Original Research Article

Effect of polysaccharide from the root of *Bupleurum Chinese* DC and *Bupleurum scorzonerifolium* Willd on hydrogen peroxide-induced myocardial apoptosis

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Abstract

Purpose: To investigate the protective effect of polysaccharide (BRP) from the root of Bupleurum Chinese DC. and Bupleurum scorzonerifolium Willd. on cardiomyocyte cells.

Methods: Response surface methodology (RSM) based on Box-Behnken Design (BBD) was performed to optimize the extraction conditions for BRP. The effect of BRP on cardiomyocyte cell apoptosis was evaluated in H9c2 cells treated with hydrogen peroxide (H_2O_2). Cell viability was determined by CCK-8 assay, while oxidative stress levels in H9c2 cells, including lactate dehydrogenase (LDH), superoxide dismutase (SOD), catalase (CAT) and creatine kinase (CK) were determined using commercial kits following the manufacture's instruction. mRNA expressions (caspase-3, caspase-8, caspase-9 and Fas) were determined by quantitative real time-polymerase chain reaction (RT-qPCR).

Results: The obtained optimal extraction conditions for BRP was as follows: extraction time (1.43 h), ratio of water to the raw material (30 mL/g) and extraction times (2 times). BRP (200, 400, 600 and 800 µg/mL) significantly increased the cell viability of H_2O_2 induced H9c2 cells ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.01$, respectively). BRP (200, 400 and 800 µg/mL) significantly decreased LDH and CK levels ($p < 0.01$, $p < 0.01$, $p < 0.01$, respectively). However, BRP increased levels of SOD (200, 400 and 800 µg/mL, $p < 0.05$) and CAT (400 and 800 µg/mL, $p < 0.05$) in H9c2 cells. BRP significantly down-regulated mRNA expressions of Caspase-3, Caspase-8, Caspase-9 and Fas (200, 400 and 800 µg/mL, $p < 0.01$) in H9c2 cells induced by H2O2.

Conclusion: BRP protects cardiomyocyte against apoptosis via inhibition of oxidative stress and mitochondria-mediated intrinsic apoptosis, and thus, may be potential therapeutic agent for the management of cardiovascular diseases.

Keywords: Bupleurum Chinese, Bupleurum scorzonerifolium Willd., Polysaccharide, Cardiomyocyte, Apoptosis, H9c2 cell, Biochemical parameters

INTRODUCTION

Incidence of cardiovascular disease is increasing every year, and myocardial infarction has become a leading cause of death worldwide [1]. Several studies have shown that oxidative stress, caused by several pathological factors, is the common mechanism underlying cardiovascular injury [2].

Bupleuri radix (BR) is the root of *Bupleurum Chinese* DC and *Bupleurum scorzonerifolium* Willd, and is used commonly in traditional Chinese medicine for the treatment of cold and fever, chest and hypochondriac pain and irregular menstruation [3]. Modern Pharmacology has demonstrated that BR possess antipyretic, analgesic, anti-inflammatory, anti-bacterial, enhancing immunity, anti-depression and anti-cancer effects and others [4]. Phytochemical studies have revealed the presence of saponins, volatile oils, polysaccharides, flavonoids, and sterols in BR [5].

It is well-known that polysaccharides are important biological macromolecules isolated from natural products [6]. polysaccharides derived from naturally occurring medicinal plants have been evaluated as drug candidates for treating various diseases due to their wide-spectrum of therapeutic activities, relative low toxicities, and minor side-effects [7,8]. However, no reports exist on the protective effect of *Bupleuri Radix* polysaccharide (BRP) on cardiomyocyte cells and its possible mechanisms of action on myocardial cell preservation. Therefore, we aimed to evaluate the protective effect of BRP on the H_2O_2-induced H9c2 cell model.

EXPERIMENTAL

Cells culture

The cardiomyoblast H9c2 cell line was obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in 90 % Dulbecco's modified Eagle's medium (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS) and antibiotics (1 % penicillin and streptomycin). The cells were kept in a 37°C humidified incubator with 5 % CO_2 and 95% air.

Chemicals

CCK-8, H_2O_2 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The DMEM media and FBS were obtained from Gibco Co. (Grand Island, NY, USA). The assay kits for lactate dehydrogenase (LDH), superoxide dismutase (SOD), creatine kinase (CK) and catalase (CAT) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RNA Trizol Reagent was purchased from Servicebio Company (Wuhan, China). The RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher (MO, USA). All reagents and chemicals used in this study were of analytical grade.

