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Induction of Apoptotic Cell Death by Red Pericarp Rice (Jakwangchalbyeo) Extracts

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Abstract The effects of ethanol fractions of three different rice grain extracts, Jakwangchalbyeo, Hwasunchalbyeo, and Ilpumbyeo, on apoptotic cell death in the rat hepatoma H4IIE cell line were investigated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay. One hundred mg/mL Jakwangchalbyeo extract significantly reduced cell viability to 69.5, 57.2, and 46.1% within 24, 48, and 72 hr, respectively. Fluorescence-activated cell sorting (FACS) analyses were also performed to characterize the cell death pattern caused by treatment with the rice grain extracts. Apoptotic cell death was clearly observed with time after treatment with the Jakwangchalbyeo extract. In Western blotting analysis, degradation of the 116 kDa poly-ADP-ribose polymerase (PARP) molecule was observed with concomitant formation of an 89 kDa product 24, 48, and 72 hr after treating cells with the Jakwangchalbyeo extract. This indicates that an apoptotic process caused cell death in these cells. In conclusion, red-pericarp Jakwangchalbyeo extract induced apoptotic cell death in H4IIE cells to a larger extent than the other rice extracts.

Keywords: Jakwangchalbyeo, H4IIE cells, MTT cell viability assay, Western blotting analysis, FACS, PARP, apoptosis.

Introduction

Rice (*Oryza sativa* L.) grain is consumed by nearly half of the world's population and is a major staple food in Korea. Many attempts have been made to develop rice varieties that are rich in certain biologically active compounds, such as immune stimulants (1) or antioxidants (2-5). To that end, we are interested in determining the anti-cancer effects of rice grain extracts.

The cell, the standard unit of living organisms, maintains homeostasis in the organism through its control of cell division and death. Active cell death is called apoptosis; it can be clearly distinguished from necrosis in many aspects and plays an important role in the maintenance of the living system. During developmental processes, this form of ideal cell death removes unneeded cells without causing inflammatory reactions (6). In addition, the induction of apoptosis is also significant in treating various types of cancer. For example, some bioactive substances with anti-tumor activity act directly on tumor cells to inhibit cell growth or to induce cell death via the apoptotic process. On the other hand, other bioactive substances exert anti-tumor activity by strengthening the immune response of the body, thereby improving the body's defensive capability against cancer cells, which can eventually induce apoptosis of the cancer cells.

In general, apoptotic pathways are initiated by the activation of a series of proteases called caspases and are composed of various cellular signaling compounds (7). Caspases lead to the degradation of cellular proteins and, in turn, cause cell death. When apoptosis occurs, cytochrome c, located in the mitochondrial membrane, is released through a channel composed of, and regulated by, the Bcl-2 family of proteins (8-10). The released cytochrome c binds to Apaf-1, caspase-9, and dATP to form apoptotic bodies that can activate caspase-9. This subsequently activates downstream executioner caspase-3, which ultimately induces apoptotic cell death (11). Activated caspase-3 subsequently causes the cleavage of poly-ADP-ribose polymerase (PARP) in the nucleus, inducing DNA fragmentation and chromatin condensation, the typical biomarkers of apoptotic cell death (12).

In the search for bioactive molecules that display anti-cancer activity, we were particularly interested in potential compounds found in rice, Therefore, experiments were performed to investigate the effects of ethanol extracts of three rice (*Oryza sativa* L. var. *japonica*) grain varieties, Jakwangchalbyeo (red glutinous rice), Hwasunchalbyeo (white glutinous rice), and Ilpumbyeo (white non-glutinous rice), on apoptotic cell death. To this end, the effects of rice grain extracts on cell death were evaluated in a cancerous rat liver cell line, H4IIE cells, with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay. Fluorescence-activated cell sorting (FACS) analyses and Western blotting were also performed to characterize the pattern of cell death caused by treatment of the H4IIE cells with each rice grain extract.

Materials and Methods

Preparation of rice grain extracts Jakwangchalbyeo (red pericarp glutinous rice), Hwasunchalbyeo (white pericarp glutinous rice), and Ilpumbyeo (white pericarp non-glutinous rice) (Fig. 1) were cultivated at the Konkuk University Experimental Farm in 2002.

Jakwangchalbyeo is an F_9 breeding line generated by mating Jakwangdo (a local rice variety with a red pericarp)

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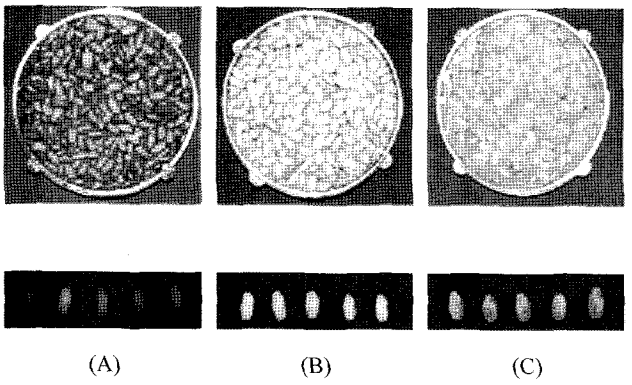

(A) (B) (C)

Fig. 1. Photographs of the three brown rice (*Oryza sativa* L.) varieties. A: Jakwangchalbyeo; B: Hwasunchalbyeo; C: Ilpumbyeo.

with Jinbuchal (an improved and early maturing variety). Although rice varieties with red pericarps are generally non-glutinous, Jakwangchalbyeo is a glutinous rice variety with a red pericarp.

