

# Methanolic Extract of Red Ginseng Marc Induces Apoptosis on Human Oral Squamous Cell Carcinoma HSC-3

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**Abstract**— Amino acid contents of glutamic acid leucine in red ginseng marc (RGM) were higher than in ginseng body and root, Red ginseng, but the amount of arginine in RGM contains high amount of fiber and polysaccharides. This study examined whether or not methanolic extract of red ginseng marc (RGME) induces apoptosis in the human oral squamous cell carcinoma (HSC-3) along with the possible mechanism (s) of the RGME-mediated cytotoxicity. Cytotoxicity and apoptosis induction of HSC-3 cells were evidenced by MTT assay, cell morphology alteration, apoptosis enzyme-linked immunosorbent assay, flow cytometric analysis, caspase-3 activity, and protein expression by Western blotting after RGME treatment for 24 h. The RGME induced the cell death of HSC-3 cells via apoptosis, as evidenced by the increased cell population in the sub-G1 phase, the appearance of condensed and/or fragmented nuclei, and the generation of a cleaved PARP product and characterized by activation of caspase-3. The efficacious induction of apoptosis was observed as a dose-dependent manner. The treatment of the cells with the RGME also induced changes in the mitochondrial level of the Bcl-2 family proteins such as Bcl-2 and Bax. Furthermore, the RGME increased the phosphorylation of ERK, and phospho-p38 MAPK at the same concentrations. The RGME inhibited the nuclear translocation of NF- $\kappa$ B by suppressing the degradation of I $\kappa$ B- $\alpha$ . Our findings clearly demonstrate that RGME induces G1 arrest, activates the MAPKs, inhibits NF- $\kappa$ B, and induces apoptosis of HSC-3 cells. These results strongly suggest that red ginseng marc might have cancer inhibition and therapeutic potential.

**Keywords**— Apoptosis, Mitochondrial pathway, Caspases, MAPKs signal pathway, Cell cycles, NF- $\kappa$ B, Red ginseng marc

## I. INTRODUCTION

Apoptosis is a programmed cell death activated by regulation of protein activity and gene expression according to the signal inside the cell, and does not cause inflammation occurring by macrophage phagocytosis of apoptotic cells without the destruction of the surrounding cells [1, 2]. Unlike necrosis, while maintain the normal structure of cell organelles, cell shrinkage, chromosome condensation, DNA fragmentation, and apoptosis corpuscles formation is accompanied by morphological changes [3, 4]. Prosurvival members of the Bcl-2 family have been shown to regulate the activation of caspases, providing a mechanism by which these proteins may inhibit apoptosis [5-8]. In addition, prosurvival Bcl-2 family members localized in the outer membrane of the mitochondria can inhibit the release of cytochrome c that is induced by specific apoptotic stimuli [7, 8]. This effect provides another mechanism by which anti-apoptotic Bcl-2 family members might inhibit caspase activation and apoptosis, as cytochrome c is required for Apaf-1 to bind to and activate procaspase-9 [9, 10]. Bax, another proapoptotic member of the Bcl-2 family, has also been shown to bind mitochondria and to induce cytochrome c release [11, 12]. Mitogen activated protein kinases (MAPKs) are serine/threonine kinases that activate numerous other protein kinases, nuclear proteins, and transcription factors, leading to downstream signal transduction [13]. Extracellular-signal-regulated kinase (ERK) is primarily involved in proliferation, transformation and differentiation [13, 14]. JNK and p38 can be activated by physiologic stress, endotoxin, osmotic stress, ultraviolet exposure, TNF- $\alpha$  and oxidative stress such as ROS [13, 14]. NF- $\kappa$ B downstream gene over-expressions are involved in apoptosis resistance, angiogenesis, enhanced invasion and metastasis, which intensify the aggressiveness of this disease and further complicate its treatment [15, 16].

Red ginseng is made from steam heat treatment of fresh ginseng roots. The red ginseng contains specific ginsenoside-Rh1, ginsenoside-Rh2, 20(S)-ginsenoside Rg2, 20(S)-ginsenoside Rg3, and these are not detected or even as trace amount in fresh and dried ginseng roots [17, 18]. These specific ginsenosides of red ginseng can be used for important medicine. Ginsenosides have a radio protective effect against radiation-induced double-strand breaks in DNA and immune modulatory activity, as evidenced by its stimulation of natural killer cells [19]. Ginsenosides exhibit less potent but broad-spectrum antibacterial activity against gram-positive and gram-negative bacterial strains, including the clinical isolates of MRSA [20,

21]. Ginseng marc is a fibrous and insoluble by-product remaining after production of ginseng extract. Red ginseng marc (RGM) is the by-product of red ginseng extract, RGM was increased with the red ginseng products increase, but there is limit to produce ginseng marc [22]. Amino acid contents of glutamic acid leucine in RGM were higher than in ginseng body and root, Red ginseng, but the amount of arginine in RGM contains high amount of fiber and polysaccharides [23, 24].

As the above, anti-cancer effect of 90% methanol extract of red ginseng marc (RGME) has been reported in several types of cancer, but the effect of RGME on oral squamous cell carcinoma has not been reported. So this study reports the mechanisms involved that RGME induced apoptosis of oral squamous cell carcinoma, HSC-3.

## II. MATERIALS AND METHODS

### 2.1 Cell culture and treatment experiments

HSC-3 established from a squamous cell carcinoma located on the tongue was provided by the Japanese Cancer Research Resources Bank (JCRB). They were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml of penicillin, and 100 µg/ml of streptomycin, in an atmosphere of 5% CO<sub>2</sub> in air at 37°C. One million cells per milliliter were resuspended in either 2 mL or 100 µL of the media and spread onto either 6-well or 96-well flat-bottomed plates, respectively. When the cells had reached 90% confluence, a fresh batch of serum-free DMEM was added to the cultures, and the HSC-3 cells were then exposed to different concentrations of RGME. At various times after the treatment, the cells were examined for any signs of cytotoxicity and apoptosis.

