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Original Article

Protective Effects of *BCC* Against Oxidative Stress in Cardiomyocyte Cells

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Oxidative stress caused by elevated reactive oxygen species (ROS) in the heart causes various heart diseases. Oxidative stress is known as a factor that causes diseases in various organs as well as the heart. Diseases such as heart failure, myocardial infarction, and cardiomyopathy caused by oxidative stress in the heart can be treated with medication or surgery. Recently, blood cells concentrate (*BCC*) is used in various treatment areas such as orthopedics, gynecology, and urology. *BCC* therapy is applied to treatment by concentrating platelets and white blood cells necessary for regeneration through simple centrifugation using autologous blood. As the platelets are activated, many growth factors are released from alpha granules of the platelets. Growth factors such as TGF-β1, PDGF, VEGF, and EGF derived from platelets are involved in various cell signaling pathway. Due to these growth factors, *BCC* can contribute to tissue regeneration and can treat various diseases. CD34+ cells contained in *BCC* may also play an important role in tissue regeneration. In this study, we investigated whether *BCC* has a regenerative effect on heart disease, and if so, what mechanism causes the effect. To observe this, cardiomyocyte cells were treated with H_2O_2 to induce oxidative stress. And the effect was confirmed in the presence or absence of *BCC*. As a result, in the presence of *BCC*, the oxidative stress of cardiomyocyte cells was reduced and cell damage was also reduced. These results suggest that *BCC* therapy can be a new treatment alternative for heart disease.

Key Words: ROS, Oxidative stress, Heart disease, Platelet, Growth factor

INTRODUCTION

The level of reactive oxygen species (ROS) in the human body is very important. ROS can be generated by metabolic pathways (oxidative phosphorylation), and cells control elevated ROS levels through various defense mechanisms (Zhang et al., 2021; Apel and Hirt, 2004). However, ROS levels above physiological levels can cause various diseases due to oxidative stress. In particular, the heart is an organ that requires high energy, and the activity of metabolic pathways is high (Chistiakov et al., 2018). In a heart that requires such high energy, ROS levels can easily increase if they are not well controlled. And Research results show that oxidative stress caused by elevated ROS levels causes heart diseases (D'Oria et al., 2020; Steinhorn et al., 2018; Singal et al., 1998). It is known that ROS-induced oxidative stress can cause diseases such as heart failure, myocardial infarction, cardiomyopathy, etc. Continuous oxidative stress activates the apoptosis pathway in cardiomyocytes and eventually

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causes heart diseases. From this perspective, ROS-induced oxidative stress can be used as a target for the treatment of heart diseases. By reducing abnormally high ROS levels in the heart, heart diseases may be treated (Hargrave and Li, 2012).

Blood Cells Concentrate (*BCC*) is a concentrated buffy coat excluding plasma and RBCs, which make up most of the components of peripheral blood (Gutierrez et al., 2017; Dhurat and Sukesh, 2014). Generally, *BCC* is defined as plasma with platelets increased by approximately 3-fold or more. *BCC* can regenerate tissues by inducing proliferation and differentiation of stem cells, due to growth factors such as TGF-β1, PDGF, VEGF, EGF, etc. released when platelets are activated (Marck et al., 2019; Etulain et al., 2018). Initially, this *BCC* was mainly used for musculoskeletal diseases. However, many studies have been conducted, and now *BCC* is also applied to skin care, gynecological diseases, and hair loss (Deshmukh and Belgaumkar, 2019; Gkini et al., 2014; Sfakianoudis et al., 2018). Currently, in addition to the manual method for extracting this *BCC*, various types of products exist and are being used (Kreuz et al., 2015).

In this study, we investigated to determine whether *BCC* could be applied to treat heart disease. For this purpose, cardiomycyte cells (H9C2 cells) were treated with H_2O_2 to induce oxidative stress and the effects were confirmed. When *BCC* was treated with H9C2 cells induced by oxidative stress, it showed a reduction in ROS levels and cell death. These results suggest that *BCC* is also effective in treating heart diseases.

