Background: *Agaricus blazei* Murill (ABM) is a widely accepted health food and is known to present anti-tumorigenic activities. ABM has also been reported to modulate oxidative stress after inflammation and infection. Reactive oxygen free radicals have been shown to be critically involved in ischemia-reperfusion injury cascades. We aimed to study the antioxidant activities of ABM against tissue injury caused by myocardial ischemia-reperfusion processes.

Methods: A rat cardiomyocyte H9c2 cell line was set up and used for the study. Cell viabilities after different dosages of ABM extract treatment and hydrogen peroxide injuries were checked. A cardiac ischemia-reperfusion injury model was set up to elucidate the cardioprotective roles of ABM extract. Different doses of 22.5 mg/kg of body weight and 45 mg/kg of body weight ABM extract were pre-treated at 24 and 48 hours before inducing the ischemia-reperfusion injury.

Results: ABM extract treatment increased the survival of cardiomyocytes without cytotoxicity. Pretreatment with ABM extract reduced hydrogen peroxide-induced cell damage and increased cell survival (0.86 ± 0.15 in ABM 6.0 mg/ml vs. 0.45 ± 0.03 in control, p = 0.019). ABM-pretreated rats that underwent myocardial ischemia-reperfusion had greatly reduced infarct areas compared to those in the control group (14.3 ± 3.3% in control group, 4.6 ± 1.9% in 22.5 mg/kg group, 4.9 ± 2.1% in 45 mg/kg group, p < 0.05).

Conclusion: ABM increases antioxidant activity, which greatly ameliorates myocardial injuries caused by myocardial ischemia-reperfusion injuries. Using ABM as a health food may provide cardioprotective effect against ischemia-reperfusion injuries.

Key Words: *Agaricus blazei* Murill • Antioxidant • Ischemia-reperfusion • Myocardial infarction

INTRODUCTION

*Agaricus blazei* Murill (ABM), an edible Basidiomycota fungus found primarily in South America, is well known for its medicinal properties in diabetes, osteoporosis, and cancer.1,2 The ABM mushroom is anticas- togenic in mice with tumor xenografts,3,4 which indicates that ABM is tumoricidal. ABM also likely plays a role in preventing tumor initiation by reducing genotoxicities caused by DNA-damaging agents.5 ABM has also been shown to enhance innate as well as adaptive immune responses in mice.6,7 It stimulates the production of interleukin and cytokines, which increase host immunities against bacterial and viral infections.8-10 It also induces interleukin-12- and interferon-γ-mediated natural killer cell activation.11 These findings invariably suggest that ABM is a promising “health food” that protects hosts from infections and cancers. One recent study12 reported that ABM prevented diethylnitrosamine,
a strong hepatic cytotoxin and genotoxin, from causing liver cell injury; this indicated that ABM helps maintain tissue integrity. The dehydrated ABM fructification body is rich in proteins and carbohydrates. The polysaccharide component, β-glucans, is key to the medicinal effects of ABM.1 The β-glucans extracted from ABM modulated immune responses by stimulating the release of cytokines, which comprise ABM’s immune regulatory effects.13

Two parallel but somewhat contradictory immune regulatory effects of ABM have been reported. One study7 reported that, in macrophages, ABM stimulated nitric oxide production and increased inflammation, which creates cellular oxidative stress; a second study,14 in liver and lung cells, reported that ABM reduced the production of reactive oxygen species (ROS) and maintained DNA and cellular integrity. The molecular mechanisms to reconcile these two distinct phenomena have not yet been identified. However, ABM may affect oxidative responses in different tissue-types in distinct ways.

In the present study, we wanted to know whether ABM is an antioxidant in the cardiac system and whether it protects cardiomyocytes from permanent damage caused by acute myocardial infarction, which usually occurs suddenly and causes extremely high mortality.15,16 Early reperfusion of an acutely occluded coronary artery improves outcomes in patients with acute myocardial infarction. Although reperfusion therapy ameliorates overall myocardial injury, reperfusion injury reduces its therapeutic benefits.17 Ischemia-reperfusion injury causes myocardial damage and leads to cardiac failure and mortality. Reactive oxygen free radicals are critically involved in ischemia-reperfusion injury cascades.18 Suppressing the generation of ROS can improve cardiac function after myocardial infarction.19-21 Given the antioxidant properties of ABM, we also wanted to know whether ABM protects cardiomyocytes from the oxidative stress caused by reperfusion therapy.