Preparation of BRP

BRP was extracted as described earlier [8]. The dried *Bupleuri Radix* (BR) was processed to powder and exhaustively extracted with 95% ethanol for 12 h to remove the colored materials, mono-saccharides, oligosaccharides and small molecule materials. After filtration, the residue was air dried and extracted with distilled water by reflux. The extract was left to cool at room temperature, filtered, and concentrated until it was reduced to one-tenth of the original volume. Then, ethanol was added slowly to reach a final concentration of 80%. After leaving overnight at 4 °C, the precipitate was collected by filtration. Then, it was washed thrice with pure ethanol and acetone and dried at 50 °C to obtain the crude BRP.

Determination of BRP yield

BRP content was determined by the phenol-sulfuric method using D-glucose as standard reference. The percentage of polysaccharide yield (%) was calculated as shown in Eq 1.

Yield (%) = W_1/W_0 .... (1)

where W_1 was the content of crude polysaccharides and W_0 was the weight of dried *Bupleuri radix* used.

Experimental design and statistical analysis

Based on previous relevant single factor experimental results, three significant factors influencing BRP extraction, which include extraction time (A), water to the raw material ratio (B), and extraction times (C) were confirmed. To obtain an optimal extraction of BRP, response surface methodology (RSM) based on Box-Behnken Design (BBD) was carried out in the present study. The three factors were designated as A (1, 1.5 and 2 h), B (20, 30 and 40 mL/g), C (1, 2 and 3n), and arranged into three levels (-1, 0 and 1). As shown in Table 1, seventeen experiments based on BBD with three centre points were performed in random order.

All tests were repeated three times, and the one-way analysis of variance (ANOVA) was used to analyse the BBD results. Design Expert (version 8.0.6, Stat-Ease, Inc., Minneapolis, MN, USA) software was used to estimate the response of independent variables. Response surfaces were drawn to determine the individual and interactive effects of the test variable on the response. Additional confirmatory experiments were

conducted subsequently to verify the validity of the statistical experimental design.

CCK-8 assay

Cytotoxicity was evaluated using the CCK-8 assay. A 100 µL cell suspension (5 x 10^5 cells/ml) was seeded in a 96-well plate and incubated in an atmosphere of 5% CO_2/95% air at 37 °C for 24 h. BRP at varying concentrations (50, 100, 200, 400, and 800 µg/mL) was added to the cells and incubated for 24 h at 37 °C. The control cells were treated with 10 µL of DMEM for 24 h at 37 °C. The cell viability (n = 4) in the CCK-8 assay was determined by measuring the absorbance (optical density, OD) at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Determination of LDH, CK, SOD and CAT in H9c2 cells

Cells were inoculated into the 24-well plates for 24 h. BRP at varying concentrations (200, 400 and 800 µg/mL) was added to the cells and incubated for 24 h at 37 °C. Next, H_2O_2 at the final concentration of 100µmol/L was added and cultured for 4 h. After that, H9c2 cell supernatants were collected, and the LDH, CK, SOD and CAT levels measured using the commercial kits following the manufacturer's instructions.

Quantitative real time-polymerase chain reaction (RT-qPCR)

H9c2 cell total RNA was extracted following the manufacturer's protocols, and the purity and concentration were determined by measuring the absorbance at 260 and 280 nm. Next, 2 µg of RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA synthesis kit. RT-qPCR was performed using an ABI StepOnePlus system (Applied Biosystems, CA, USA). Reaction conditions for the RT-qPCR were as follows: 95 °C for 30 s, followed by cycles of 95 °C for 5 s, 55°C for 30 s, and 72 °C for 30 s. And sequence of primers was showed in Table 2. The caspase-3, caspase-8, caspase-9, and Fas gene expression levels were normalised to β-actin and analysed using the $2^{-\triangle\triangle CT}$ method.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and group comparison was performed by ANOVA (SPSS 16.0, SPSS Inc., USA). $P < 0.05$ was considered statistically significant.