After drying the harvested rice, the seeds were hulled with a milling machine and then stored at room temperature until needed. The brown rice cultivated from each of these varieties was used as the raw material for the preparation of the ethanol fraction of crude rice grain extracts. First, fatty compounds were removed from the rice grain by extraction with benzene, and the rice grain remnants were then soaked in 80% ethanol for 2-3 days at 4°C. The supernatant was subsequently filtered through Whatman #4 filter paper, concentrated, and dried under vacuum. The resultant crude extracts were re-extracted six times with 300 mL of ethanol. Finally, the subsequent extracts were concentrated and freeze-dried under vacuum to provide the samples that were used in these experiments.

Culture of H4IIE cell lines The rat hepatoma H4IIE cell line was obtained from the College of Pharmacy, Seoul National University, Korea. Rat H4IIE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin in a- humidified atmosphere containing 5% CO_2 at 37°C. H4IIE cells were passaged every 3-4 days (13).

Cell viability assay Rat H4IIE cells were plated at a density of 4×10^4 cells/well on a 96-well plate. Cells were then synchronized by culturing in serum-free media overnight, after which various concentrations (10, 25, 50, and 100 g/mL) of Jakwangchalbyeo, Hwasunchalbyeo, and Ilpumbyeo extracts were added into the wells and further incubated for 24, 48, and 72 hr. Cell viability was assessed by measuring the degree of formazan crystal formation in the MTT assay (14, 15). In brief, after incubation of cells for the indicated time, viable cells were stained with MTT (0.2 g/mL) for 4 hr. The media was then removed, and any formazan crystals that had formed in the wells were dissolved by the addition of 100 L of dimethylsulfoxide (DMSO). Absorbance was measured at 540 nm using an ELISA microplate reader (Multiskan EX; Thermo Labsystems, Walthan, MA, USA). Cell viability was calculated relative to untreated control cells [i.e., viability (% control) = (absorbance of treated sample) / (absorbance of control)×100].

Analysis of apoptosis by FACS Jakwangchalbyeo, Hwasunchalbyeo, and Ilpumbyeo extracts that induced cell death were analyzed by FACS. Briefly, H4IIE cells were plated at a density of 1×10^6 cells/well in six-well cell culture plates. Cells were synchronized by culturing in serum-free media overnight, followed by the addition of 100 g/mL of Jakwangchalbyeo, Hwasunchalbyeo, or Ilpumbyeo extract to the culture media. They were then incubated further for 1, 3, 6, 15, 18, 24, 48, or 72 hr. Following this incubation period, the H4IIE cells were washed twice with cold phosphate buffered saline (PBS) and resuspended in 1× binding buffer at a cell density of 1×10^6 cells/mL. Cell suspensions (100 μL) containing 10^5 cells were then transferred to 5 mL culture tubes, and 5 μL of Annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit 1; BD Biosciences pharmingen, San Diego, CA, USA) and 5 μL of propidium iodide (PI) were added. The reaction mixture was gently stirred and incubated for 15 min at room temperature (25°C) in the dark; 400 L of 1× binding buffer were then added to each tube, and the reaction mixtures were subjected to flow cytometry with FACS analysis (Becton Dickinson, NJ, USA) (16-19). Data were analyzed with Cell Quest analytical software.

Microscopic observation of cell death In order to verify the time-dependence of the observed cell death, H4IIE cells were plated at a density of 1×10^6 cells/well in six-well cell culture plates. Cells were synchronized by culturing in serum-free media overnight, followed by 30-min incubation in the presence (1 μM final concentration) or absence of PMA (phorbol 12-myristate 13-acetate), then addition of 100 g/mL Jakwangchalbyeo extract for 24, 48, and 72 hr. As a control, cells were incubated in the presence or absence of PMA for 24, 48, and 72 hr. The time-dependent cell death process was observed under a camera-equipped (Olympus, Japan) microscope, with pictures taken at various time points (20).