### 2.2 Measurement of cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) was used to examine the cell viability. Briefly, the cultured HSC-3 cells were exposed to several concentrations of RGME. At various exposure times, 100 µL of a MTT solution (5 mg/mL in PBS as stock solution) was added to each well, and the cells were incubated for a further 4 h at 37 °C. DMSO was then added to each well, and the absorbance of the plates was read at 560 nm using a ELISA reader (Bio-TEK, Winooski, VT, USA).

### 2.3 Cell cycle analysis

The progression of the cell cycle was determined using flow cytometric analysis after staining with propidium iodide (PI). Initially, the suspension ( $2 \times 10^6$  cells) of several concentrations of RGME treated HSC-3 cells was fixed with 80% ethanol at 4 °C for 24 h, and incubated overnight at 4 °C with 1 mL of a PI staining mixture (250 µL of PBS, 250 µL of 1 mg/mL RNase in 1.12% sodium citrate, and 500 µL of 50 µg/mL PI in 1.12% sodium citrate). After staining,  $1 \times 10^4$  cells were analyzed using the MACSQuant (Miltenyi Biotec, France)

### 2.4 Annexin V assay

Induction of apoptosis was measured by Annexin V-FITC (Fluorescein Isothiocyanate)/PI (Propidium Iodide) assay using flow cytometry (Wilkins et al., 2002). Briefly, HSC-3 ( $4 \times 10^5$  cells, 60 mm dish, at 60–70% confluency) were treated with REME corresponding to their IC<sub>50</sub> values (Table 1) for 24 h. At the end of the treatment, cells were harvested, centrifuged at 2000 rpm for 5 min at RT, washed with ice cold HBSS, and re-suspended in 100 µL of annexin binding buffer (10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or HEPES), 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4 in milli Q water). Cells were stained with Annexin V-FITC and PI for 15 min in dark at RT. Thereafter, annexin binding buffer was added and maintained at 4 °C. Cells were analyzed by flow cytometry (MACSQuant, Miltenyi Biotec) and the percentage live, necrotic, early apoptotic, and late apoptotic cells were determined.

### 2.5 Caspases activity assay

The effect of RGME or/and MAPK inhibitors (SB203580, SP600125, and PD98059) on caspase-3, -8, and -9 activity in HSC-3 cells were determined using a commercially available caspase-3, -8, and -9 (active) ELISA kit (eBioScience Corporation, CA, USA). Active caspase-3, -8- and -9 (ng/mg total protein of cell lysate) were determined and results were expressed as folds of caspase-3, -8, and -9 activity in RGME-treated cells relative to DMSO-treated control. Briefly,  $4 \times 10^5$  cells were plated in tissue culture dishes, allowed to attach by overnight incubation and exposed to DMSO or RGME for desired time period. Cells were collected and lysed in cell extraction buffer mixed with protease inhibitor cocktail and 5 mM of PMSF (phenyl methane sulfonyl fluoride, Sigma). ELISA assay for caspase-3, -8, and -9 activities was carried out

according to manufacturer's instructions. Briefly, 100  $\mu$ l of cell lysates were incubated in the microplate wells provided in the kit and incubated at room temperature for 2 h. The samples were aspirated and washed 4 times with washing buffer and incubated with 100  $\mu$ l of detection antibody (caspase-3, -8 and -9) for 1 h at room temperature. After removal of the antibody solution, the wells were washed again and incubated with 100  $\mu$ l of HRP anti-rabbit antibody for 30 min at room temperature. After the aspiration of the anti-rabbit antibody, blue color was developed by adding 100  $\mu$ l of stabilized chromogen solution for 15-20 min at room temperature. The reaction was stopped adding 100  $\mu$ l of stopping solution and the yellow color developed was read using a microplate reader at 450 nm (Bio-TEK).

## 2.6 Preparation of cell lysates

At various times after treating HSC-3 cells with RGME, the cells were collected and resuspended in a lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1  $\mu$ g/mL aprotinin, 100  $\mu$ g/mL PMSF, and 250 mM sucrose). After centrifugation at  $700 \times g$  for 10 min, the supernatant was transferred into a new tube and further centrifuged at  $10,000 \times g$  for 30 min to isolate the cytosolic fraction. The pellets were then used as the mitochondrial fraction. In addition, the nuclear extracts were prepared according to the manufacturer's instruction using a NE-PERTM nuclear and cytoplasmic extraction reagents Kit (Pierce Rockford, IL, USA). Protein content of the cell lysates was quantified using the bicinchoninic acid assay (BCA, Pierce Rockford) method.

## 2.7 Western blot analysis

Equal amount of protein (20-40  $\mu$ g/sample) was separated electrophoretically by 6-12% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with the primary antibodies and incubated with a horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, CA, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The antibodies specific to Bcl-2, Bax, cytochrome c, ERK, p-ERK, and p-JNK were purchased from Santa Cruz Biotechnology. The polyclonal antibody specific to caspase-3 and poly (ADP ribose) polymerase (PARP) was purchased from BD,  $\beta$ -actin from Sigma Chemical Co, and p-p38, p38, JNK, I $\kappa$ B- $\alpha$ , p-I $\kappa$ B- $\alpha$ , and p65 from Cell Signaling (Cell Signaling Co., MA, USA).

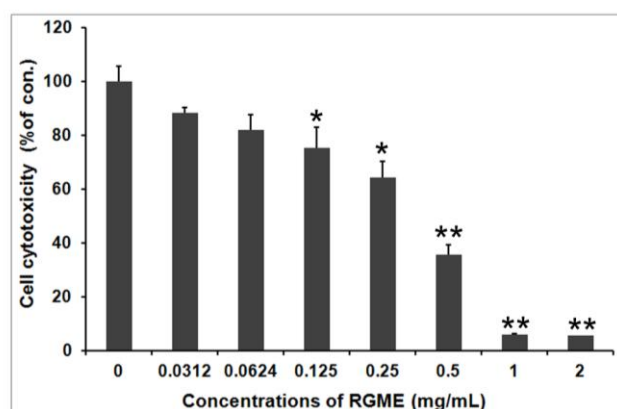
## 2.8 Statistical analysis

All the data are expressed as a mean  $\pm$  standard error (SE). One-way ANOVA using SPSS ver. 19.0 software was used for multiple comparisons. A  $P < 0.05$  was considered significant.

