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The Anti-carcinogenic Effect of Korean Cabbage Extracts on the Inhibition of Gap Juctional Intercellular Communication by Hydrogen Peroxide and 12-*O*-Tetradecanoylphorbol-13-acetate

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Modulation of gap junctional intercellular communication (GJIC) is a known cellular event associated with tumor promotion. We examined the anti-carcinogenic properties of the Korean cabbage extracts (DF-01, DF-02, DF-03, Seoul, GM-A, GM-B) on the inhibition of GJIC by hydrogen peroxide (H_2O_2) or phorbol ester (12-*O*-Tetradecanoylphorbol-13-acetate; TPA) in WB-F344 rat liver cell (WB cells). The cells were pre-incubated with Korean cabbage extracts within non-cytotoxic concentration for 24 h followed by co-treatment with extracts and H_2O_2 (750㎛M) or TPA (10 ng/ml) for 1 h. DF-01, DF-02, and DF-03 prevented the inhibition of GJIC by H_2O_2. Inhibition by TPA of GJIC was not prevented by any extracts. To find out the action mechanism of Korean cabbage extracts, western blot analysis was performed to show whether, DF-01 and DF-03 block connexin 43 hyperphosphorylation or inhibit extracellular signal-regulated protein kinases (ERK) 1/2 activation by H_2O_2 in WB cells. Korean cabbage extracts such as DF-01 and DF-03 prevented H_2O_2 induced GJIC inhibition through inactivation of ERK1/2 mitogen-activated protein (MAP) kinases. Therefore, it is suggested that the Korean cabbage extracts might have the potential chemopreventive effects by the inhibition of GJIC.

Key Words: Korean cabbage extracts, Gap junctional intercellular communication (GJIC), WB-F344 rat liver cells (WB cells), Connexin 43, Extracellular signal-regulated protein kinases (ERK) 1/2

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INTRODUCTION

Consumption of *Brassica* vegetables, which are members of a genus in the family *Cruciferae*, is associated with the reduced risk of cancer in human populations.[1] These vegetables and their constituents, such as sulfur-containing glycosides and their biologically active products including the isothiocyanates and other glucosinolate derivatives, have been shown to inhibit the formation of tumors in some studies using animal models.[2] The mechanisms by which these chemicals might act such as triggering apoptotic pathways in cancer cells or inhibiting metastasis have been widely elucidated so far.[3,4]

Gap junction channels play an important role in intercellular communication by providing a direct pathway for the movement of molecular information, including ions and polarized or non-polarized molecules up to a molecular mass of 1 kDa between adjacent cells.[5,6] Although the mechanisms of gap junction formation and channel permeability regulation are poorly understood, the inhibition of gap junction intercellular communication (GJIC) is believed to be involved in the mechanism of tumor promotion.[7] Most tumor promoters including phorbol esters (12-*O*-tetradecanoylphorbol-13-acetate; TPA) and hydrogen peroxide (H_2O_2) inhibit GJIC[8,9] via mitogen-activated protein (MAP) kinases-dependent pathways, and the transfection of GJIC deficient cells with connexins (Cx) suppresses tumor formation.[10~16] Recently, chemopreventive natural chemicals, which have antioxidative activities, such as vitamin C,[17] germanium dioxide,[18] honeybee propolis,[19] green tea and Korean ginseng components[20] and mushroom extract[21] could prevent or recover the inhibition of GJIC induced by cancer promoters. Therefore, recovery of the inhibition of GJIC by cancer promoters has been proven to be a useful tool for the screening and assay of chemopreventive agents, as well as mechanistic studies.

H_2O_2 and TPA are well-known cancer promoters that inhibit GJIC in WB-F344 (WB) rat liver cells in both dose- and time-dependent manners[22,23] by reducing its number and size, as well as increasing hyper-phosphorylation of Cx 43. These carcinogenic effect of these cancer promoters have been shown to be mediated by modulation of MAPkinases,[22,23] which include the extracellular signal-regulated protein kinases (ERK), the c-jun N-terminal kinase (JNK) and the p38 subfamilies.[24] In particular, it has been well documented that phospho-ERK (activated form) can inhibit GJIC in several kinds of cell lines, including WB cells, by aberrant increase of Cx 43 hyper-phosphorylation.[25,26]

In the present study, we assessed the anti-carcinogenic effects of several Korean cabbage extracts by surveying the recovery or preventive effects on the H_2O_2 and TPA induced GJIC inhibition in WB-F344 rat liver cells.