Table 1: BBD experimental design with independent variables

Run	A	B	C	Yield (%)
1	0.00	1.00	-1.00	11.18
2	0.00	0.00	0.00	12.22
3	0.00	-1.00	1.00	11.06
4	-1.00	-1.00	0.00	11.51
5	1.00	0.00	1.00	10.46
6	0.00	-1.00	-1.00	11.43
7	0.00	0.00	0.00	12.29
8	1.00	0.00	-1.00	11.53
9	0.00	0.00	0.00	12.37
10	-1.00	0.00	-1.00	11.48
11	1.00	-1.00	0.00	11.29
12	1.00	1.00	0.00	11.17
13	0.00	0.00	0.00	12.28
14	0.00	0.00	0.00	12.11
15	-1.00	1.00	0.00	11.46
16	0.00	1.00	1.00	10.59
17	-1.00	0.00	1.00	11.25

BBD = Box-Behnken Design

Table 2: Sequence of primers

| Gene name	Forward primer	Reverse primer
β-actin	5'-TTCCTACCCCCAATGTATCCG-3'	5'-CCACCCTGTTGCTGTAGCCATA-3'
caspase-3	5'- ACTGGAAAGCCGAAACTCTTCATCA -3'	5'- GGAAGTCGGCCTCCACTGGTATC -3'
caspase-9	5'- AGCCAGATGCTGTCCCATACCA-3'	5'- GGGAAGGTGGAGTAGGACACAAGG -3'
caspase-8	5'-GATGAGGCAGACTTTCTGCT-3'	5'-GATGAGGCAGACTTTCTGCT-3'
Fas	5'-GTTGGAAAGAACCGAAGGACAA-3'	5'-CCTCAAAGTAGGCACAGGATGT-3'

RESULTS

Response surface analysis

Using multiple regression analysis of the experimental data, the predicted response Y for BRP (%) extraction yield was obtained by the following second-order polynomial equation: Yield = 12.25 - 0.16 A - 0.11 B - 0.28 C - 0.018 AB - 0.21 AC - 0.055 BC - 0.39 A^2 - 0.51 B^2 - 0.68 C^2. The F-test was used to test the statistical significance of the regression equation, and the effects of independent variables on the extraction yield were checked for adequacy and fitness by ANOVA.

The results from response surface analysis are shown in Table 3 and Figure 1. The determination coefficient (R^2) confirmed that the fitted model could explain 98.34% of the variations. The adjusted determination coefficient (R^2adj = 0.9620) also indicated that the model was highly significant. In addition, it also showed a high degree of correlation between the observed and predicted values. A relatively low value of coefficient of variation (CV = 2.71 %) indicated a high degree of reliability and precision of the experimental values. The experiment had a very low P-value ($P < 0.0001$) and a very high F-value (F = 42.13), suggesting that the proposed model was highly significant. In addition, F-value (2.10) and P-value (0.2425) lack-of-fit, showed no significance relative to the pure error, indicating that the model equation was adequate for predicting the BRP yield under any combination of variable values.

The 2D contour plots and 3D response surface are presented in Figure 1 and show the effects of independent variables and their mutual interaction on the BRP yield. The optimum conditions for the BRP yield were: an extraction temperature of 86.57°C, an extraction time of 2.23 h, and water to the raw material ratio of 10.77 mL/g. Therefore, the theoretically highest yield of BRP was predicted as 6.10 % by the developed model. Verification experiments were carried out using the modified conditions, with an extraction temperature of 86.6°C, an extraction time of 2.23 h, and water to the raw material ratio of 10.8 mL/g with three replicates. The average BRP yield from the actual experiments was 6.08%, thus demonstrating the RSM model validity.
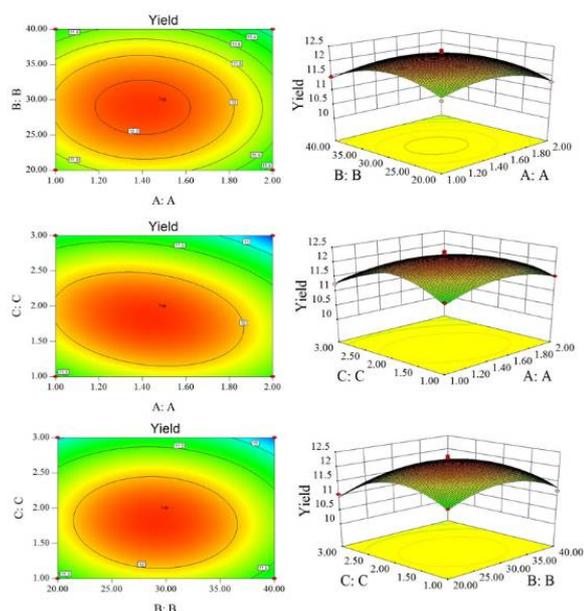