MATERIALS AND METHODS

Blood collection

Blood was collected from donor volunteers. All donors gave informed consent and were healthy. Whole Blood (W.B.) was collected by venipuncture. Anticoagulant was used acid citrate dextrose solution, Solution A (ACD-A) (DAE HWA PHARMACEUTICAL CO., LTD., Korea). The volume ratio of blood to ACD-A was applied at 9 to 1 (27 mL : 3 mL). W.B. and *BCC* samples were prepared in MODELLINE CLINIC (Bundang, Korea).

Fig. 1. Process steps for *BCC* KIT.

BCC preparation & Hematological analysis

BCC was extracted using the REV-MED Inc.'s *BCC* KIT (Model No., RM-142) according to the manufacturer's instructions (Fig. 1). Briefly, 30 mL of W.B. is injected into KIT. After performing first and second centrifugation, 3 mL of *BCC* is extracted. After *BCC* extraction, W.B. and *BCC* were sufficiently incubated on the shaking plate for about 5 minutes. The cell counts of W.B. and *BCC* were analyzed using a Mythic^{TM}22AL (Orphee S.A., Switzerland).

BCC activation

To manufacture activated *BCC*, $CaCl_2$ (Sigma-Aldrich Co., St. Louis, USA) was used as an activator (Nam and Kim, 2018). *BCC* and 0.25 M $CaCl_2$ were mixed at 9:1 (vol: vol). A mixture of *BCC* and 0.25 M $CaCl_2$ was incubated for 1 hour at room temperature. After incubation for 1 hour, supernatant was harvested by centrifugation (2,500 xg, 15 minutes). The supernatant was stored at -80℃ until its use.

Cell culture & treatment

The rat cardiomyocyte H9C2 cells were purchased from the Korean Cell Line Bank (21446, Korea). H9C2 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) at 37℃ in a 5% CO_2. H9C2 Cells were seeded in a 6 well plate incubated overnight. Next day, Cells were replaced by serum-free medium. Oxidative stress was induced by treating cells with 600 μM H_2O_2 (Sigma-Aldrich Co.) for 5 hours. And then, Cells were treated with 1% a*BCC* or 0.5 mM N-acetylcysteine (NAC) (Sigma-Aldrich Co.) under oxidative stress condition.

Cell viability assay

Cell viability assay was performed using the EZ-CYTOX kit (DoGenBio Co., LTD, Korea) according to the manufacturer's instructions.

Intracellular ROS assay

Intracellular ROS levels were measured in H9C2 cells using 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Abcam, MA, USA). H9C2 Cells were treated with H_2O_2 or a*BCC* alone, combination of H_2O_2 and a*BCC*. Untreated cells were used as control cells. For DCFH-DA staining, H9C2 cells were treated with 10 μM DCFH-DA solution for 45 minutes in dark. After 45 minutes of treatment, H9C2 cells were washed 2 times with 1X buffer. Fluorescent intensity was detected by fluorescent microscopy (Axiovert 200 M, Carl Zeiss, Germany).

Western blot analysis

Cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.5% Sodium deoxycholate; 0.1% SDS; 2 mM EDTA) supplemented a protease inhibitor cocktail and a phosphatase inhibitor cocktail (GenDEPOT, TX, USA). And the supernatants were harvested by centrifugation at 12,000 xg for 10 minuts at 4℃. Protein samples (20~50 μg) were resolved by SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., USA). Membranes were blocked with 5% skim milk diluted in TBS-T (Tris-Buffered Saline containing 0.1% tween-20) for 1 hour. After blocking, membranes were incubated with primary antibodies diluted in blocking buffer overnight at 4℃, followed by incubation with HRP-labeled secondary antibodies for 1 hour at room temperature. Immunoreactivity was detected by ECL Western Blotting Detection Reagent (Thermo Scientific™, IL, USA). β-actin was used as the internal control. The following antibodies were used. β-actin (Santa Cruz Biotechnology, CA, USA), pro- and cleaved caspase-9 (Cell Signaling Technology, Inc., Danvers, MA, USA), Bax (Cell Signaling Technology, Inc.), Bcl-xL (Cell Signaling Technology, Inc.).

Statistical analysis

Data are presented as mean ± standard deviations (SD). Significant differences between two groups were determined using Student's *t*-test. *P* values < 0.05 were considered significant. Statistical analyses were conducted by the SAS software package v.9.13 (SAS Inc., Cary, NC, USA).