MATERIALS AND METHODS

Cell lines and the ABM mushroom

We used rat cardiomyocyte H9c2 cells for this study. The cells were maintained in regular Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 × non-essential amino acid, and 1 × antibiotic/antimycotic mixture (Gibco BRL, Grand Island, NY). We grew the cells at 37 °C in a 5% CO2 atmosphere.

The dried ABM powder was offered by Simpson Biotechnologies, Inc. (Taoyuan, Taiwan). We mixed 2.5 grams of ABM powder with 100 mL of phosphate buffered saline (PBS) [pH 7.4] and shook it at room temperature for 1 h. The ABM solution was filtered through Whatman No. 1 filter paper. The filtrate, which comprised the water-soluble components of ABM, was sterilized by passing it through a 0.22-µm-syringe filter. It was then stored at -20 °C until use.

MTT assay

A 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) cell proliferation assay was used to detect cell viability by evaluating the mitochondrial reductase activity after treatments with ABM extract and hydrogen peroxide. The protocols basically followed those previously described.22 Briefly, the cells that were grown to 5 × 10⁵/well in a 24-well culture plate were treated with various doses of ABM extract for 24 h at 37 °C and then treated with 100 µM hydrogen peroxide for 24 h or else were mock-treated. After the treatments, 250 µL of cell medium was sucked from each well and then mixed with 0.5 mg/mL of MTT labeling reagent (Sigma-Aldrich Chemical Co., St Louis, MO) at 37 °C for 4 h, and then with 10% sodium dodecylsulfate (SDS)/0.01 M HCl solubilization solution overnight. On the next day, the optical density (OD)₅₉₀ nm was measured. The cell survival percentage was calculated as the ratios of (OD)₅₉₀ nm in each ABM-treated experiment to that in the mock-treated experiment (n = 6 for each study).

Intracellular reactive oxygen species (ROS) measurement

Intracellular ROS level was studied by measuring the changes in fluorescence resulting from intracellular probe oxidation. The probe 2',7'-dichlorofluorescin diacetate (DCFH-DA, 5.0 mM, Sigma, St. Louis, MO, U.S.A.) entered the cell and the acetate group on DCFH-DA was cleaved by cellular esterases. Subsequent oxidation by ROS, particularly hydroxyl radical,
yielded the fluorescent product DCF. ABM extract-treated and controlled H9c2 cells \((1 \times 10^6)\) were loaded with 10 ml DCFH-DA at 4 hours after treatment with \(\text{H}_2\text{O}_2\), and analyzed by FACSscan (Becton Dickinson Heidelberg, Germany).

**Cell damage assays by flow cytometry analysis**

Damaged cells were detected by propidium iodide (PI, 50 mg/ml) after treatment with \(\text{H}_2\text{O}_2\) for 24 h as described previously. Labeling was performed with the staining analyzed on a FACSscan (Becton Dickinson Heidelberg, Germany). When nuclear membrane disrupted, which is a sign of advanced cell damaged, the PI went into the nuclei and showed the positive staining.

**Treating rats with ABM**

Eighteen 8-week-old Wistar rats weighing approximately 350 g were divided into three groups: a control group \((n = 6)\) and two groups treated with ABM 22.5 mg/kg of body weight \((n = 6)\) and ABM 45 mg/kg of body weight \((n = 6)\) group. The ABM was administered by using orogastric tubes at 24th hour and 48th hour before ischemia-reperfusion injuries were induced. The control group received the equivalent amount of water at the same time points as the treatment groups did.

**Cardiac ischemia-reperfusion animal model**

After we had pretreated the rats with ABM, we induced myocardial ischemia by completely ligating the marginal coronary artery, using protocols described elsewhere. Briefly, the rats were intravenously anesthetized during the experiment by using 30 mg/kg pentobarbital. An intratracheal tube was inserted, and all rats were given intermittent positive-pressure ventilation using a small animal respirator (Harvard Apparatus, Inc., Holliston, MA) adjusted to maintain arterial blood gases within the physiological range. Each rat was given a midline sternotomy, after which the pericardium was incised and the heart exposed. A 5-0 silk suture on a small curved needle was passed through the myocardium beneath the major marginal branch (2 mm distal to its origin) of the left anterior descending coronary artery on the dorsal surface of the heart. A reversible tie was subsequently made and loosely placed on the myocardial surface. After a 20-min post-thoracotomy stabilization period, myocardial ischemia reperfusion was initiated by completely ligating the marginal coronary artery for 30 min. After 30 min of ischemia, the surgical tie was released and the ischemic myocardium was reperfused for 2.5 h. Myocardial ischemia was confirmed visually by regional cyanosis of the myocardial surface.

