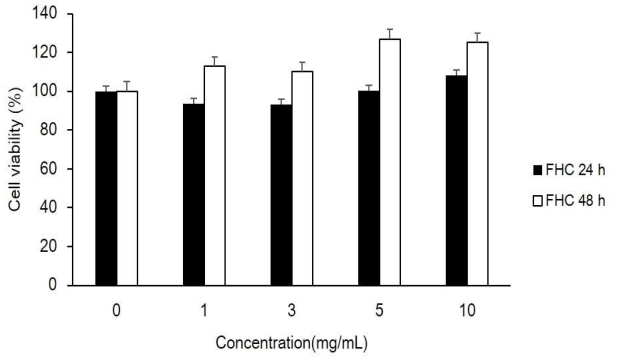

Fig. 1. Cell viability of Bibimbap on FHC (normal human colon epitherial cells) in a dose-dependent manner at 24, and 48 h.

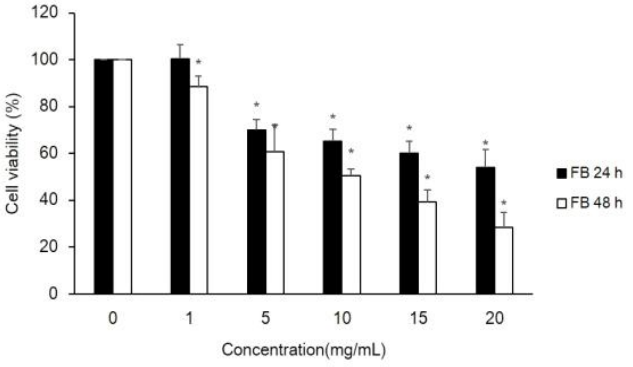

Fig. 2. Cell viability of Bibimbap on HT-29 cells in a dose-dependent manner at 24, and 48 h. $^{*}p<0.05$, significantly different from control cells.

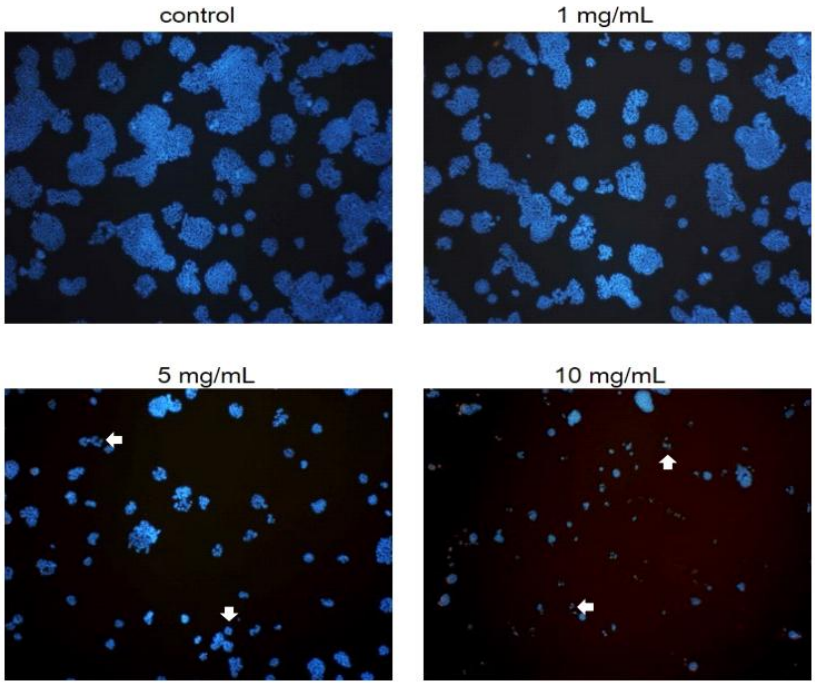

Fig. 3. Microscopy image of Bibimbap-treated HT-29 cells. After incubation with 0, 1, 5, and 10 mg/mL of Bibimbap for 48 h, the cells were observed by fluorescent microscopy (magnification, ×400) using Hoechst 33342 staining (arrow indicates the formation of bodies).