# III. RESULTS

## 3.1 RGME induces cytotoxicity

Initially, a MTT assay was used to determine if RGME had any cytotoxic effect on HSC-3 cells (Fig. 1).



**FIG. 1. RGME CAUSES CYTOTOXICITY TO HSC-3 CELLS IN A DOSE-DEPENDENT MANNER. HSC-3 CELLS WERE TREATED WITH THE INDICATED CONCENTRATIONS OF THE RGME FOR 24 h AND THEN PROCESSED FOR MTT ASSAY. THE RESULTS ARE REPORTED AS A MEAN  $\pm$  SE OF TRIPPLICATE EXPERIMENTS, AND THE DIFFERENT SUPERSSCRIPTS REPRESENT THE SIGNIFICANT DIFFERENCES BETWEEN THE GROUPS USING THE SCHEFFE'S MULTIPLE RANGE TEST.**

As shown in the figure, the addition of RGME significantly reduced the viability of HSC-3 cells in a dose-dependent manner. After incubating the cells with 0.5 mg/mL RGME for 24-h, cell viability was found to be  $51.35 \pm 1.23\%$ . However, when 1 mg/mL of RGME in was added, only approximately 22% of the cells were viable.

3.2 RGME-induced cell cycle arrest and apoptosis in HSC-3

The RGME-treated HSC-3 cells were subjected to apoptosis assays to determine if the RGME induces apoptosis (Fig. 2). Initially, PI staining revealed a dose-dependent increase in the cell populations in sub-G1 phase of the cell cycles after the RGME treatment. Compared with the control, RGME treated with 1 mg/mL resulted in a significant accumulation of cells in G1 phase (from 1.35 to 39.26%), accompanied by a decrease in G2/M (from 28.73 to 10.58%) and S-phase (from 13.62 to 9.76%) cells.

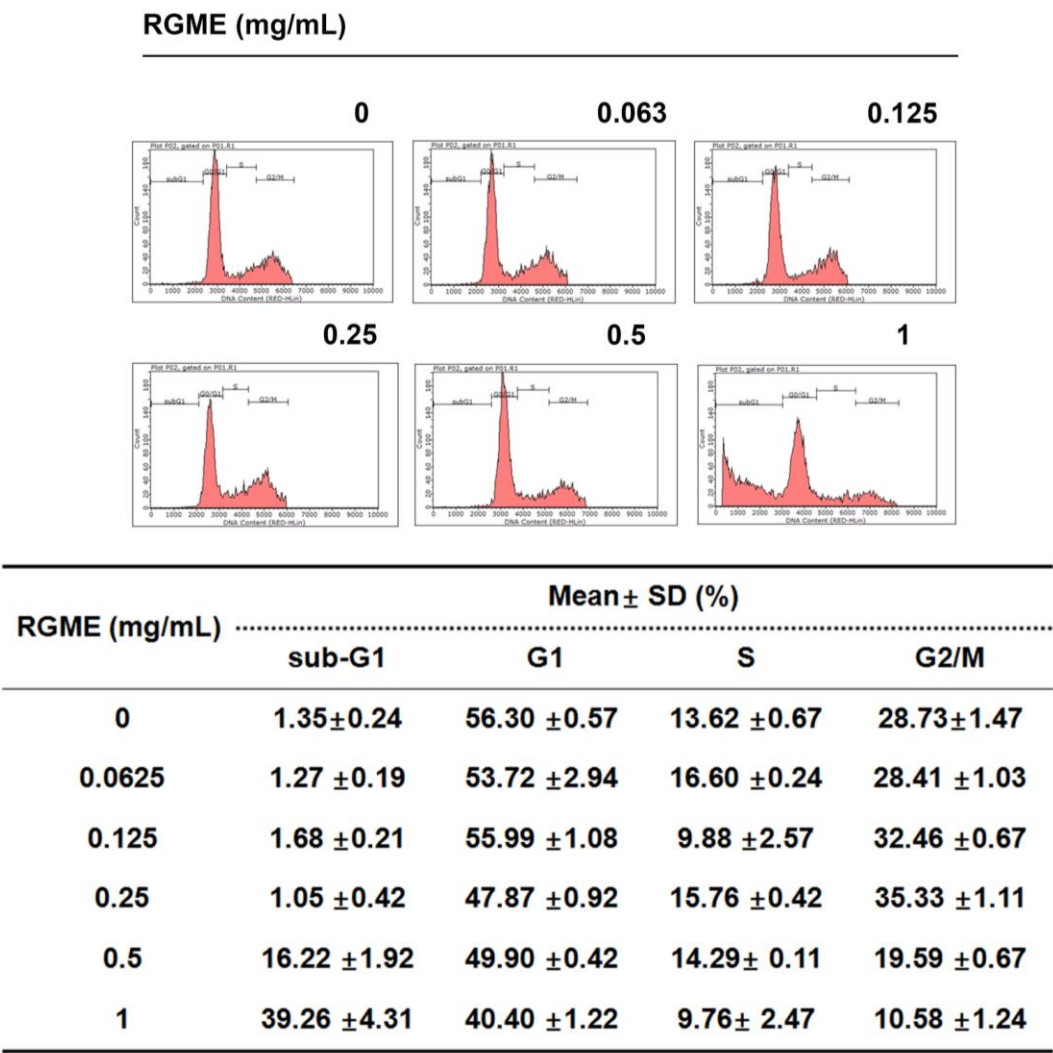
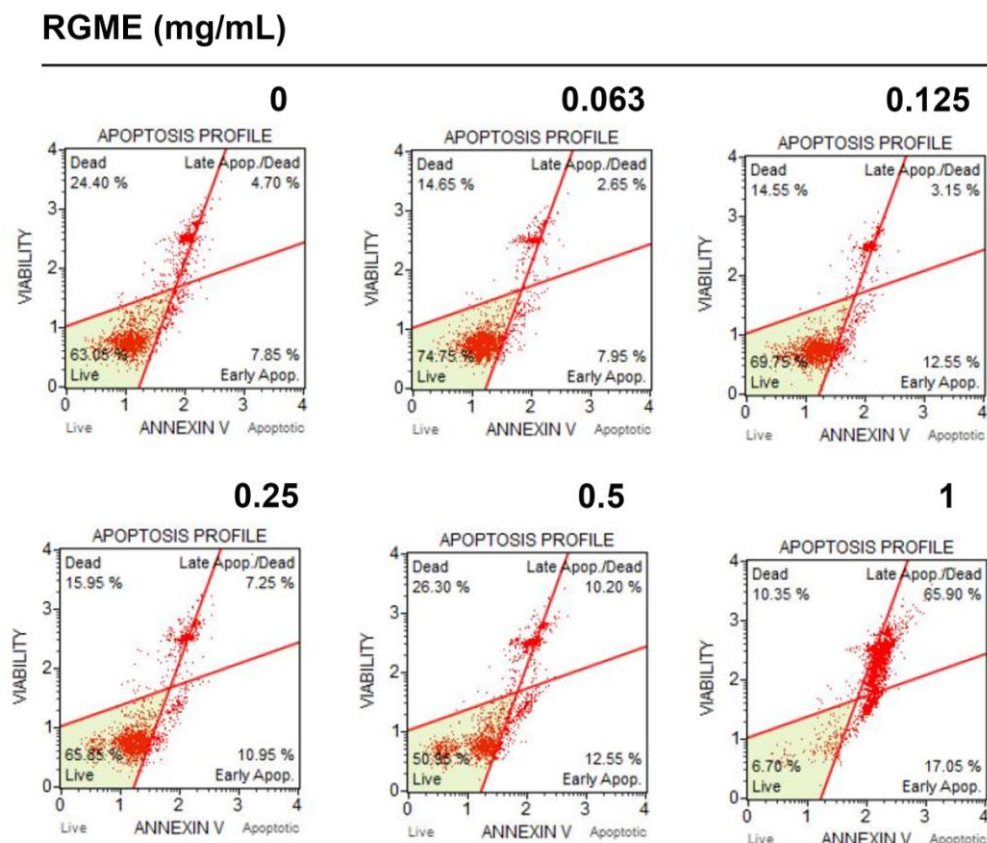