Western blotting Western blotting analysis was used to verify apoptosis by detecting apoptotic markers such as PARP. H4IIE cells were plated at a density of 5×10^6 cells/well in six-well cell culture plates and synchronized by culturing in serum-free media overnight. Jakwangchalbyeo, Hwasunchalbyeo, or Ilpumbyeo extracts (100 g/mL) were added to the cells and incubated for 24, 48, or 72 hr. After this incubation period, the cells were held on ice for 30 min and subsequently washed twice with PBS. Cell lysates were collected in radioimmunoprecipitation (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/mL Aprotinin, 2 μg/mL Leupeptin, 250 uM vanadate, and 0.1% β-mercaptoethanol in PBS) and the amount of protein in each sample was quantified with a Bradford assay. Fifteen μM 4× Laemmli's buffer was then added, and the cell lysate was boiled. Samples were then subjected to SDS gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Waters, Millipore, MA, USA). The membrane was washed with Tris-buffered saline (TBS) containing 0.1% Tween-20

(TBST) and the protein bands were visualized with Ponceau S solution. Once again, the membrane was washed with TBST, then blocked for 1 hr in TBST containing 5% skim milk. After washing the membranes with TBST, the antibodies against PARP (Zymed, San Francisco, CA, USA) were added at a dilution of 1:1,000 to TBST containing 5% bovine serum albumin (BSA) and incubated with the membranes overnight at 4°C. The membranes were washed with TBST and incubated with anti-mouse IgG (diluted to 1:5,000) in TBST containing 5 % skim milk for 2 hr. The membrane was then treated with the Western blotting detection reagents (Amersham Biosciences, Uposala, Sweden), and chemiluminescence was detected with X-ray film.

As a control experiment, H4IIE cells were treated with PMA (1 μM final concentration). Using the same culturing and synchronization conditions as above, the cells were incubated for 30 min in the presence or absence of 1 μM PMA, followed by treatment with 100 μg/mL Jakwangchalbyeo extract for 1, 3, 6, 15, 18, 24, 48, and 72 hr. Cell lysates were prepared as described above, subjected to SDS-gel electrophoresis, transferred onto PVDF membrane, and then blocked for 1 hr in TBST containing 5% skin milk. After washing the membranes with TBST, the primary antibodies against phospho-ERK (p-ERK) or total ERK (t-ERK) (Cell signaling, Denver, CO, USA) were added at a dilution of 1:1,000 in TBST and incubated overnight at 4°C. Again, the membranes were washed with TBST, and then treated with anti-rabbit IgG (diluted to 1:5,000) in TBST for 2 hr. Chemiluminescence was detected with X-ray film, following incubation with Western blotting detection reagents (Amersham Biosciences). The amount of protein in each sample was quantified by Bradford assay (21).

Results and Discussion

Cell viability assay The effects of rice grain extracts on cell death were investigated in rat H4IIE cells using MTT assays, and the results showed time-dependent H4IIE cell survival following treatment with rice grain extracts.

Figure 2 exhibits the extent of cell viability following treatment with Jakwangchalbyeo extracts. At concentrations of 10, 25, and 50 μg/mL, there was no change in cell viability after incubation for 24 hr. In fact, after a 24-hr incubation period, cell viability appeared to increase slightly to 102.1, 102.5, and 114.9% with 10, 25, and 50 μg/mL Jakwangchalbyeo extract, respectively. When incubated for 48 and 72 hr at concentrations of 10, 25, and 50 μg/mL, the viability of H4IIE cells decreased slightly. At 10 μg/mL, cell viability decreased to 86.6 and 71.7% within 48 and 72 hr, respectively. At 25 μg/mL, cell viability decreased to 83.7 and 69.4% within 48 and 72 hr, respectively. At 50 μg/mL, cell viability decreased to 87.9 and 67.3% within 48 and 72 hr, respectively. However, at a concentration of 100 μg/mL, cell viability decreased significantly to 69.5, 57.2, and 46.1% within 24, 48, and 72 hr, respectively.

Figure 3 shows the extent of cell viability following treatment with Hwasunchalbyeo extracts. At concentrations of 10, 25, 50, and 100 μg/mL, there was no change in cell viability after 24 hr. When incubated for 48 and 72 hr at concentrations of 25, 50, and 100 μg/mL, the viability of H4IIE cells decreased slightly. At a concentration of 10 μg/mL, cell viability decreased to 95.3, 82.5, and 79.9% within 24, 48, and 72 hr, respectively. At a concentration of 25 μg/mL, cell viability decreased to 98.0, 82.1, and 82.5% within 24, 48, and 72 hr, respectively. At a concentration of 50 mg/mL, cell viability increased to 102.0% after 24 hr, and then decreased to 80.3 and 74.1% within 48 and 72 hr, respectively. At a concentration of 100 μg/mL, cell viability decreased to 97.8, 78.3, and 69.3% within 24, 48, and 72 hr, respectively.