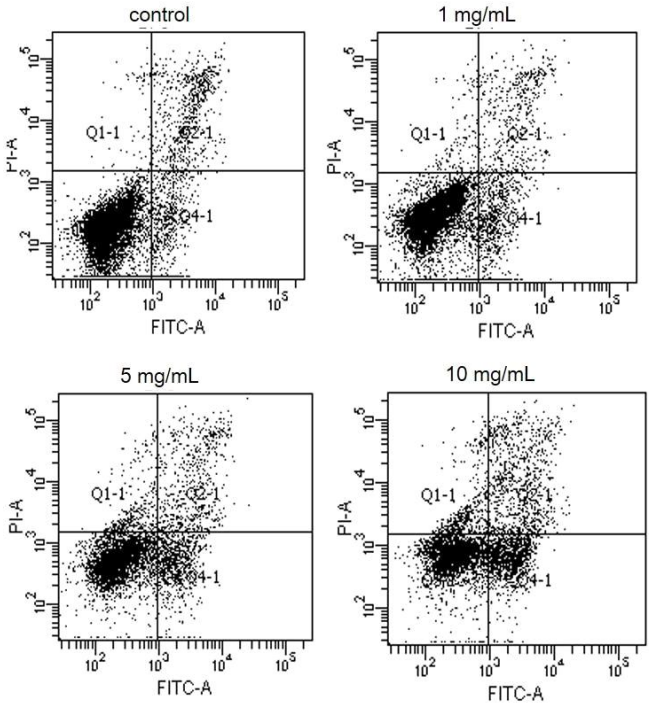

Fig. 4. Effects of Bibimbap (FB) on apoptosis on HT-29 cells. Flow cytometric analysis of HT-29 cells incubated with 0, 1, 5, and 10 mg/mL of Bibimbap for 48 h. The right bottom quadrant represents Annexin V-stained cells (early-phase apoptotic cells). The top right quadrant represents PI- and Annexin V-stained cells (late-phase apoptotic cells).

side of the cell membrane to its outer side (Kumar et al. 2021). PS exposure on the external leaflet of the plasma membrane is widely observed during apoptosis and forms the basis for the Annexin V binding assay to detect apoptotic cell death. In the early stages of apoptosis, there are alterations of PS, which translocate the outer layer from the inner side of the plasma membrane. This allows early recognition of dead cells by which PS exposes at the external surface of the cell. This is followed by an enabling characteristic patterns can cause DNA damage in the short term (Lee et al. 2013; Darbre & Harvey 2014), but more study is needed to figure out long-term and low-dose mixtures.

4. Effects of Bibimbap on the expressions of Bcl-2 family and caspase proteins

To study the apoptotic effects of Bibimbap on HT-29 cells, we examined the expression levels of a number of apoptosis regulatory proteins, including Bcl-2, Bax, caspases-3, -8, -9 and PARP. Bcl-2 interacts with the mitochondrial plasma membrane and prevents mitochondrial membrane pores from opening during apoptosis, blocking the signals of apoptotic factors, such as Bax (Ryu et al. 2013). The mitochondrial pathway is an important apoptosis pathway as it regulates the apoptotic cascade via a convergence of signaling at the mitochondria. As a result, Bibimbap increased Bax expression but decreased the expression of Bcl-2, each in a dose-dependent manner (Figs. 5 and 6). Nuclear morphological changes during apoptosis are very distinct and effector caspases have been implicated to play a central role in these processes (Johnson et al 2020). To study the apoptotic effects of Bibimbap on HT-29 cells, we examined the expression levels of caspases-3, -8, and -9 including Bcl-2, Bax, and PARP. As shown in Fig. 7, Bibimbap was followed by the activation of caspases-3, -8, and -9 and its target, PARP with a dose-dependent manner. The mitochondrial plasma membrane disruption increased the activation of caspases-3, -8, -9, and its target, PARP (Kim et al. 2012; Nagappan et al. 2012). These results suggested that Bibimbap can induce apoptosis through the regulation of apoptosis-related protein expressions on HT-29 cells. Caspases are cysteinyl aspartate proteinases, which is essential phase in apoptosis. While the upstream caspases for the intrinsic pathway is caspase-9, of the extrinsic pathway is caspase-8. The intrinsic and extrinsic pathways converge to caspase-3. Caspase-3, then cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis (Li et al. 1997;

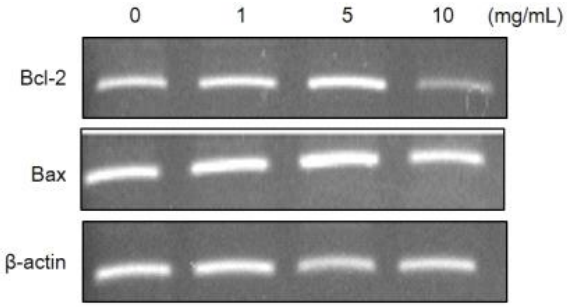

Fig. 5. Effects of Bibimbap on the expressions of Bax mRNA on HT-29 cells in a dose dependent manner. Cells were treated with 0, 1, 5, and 10 mg/mL of Bibimbap for 48 h.