FIG. 2. RGME INDUCES CELL CYCLE ARREST IN HSC-3 CELLS. HSC-3 CELLS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF RGME FOR 24 H. THE CELL CYCLE DISTRIBUTION OF TREATED CELLS WAS DETERMINED USING FLOW CYTOMETRY. THE DATA ARE EXPRESSED AS THE MEAN ± SD, WITH RESULTS REPRESENTATIVE OF 3 INDEPENDENT EXPERIMENTS SHOWN.

### 3.3 RGME-induced HSC-3 cell apoptosis tested by PI-annexin V assays

To determine whether this reduced cell viability was due to apoptosis, PI-annexin V double staining was used. As shown in Fig. 3, HSC-3 cells treated with various concentrations of RGME showed increased percentages of total and late apoptotic cells of 12.55, 18.20, 22.75, and 82.95% and 4.70, 7.25, 10.20, and 65.90%, respectively, at 24 h. These data showed that RGME could induce apoptosis in HSC-3 cell line in a dose-dependent manner, especially during late apoptosis.



**FIG. 3. RGME INDUCES APOPTOSIS IN HSC-3 CELLS. HSC-3 CELLS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF RGME FOR 24 H. FLOW CYTOMETRIC ANALYSIS OF RGME-INDUCED APOPTOSIS IN HSC-3 CELLS USING ANNEXIN V-FITC/PI STAINING. CELLS IN THE LOWER RIGHT QUADRANT REPRESENT EARLY APOPTOTIC CELLS, AND THOSE IN THE UPPER RIGHT QUADRANT REPRESENT LATE APOPTOTIC CELLS. THE DATA ARE REPRESENTATIVE OF 3 SIMILAR EXPERIMENTS.**

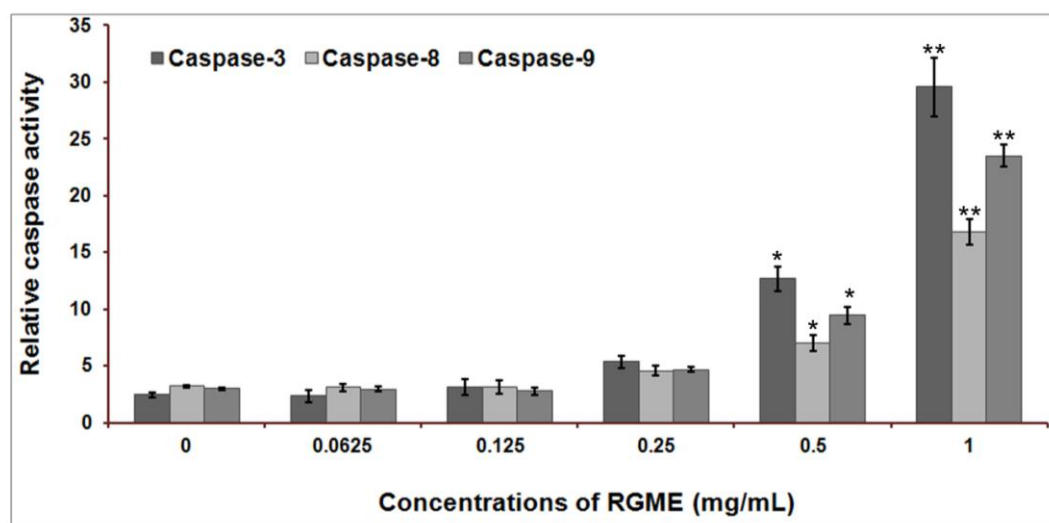
### 3.4 RGME induces apoptosis by the activation of Caspase-3, -8, and -9