Figure 1: Results from response surface studies

Table 3: Analysis of variance (ANOVA) of experimental results from BBD

Source	Sum of squares	df	Mean square	F -value	*P*-value
Model	5.21	9	0.58	42.13	< 0.0001
A	0.20	1	0.20	14.21	0.0070
B	0.099	1	0.099	7.21	0.0313
C	0.64	1	0.64	46.46	0.0002
AB	1.225^{E-003}	1	1.225^{E-003}	0.089	0.7739
AC	0.18	1	0.18	12.84	0.0089
BC	0.012	1	0.012	0.88	0.3793
A^2	0.64	1	0.64	46.78	0.0002
B^2	1.08	1	1.08	78.37	< 0.0001
C^2	1.97	1	1.97	143.03	< 0.0001
Residual	0.096	7	0.014		
Lack of Fit	0.059	3	0.020	2.10	0.2425
Pure Error	0.037	4	9.330E-003		
Cor Total	5.31	16			

| Standard Deviation	Mean	C.V.%	Press	R^2	R^2 Adj	R^2 Pred	Adequate precision
0.17	6.43	2.71	2.52	0.9834	0.9620	0.8030	19.187

BBD = Box-Behnken Design.

CCK-8

The results of the CCK-8 assay are shown in Figure 2. H9c2 cell viability decreased sharply after H_2O_2 induction (100 µmo1/L) for 4 h, compared to normal H9c2 cells ($p < 0.01$). However, the H_2O_2-induced H9c2 cell viability increased significantly after BRP treatment (200, 400, and 800 µg/mL) when compared to the H9c2 cells induced by H_2O_2 ($p < 0.05$, $p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively). These results demonstrated that BRP could enhance the viability of H_2O_2 stimulated cardiomyocyte.
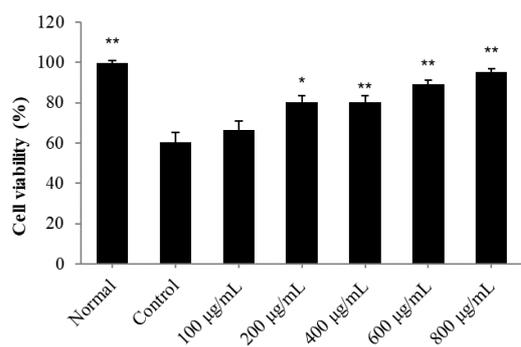
Figure 2: Effects of BRP on the cell viability rate of H_2O_2 stimulated H9c2 cells. Data are expressed as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

LDH and CK contents

As shown in Figure 3, it could be seen that the LDH and CK contents in H9c2 cells induced by H_2O_2 increased ($p < 0.01$), compared to the normal cells. After treating with BRP (400 and 800 µg/mL), LDH and CK levels significantly decreased ($p < 0.01$ and $p < 0.01$, respectively), when compared to the control group. Above results indicated that BRP could reduce the levels of LDH and CK from the cytoplasm of cells induced with H_2O_2.
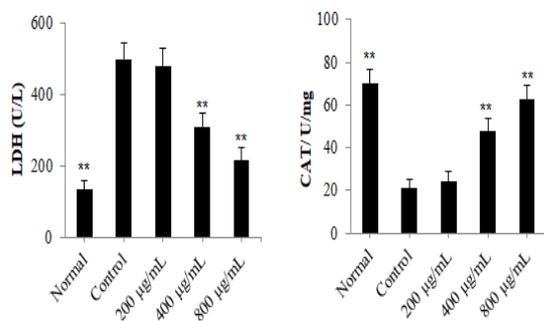
Figure 3: Effects of BRP on LDH and CK contents in H_2O_2-stimulated H9c2 cells. Data are expressed as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