RESULTS

Cell counts of *BCC*

After preparing *BCC*, the levels of platelets (PLTs) and white blood cells (WBCs) were analyzed. The cell count of whole blood (W.B.) and *BCC* was analyzed using a Mythic™22AL (Orphee S.A., Switzerland). Compared to W.B., the platelets count was measured to be 9 times higher in *BCC*, and the WBCs count was measured to be 4 times

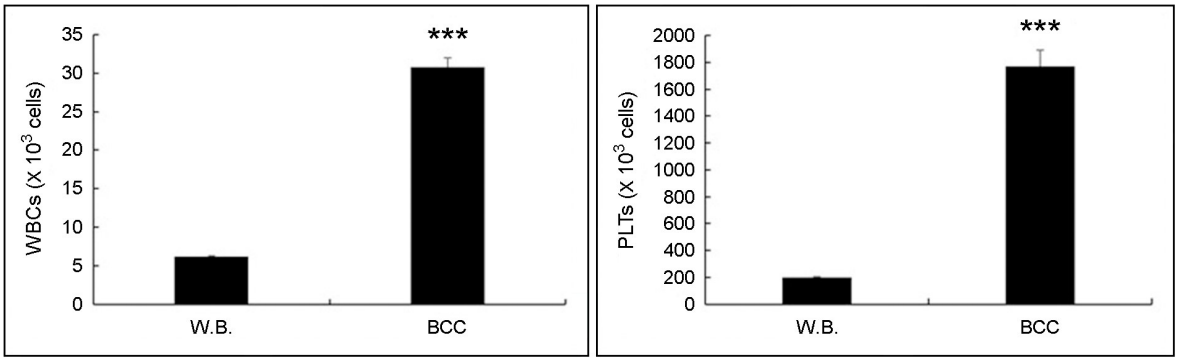

Fig. 2. Counts of white blood cells (WBCs) and platelets (PLTs) in *BCC*. After *BCC* extraction, the cell counts of W.B. and *BCC* were analyzed using a Mythic™22AL. Data are ± mean SD. ****P*<0.001 compared to W.B.

higher (Fig. 2). In the extracted *BCC*, the counts of WBCs and PLTs were significantly increased compared to W.B. And the red blood cells were depleted by more than 95%. We concluded that these blood cell counts were suitable for *BCC*. Activated *BCC* (a*BCC*) was obtained by treating this *BCC* with $CaCl_2$.

Effects of H_2O_2-induced oxidative stress in H9C2 cells

To confirm the effects of oxidative stress caused by ROS levels in the heart, H9C2 cells were treated with H_2O_2 at a concentration of 0 μM to 1,000 μM for 24 hours. As a result of cell viability analysis, dead cells increased in a dose-dependent manner of H_2O_2 (Fig. 3-A). And dead cells were significantly increased in 600 μM treated cells, which was confirmed to be an appropriate dose of H_2O_2. Therefore, oxidative stress was induced in H9C2 cells by treatment with 600 μM H_2O_2, and the effects of *BCC* were confirmed.

Protective effects of *BCC* on H_2O_2-induced oxidative stress in H9C2 cells

Protective effects of *BCC*: To evaluate the effect of *BCC* on ROS-induced oxidative stress, H9C2 cells were treated with 600 μM H_2O_2 for 5 hours in the presence or absence of a*BCC*. Similar to the results in Fig. 3-A, it was confirmed that cells treated with H_2O_2 received significantly more damage compared to the control group. It was confirmed that this cell damage was reduced by treating a*BCC* (Fig. 3-B). However, there was no significant difference depending on the dose of a*BCC* (1% or 5%). The results of the cell viability assay were similar to the microscope image (Fig. 3-C). Based on these results, *BCC* was evaluated to have a protective effect against cell damage caused by H_2O_2. Taken together, these data suggest that various growth factors, such as EGF, PDGF, VEGF, and TGF-β, secreted when platelets are activated, played an important role in this protective effect (Bader et al., 2020). Additionally, it is thought that CD34+ cells concentrated in *BCC* may have played an important role.