The RGME-treated HSC-3 cells were investigated for caspase-3, caspase-8, and caspase-9 activities by colorimetric enzymatic assay. As shown in Fig. 4, caspase-3, -8, and -9 activities increased at dose-dependent manner after RGME treatment. In particular, the RGME-induced apoptosis was showed the strongest activity of caspase-3. To investigate the relevance of MAPKs and caspases, HSC-3 cells were then treated with several concentrations of RGME in the presence or absence of 10  $\mu$ M of MAPKs inhibitors, SB203582, SP600125, and PD98059 for 24 h. These inhibitors significantly inhibited the RGME-induced caspase-3, -8, and -9 activity in the cells at dose-dependent manner (Fig. 9).

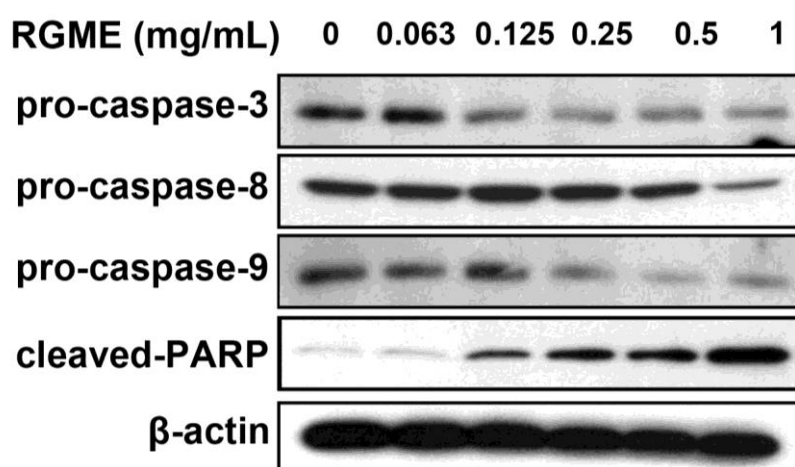
### 3.5 RGME-mediated apoptosis of HSC-3 cells involves a caspases and cleavage of PARP

HSC-3 cells were treated with different concentrations of the RGME for 24 h, and processing of procaspases-3 and PARP was monitored by Western blotting (Fig. 5). At concentrations more than 0.125 mg/mL, the RGME induced processing of procaspase-3 into active forms. In contrast, the RGME-mediated degradation of procaspase-3 or procaspase-9 was more apparent than that of procaspase-8. To confirm further the apoptosis induced by the RGME, we investigated the cleavage of

PARP in HSC-3-treated cells. Treatment of HSC-3 cells with RGME caused a proteolytic cleavage of PARP, with accumulation of the characteristic 85 kDa fragments and a concomitant disappearance of the full-length 116 kDa protein.



**FIG. 4. EFFECT OF RGME ON THE ACTIVATION OF CASPASE-3, -8, AND -9.** HSC-3 CELLS WERE TREATED WITH INCREASING DOSES OF RGME FOR 24 H. EQUAL AMOUNTS OF WHOLE CELL EXTRACTS WERE MIXED WITH 100 mM OF THE CASPASE-3, -8, AND -9 SUBSTRATES, AC-IETD-AMC AND AC-LEHD-AMC, RESPECTIVELY, AND INCUBATED FOR 1 H AT 37°C. FREE AMC WAS DETERMINED AS ABOVE. DATA ARE MEAN  $\pm$  SD OF THREE INDEPENDENT EXPERIMENTS, AND EACH EXPERIMENT WAS CONDUCTED IN TRIPLICATE.



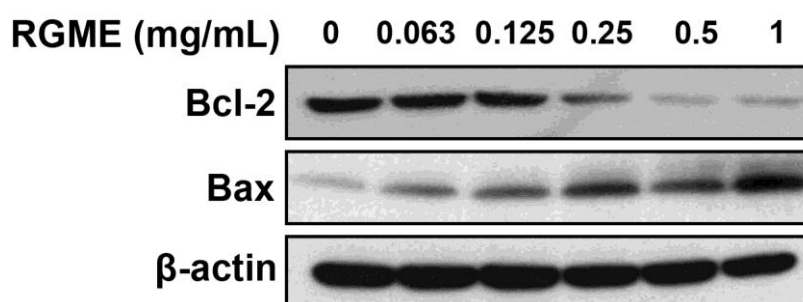
**FIG. 5. EFFECT OF RGME ON THE ACTIVATION OF CASPASE-3, -8, AND -9 AND PARP.** HSC-3 CELLS WERE EXPOSED TO THE INDICATED CONCENTRATIONS OF RGME FOR 24 H. EQUAL AMOUNTS OF WHOLE CELL EXTRACTS WERE SEPARATED ON SDS-PAGE GELS, AND CASPASE-3, -8, AND -9 AND PARP WERE DETECTED BY WESTERN BLOT ANALYSIS. B-ACTIN WAS USED AS A CONTROL. DATA ARE MEAN  $\pm$  SD OF THREE INDEPENDENT EXPERIMENTS, AND EACH EXPERIMENT WAS CONDUCTED IN TRIPLICATE.

