Germinated Brown Rice Protects H9c2 Cardiomyocyte against Simulated Ischemic/Reperfusion Injury via Mediated Mitochondria Function

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Abstract

Background

Ischemia/reperfusion (I/R) injury is the major mechanism during Ischemic Heart Disease (IHD). The key modulator of I/R injury is dysregulation of mitochondria function. Germinated Brown Rice (GBR) has recommended as a bio-functional food and has clarified the potential properties in several effects. However, the effect of GBR mediated cardioprotective properties, focusing on the role of mitochondrial function, remains unexplored. Thus, this study aims to investigate the cardioprotective effects of GBR pretreatment against simulated I/R injury.

Results

H9c2 cardiomyocytes were incubated with GBR at a concentration of 5 μg/ml for 24 hours and/or simulated I/R (sI/R) for 40 minutes. Cell viability and cell apoptosis were assessed by 7-AAD staining and AnnexinV/PI staining, respectively. For evaluation of mitochondrial functions, not only mitochondrial membrane potential was determined by JC-1 staining but also mitochondrial respiration was represented by oxygen consumption rate (OCR) using Seahorse Flux analyzer. The results revealed that administration of GBR prior to sI/R significantly decreased the percentage of cell death and total cell apoptosis in H9c2 during stimulation of ischemic/reperfusion. In addition, pretreatment of cardiomyocytes with GBR remarkably stabilized mitochondrial membrane potential and improved impaired mitochondrial respiration in simulated-H9c2 injury.

Conclusion

the present research is the first study to report the effective cardioprotection of GBR. Pretreatment of GBR potentially protects H9c2 cardiomyocytes against sI/R injury through mitochondrial function. The underlying therapeutic activities are possibly associated with its bio-functional compounds. However, the underlying mechanism on cardioprotective effects of GBR needs further studies.

Background

Ischemic heart disease (IHD) is considered to be the single largest cause of death worldwide and is estimated to increase both in morbidity and mortality in the next coming decade (Benjamin et al., 2019). Principally, IHD is caused by an insufficient blood supply to heart tissues due to occlusion of the arterial blood flow. Inadequate tissue perfusion leads to ischemia-induced tissue damage and/or cell death (Kalogeris, Baines, Krenz, & Korthuis, 2012). The presence of attenuated blood flow in the heart response to myocardial ischemia can lead to myocardial infarction (MI) (Jennings, 2013; Jennings & Reimer, 1991). Not only ischemia that occurred on myocardium damage but also reperfusion. Reperfusion is a rapid restoration of arterial blood flow to the ischemic myocardium (Jennings & Reimer, 1994). The

adverse result of ischemic and reperfusion processes are called ischemia/reperfusion (I/R) injury (Ibanez, Heusch, Ovize, & Van de Werf, 2015). The key mechanism of I/R injury reveals that ischemia derogates ATPase-dependent ion-exchange channels, interrupts regulation of cell volume, which lead to lysis of organelle and plasma membranes and disrupted enzymatic activity (M. Y. Wu et al., 2018). In addition, reperfusion enhances reactive oxygen and nitrogen species production by impairing mitochondria function and triggers series of pro-inflammatory cytokines release, endoplasmic reticulum stress, and development of tissue damage (Kalogeris et al., 2012; M. Y. Wu et al., 2018). The well-known of end-effector of I/R-induced cell injury and death is the opening of mitochondrial permeability transition pores (MPTPs) (Kalogeris et al., 2012). Many recent studies emphasize the role of mitochondrial dysfunction which is associated with I/R injury (Carpi et al., 2009; Chen et al., 2009; Hausenloy & Yellon, 2013; Ibanez et al., 2015; Kalogeris et al., 2012; Li et al., 2016; Solaini & Harris, 2005; Veloso et al., 2019; M. Y. Wu et al., 2018). Therefore, maintaining of impaired cardiac mitochondrial function could be an effective therapeutic target of I/R injury.