Figure 4 shows the extent of cell viability following treatment with Ilpumbyeo extracts. At concentrations of 10, 25, and 50 μg/mL, there were very small decreases in cell viability after 24 hr, with 97.3, 98.9, and 91.9% viability, respectively. When incubated for 48 and 72 hr at concentrations of 10, 25, and 50 μg/mL, the viability of H4IIE cells decreased slightly. At a concentration of 10 μg/mL, cell viability decreased to 94.5 and 89.6% within 48 and 72 hr, respectively. At a concentration of 25 μg/mL, cell viability decreased to 96.4 and 87.6% within 48

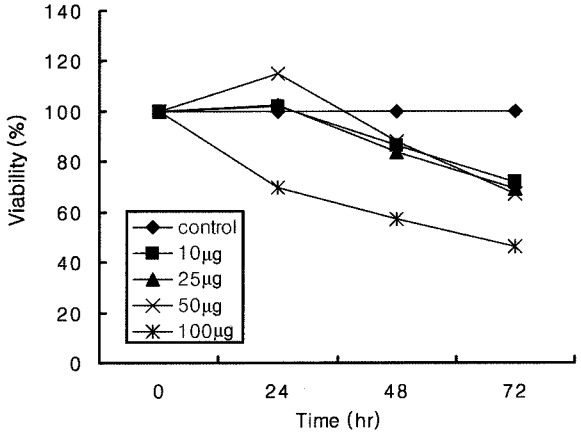

Fig. 2. Concentration- and time-dependent effects of Jakwangchalbyeo extract on viability in H4IIE cells. Cell viability is expressed relative to untreated control cells.

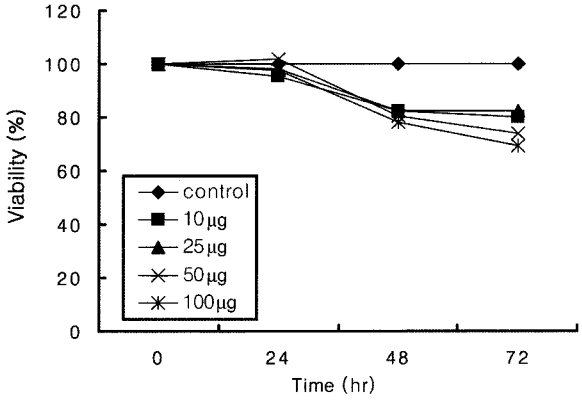

Fig. 3. Concentration- and time-dependent effects of Hwasunchalbyeo extract on viability in H4IIE cells. Cell viability is expressed relative to untreated control cells.

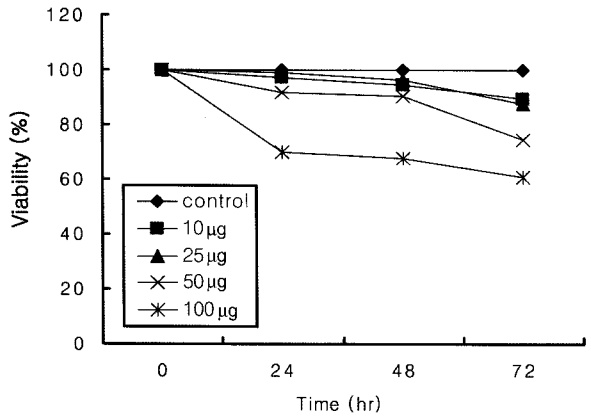

Fig. 4. Concentration- and time-dependent effects of Ilpumbyeo extract on viability in H4IIE cells. Cell viability is expressed relative to untreated control cells.

and 72 hr, respectively. At a concentration of 50 μg/mL, cell viability decreased to 90.4 and 74.6% within 48 and 72 hr, respectively. However, at a concentration of 100 μg/mL, cell viability decreased significantly to 70.1, 67.8, and 60.9% within 24, 48, and 72 hr, respectively.

In spite of the low concentration (100 μg/mL) of our crude sample, which was not a pure compound, the Jakwangchalbyeo extract had greater anti-cancer effects than the other rice grain extracts used in these experiments. These results are similar to those observed by Sung and Park (22), who reported that brown rice grain extracts caused significant cell death in breast cancer cell lines, indicative of anti-cancer activity.

Many studies have been performed to determine anti-cancer effects of commercially available, pure flavonoids *in vitro* (23-26). Further experiments will also be necessary to assess the biological activity in Jakwangchalbyeo, Hwasunchalbyeo, and Ilpumbyeo extracts.

Analysis of apoptosis by FACS Apoptosis and necrosis are two distinct processes leading to cell death and morphological and biochemical features allow each to be clearly distinguished from the other (27). Apoptosis is a normal physiological process that occurs during embryonic development, as well as in the maintenance of tissue homeostasis. Certain morphological features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA, characterize the

