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죽력의 Nrf2 활성화를 통한 장상피세포 보호 효능

김재민[1], 양지혜[1]*

동신대학교 한의과대학 한의예과

Efficacy of Bambusae Caulis in Liquamen to protect intestinal epithelial cells via Nrf2 activation

Jae Min Kim[1], Ji Hye Yang[1]*

[1]College of Korean Medicine, Dongshin University

ABSTRACT

Objectives : Intestinal epithelial cell damage is closely associated with various intestinal diseases, such as Inflammatory Bowel Disease (IBD), Celiac Disease and Gastroenteritis, and it plays a crucial role in the development and progression of intestinal diseases. Therefore, it is important to develop drugs that target protection of intestinal epithelial cells. Here, we aimed to investigated whether Bambusae Caulis in Liquamen (BCL) against t-BHP induced oxidative stress injury in human intestinal epithelial cells and to explore the underlying molecular mechanism.

Methods : In this study, we performed MTT assay, measurement of ROS generation, and immunoblot analysis to determine the cytoprotective efficacy in HT29 cells (human colorectal adenocarinoma cell line with epithelial morphogy).

Results : First, we checked that BCL was not cytotoxic up to concentration 30 μg/mL in HT29 cells. Then, we confirmed that BCL inhibited t-BHP-induced ROS and cell death. BCL also reversed the expression of proteins associated apoptosis. Next, to confirm the relationship between efficacy of BCL and Nrf2, we conducted experiments using siNrf2. As result, the effects of inhibiting ROS production and cell death of BCL was reversed by siNrf2.

Conclusion : BCL prevents t-BHP-induced oxidative stress and apoptosis. And the efficacy of BCL is related to Nrf2 activation.

Key words : *Bambusae Caulis* in Liquame, Intestinal epithelial cells, ROS, Apoptosis, Nrf2

1. Introduction

Intestinal epithelial cells are the cells that make up the lining of the intestines. They form a protective barrier between the internal environment of the body and the contents of the intestines, playing a crucial role in absorption, secretion, and protection[1]. Intestinal epithelial cells are connected by tight junctions, which are protein complexes that seal the space between cells. These junctions help maintain the integrity of the intestinal barrier. Intestinal epithelial cell damage is closely associated with various intestinal diseases, such as Inflammatory Bowel Disease (IBD), Celiac Disease and Gastroenteritis, and it plays a crucial role in the development and progression of intestinal diseases[2]. Therefore, it is important to develop drugs that target protection of intestinal epithelial cells.

Maintaining a balance in Reactive Oxygen Species (ROS) levels in cells is crucial for nornal cellular function[3]. Cells have antioxidant systems in place to neutralize ROS and prevent excessive damage[4]. However, in certains or disease, this balance can be disrupted, leading to oxidative stress and cell death. Nrf2 (Nuclear-factor erythroid 2-related factor 2) is a transcription factor that play a primary role in the cellular defense against oxidative stress[5]. The activation of Nrf2 and subsequent upregulation of these genes enhance the ability of cells to neutralize ROS and repair oxidative damage[6]. So, Nrf2 activation has been explored as a potential therapeutic strategy in various disease associated with oxidative stress.

Bambusae Caulis in Liquamen (BCL), which has been used as a traditional herbal to treat coughs and asthma, is a liquid extracted from heat-treated fresh bamboo stems. Recent studies were determined that BCL has anti-inflammatory and anti-allergenic and anti-oxidative effects[7]. We have also found in previous study that BCL has the effect of protecting hepatocytes[8]. In addition, Xu-Feng_Qi et al. found that BCL significantly suppresses the development of DNCB-induced atopic dermatitis -like skin lesions in hairless mice, a murine model of atopic dermatitis[9] and BCL Suppresses the Expression of Thymus and Activation-Regulated Chemokine and Macrophage-erived Chemokine in Human Keratinocytes due to Antioxidant Effect[10]. However, Efficacy of BCL to protect intestinal epithelial cells has not been evaluated.

Here, we aimed to investigated whether BCL against tert-Butyl hydroperoxide (t-BHP) induced oxidative stress injury in human intestinal epithelial cells and to explore the underlying molecular mechanism.

2. Materials and Methods

2.1. Materials

MTT and DCFH-DA were sourced from Sigma Chemicals (St. Louis, MO, USA). Antibodies against Bax, Bcl-Xl, and Nrf2 were obtained from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). Caspase-3 antibody was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). β-Actin antibody was sourced from Sigma Chemicals (St. Louis, MO, U.S.A.).