In the recent few years, an attractive alternative medication such as the usage of herbal therapies and dietary supplements has remarkably increased because of their variety of remediable properties (Chung, 2004). Many of evidence recommended the therapeutic effects of bio-functional components in a variety of daily diets such as antioxidant (Caceres, Martinez-Villaluenga, Amigo, & Frias, 2014; Walter et al., 2013), antimicrobial property (Rahman, 2003), cardioprotective effects and diminishing the risk factors of cardiovascular diseases such as hypertension, hyperinsulinemia, dyslipidemia, or arteriosclerosis *in vitro* and animal models (Asdaq & Inamdar, 2010; Kris-Etherton et al., 2002; Rahman & Lowe, 2006; Valli & Giardina, 2002). Several current studies demonstrated that population in many Asian countries tend to have a lower risk of cardiovascular diseases than Europe and American countries regarding to their consumer behavior and cultivated areas (Ling, Cheng, Ma, & Wang, 2001). One such plant food is rice which is mostly cultured in Asia for consuming around the world. Brown rice is an important rice that contains lots of basic nutritional contents and bioactive compounds. However, it has been reported that bioactive components gained more during the germination process (Cho & Lim, 2016). Germinated brown rice (GBR) contains a lot of bio-functional components such as ferulic acid, γ -oryzanol, and gamma-aminobutyric acid (GABA) (Gong et al., 2017; Ohtsubo, Suzuki, Yasui, & Kasumi, 2005; Tian, Nakamura, & Kayahara, 2004). Several physiological effects of GBR have been demonstrated as an antihyperlipidemia by increasing cholesterol catabolism, antihypertension, and exhibited to lower risk of chronic diseases, including cancer, diabetes, cardiovascular diseases, and Alzheimer's disease (F. Wu, Yang, Toure, Jin, & Xu, 2013). Hence, it may be possible that GBR could be biologically active on human health due to its benefits. By the way, the underlying mechanism of GBR, especially cardiovascular diseases focusing on mitochondria function, is not revealed. Therefore, this study aims to investigate the cardioprotective effect of GBR during myocardial I/R injury.

Results

1. Bioactive compounds of GBR

The active principles compounds of germinated brown rice such as of phenolic contents, total flavonoid content and GABA are shown in Table 1. The total phenolic, flavanoid and GABA contents were 155.95-146.71 mg, 44.59–38.73 and 13.35–12.65, respectively. Germinated brown rice contained the highest phenolic contents among the three main contents.

Table 1
Bioactive compounds of GBR

Phenolic contents	Total flavonoid content	GABA
(mgGAE/100 gDW)	(mgCE/100gDW)	(mgGABA/100 g DW)
151.33 ± 4.62	41.66 ± 2.93	13.00 ± 0.35

2. The protective property of GBR against sI/R in H9c2 cardiomyocyte

We firstly determined the protective effect of GBR on sI/R induced-H9c2 cardiomyocyte cell death. The results demonstrated that GBR pretreatment remarkably decreased cell death when compared to sI/R condition (8.31% vs 11.99%, respectively) (Fig. 1A). While, induction of sI/R significantly increased the percentage of dead cells when compared to control (11.99% vs 9.51%, respectively). A summary of the findings on percentage of cell death are shown in Fig. 1B. This result suggested that GBR pretreatment reduced sI/R-induced cardiomyocyte cell death.

H9c2 cardiomyocytes were incubated with or without GBR for 12 hours and then exposed to sI/R for 40 minutes. (A) Flow cytometer plot for 7-AAD staining. (B) The percentage of 7-AAD-stained cells was analyzed by flow cytometry. All data was analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The mean ± SEM of six individual experiments are shown. *P < 0.01, **P < 0.001, ***P < 0.0001 significant different from DMSO; # P < 0.05 significant different from control; † P < 0.01, †† P < 0.001 significant different from sI/R

3. Anti-apoptotic effect of GBR on H9c2 cardiomyocyte after sI/R induction for 72 hours.

To further determine the anti-apoptotic effect of GBR, H9c2 cardiomyocyte cells were incubated with or without 5 μg/ml of GBR for 72 hours and followed by sI/R induction for 40 minutes. Then, total cell apoptosis was assessed by AnnexinV/PI staining and displayed on dot plot (Fig. 2A). The results showed that treatment with GBR significantly reduced H9c2 cardiomyocyte cells apoptosis (9.57%) as same as observed in GBR co-cultured with sI/R group (8.36%). Whereas, higher percent of total cell apoptosis was observed in sI/R group, compared to control (15.47% vs 9.94%, respectively) (Fig. 2B). This finding suggested that GBR treatment exhibited the cardioprotective effect on sI/R induced cardiomyocyte apoptosis.

4. Determination of The Effect of GBR on Mitochondrial Transmembrane Potential (ΔΨm) Alteration against sI/R in H9c2 cardiomyocyte.