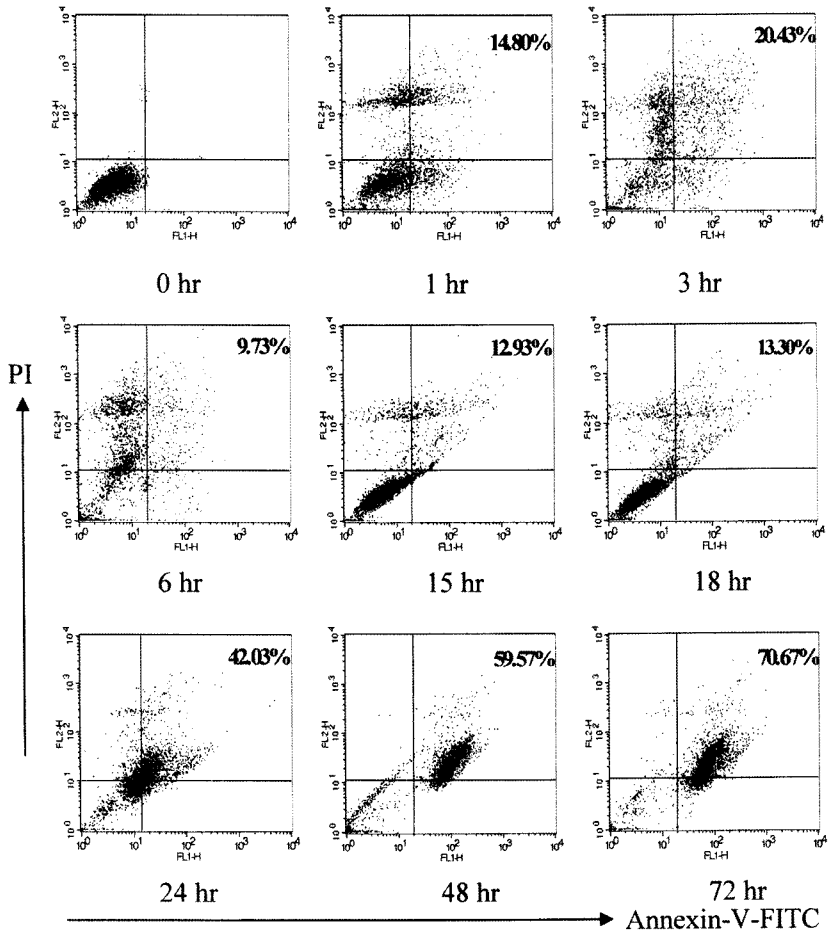

Fig. 5. FACS analysis of apoptotic death following treatment with 100 μg/mL Jakwangchalbyeo extract in H4IIE cells. Graphs are representative of data analysis by Cell Quest analytical software.

apoptotic process. Loss of the plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. This can be detected through the use of Annexin V, a 35-36 kDa Ca^{2+}-dependent, phospholipid binding protein that can be conjugated to a fluorochrome such as PI. In this format, Annexin V retains its high affinity for PS, and thus serves as a sensitive probe for the flow cytometric analysis of cells undergoing apoptosis (19, 28, 29).

The other mechanism of cell death is necrosis. Necrosis is a pathological process that occurs when cells are exposed to a variety of detrimental stimuli (30). It begins with damage to the plasma membrane and causes impairment in the influx of water and extracellular Ca^{2+} ions. With the breakdown of the plasma membrane, the cytoplasmic contents leak into the extracellular fluid. As a result, necrotic cell death is often associated with extensive tissue damage and an intense inflammatory response (31).

FACS analyses were performed to characterize the pattern of cell death caused by the treatment of Jakwangchalbyeo, Hwasunchalbyeo, and Ilpumbyeo extracts in H4IIE cells. Time-dependent cell death patterns are shown in Fig. 5-7. As shown in Fig. 5, apoptosis increased with time in the cells treated with Jakwangchalbyeo extract, with apoptotic cell death representing 42.0% at 24 hr, 59.6% at 48 hr, and 70.7% at 72 hr. However, apoptotic changes were scarcely observed in cells treated with Hwasunchalbyeo (Fig. 6) or Ilpumbyeo extracts (Fig. 7). After 72 hr, apoptotic cell death following treatment with the Hwasunchalbyeo and Ilpumbyeo extracts was 13.0 and 18.4%, respectively. Therefore, Jakwangchalbyeo extracts induced apoptotic cell death to a larger extent than those of Hwasunchalbyeo and Ilpumbyeo. As a variety of cellular apoptotic signaling pathways can be activated when cells are affected by stress, the detailed mechanism of rice grain extract-induced apoptosis should be studied in future experiments.

Microscopic observation of cell death The time-dependent cell death process was observed under a microscope following treatment with Jakwangchalbyeo extracts to detect any morphological changes in the treated cells. As shown in Fig. 8, the population of H4IIE cells treated with Jakwangchalbyeo extracts decreased significantly with time compared to the control group. In addition, the number of H4IIE cells treated concurrently with Jakwangchalbyeo extract and PMA also decreased in a time-dependent manner compared to cells treated with