The infarct size was determined as previously described using 1% triphenyl-tetrazolium chloride (Sigma-Aldrich, U.S.) in phosphate buffer [pH 7.4]. The left ventricular area at risk and the area of infarcted tissue were measured by an independent, blinded observer using computer planimetry.

**Statistical analysis**

Data are presented as mean ± standard deviation. ANOVA test with post-hoc analysis by Bonferroni method was used to evaluate statistical significance of differences among different groups. A value of \(p < 0.05\) was considered statistically significant.

**RESULTS**

**ABM increased cell proliferation and was not cytotoxic**

In the H9c2 cell line treated with ABM extract only without hydrogen peroxide, the cell numbers were significantly higher in the ABM-treated cells than in the controls \((1.47 \text{ fold in ABM 6.0 mg/ml compared to control, } p = 0.047, \text{ Figure 1})\), which suggested that ABM raised cell proliferation rates and were not cytotoxic.

**Antioxidant activity in ABM**

The H9c2 cells pretreated with ABM extract were more resistant to hydrogen peroxide \((100 \mu\text{M})\) than those not pretreated with ABM by MTT assays. The cell survival rate was significantly higher in the ABM extract-pretreated cells against hydrogen peroxide damage \((0.86 \pm 0.15 \text{ in ABM 6.0 mg/ml compared to 0.45 \pm 0.03 in control, } p = 0.019)\). Under treatment of 4 mg/ml ABM extract, the cell survival was also improved compared to the control group \((0.87 \pm 0.18 \text{ in ABM 4.0 mg/ml compared to 0.45 \pm 0.03 in control, } p = 0.045)\). The survival rate was improved under the treatment of 2 mg/ml ABM extract, however, it did not reach statistical significance. The cell survival rates were highly correlated to ABM dosages (Figure 2), suggesting that ABM contributes to
antioxidant activities, which reduced the hydrogen peroxide-induced oxidative stress.

The anti-oxidant activity of decreasing intracellular ROS by ABM extract was checked. The level of ROS increased after H2O2 treatment (0.41 ± 0.33 vs. 1.00 ± 0.01, p < 0.05 by fold change, Figure 3). The ABM extract treatment with concentration of 6 mg/ml attenuated the ROS intensity significantly (0.82 ± 0.14 vs. 1.00 ± 0.01, p < 0.05). There were mild decreases of ROS intensity under 2 mg/ml and 4 mg/ml, however, the differences were not statistically significant.

By using P.I. staining to detect the cell damage, we found the ABM extract pre-treatment ameliorated the H9c2 damage against the oxidative injuries by hydrogen peroxide at different concentrations (Figure 4). The number of P.I. positive-stained nuclei was significantly reduced under ABM extract pretreatment (0.31 ± 0.18 in ABM 3.0 mg/ml compared to 0.40 ± 0.19 in control, p = 0.049).

**ABM prevented myocardial damage caused by ischemia-reperfusion injuries**

We tested the protective effects of ABM against myocardial injuries caused by ischemia-reperfusion ther-
apy used after acute myocardial infarction. We found that rats pretreated with ABM (both 22.5 mg/kg and 45 mg/kg doses) had significantly smaller infarct areas compared to the control group (14.3 ± 3.3% in control group, 4.6 ± 1.9% in 22.5 mg/kg group, 4.9 ± 2.1% in 45 mg/kg group, p < 0.05 by ANOVA test, Figures 5A and B). These findings indicated that the ABM pretreatment ameliorated the myocardial injury by reducing the infarct area after myocardial infarction.

**DISCUSSION**

ABM has cardioprotective effects against oxidative stress injuries, as shown in this study. Pretreatment of ABM can ameliorate hydrogen peroxide injuries, which is a strong donor of oxidative stress, to cardiomyocytes. The cell damage as shown by the decrease of mitochondrial reductase activity and increase of nuclear staining by PI was less under ABM pretreatment in cardiomyocytes. ABM pretreatment as a nutrient in the food could decrease the myocardial infarct size in experimental animals, implying the ABM could be helpful to prevent the myocardial damage in acute cardiac event.