### 3.6 Mitochondrial stress is an important event in RGME-mediated apoptosis of HSC-3 Cells

In particular, the release of cytochrome c into the cytosol plays an important role in the execution of apoptosis in a number of different cell types, which is tightly regulated by the equilibrium between the anti-apoptotic Bcl-2 and pro-apoptotic Bad and



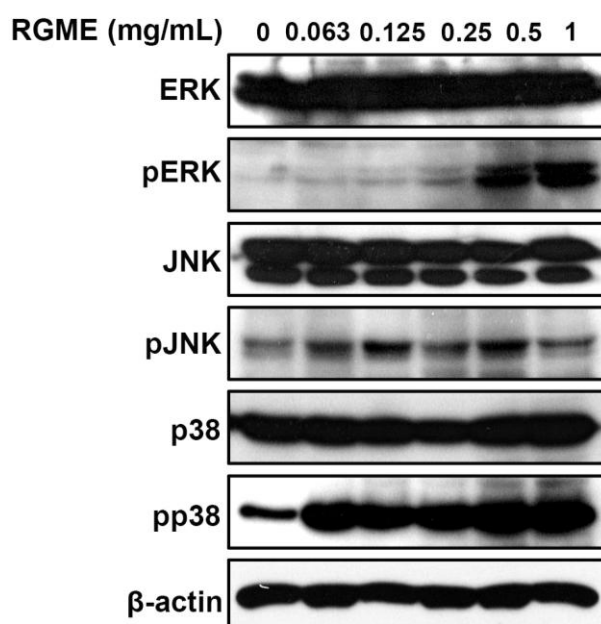
Bax [8, 9]. To evaluate the effect of RGME on the mitochondrial membrane potential, Bax and Bcl-2, respectively, we performed Western blot analysis for Bax and Bcl-2. RGME treatment increased the intensity of the bands corresponding to Bax protein in mitochondrial fractions but significantly reduced the level of Bcl-2 protein (Fig. 6).



**FIG. 6. INVOLVEMENT OF MITOCHONDRIAL STRESS IN THE RGME-MEDIATED APOPTOSIS OF HSC-3 CELLS. THE CELLS WERE TREATED WITH THE INDICATED DOSES OF RGME FOR 24 H. CELL LYSATES WERE ANALYZED BY 12% SDS-PAGE FOLLOWED BY IMMUNOBLOT ANALYSIS. A REPRESENTATIVE RESULT FROM THREE INDEPENDENT EXPERIMENTS IS SHOWN.**

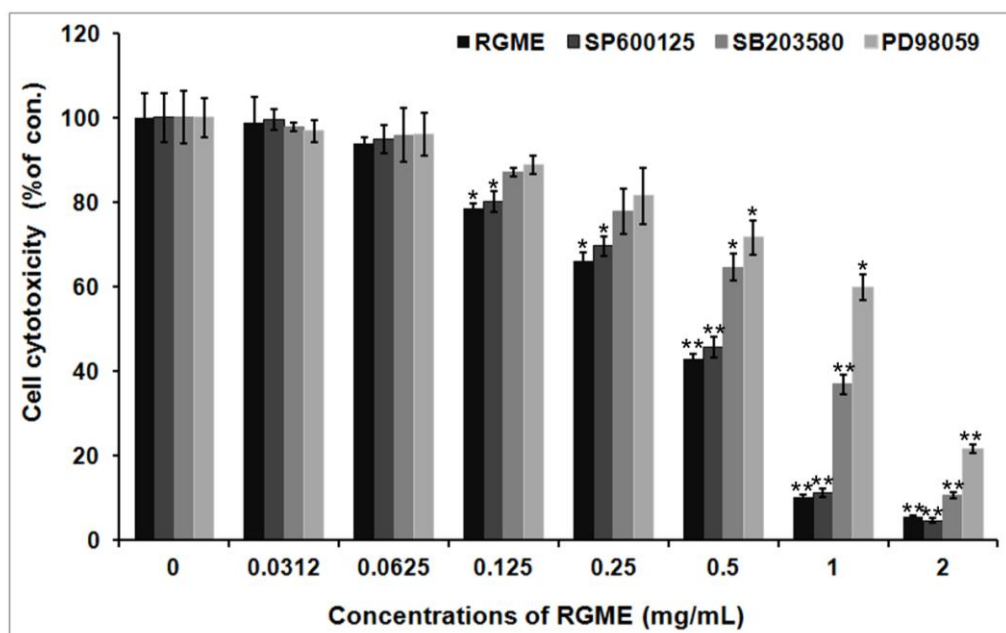
### 3.7 MAPK activation is a key step in the apoptotic process in RGME-treated HSC-3 cells

MAPKs including JNK, ERK, and p38 kinase are activated by various extracellular stimuli, and mediate the signal transduction cascades that play an important role in regulating apoptosis [13, 14]. Therefore, we measured the level of phosphorylated protein of these MAPKs using Western blot analysis. A dose-dependent increase in the phosphorylated forms of MAPKs was observed by treating the HSC-3 cells with RGME (Fig. 7). The pattern of ERK and p38 phosphorylation after the RGME treatment was quite different from the cases in the JNK. The p-ERK and p-p38 level returned to the basal level after being treated from 0.25 mg/mL of RGME for 1.5 h, and increased 10 fold after 0.5 mg/mL of the treatment, compared with the untreated control cells.



**FIG. 7. EFFECT OF RGME ON THE PHOSPHORYLATION OF MAPKS IN HSC-3 CELLS. THE CELLS WERE EXPOSED TO INDICATE CONCENTRATIONS OF RGME FOR 3 H AND EXAMINED BY WESTERN BLOT ANALYSIS. THE RESULTS FROM THREE INDEPENDENT EXPERIMENTS WERE QUANTIFIED THROUGH DENSITOMETRY AND A REPRESENTATIVE DATA IS SHOWN**

To determine if MAPK phosphorylation plays an important role in regulating RGME-mediated apoptosis, we treated the HSC-3 cells with various concentrations of RGME and MAPK specific inhibitors. In the cells treated with several concentration of RGME for 24 h, the cells stained positively for trypan blue, which was increased significantly by treating the cells with the MAPK inhibitors (Fig. 8). The most significant inhibition of RGME-induced cytotoxicity was observed when the cells were treated with PD98059 rather than SP600125 and SB203580. This suggests that ERK is a key regulator in the RGME-induced apoptosis of HSC-3 cells.