MATERIALS AND METHODS

1) Chemicals

H_2O_2, TPA, Lucifer yellow, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and monoclonal β-actin antibodies were from Sigma Chemical Co. (St. Louis, MO, USA), mouse monoclonal anti-Cx43 antibody from Chemicon International, rabbit anti-JNK1 and mouse monoclonal IgG against p38 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-MAP kinase, rabbit polyclonal anti-Cx 43, horse radish peroxidase (HRP)-goat anti-mouse IgG conjugate and HRP-goat anti-rabbit conjugate antibody were all from Zymed Laboratories (San Francisco, CA). Anti-active MAP kinase, JNK1/2 and p38 rabbit polyclonal antibodies were purchased from Promega (Madison, CA).

2) Cabbage extraction

Cabbages used in this study (DF-01, DF-02, DF-03, Seoul, GM-a. GM-B) were kindly gifted by Dongbu Hannong Chemical (Seoul, Korea) and extracted with 100% methanol. Briefly, 500 g of each cabbage

sample were extracted with 1000 ml of absolute methanol, and filtered through mesh filter followed by concentration using rotary evaporator. These methanol fractions from each cabbage were diluted with DMSO at 10 mg/ml and used in this study.

3) Cell culture

WB rat liver epithelial cells were kindly provided by Dr. J. E. Trosko at Michigan State University (USA). The procedure used for characterizing these cell lines has been described previously.[22] Cells (passage 8～12) were cultured in D-media (Formula No. 78～5470 EF, Gibco BRL, Grand Island, NY) containing 3 ml/l PSN mixture (Gibco BRL) in the presence of 5% fetal bovine serum (Gibco BRL). Cells were incubated in a 37°C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 75 mm tissue culture plates and the culture medium was changed every other day.

4) Bioassay of cytotoxicity

Cytotoxicity was determined by MTT assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide according to the method which was offered by manufacturer. Briefly, cells were treated with each cabbage extract for 24 h with addition of MTT stock solution (5 mg/ml) for one tenth the original culture for the last 4 h of incubation. At the end of the incubation period the media containing converted dye which may be solubilized with DMSO were removed. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630～690 nm using an ELISA reader.

5) Bioassay of GJIC

The scrape loading/dye transfer (SL/DT) technique was adapted using the method of El-Fouly *et al.*[27] Cells were pre-treated with each Korean cabbage extract for 24 h prior to the addition of H_2O_2 or TPA for 1 h. The GJIC assay was conducted at non-cytotoxic dose levels of the samples, as determined by the cytotoxicity assay using MTT.

Following incubation, the cells were washed twice with 2 ml of phosphate-buffered saline (PBS). Lucifer yellow was added to the washed cells and three scrapes were made with a surgical steel-bladed scalpel at low light intensities. These three scrapes were performed to ensure that the scrape traversed a large group of confluent cells. After a 3-min incubation period the cells were washed with 10 ml of PBS and then fixed with 2 ml of a 4% paraformaldehyde solution. The distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted fluorescence microscope (Olympus IX70, Okaya, Japan).

6) Western blot analysis

Cells were grown in a 100 mm tissue culture dish (Nunc, Rochester, NY) to the same confluency as in the SL/DT assay. The cells were then treated with each test in the same way as described in the SL/DT assay. Western blot analysis of Cx 43 was performed, as described previously.[28,29] Proteins were extracted with 20% SDS solution containing 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor), 10 mM iodoacetamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride. Protein content was determined using the DC assay kit (Bio-Rad, Hercules, CA), and separated on 12.5% SDS-PAGE according to the method of Laemmli.[30] They were then transferred to nitrocellulose membranes at 100 V, 350 mA for 1 h. All antibodies were used according to the manufacturer's instructions and protein bands were detected using an ECL detection kit (Amersham, Piscataway, NJ).