2.2. Preparation of Bambusae Caulis in Liquamen (BCL)

Bambusae Caulis in liquid (BCL) was prepared as described in a previous study[8]. A total of 18 L of BCL, purchased from Bamboo Forest Foods Co., Ltd. (648 Samdari, Damyang-eup, Damyang-gun, Jeollanam- o, Republic of Korea), was concentrated using a decompression concentrator. For treatment in cells, BCL was dissolved in DMSO (dimethyl sulfoxide).

2.3. Cell Culture

HT29 cells (human colorectal adenocarinoma cell line with epithelial morphogy) from the American Type Culture Collection (Manassas, Virginia, USA). Cells were grown in Roswell Park Memorial Institute

*Corresponding author: Ji Hye Yang. College of Korean Medicine, Dongshin University, 67, Dongshindae-gil, Naju-si, Jeollanam-do, 58245, Republic of Korea.
Tel : +82-61-330-3518, E-mail : uranus2k@dsu.ac.kr
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(RPMI) media supplemented with 10% fetal bovine serum (FBS) and 50 U/mL penicillin/streptomycin at 37° C in a humidified 5% CO_2 atmosphere.

2.4. MTT Assay

To measure cell viability, cells were plated in 48-well plates (2×10^5 cells/well) and treated for 24 h or 6 h. Viable cells were then stained using 0.2 mg/mL MTT for 1 h and the media was then removed. Then, formazan crystals produced in the wells were dissolved using 200 μL of dimethyl sulfoxide. The absorbance at 540 nm was measured using a microplate reader (Spectramax; Molecular Devices, Sunnyvale, CA, USA).

2.5. Measurement of ROS Generation

Diacetyldichlorofluorescein (DCFH-DA) is a cell-ermeable, non-fluorescent probe that is cleaved by intracellular esterases and converted to highly fluorescent dichlorofluorescein upon reaction with H_2O_2. After treatment with t-BHP (500 μM, 1 h), HT29 cells were stained with 10 μM DCFH-DA for 30 min at 37° C. H_2O_2 generation was determined by measuring the fluorescence intensity of dichlorofluorescein using a fluorescence microscope (Zeiss, Jena, Germany) or fluorescence microplate reader (Jemini; Molecular Devices) with excitation and emission wavelengths of 485 and 530 nm, respectively.

2.6. Immunoblot Analysis

Protein extraction, subcellular fractionation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described[11]. Briefly, the samples were separated using 7.5% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were visualized using an ECL chemiluminescence detection kit (Amersham Biosciences). Equal protein loading and the integrity of subcellular fractionation were verified by b-actin expression in immunoblots.

2.7. Statistical Analysis

The statistical significance of differences among groups was assessed using one-way analysis of variance (ANOVA) For each statistically significant treatment effect, the Newman-Keuls test was employed for comparisons between multiple groups. The data were presented as means ± standard error (S.E.).

3. Results

3.1. Evaluation of cytotoxicity of BCL in HT29 Cell

In many studies, human colorectal adenocarinoma cell line was used as oxidative damage models [12-14]. Because HT29 cells have epithelial morphology, these cells were selected to construct oxidative damage models in the present study. First of all, we performed MTT assay to set the concentration of BCL for cell experiments. We found that BCL was not cytotoxic up to concentration 30 μg/mL in HT29 cells (Figure 1).

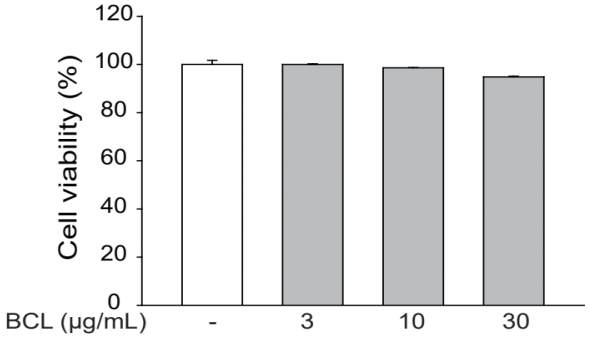

Figure 1. The cytotoxicity measurement of BCL in HT29 cells using MTT assay. BCL was treated at concentrations from 3 μg/mL to 30 μg/mL, and incubated for 24 h. Data represents the mean ± S.E. of three replicates (compared to vehicle-treated control).