Protective effects of *BCC* on H_2O_2-induced oxidative stress in H9C2 cells

Attenuation of the cell damage via ROS scavenging: To

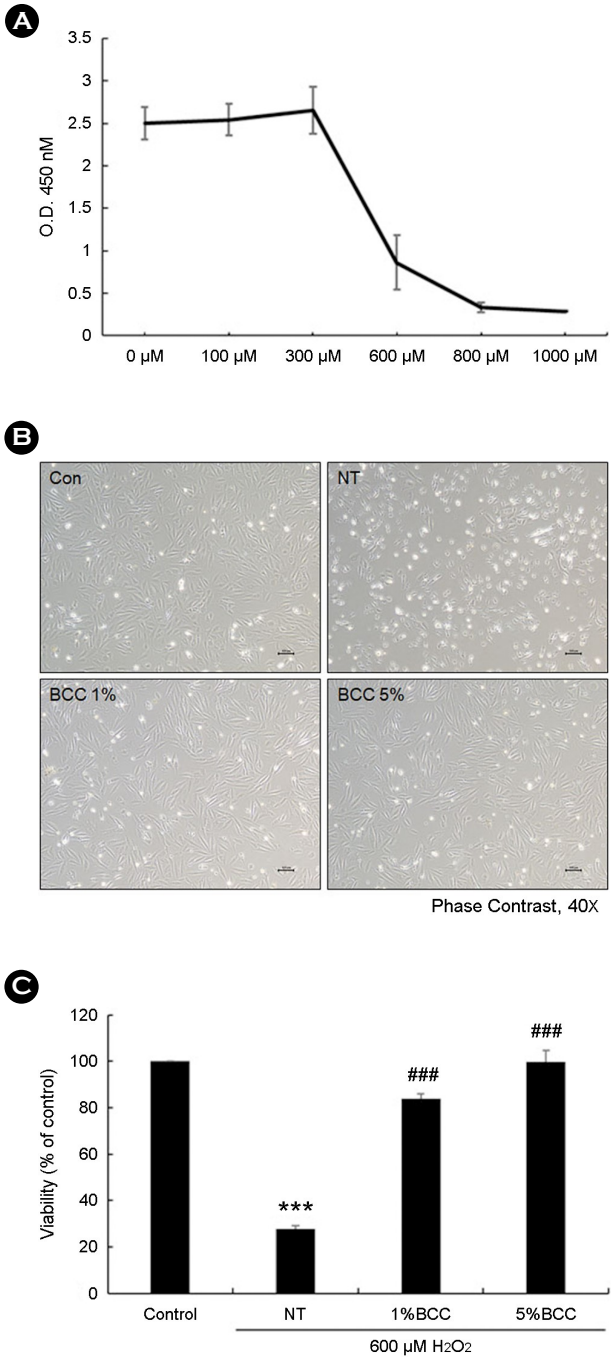

Fig. 3. Protective effects of *BCC* against H_2O_2-induced oxidative stress in H9C2 cells. (A) H9C2 cells were treated with 0~1,000 μM H_2O_2 for 24 hours. Cell viability was measured using the EZ-CYTOX kit. Absorbance was measured at 450 nm using microplate reader. Data are ± SD. (B) H9C2 Cells were treated with 600 μM H_2O_2 alone, combination of H_2O_2 and a*BCC* (1% or 5%) for 5 hours. Cells were observed with a phase contrast microscopy. (C) H9C2 Cells were treated with 600 μM H_2O_2 alone, combination of H_2O_2 and a*BCC* (1% or 5%) for 5 hours. Cell viability was measured using the EZ-CYTOX kit. Data are ± mean SD. $^{***}P<0.001$ compared to control. $^{###}P<0.001$ compared to NT. NT, 600 μM H_2O_2 alone.