RESULTS

1) Cytotoxicity assay of korean cabbage extracts

The optimal concentrations of H_2O_2 (750㎛M) and TPA[31] (10 ng/ml) were obtained by time- and dose-dependent GJIC assays using SL/DT and also was proved to be non-cytotoxic by MTT assay (data not

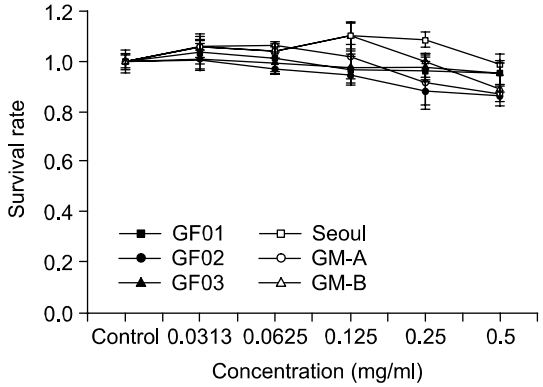

Fig. 1. Cytotoxic effect of Korean cabbage extracts. Cells were treated with each Korean cabbage extract for 24 h followed by addition of MTT stock solution (5 mg/ml) for one tenth the original culture for the last 4 h of incubation. Cell viability was measured by ELISA reader according to the method offered by manufacturer. Each bar represents the mean±SD.

shown). The highest non-cytotoxic concentrations of cabbage extracts (0.5 mg/ml for DF-01, DF-03 and Seoul, 0.125 mg/ml for DF-02, 0.25 mg/ml for GM-A and GM-B) were chosen within the level where the survival rate was higher than 90%, and applied by double dilution (Fig. 1).

2) Effect of Korean cabbage extracts on GJIC

The GJIC of WB cells were assessed using SL/DT assay after treatment with the test compounds. The GJIC of control cells did not decrease during the experimental incubation period. After exposing the cells to 750㎛M H_2O_2 or 10 ng/ml TPA for 1 h, an obvious inhibition was detected. The cells pre-treated for 24 h with DF-01 (0.25 mg/ml), DF-02 (0.0625 and 0.0125 mg/ml) and DF-03 (0.5 mg/ml) showed increased GJIC, leading to effective blocking the inhibition by H_2O_2 treatment. However, the cells pre-treated with other extracts (Seoul, GM-A and GM-B) did not show preventive effects on GJIC inhibited by 750㎛M H_2O_2 (Fig. 2A). None of the extracts effectively defended TPA induced GJIC inhibition (Fig. 2B).

3) Western blot analysis for a linkage protein group of GJIC

Western blot analysis, with antibodies specific to Cx 43, was used to assess the phosphorylation status of the gap junction proteins. The typical three bands (P0, P1 and P2) of Cx 43 were detected in the control cells, and these were separated according to their degree of phosphorylation. H_2O_2 treatment caused the P0 band to disappear and induced the P2 band. Treatment of DF-01 or DF-03 in combination with H_2O_2 decreased the phosphorylation ratio (P2 : P0) of Cx 43 as induced by H_2O_2 (Fig. 3) while DF-02 did not.

4) Western blot analysis of MAP kinases activation

ERK1/2, JNK and p38 kinase were examined using western blot analysis to identify the protective action of cabbage extracts on the inhibition of GJIC. H_2O_2 activated p38 kinase, ERK1/2 and JNK. DF-01 and DF-03 (0.5 mg/ml) remarkably decreased the phosphorylated ERK1/2, even though the proteins were constitutively expressed (Fig. 4A). However, pre-treatment of these effective cabbage extracts did not inhibit H_2O_2-induced activation of the p38 or JNK pathway (Fig. 4B, C).

DISCUSSION

Most tumor-promoting agents inhibit intercellular communications, and reduced GJIC capacity has been observed frequently during carcinogenesis.[8,9] Moreover, tumor promoters or reactive oxygen species such as TPA and H_2O_2 almost immediately disrupt GJIC *in vitro* system and directly promote transformation in many *in vivo* and *in vitro* model systems, which play important roles in the multistep process of carcinogenesis.[22,32] Therefore, it is very promising that GJIC can be a target of anti-cancer research.