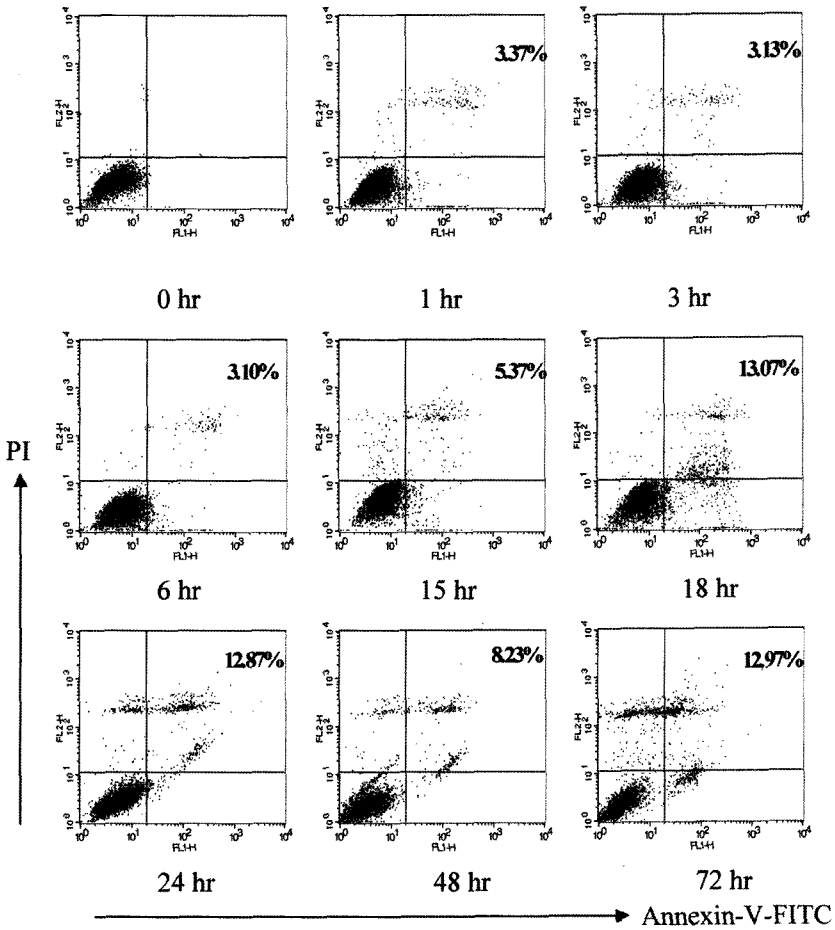

Fig. 6. FACS analysis of apoptotic death following treatment with 100 μg/mL Hwasunchalbyeo extract in H4IIE cells. Graphs are representative of data analysis by Cell Quest analytical software.

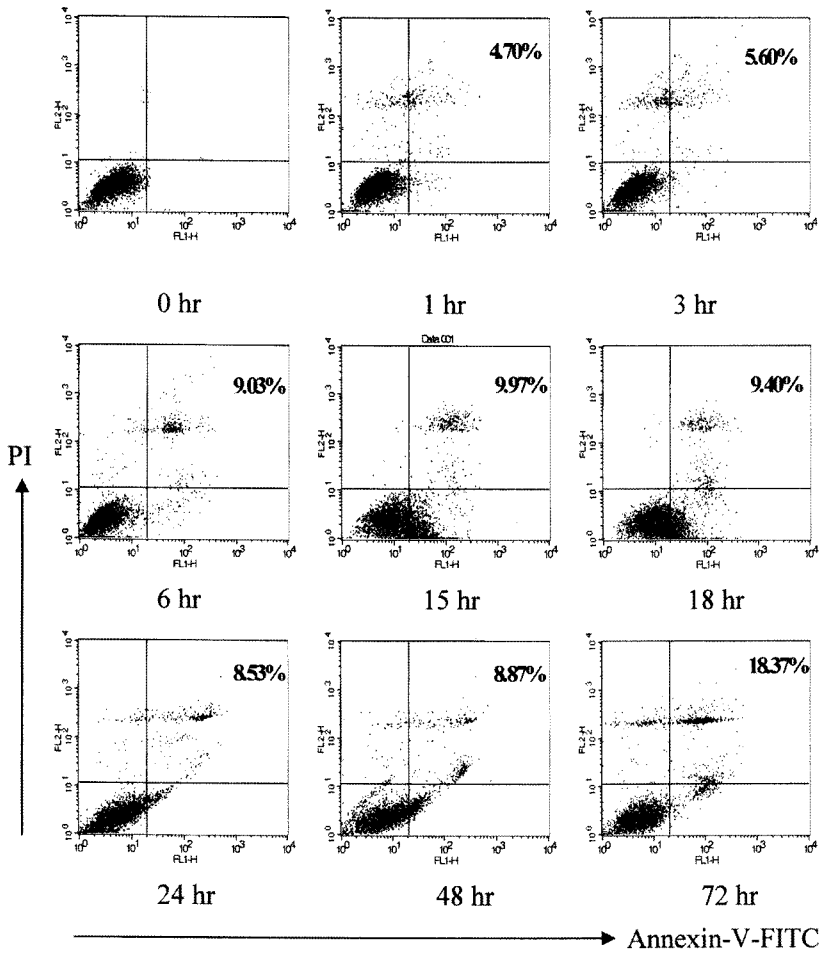

Fig. 7. FACS analysis of apoptotic death following treatment with the 100 μg/mL Ilpumbyeo extract in H4IIE cells. Graphs are representative of data analysis by Cell Quest analytical software.