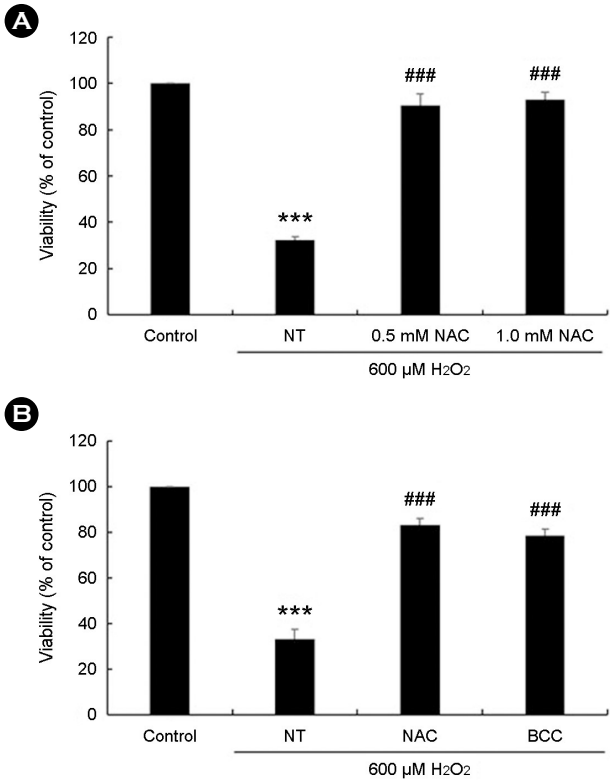

Fig. 4. Protective effects of NAC against H_2O_2-induced oxidative stress in H9C2 cells. (A) H9C2 Cells were treated with 600 μM H_2O_2 alone, combination of H_2O_2 and NAC (0.5 mM or 1 mM) for 5 hours. (B) H9C2 Cells were treated with 600 μM H_2O_2 alone, combination of H_2O_2 and 1% a*BCC* (or 0.5 mM NAC) for 5 hours. Cell viability was measured using the EZ-CYTOX kit. Data are ± mean SD. $^{***}P<0.001$ compared to control. $^{###}P<0.001$ compared to NT. NT, 600 μM H_2O_2 alone; NAC, N-acetylcysteine.

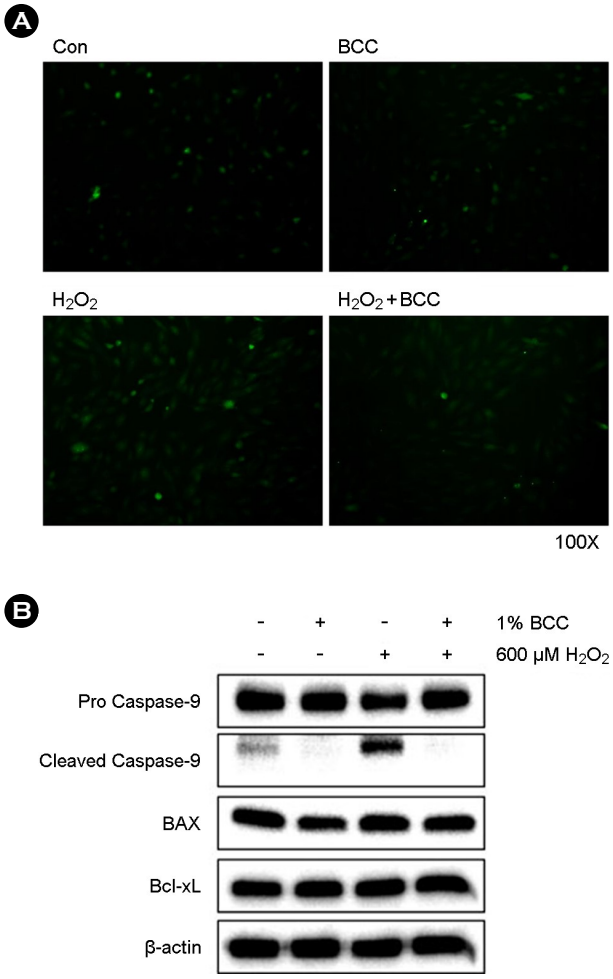

Fig. 5. Suppression of ROS levels and apoptosis of *BCC* in H_2O_2 treated H9C2 cells. (A) H9C2 Cells were treated with 600 μM H_2O_2 alone, combination of H_2O_2 and NAC (0.5 mM or 1 mM) for 5 hours. Intracellular ROS levels were measured using DCFH-DA method. DCF fluorescence was observed with the Zeiss Axiovert 200 M. (B) Expression levels of Pro-Casepase-9, cleaved Caspase-9, Bax, and Bcl-xL were detected by western blot. β-actin was used as a loading control. DCFH-DA, 2',7'-dichlorofluorescin diacetate.