Also, it is very obvious that increasing interests are getting focused on naturally acquirable chemicals such as phytochemicals and some inorganic minerals (sele-

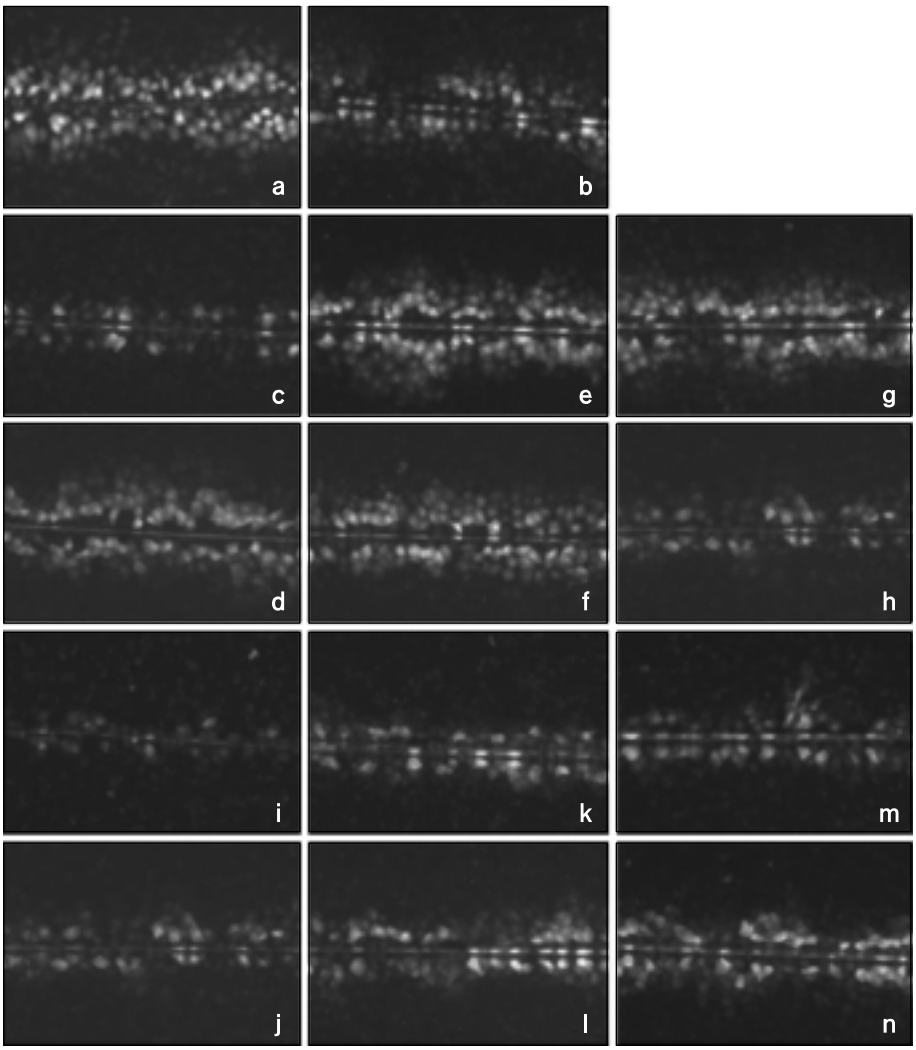

Fig. 2A. Effects of Korean cabbage extracts on H_2O_2 induced GJIC inhibition assessed by SL/DT assay. Cells were treated with (a) control, (b) H_2O_2 750㎛M, (c) H_2O_2 plus DF-01 extract 0.5 mg/ml, (d) H_2O_2 plus DF-01 extract 0.25 mg/ml, (e) H_2O_2 plus DF-02 extract 0.125 mg/ml, (f) H_2O_2 plus DF-02 extract 0.0625 mg/ml (g) H_2O_2 plus DF-03 extract 0.5 mg/ml, (h) H_2O_2 plus DF-03 extract 0.25 mg/ml, (i) H_2O_2 plus Seoul extract 0.5 mg/ml, (j) H_2O_2 plus Seoul extract 0.25 mg/ml, (k) H_2O_2 plus GM-A extract 0.25 mg/ml, (l) H_2O_2 plus GM-A extract 0.125 mg/ml (m) H_2O_2 plus GM-B extract 0.25 mg/ml, (n) H_2O_2 plus GM-B extract 0.125 mg/ml.

nium derivatives *etc.*) for chemoprevention. Therefore, in this study, we investigated the anticarcinogenic effect of cabbage extracts on WB cells after treatment with TPA or H_2O_2 using the SL/DT and western blotting assasy.