3.2. BCL inhibits t-BHP induced-Oxidative Stress in HT29 Cell

Intestinal epithelial cells play a crucial role in forming a protective barrier against harmful substances present in the gut[1]. Disruption of this barrier, often associated with oxidative stress, can lead to increased permeability and the translocation of bacteria and toxins[15]. So, we checked whether BCL has inhibitory effects against ROS in intestinal epithelial cells. As result of experiments, pre-treatment of BCL inhibited t-BHP induced ROS production in HT29 Cell (Figure 2). These results demonstrate that BCL effectively inhibited t-BHP-induced oxidative stress in HT29 Cell.

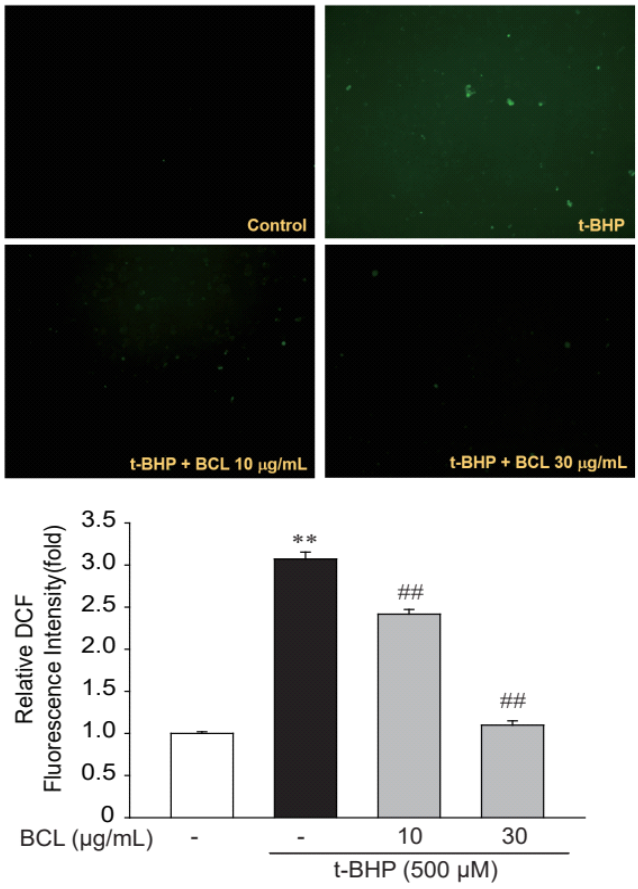

Figure 2. The measurement of ROS production using DCFH-DA in HT29 cells. BCL was pre-treated at concentrations from 10 μg/mL to 30 μg/mL for 1 h, and then t-BHP (500 μM) was treated for 1h. Cells were recorded by fluorescence microscope. Data represents the mean ± S.E. of three replicates; ** $p < 0.01$ significant versus vehicle-treated control; $^{\#\#}$ $p < 0.01$, significant versus t-BHP alone.

3.3. BCL suppresses t-BHP induced-Cell Death in HT29 Cell

Intestinal epithelial cells death is associated with various intestinal diseases, such as Inflammatory Bowel Disease (IBD), Celiac Disease and Gastroenteritis[16]. So, inhibitory effects about intestinal epithelial cells death is crucial for developing targeted therapeutic strategy of intestinal diseases. We pre-treated with BCL for 3 h, and utilized t-BHP to induce HT29 cell death. As result, t-BHP alone-treated cells were killed, while BCL pre-treatment inhibited cell death (Figure 3). These findings strongly indicate that BCL suppressed t-BHP-induced cell death in HT29 cells.