**FIG. 8. EFFECT OF MAPKS INHIBITORS ON THE CELL VIABILITY. THE CELLS WERE EXPOSED TO SEVERAL CONCENTRATIONS OF RGME FOR 24 h IN THE PRESENCE OR ABSENCE OF MAPK INHIBITORS (10 mM). THE VIABILITY REDUCING ACTIVITY OF THE CELLS WERE DETERMINED USING MTT ASSAY, AS DESCRIBED IN MATERIALS AND METHODS. EACH BAR SHOWS THE MEAN  $\pm$  SE OF THREE SEPARATE EXPERIMENTS.**

### 3.8 Suppression of NF- $\kappa$ B in RGME-mediated apoptosis of HSC-3 Cells

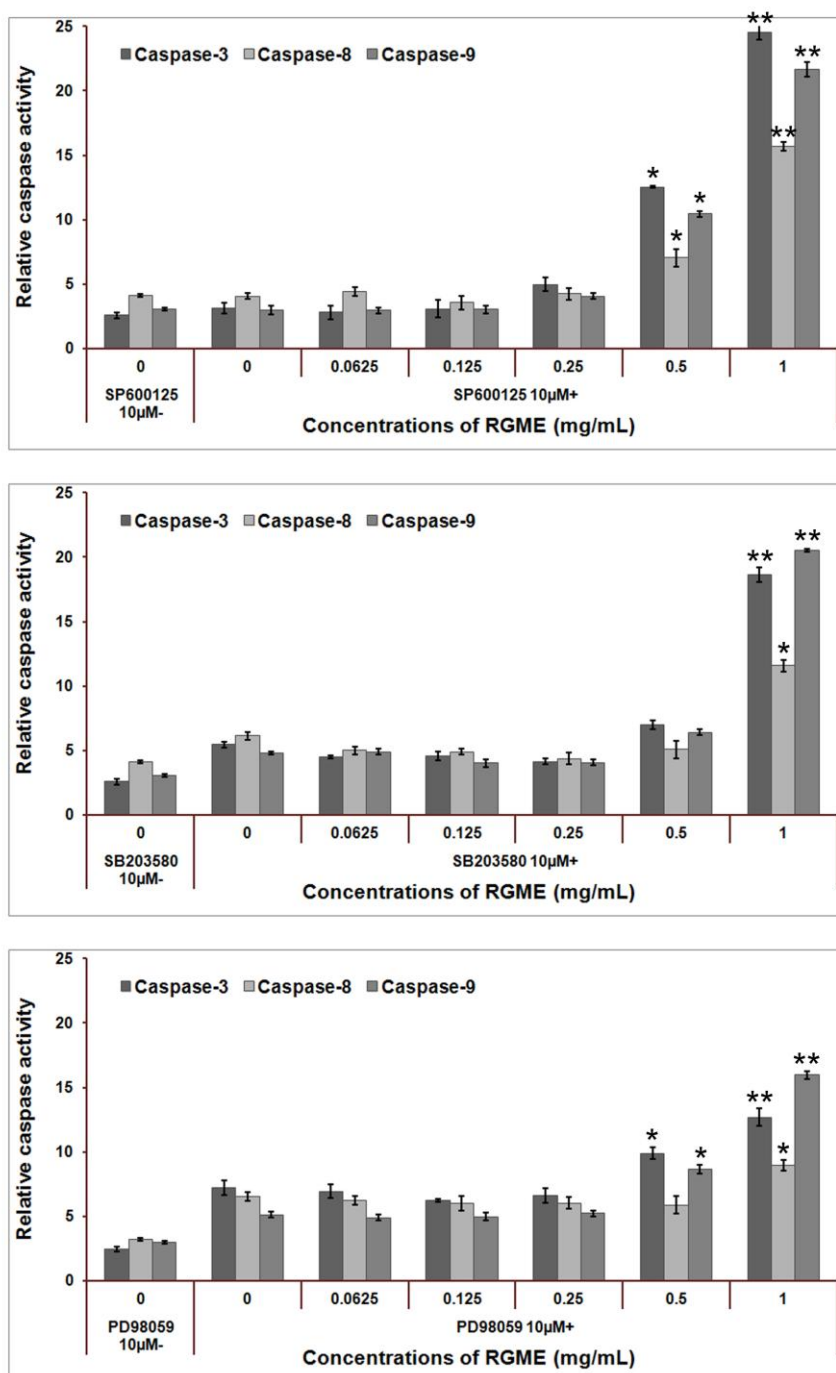
We estimated the expression levels of NF- $\kappa$ B and I $\kappa$ B- $\alpha$  in RGME-mediated apoptosis of HSC-3 cells using western blotting. The RGME treatment increased the nuclear translocation of NF- $\kappa$ B (p65) protein and treating the cells with the essential also degraded the cytosolic I $\kappa$ B- $\alpha$  (Fig. 10)