Significant prevention against the H_2O_2 induced inhibition of GJIC was achieved with Korean cabbage extracts DF-01, DF-02 and DF-03 between the concentrations of 0.25 and 0.5 mg/ml (Fig. 2A) as assessed by SL/DT, while these extracts were not effective on TPA induced inhibition (Fig. 2B).

Although it has been reported that cabbage extracts

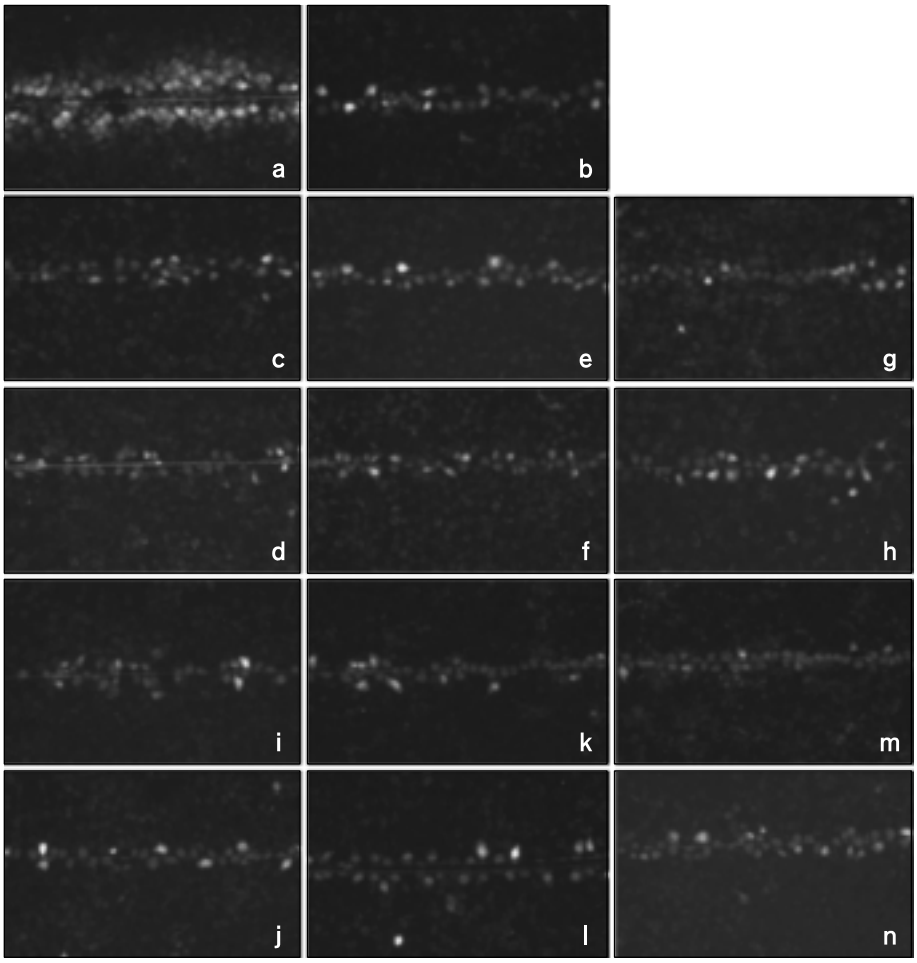

Fig. 2B. Effects of Korean cabbage extracts on TPA induced GJIC inhibition assessed by SL/DT assay. Cells were treated with (a) control, (b) TPA 10 ng/ml, (c) TPA plus DF-01 extract 0.5 mg/ml, (d) TPA plus DF-01 extract 0.25 mg/ml, (e) TPA plus DF-02 extract 0.125 mg/ml, (f) TPA plus DF-02 extract 0.0625 mg/ml (g) TPA plus DF-03 extract 0.5 mg/ml, (h) TPA plus DF-03 extract 0.25 mg/ml, (i) TPA plus Seoul extract 0.5 mg/ml, (j) TPA plus Seoul extract 0.25 mg/ml, (k) TPA plus GM-A extract 0.25 mg/ml, (l) TPA plus GM-A extract 0.125 mg/ml (m) TPA plus GM-B extract 0.25 mg/ml, (n) TPA plus GM-B extract 0.125 mg/ml as described in the Materials and Methods section.

and their ingredients including the isothiocyanates inhibits tumor growth by several anti-carcinogenic events,[1~4] it is very rare that the cabbage extracts prevent the tumor promoter-induced inhibition of GJIC.