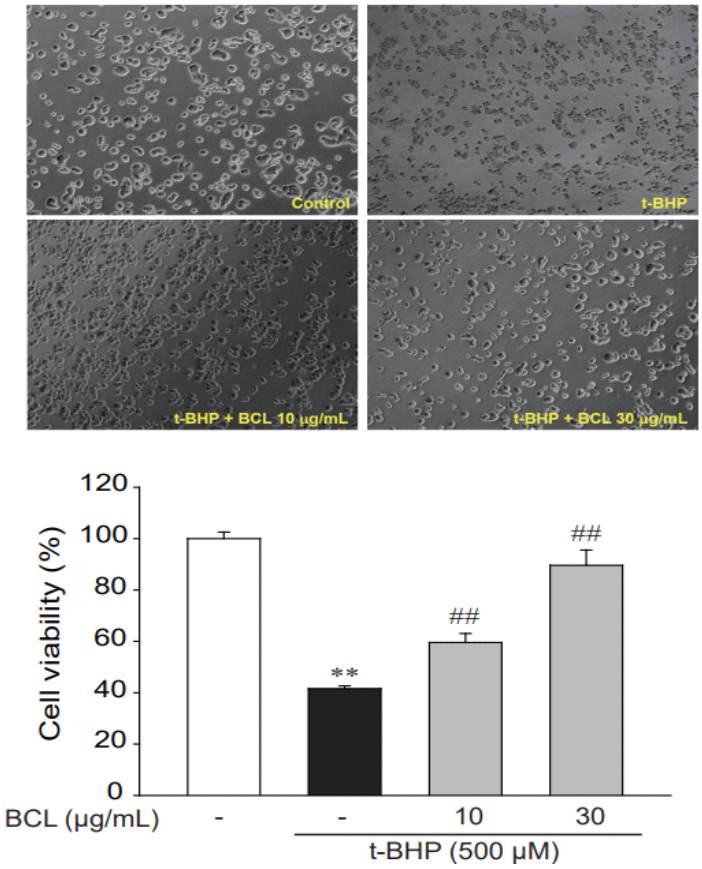

Figure 3. The cytoprotective efficacy of BCL using MTT assay in HT29 cells. Cells were treated with t-BHP (500 μM) and/or 10-30 μg/mL BCL for 6 h. Cells were recorded by microscope (50X). Data represent the mean ± standard error (S.E.) of three experiments; ** $p < 0.01$ vs. vehicle-treated control; $^{\#\#}$ $p < 0.01$ vs. t-BHP treatment alone.

3.4. BCL reverses t-BHP induced-apoptosis proteins in HT29 Cell

Apoptosis, also known as programmed cell death, is essential for maintaning tissue homestasis by removing unwanted or damaged cells[17,18]. However, excessive apoptosis is associated with various diseases. So, we checked whether BCL can affect apoptosis pathway. The Bcl-2 family regulates apoptosis, with

pro-apoptotic (Bax) and anti-apoptotic (Bcl-xL) members[19]. And caspase-3 targets structural proteins, leading to cellular chages associated with apoptosis[20]. t-BHP treatement induced apoptosis proteinsm but BCL pre-treatment reversed the expression of them (Figure 4).