Oxidative stress plays an important role in cardiac ischemia-reperfusion injuries. The excessive oxygen molecules go into the cardiomyocytes during the reperfusion stage. These oxygen molecules will compromise the functions of complex I and complex III of electron transport chains in the mitochondria, then superoxides are generated excessively. The superoxides bind to the various protein and damage their functions. In another way, the superoxides are converted into hydrogen peroxide and free radicals which can induce more potent and severe oxidative injuries. The free radicals and cytochrome C enter cytosol after damaging the mitochondria permeability transition pores leading to cardiomyocyte necrosis and apoptosis. The anti-oxidant effects of ABM have been studied in blood cells. The ABM extract shows a significant dose-dependent protective effect against DNA damage induced by hydrogen peroxide in human lymphocytes. Hot water extract of ABM can significantly decrease chemically induced lipid-peroxidation in liver tissue. In our study, we found ABM could protect cardiomyocytes from oxidative injuries by hydrogen peroxide. It could be one of the major mechanisms by which ABM could ameliorate the myocardial ischemia-reperfusion injury in animal model.

Post-myocardial infarction injuries are related to the up-regulated local neurohumoral factors, including pro-inflammatory cytokines and reactive oxygen species. Reactive oxygen species may contribute to myocardial damage via various processes, including direct cytotoxic, negative inotropic, and cytokine-stimulating effects, and cause apoptosis. Some antioxidant drugs such as probucol (an antihyperlipidemic drug), dimethylthiourea (DMTU; a hydroxyl radical scavenger), and vitamin E have been tested in animal models of myocardial ischemia-reperfusion and significantly attenuated oxidative stress, inhibited the activation of pro-inflam-
flammatory cytokines, and preserved ventricular function after myocardial infarction. The present study showed that ABM treatment reduced infarcted areas after cardiac ischemia-reperfusion, and that the antioxidant activity of ABM likely plays the key protective role. Based on a previous report that ABM contributes to anti-inflammatory effects, ABM might also protect myocardial cells from inflammation-induced oxidative tissue damage. However, numerous studies have pointed out that β-glucans are the major components of antioxidant and anti-inflammatory activities. Whether β-glucan components are the key players contributing to these effects was not clearly demonstrated in the present study. β-glucans are polysaccharides of D-glucose monomers which are linked by β-glycosidic bonds. They occur most commonly as cellulose in plants, the cell wall of certain fungi, mushrooms and bacteria. Glucans exhibits their protective effect against oxidative damage as a consequence of scavenging both OH radicals and singlet oxygen. Therefore, we speculate that β-glucans could be the key contributors in ABM’s myocardial protection. It has become an important issue whether natural ingredients and nutrients can lower the severity and chances of developing heart diseases in the preventive medicine. Some ingredients, such as red yeast rice (Monascus purpureus) and fish oil are proved for their lipid-lowering effects. Mushroom extract, such as Ganoderma lucidum (Reishi mushroom) is reported for its anti-oxidant and cardioprotective effects against ethanol and ischemia-reperfusion cardiac injuries through mitochondria-dependent pathways. ABM has immune-modulating effects. In our study, we proved anti-oxidant and cardiac protective effects of pretreated ABM against ischemia-reperfusion injuries. ABM also showed no cytotoxicity in the cardiomyocyte culture study. This implies ABM may have potential as a nutrient ingredient for cardiac health. The study has limitations. Firstly, the ABM extract may contain some compounds rather than a single chemical compound. The real active ingredient of ABM extract for cardioprotection is not clear so far. However, as a nutrient for daily use, the mixture of some compound is acceptable and convenient in use. Secondly, the comparison of anti-oxidant activity among ABM extract and other anti-oxidants was not checked in the study. Thirdly, the animals used for inducing ischemia-reperfusion injuries model in the study were healthy animals. The responses to the injuries and treatment may be different in the clinical setting. Human studies are needed before clinical application.

CONCLUSION

In this study, we found that ABM reduced oxidative stress in cells and protected them from permanent cell damage. Using ABM as a health food may help decrease myocardial injury after acute myocardial infarction. Based on the findings in the present study, we conclude that ABM is a promising nutritional supplement for individuals with a high risk of myocardial infarction.

REFERENCES