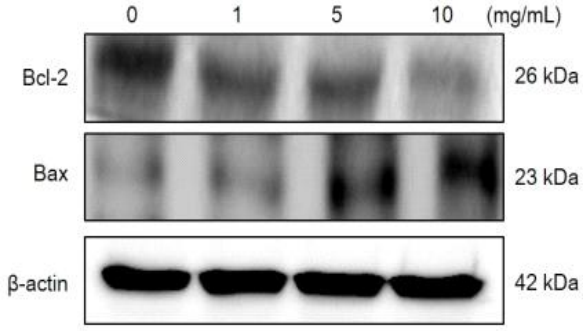

Fig. 6. Effects of Bibimbap on the expressions of Bcl-2 family on HT-29 cells in a dose dependent manner. Cells were treated with 0, 1, 5, and 10 mg/mL of Bibimbap for 48 h.

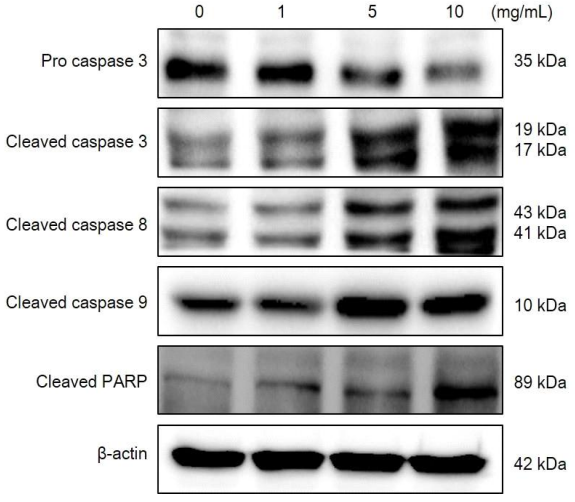

Fig. 7. Effects of Bibimbap on the expressions of apoptosis-related proteins on HT-29 cells. Cells were treated with 0, 1, 5, and 10 mg/mL of Bibimbap for 48 h. The cell lysates were electrophoresed, and Western blotting with Bax, Bcl-2, caspases-3, -8, -9, and cleaved PARP antibodies.

Acehan et al. 2002). The apoptosome-bound caspase-9 cleaves and activates caspase-3. Caspase-3 is one of the key protagonists of apoptosis, because it is either partially or completely responsible for the proteolytic cleavage of many key proteins, such as PARP. PARP is important for cell viability, but its cleavage facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Trebinska et al. 2014; Liu et al. 2015). Plant-derived natural compounds have the attention owing to health benefits, safety, and low side effects (Garcia-Oliveira et al. 2021) for the treatment of cancers followed by caspase-related proteins. Our experiments indicated that caspases-3, -8, -9 appear to be activated on Bibimbap- induced HT-29 cells. These results suggest that HT-29 cells are highly sensitive to growth inhibition by Bibimbap consisting with vegetables via the activation of apoptosis, as evidenced by activation of Bcl-2-mediated signaling, as well as alteration in caspases-3, -8 and -9 respectively.

Conclusion

In the present study, we investigated anti-proliferative activity on HT-29 human colorectal adenocarcinoma cells which were treated with various concentrations of Bibimbap (0~20 mg/mL) made with purple rice and *Glycine max* L. *Merr*, *Brassica rapa* var. *glabra*, *Agaricus bisporos*, *Pleurotus eryngii*, *Lentinula edodes*, and *Brassica oleracea* var. *capitata* L. Bibimbap suppressed the growth of H-29 cells in dose- and time-dependent manners (IC_{50}, 10.1±0.17 mg/mL for 48 h). The population of live cells dropped slightly, and morphology changed with reduction in cell volume (pyknosis) with Bibimbap. Treatment with 5 mg/mL of Bibimbap resulted in slight cell shrinkage. Furthermore, increasing Bibimbap dose to 10 mg/mL, these characteristics were more evident and the HT-29 cells exhibited partial detachment via staining with the DNA-binding dye Hoechst 33342. To investigate the extent of apoptotic cells, HT-29 cells were subsequently subjected to staining with Annexin V and PI double staining, and were analyzed by flow cytometry analysis. On exposure of HT-29 cells to Bibimbap, total apoptotic cells increased in a dose-dependent fashion. The results showed that treatment with Bibimbap significantly increased the percentage of apoptotic cells compared with untreated control cells. These results indicated that Bibimbap effectively induces apoptosis in HT-29 cells with the up-regulation of Bax protein as well as the down-regulation of Bcl-2 expression, each in a dose dependent manner in RT-PCR. In addition, Western blotting experiments indicated that caspases-3, -8, -9, and cleaved form of PARP appear to be activated on Bibimbap-induced HT-29 cells. Outcomes from our study provide the implications for the preparation of healthy Korean traditional dish, Bibimbap with respect to anti-proliferative activity against human colorectal cancer cells. Identification of bioactive components and animal experiments of Bibimbap are needed further research.

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