SOD and CAT levels

Effects of BRP on the SOD and CAT levels in H9c2 cells induced with H_2O_2 are presented in Figure 4. After H_2O_2 stimulation, levels of SOD and CAT in H9c2 cells significantly decreased ($p < 0.01$) compared with normal H9c2 cells. And the SOD ($p < 0.01$ and $p < 0.01$) and CAT ($p < 0.01$ and $p < 0.01$) levels in H9c2 cells treated with BRP (400 and 800 µg/mL) were significantly increased, compared to the control group.
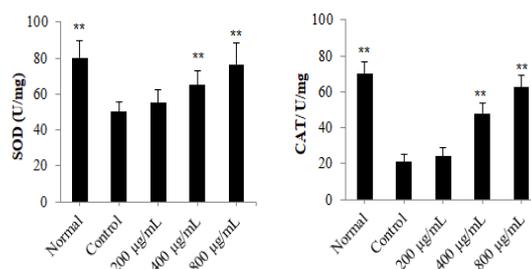
Figure 4: Effect of BRP on SOD and CAT contents in H_2O_2 stimulated H9c2 cells. Data were expressed as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

RT-qPCR results

As shown in Figure 5, the mRNA expressions of caspase-3, caspase-8, caspase-9, and Fas were up-regulated ($p < 0.01$) in the model cells. Compared to the model cells, the caspase-3, caspase-8, caspase-9, and Fas mRNA expressions decreased significantly in all BRP-treated (200, 400, and 800 µg/mL) cells.
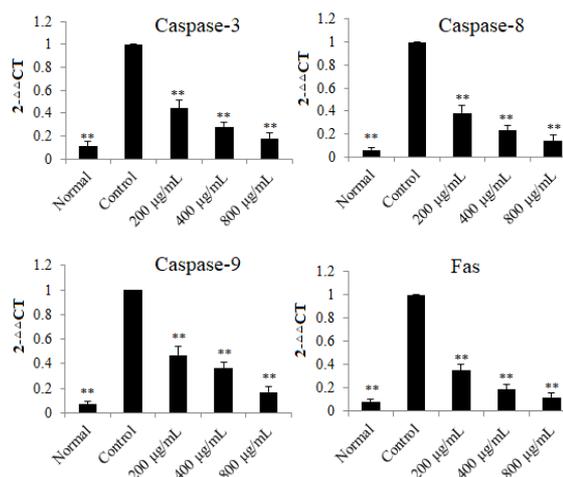
Figure 5: Effect of BRP on expressions of caspase-3, caspase-8, caspase-9 and Fas in H_2O_2 stimulated H9c2 cells. Data were expressed as mean ± SD (n = 6); **$p < 0.01$, *vs* control group

DISCUSSION

Myocardial infarction could lead to myocardial ischemia and irreversible myocardial necrosis if not perfused timely with blood [1,9]. Myocardial ischemia is reported to occur due to an imbalance in the heart's oxygen supply and demand [10,11]. H_2O_2 may induce ROS generation in the mitochondria [11]. The myocardial apoptosis model of H_2O_2-induced H9c2 cells is used routinely to study the underlying pathological processes and molecular mechanisms of myocardial ischemic injury [11,12]. The results of the present investigation showed that BRP could alleviate H_2O_2-induced myocardial apoptosis. In addition, BRP could also decrease CK and LDH levels in the cytoplasm.

CAT and SOD are critical anti-oxidative enzymes and generally regarded as the antioxidant defence system of the body that play a crucial role in suppressing oxidative stress [13]. The present results show that BRP increased levels of CAT and SOD, and inhibited the oxidative stress level in H_2O_2-induced H9c2 cells. Caspase proteins, a group of aspartate specific cysteine proteases, play critical roles in regulating apoptosis induced by a variety of stimuli, such as oxidative stress, and caspase-3 is the most important effector in the apoptotic process [14]. Caspase-9 is the initial caspase in the caspase cascade reaction [15]. The combination of the death receptor Fas with the FasL ligand, initiates signal transduction and activation of caspase-8 and caspase-3 [16]. In the present study, we found that BRP down-regulated the mRNA expressions of caspase-3, caspase-8, caspase-9, and Fas, which demonstrate the protective effect of BRP on myocardial cell apoptosis.

CONCLUSION

The findings of the present investigation show that the polysaccharide from *Bupleuri radix* (BRP) alleviates myocardial apoptosis in H9c2 cell induced with H_2O_2. The possible mechanism of action might be related to inhibition of oxidative stress and apoptosis. These findings may be useful in the development of novel medicine for the treatment of cardiovascular disease.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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