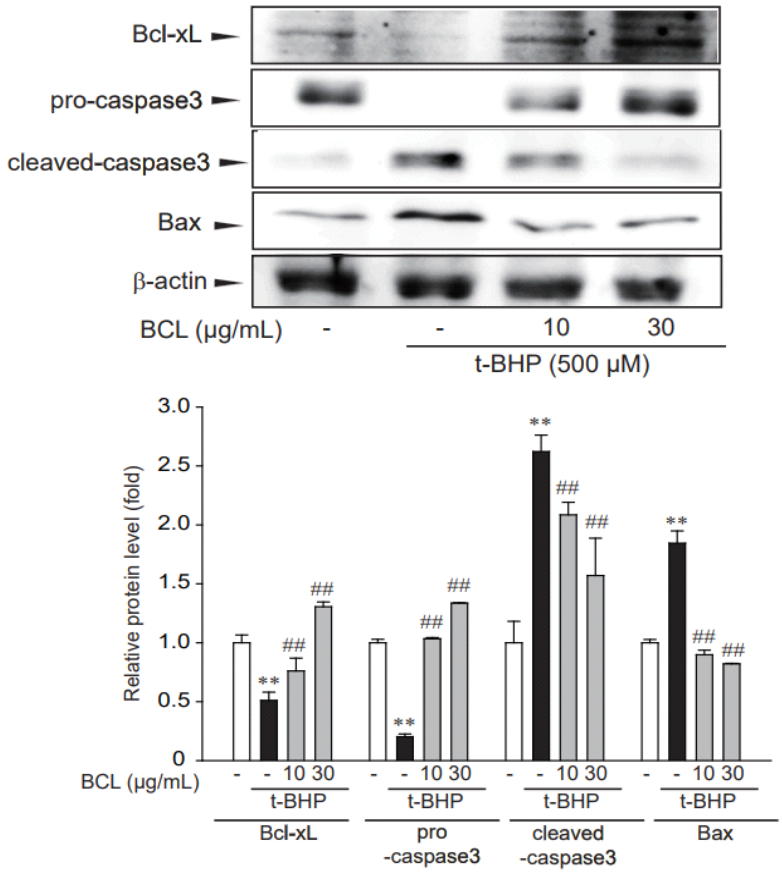

Figure 4. The levels of marker proteins (Bcl-xL, pro-caspase3, cleaved-caspase3 and Bax) of apoptosis using immunoblotting in HT29 cells. BCL was pre-treated at concentrations from 10 μg/mL to 30 μg/mL for 1 h, and then t-BHP (500 μM) was treated for 4h. Data represent the mean ± standard error (S.E.) of three experiments; ** $p < 0.01$ vs. vehicle-treated control; ## $p < 0.01$ vs. t-BHP treatment alone.

3.5. Effects of BCL in HT29 Cell were associated with Nrf2

Nrf2, as a master regulator, orchestrates the cellular defense against oxidative stress by governing the expression of genes involved in antioxidant pathways[21]. Through the experiments in this study, we found that BCL can suppress ROS production and apoptosis. However, we are yet to determine whether the effects of BCL are related to Nrf2 activation. To investigate the activation of Nrf2 by BCL, we examined Nrf2 nuclear translocation. The results unequivocally showed that nuclear translocation increased in a time-dependent manner following BCL treatment (Figure 5A). Also, we used siRNAs for Nrf2 (siNrf2) to confirm the association between BCL and Nrf2 activation. As result, ROS production and cell death reduced by BCL were reversed by siNrf2 (Figures 5B and C).