determine the mechanism of the protective effect of *BCC*, it was compared with N-acetylcysteine (NAC). NAC is a ROS scavenger and can reduce intracellular ROS levels. H9C2 cells were treated with 600 μM H_2O_2 for 5 hours in the presence or absence of NAC (0.5 mM, 1 mM). In NAC-treated cells, cell viability was recovered to the level of H_2O_2-untreated cells (Fig. 4-A). There was no significant difference in NAC dose. Next, H9C2 cells were treated with 600 μM H_2O_2 for 5 hours in the presence or absence of a*BCC* (or NAC). a*BCC* and NAC recovered cell viability, and the difference was not significant (Fig. 4-B). Additionally, to verify intracellular ROS levels, DCFH-DA assay was performed. The green fluorescence that increased with H_2O_2 treatment in H9C2 cells was decreased by a*BCC* (Fig. 5-A). These results suggest that the growth factors secreted by platelets reduce ROS levels, resulting in a protective effect against H_2O_2-induced oxidative stress.

Protective effects of *BCC* on H_2O_2-induced oxidative stress in H9C2 cells

Attenuation of the cell damage via suppressing apoptosis: The protective effects of *BCC* against H_2O_2-induced oxidative stress in H9C2 cells were verified for their relationship with apoptosis. To verify the expression level of apoptotic proteins, western blot analysis was performed. H9C2 cells

were treated with H_2O_2 in the presence or absence of a*BCC*, and western blot analysis was performed. The expression levels of cleaved caspase-9 and Bax were increased by H_2O_2 treatment and decreased by the addition of a*BCC* (Fig. 5-B). These results suggest that the protective effects of *BCC* against H_2O_2-induced oxidative stress in H9C2 cells are induced by suppressing apoptosis.

DISCUSSION

High ROS levels in the human body cause oxidative stress and mediate various diseases (Singh et al., 2019; Adeoye et al., 2018). Oxidative stress in the heart causes diseases such as heart failure, cardiomyopathy, myocardial infarction, etc. To treat these heart diseases, pharmacological and surgical treatments are being implemented. Recently, stem cell therapy has also been implemented (Muller et al., 2018). *BCC* therapy still remains controversial. However, *BCC* can be easily obtained using autologous blood, and research data on the effectiveness and safety of treatment is accumulating. Recently, *BCC* therapy has been used to treat various diseases such as osteoarthritis, erectile dysfunction, androgenic alopecia, etc (Burchard et al., 2019; Poulios et al., 2021; Gkini et al., 2014).

In this study, to determine the effects of ROS on heart diseases, H9C2 cells were treated with H_2O_2 and mimicked *in vitro*. After H_2O_2 treatment of H9C2 cells, it was confirmed that H9C2 cells were damaged and the number of dead cells increased. This cell damage was suppressed by the addition of a*BCC*. When H9C2 cells is treated with a*BCC*, there is a possibility of protective effects by reducing intracellular ROS levels (Bader et al., 2020). DCFH-DA assay was performed. The results confirmed that the protective effects of *BCC* were due to decreased intracellular ROS levels. Additionally, H_2O_2-induced oxidative stress was confirmed to cause cell damage, and eventually cell death occurred. And *BCC* suppressed cell damage and cell death. In this regard, we performed apoptosis pathway analysis. In the presence of a*BCC*, the expression levels of cleaved caspase-9 and Bax decreased. It was also confirmed that the protective effects of *BCC* against H_2O_2-induced oxidative stress were regulated through the apoptosis pathway.

As an *in vitro* model of heart disease, it was experimentally proven that *BCC* can play a protective role against ROS-induced oxidative stress. We observed that *BCC* plays a protective role through regulation of intracellular ROS levels and Apoptosis pathway. The findings of our study show that *BCC* therapy may be a new treatment option for heart diseases. For *BCC* therapy to become a treatment option for heart disease, further research on more specific mechanisms will be needed. And reports of clinical research will need to be accumulated. Currently, there is no standard protocol for the preparation of *BCC*. Further studies should expand understanding of the standard protocol for *BCC* preparation.

Recently, *BCC* therapy is used in various treatment areas such as orthopedics, gynecology, and urology. In this study, it was determined that H_2O_2-induced oxidative stress was suppressed by *BCC* in H9C2 cells. *BCC* reduced intracellular ROS levels and blocked the apoptosis pathway. Taken together, we suggest that *BCC* therapy can effectively treat heart diseases.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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