PMA alone. PMA is known to function as a tumor promoter.

Under the light microscope, there were no observable morphological changes in the cells that did not undergo any treatment (control), in the cells treated with the Jakwangchalbyeo extract, or in the cells treated with the Jakwangchalbyeo extract in the presence of PMA. Nevertheless, the number of cells decreased gradually with time when treated with Jakwangchalbyeo extract in conjunction with PMA as compared to the H4IIE cells treated with PMA alone. Similarly, the number of cells decreased significantly with time when treated with Jakwangchalbyeo extract alone, without prior treatment of PMA. This indicates that the Jakwangchalbyeo extract induced apoptotic cell death.

Western blotting PARP, which represents a family of related proteins with a common enzymatic activity, covalently modifies a variety of proteins involved in the metabolism of nucleic acids and the maintenance of chromatin structures (32). In general, when cells are affected by various stress signals, apoptosis is initiated and the protease caspases subsequently undergo an activation process resulting in the degradation of many of the cellular proteins. Various caspases are involved in this cell death process. In particular, active caspase-3, derived from the cleavage of inactive procaspase-3, induces cleavage of PARP, which causes DNA fragmentation and nuclear condensation in the typical apoptotic cell death phenomena (11). The PARP enzyme (116 kDa), which is located in the nucleus of the cell and is involved in repairing impaired DNA, is fragmented into an 89 kDa product during the apoptotic process. Thus, the formation of 89 kDa PARP can be used as a biological marker of apoptotic cell death. As shown in Fig. 9, the intensity of the 116 kDa PARP band decreased, and concomitant increases in the intensity of the 89 kDa PARP band were observed 24, 48, and 72 hr after treatment with the Jakwangchalbyeo extract, indicating that cell death was caused by the apoptotic process. However, there were few changes in the band intensity of either the 116 or 89 kDa PARP bands in cells treated with the Hwasunchalbyeo extract. On the other hand, the intensity of the 89 kDa PARP band increased significantly when cells were treated with the Ilpumbyeo extract for 72 hr.

Mitogen-activated protein kinases (MAPKs), which are proline-directed serine/threonine kinases, serve as mediators of cellular responses to a variety of stimuli such as growth factors, cytokines, hormones, and environmental stresses (33, 34). In mammalian cells, MAPKs have been classified