Tumor promoters can affect intercellular communication by mechanisms that distinguish between immediate and long-term responses. The immediate response of H_2O_2 and TPA on GJIC is associated with the hyper-phosphorylation of Cx 43.[22] In the present study, western blot analysis showed that the intensity of the Cx 43 P2 band increased after H_2O_2 treatment with loss of the P0 band of Cx 43, and that it decreased slightly or remarkably when cells were pre-incubated with 0.25 or 0.5 mg/ml DF-01 or DF-03, respectively (Fig. 3).

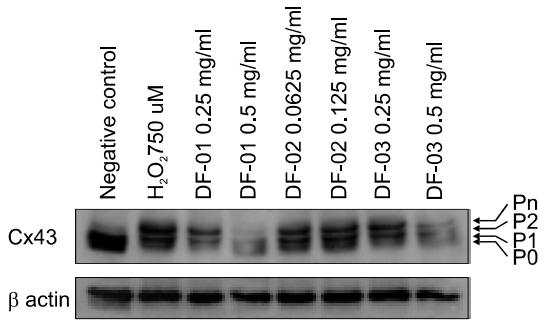

Fig. 3. Western blot analysis on the changes in the phosphorylation pattern of Cx 43. Total cellular protein extracts were prepared and western blot analysis was performed with 20㎍ protein using antibody specific for Cx 43.

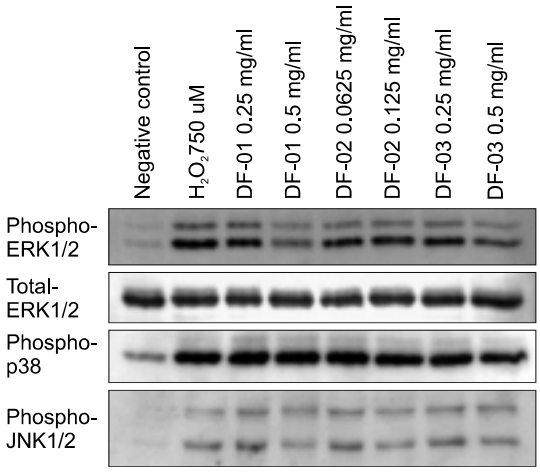

Fig. 4. Effect of Korean cabbage extracts on H_2O_2-induced ERK, p38 and JNK phosphorylation. Total cellular protein extracts were prepared and western blot analyses were performed with 20㎍ protein using antibody specific for total- or phospho-ERK, phospho-p38 and phospho-JNK, respectively.

To elucidate the preventive mechanism of cabbage extracts on GJIC, we examined MAP kinases. MAP kinases, which include the ERK, JNK/stress-activated protein kinase and p38 subfamilies, are activated in response to stimuli such as treatment with DNA-damaging agents, growth factors and cytokines.[24,33~35] MAP kinases regulate gene expression through the phosphorylation of downstream transcription factors.[24,33~35] Activation of JNK and p38 kinase is related to the stress response, growth arrest and apoptosis,[33~35] while ERK is important in mitogenesis and differentiation.[36] Recently, we found an significant relationship between MAP kinases and GJIC.[21] In that study, we reported that ERK1/2 and p38 kinase activation might be the primary mechanisms of GJIC inhibition by H_2O_2 treatment. In the present study, there was remarkable decrease only in ERK1/2 activation by cabbage extracts DF-01 and DF-03 treatment (Fig. 4). Therefore, we hypothesized that blockage of ERK1/2 activation might solely contribute to recovery of H_2O_2 induced GJIC inhibition.

In conclusion, this study shows that Korean cabbage extracts DF-01 and DF-03 increase GJIC and prevent the inhibition of GJIC by H_2O_2 through inhibition of ERK activation, which might be an important mechanism whereby the Korean cabbage extract protects against tumor promotion. In addition, a follow-up progress to find single compound in these extracts which are related with the effects remains.

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