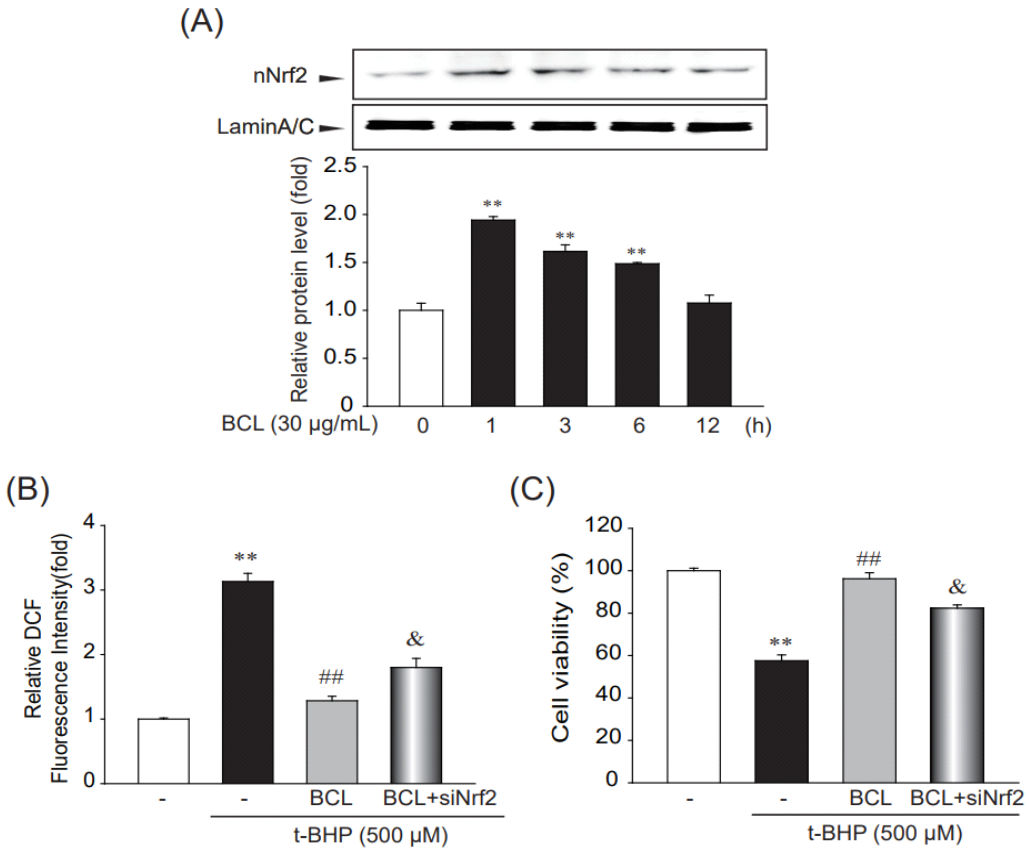

Figure 5. Effects of *Bambusae Caulis* in Liquamen is associated with Nrf2 in HT29 cells. (A) The time-course study of nuclear translocation of Nrf2 in HT29 cells treated with BCL (30 μg/mL) for 1 h to 12 h. Data represent the mean ± standard error (S.E.) of three experiments; $^{**}p < 0.01$ vs. vehicle-treated control. (B and C) After siNrf2 transfection in HT29 cells for 24 h. The measurement of ROS production using DCFH-DA. BCL was pre-treated at concentrations from 10 μg/mL to 30 μg/mL for 1 h, and then t-BHP (500 μM) was treated for 1h (B). The cytoprotective efficacy of BCL using MTT assay. Cells were treated with t-BHP (500 μM) and/or 30 μg/mL BCL for 6 h (C). Data represent the mean ± standard error (S.E.) of three experiments; $^{**}p < 0.01$ vs. vehicle-treated control; $^{\#\#}p < 0.01$ vs. t-BHP treatment alone; $^{\&}p < 0.05$ vs. t-BHP +BCL treatment.

4. Discussion

HT29 cells are a human colon adenocarcinoma cell line commonly used in scientific research of tumor[22,23]. However, many researchers use HT29 cells to investigate cell signaling pathways, drug responses, and mechanisms of cancer progression, because these cells have epithelial morphology[12-14]. So, we used HT29 human colon cancer cells to construct oxidative damage models in the present study.

Several intestinal disease involve oxidative stress and inflammation[24], and Nrf2 has been studied inrelation to these conditions such as inflammatory bowel disaseas (IBD)[25]. Nrf2 activation has been explored as

a potential therapeutic target to mitigate oxidative stress and inflammation[26]. Nrf2 is an important player in cellular defense mechnisms against oxdative stress, and its involvement in intestinal disease underscores its potential significance in therapeutic strategies for conditions affecting the gastrointestinal tract[27]. BCL is well known that it has anti-inflammatory, anti-allergenic and anti-oxidative effects[7-10]. However, Efficacy of BCL to protect intestinal epithelial cells has not been evaluated. So, this study's aime is to found out whether BCL has a cytoprotective effects in HT29 clles via Nrf2 activation.

We first, checked that BCL was not cytotoxic up to concentration 30 μg/mL in HT29 cells using MTT assay (Figure 1). Then, we confirmed that BCL inhibited t-BHP-induced ROS production and HT29 cell death (Figure 2, 3). t-BHP is a compound that is often used in laboratory studies to induce oxidative stress[28]. In the context of cell death, exposure to t-BHP is known to induce apoptosis, a form of programmed cell death. The increased oxidative stress caused by t-BHP can damage cellular components, such as DNA, proteins, and lipids, triggering signaling pathway that ultimately lead to programmed cell death[29]. Therefore, we verified that BCL influenced t-BHP-induced changes in apoptosis-related protein expression (Figure 4).

Next, Nrf2 helps maintain the redox balance within cells, ensuring that ROS levels are controlled and do not lead to excessive oxidative stress or cell death[30].

In a previous study, we confirmed that BCL increased antioxidant enzyme expression, and BCL has cell protective effect and anti-inflammatory efficacy via Nrf2 activaion in Hepatocye and Kupffer cells[8,31]. In the present study, we also checked that BCL increased Nrf2 nuclear translocation, and we used siNrf2 to confirm the association between effect of BCL and Nrf2 activation (Figure 5). These results suggested that BCL is a potential therapeutic drug candidate for intestinal injury. So, based on the results of this study, we have a plane to study the therapeutic efficacy of BCL in vivo models of bowel disease in the future.

5. Conclusions

The study results showed that BCL inhibited t-BHP-induced ROS production and HT29 cells death. BCL also reversed t-BHP-induced appoptosis proteins. In addition, the reduction in ROS production and cell death induced by BCL were reversed by siNrf2. These results suggest that BCL is effective in intestinal epithelial cells and that cytoproective effects of BCL are mediated by Nrf2. This study suggests that BCL is a promising therapeutic drug for the treatment of intestinal disease.

6. A written apology

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