This study further determined the cardioprotective effect of GBR on mitochondria function by JC-1 staining. At the end of experiment, the qualitative analysis of mitochondria membrane potential alteration was observed under a fluorescence microscopy as shown in Fig. 3A. In addition, the percentage of JC-1 fluorescence intensity in each group was calculated relative to control (Fig. 3B). Whereas, the quantitative JC-1 analysis was performed by flow cytometry (Fig. 3C, 3D). These results demonstrated that pretreatment with GBR declined the mitochondrial membrane depolarization which represented by high intensity of red fluorescence. On the other hand, sI/R treated cells clearly gained the mitochondrial membrane potential disruption as indicated by the decreased red fluorescence, however, increased green fluorescence (Fig. 3A, 3B). Thus, the fluorescence intensity of GBR pretreatment was stronger than sI/R-treated cells. Furthermore, quantitation of JC-1 staining was further confirmed by flow cytometry. These quantitative results was shown and accomplished by calculating the ratio of the red channel (living cell) with the green channel (non-living cell) (Fig. 3C, 3D). Results showed that cardiomyocyte JC-1-stained cells were conspicuously shifted from red to green fluorescence in sI/R condition compared with that in control cells, indicating mitochondria membrane potential dissipation (Fig. 3C). Interestingly, this pattern was reversed by pretreatment of GBR which was dramatically increased the red/green ratio compared with those in sI/R alone treated cells (Fig. 3D). All of these results indicated that GBR pretreatment improved the mitochondrial membrane potential disruption on H9c2 cell-induced sI/R.

H9c2 cardiomyocytes were incubated with or without GBR for 12 hours and then exposed to sI/R for 40 minutes. (A) Qualitative analysis of mitochondria membrane potential was assessed by fluorescence microscopy with JC-1 staining. (B) JC-1 Fluorescence intensity was calculated using Columbus image data storage and analysis system. (C) Scatter diagram of JC-1 staining by flow cytometer. (D) Quantitative analysis of red/green ratio. All values were analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The representative images were collected in three separated images per experiment from at least three independent experiments and all values are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 significant different from DMSO; #P < 0.01, ##P < 0.0001 significant different from control; † P < 0.05 †† P < 0.01, †††P < 0.0001 significant different from sI/R.

5. Evaluation of mitochondrial respiration on GBR treatment against sI/R induction

The mitochondria respiration was determined through oxygen consumption rate (OCR) on pretreatment of GBR in sI/R condition. In the study, we used XF Cell Mito Stress Test kit and analyzed by using the Seahorse XFp Extracellular Flux analyzer and software (Seahorse Bioscience, USA). The mitochondria respiration profile was shown in Fig. 4A. The representative mitochondrial respiration parameters indicated that GBR pretreatment significantly increased all of mitochondrial respiration parameters, including basal respiration, maximal respiration, spare respiratory capacity, mitochondrial ATP production, proton leak, and non-mitochondrial respiration to different levels when compared with sI/R treated cell. While, the sI/R induction dramatically decreased all of these parameters as shown in Fig. 4B. Over all, these data suggested that GBR pretreatment preserved the mitochondrial function on sI/R-treated H9c2 cells.

Discussion

There are several therapeutic approaches of bio-functional foods against cardiovascular diseases both in the experimental study and clinical study. Germinated Brown Rice (GBR) is also exerted its protective properties on cardiovascular diseases and related-cardiovascular metabolism dysfunction, including hypertension, hyperlipidemia, and diabetes. Here, this is the first study to represent the cardioprotective effect of GBR on cardiomyocyte-simulated I/R injury. The major findings in this study indicated that simulation of I/R promoted the percentage of cell death and total cell apoptosis. Additionally, ischemic/reperfusion induction disrupted the mitochondria membrane potential and mitochondria respiration. On the other hand, pretreatment with GBR could significantly reverse all impacts of simulated I/R injury. Therefore, GBR is able to offer the cardioprotection on I/R injury.

Rice is a major dietary food and mostly cultivation in Asian countries. Several studied suggested that brown rice contained a rich of bio-function components, including phenolic contents, gamma-aminobutyric acid (GABA), γ-oryzanol, ferulic acid, and dietary fibers (Patil & Khan, 2011). Beside from that, the health beneficial properties of brown rice were especially contributed in part of germination process mainly throughout metabolic and phytochemicals alteration of GBR (Chavan & Kadam, 1989). Consistent with the present study, we found that GBR mostly contains phenolic contents, total flavonoid content, and GABA, respectively. Focusing on reducing the risk of cardiovascular diseases, GBR treatment has been shown to decline blood pressure in spontaneously hypertensive animal models and humans (Estruch et al., 2009). Additionally, the administration of GBR has been indicated to improve the lipid profiles in obese mice (S.-H. Oh, Moon, Soh, & Cha, 2005) and decrease the level of hepatoma-induced HDL cholesterol in rats (Roohinejad et al., 2010). Moreover, GBR has found to suppress hypercholesterolemia via upregulating cholesterol catabolism (Miura et al., 2006). Similar observations in a large number of epidemiological and clinical studies suggested that higher consumption of dietary fiber is able to affect the lower cardiovascular risk through LDL cholesterol oxidation (Brown, Rosner, Willett, & Sacks, 1999; Estruch et al., 2009; Kendall, Esfahani, & Jenkins, 2010). These evidences have been documented that GBR exerts it's nutritional and health-promoting benefit properties due to its several bio-function components.