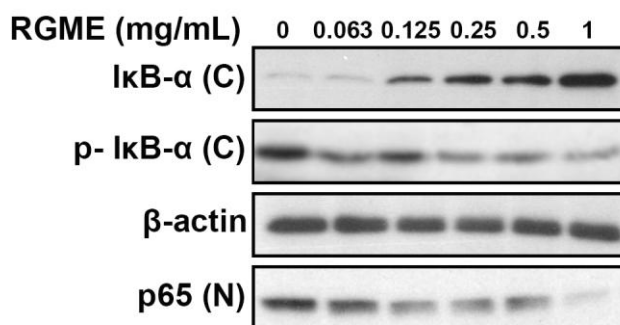
## IV. DISCUSSION

We have studied to confirm if RGME induces the apoptosis of oral squamous cell carcinoma, HSC-3 and what mechanism is involved in the apoptosis. Apoptosis, a process of cell suicide critical for development and tissue homeostasis, is controlled by an evolutionarily conserved program [3, 25]. The uniform morphological features observed in apoptotic cells from different organisms suggest that a common mechanism may operate to trigger the execution of the cell. In this study we showed that the RGME induces cell death in HSC-3 cells at a dose-dependent manner. The RGME-induced cell death was accompanied by nuclear condensation and morphologic changes such as apoptotic bodies and chromatin condensation (data not shown). Most of the anti-cancer agents target the cell cycle of the cancer cells and this property to alter the cell cycle is considered one of the important property in the anti-cancer drug development [26]. Progression of cell cycle is regulated by CDK inhibitor, p27. Increase in the expression of p27 inhibits the cell cycle entry into S phase [27]. The RGME-treated HSC-3 cells were investigated to determine the cell cycle observed during apoptosis. The result showed an increase of the sub-G1 population and decrease of S/M and S phase at a dose-dependent manner. In the Annexin V FITC apoptosis detection, the RGME treatment significantly increased the percentage of positively stained cells at a dose-dependent manner for 24 h. These data showed that RGME could induce apoptosis of 4.70, 7.25, 10.20, and 65.90% in HSC-3 cells, especially



during late apoptosis. The non apoptotic or necrotic cells concentration in the treated RGME was found to be very low compared with the control group. Although both late apoptotic and necrotic cells are Annexin V and PI positive, the presence of these cells with early apoptotic cells suggests that such dead cells resulted from the apoptosis rather than necrosis [25, 28].





**FIG. 10. RGME ACTIVATES NF- $\kappa$ B IN HSC-3 CELLS. CYTOSOLIC AND NUCLEAR EXTRACTS WERE PREPARED FROM HSC-3 CELLS THAT HAD BEEN TREATED WITH THE INDICATED CONCENTRATIONS OF RGME FOR 1 h. THE PROTEINS WERE SUBJECTED TO IMMUNOBLOT ANALYSIS IN ORDER TO MEASURE THE LEVELS OF NUCLEAR TRANSLOCATED SUBFAMILY PROTEINS CORRESPONDING TO NF- $\kappa$ B (p65) COMPOSITION. THE RESULTS FROM THREE INDEPENDENT EXPERIMENTS WERE QUANTIFIED THROUGH DENSITOMETRY.**

Caspases are activated by a variety of apoptotic stimuli, and cell death proteases have been divided into upstream (initiator) and downstream (effector) caspases based on their sites of action in the proteolytic caspase cascade [6, 29]. Of which, caspase-3 was classically divided into executioner caspases, and caspase-8 and -9 into initiator caspases according to their function and their sequences of activation [5, 30]. The caspase-3 activation cascade plays a central role in several apoptotic mechanisms [31]. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP [32]. PARP (116 kDa) is cleaved to produce an 85-KDa fragmentation during apoptosis [33]. So, this we demonstrated RGME-induced activation of caspase-3 and PARP in HSC-3. In particular, approximately 50% and 100% degradation of procaspase-3 was observed when the cells were exposed to 0.5 and 1 mg/mL of RGME for 24 h, respectively. However, the RGME-mediated degradation of procaspase-3 or procaspase-9 was more apparent than that of procaspase-8 in this study.

Caspases-8, -9, and -3 are believed to play crucial roles in mediating mitochondrion-mediated apoptosis pathways [11, 32]. Active caspase-8 can activate Bid, which then triggers the mitochondrial pathway to further activate caspase-9 and in turn activates the executioner, caspase-3, thus committing a cell to apoptosis [7, 34]. Changes in the induction of Bcl-2 family proteins are closely related to an imbalance in the mitochondrial homeostasis, which leads to apoptosis [13]. In particular, the release of cytochrome *c* into the cytosol plays an important role in the execution of apoptosis in a number of different cell types, which is tightly regulated by the equilibrium between the antiapoptotic Bcl-2 and pro-apoptotic Bad and Bax [7, 8]. The release of mitochondrial cytochrome *c* is the crucial event in caspase-9 activation [9]. In these studies, RGME-treatment increased the intensity of the bands corresponding to Bax protein in mitochondrial fractions and significantly reduced the level of Bcl-2 protein. Therefore, mitochondrial stress might play a role in RGME-mediated apoptosis of HSC-3 cells.

The family of MAPKs play central role in the signaling pathway of cell proliferation, survival, and apoptosis, including ERK 1/2, SAPK/JNK1/2 and p38 MAPK. ERK1/2 is activated by mitogens and growth factors through a Ras/Raf/MEK signaling cascade leading to cell growth and survival [13, 14]. We found that the RGME was resulted in the increased phosphorylation level of ERK and p38 MAPK, but not that of JNK. The ERK-specific inhibitor, PD98059 and JNK-specific inhibitor, SP600125, respectively reversed activation of caspases-8, -9, and -3 induced by RGME. These results suggest that activation of ERK and JNK plays important roles in RGME-induced apoptosis of HSC-3 cells via regulation of caspase-8, -9, and -3 activities. Moreover, RGME-induced cell death might also occur via a caspase-independent mechanism.

NF- $\kappa$ B is an anti-apoptotic transcription factor which plays an important role in the cell survival signaling. Interaction with I $\kappa$ B makes NF- $\kappa$ B to reside in the cytoplasm as a dimer. Cell proliferation inducers mediate rapid degradation of I $\kappa$ B, promotes translocation of NF- $\kappa$ B to the nucleus and induces the expression of several anti apoptotic protein including Bcl-2 family members [15, 35]. We estimated the expression levels of NF- $\kappa$ B and I $\kappa$ B- $\alpha$  using western blotting. Treatment of HSC-3 cells with RGME inhibited the translocation of NF- $\kappa$ B to the nucleus. RGME suppressed the degradation of I $\kappa$ B- $\alpha$ .

In conclusion, this study confirmed that RGME induces the apoptosis of oral squamous cell carcinoma, HSC-3 and the

apoptosis is involved in the caspases signal transduction and mitochondrial stress, and these process in regulated by ERK and p38. These results revealed the anti-cancer effect of RGME which may act as a promising therapeutic agent for the treatment of oral squamous cell carcinoma.

## V. CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

## ACKNOWLEDGEMENTS

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