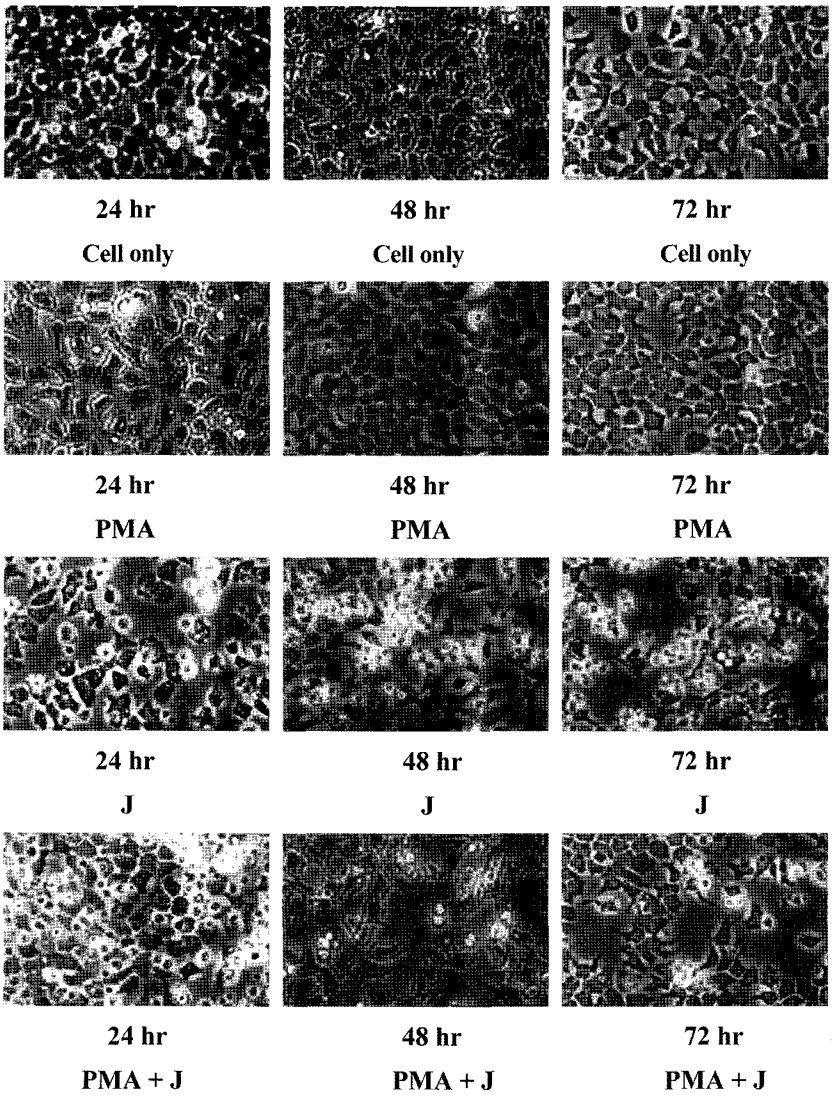

Fig. 8. Photographs of H4IIE cells treated with 100 μg/mL Jakwangchalbyeo extract in the presence or absence of 1 μM PMA for 24, 48, and 72 hr. (PMA: Phorbol 12-myristate 13-acetate, J: Jakwangchalbyeo extract)

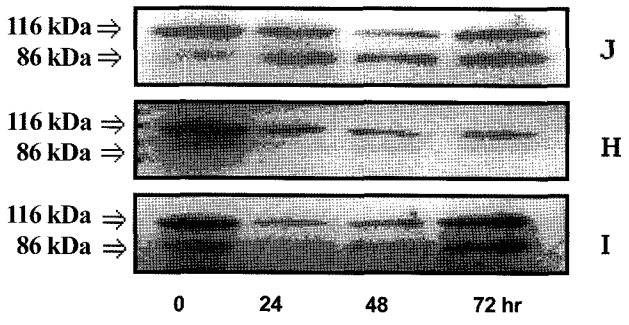

Fig. 9. Western blotting analysis of PARP degradation in H4IIE cells. PARP: Poly (ADP-ribose) polymerase, J: Jakwangchalbyeo, H: Hwasunchalbyeo, I: Ilpumbyeo extract.

into at least four subfamilies: ERK, JNK (c-Jun N-terminal kinase), p38 MAPK, and BMK1/ERK5. Among them, ERK can act as a cell survival signal and has been studied extensively in many cell types. In order to determine if changes in cellular ERK activity were among the causes of rice grain extract-induced cell death, ERK activity in H4IIE cells was pre-stimulated with 1 μM PMA. The effects of Jakwangchalbyeo extract on cellular ERK activity were then assessed by detecting the degree of ERK phosphorylation in H4IIE cells following treatment with the extract. In the preliminary experiments, ERK phosphorylation in H4IIE cells increased drastically following the application of PMA (Fig. 10).

After considering all these factors, we reached that red-pericarp Jakwangchalbyeo extract induced apoptotic cell death in H4IIE cells to a larger extent than other rice extracts. Up to now, effect of apopototic was reported in pure compound such as soy-isoflavonoid (35), resveratrol (36), and several flavonoid (37). The result of this study should be of interest to chemical analyst for further investigating the separation anti-cancer compound present in Jakwangchalbyeo extracts.

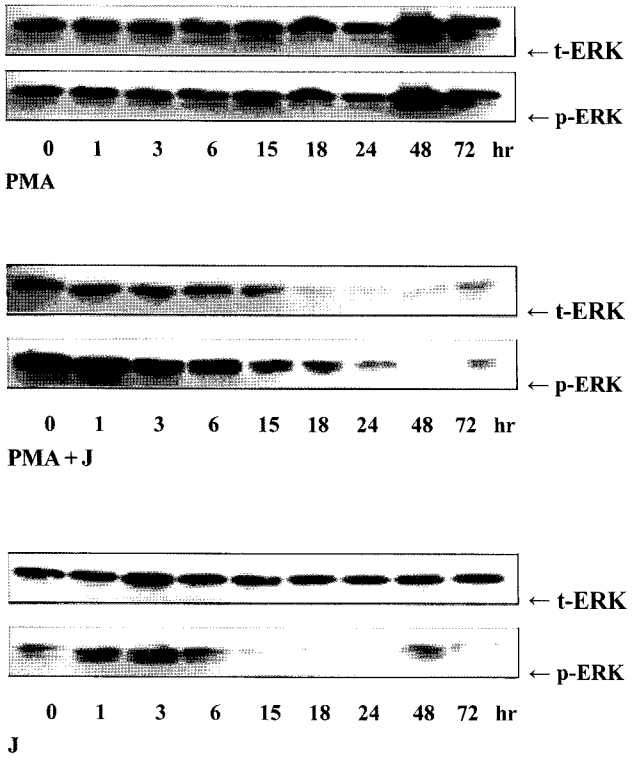

Fig. 10. Western blotting analysis of ERK phosphorylation in H4IIE cells. p-ERK: phospho-extracellular signal-regulated kinase, t-ERK: total extracellular signal-regulated kinase, PMA: Phorbol 12-myristate 13-acetate, J: Jakwangchalbyeo extract.

Acknowledgments

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