To further investigate the cardioprotective effect of GBR in the molecular level, we first determined the percentage of cell death and apoptosis. Our results indicated that GBR pretreatment significantly protected cardiomyocyte cell death and apoptosis from sI/R induction. There are several studies have been recommended that bioactive components play an important role on these effects (F. Wu et al., 2013). In addition, treatment of GBR has also been revealed the therapeutic effect on cancer cell proliferation and apoptosis (C. H. Oh & Oh, 2004).

Mitochondria function is a key role in the mechanism of myocardial I/R injury. Moreover, several cellular processes such as endoplasmic reticulum stress, intracellular calcium overload, and activated apoptotic pathway are involved in the pathophysiology of myocardial I/R injury (Chen et al., 2009; Veloso et al., 2019). Therefore, the preservation of mitochondrial functions, especially maintained the mitochondrial membrane potential and mitochondria respiration, may provide the benefit in the treatment of myocardial ischemic/reperfusion injury (Pohjoismaki & Goffart, 2017). In this study, our results demonstrated that

GBR pretreatment significantly preserved the mitochondrial membrane potential disruption and enhanced mitochondria respiration in simulated ischemic cardiomyocytes. Therefore, it is important to note that the protective effect of GBR could be explained by the bio-function components in GBR.

Even though, many current researchers play maximize attention on the mechanism of GBR on cardiovascular diseases. However, the exact underlying mechanisms of the protective effects of GBR on myocardial I/R injury are still unknown. Initially, previous observations have raised the possibility that GBR administration demonstrated a positive association with risk factors of cardiovascular diseases and GBR due to its bioactive compounds. The study in the effective natural therapeutic of honey on cardiovascular diseases has suggested that phenolic and flavonoid components potentially provided antioxidant and anti-platelet activation (Olas, 2020). Similar suggestion has been reported by Daskalova et al. They found that phenolic compounds in berry showed the antioxidant properties, anti-atherogenic effects, as well as, cardioprotective effects in aging rats (Daskalova et al., 2015). A meta-analysis of prospective cohort studies had revealed a positive correlation between higher consumption of flavonoids and lower risk of mortality in cardiovascular diseases both in men and women (Kim & Je, 2017). In the aging rat, long-term treatment of flavonoid significantly declined fibrosis, encouraged citrate synthase activity and maintained the mitochondrial membrane potential (Testai et al., 2020). Others, the dietary consumption of flavonoids in atherosclerosis has performed by macrophage RAW264.7 cells. This study highlighted that consumed flavonoids acted on prohibiting LPS-induced production of nitric oxide (NO), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β) and gene expression. In addition, the results also indicated the potential effect of flavonoid on extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), p38, p65, IκBα, IκKα/β phosphorylation, and nuclear factor-kappa B (NF-κB) nuclear translocation (Shen, Lin, Jiang, Wang, & Zhu, 2020). In addition, it has been suggested that GABA has anti-hypertension, anti-diabetes, anti-cancer, antioxidant, anti-inflammation, anti-microbial, anti-allergy, hepato-protection, reno-protection, and intestinal protection (Ngo & Vo, 2019). A study in islet β-cells has been revealed that GABA potentially exhibited its antidiabetic effects by modulating on PI3K/Akt-dependent growth and survival pathways. According to the activation of PI3K/Akt pathway, it resulted in cellular membrane depolarization and calcium influx, leading to cell growth and survival (Soltani et al., 2011). A key study of GABA-rich GBR administration has been considerably discarded hydroxyl radical and thiobarbituric acid-reactive substances in both free medium and cultured media (Md Zamri, Imam, Abd Ghafar, & Ismail, 2014). Subsequent studies have been documented that GABA protected pancreatic cells and human umbilical vein endothelial cells against H_2O_2-induced oxidative stress via reducing cell death, diminishing reactive oxygen species (ROS) production and promoting antioxidant activity (Tang, Yu, Zhou, Jiang, & Le, 2018; Zhu et al., 2019). These all given evidence support the pharmaceutical properties of GBR on I/R injury–induced cardiomyocyte cell.

Nevertheless, the substantial mechanism by which protective effect of GBR has not been established yet. Together with our results in this study offered the potential mechanism of GBR, which is associated with the process of cell death, apoptosis, mitochondria membrane potential, and mitochondria respiration on

simulated I/R cardiomyocytes. Therefore, these findings suggested that GBR offered cardioprotection against I/R injury was partly mediated through mitochondrial metabolic function. During I/R injury progression, the opening of mitochondria permeability transitional pore serves as a critical factor for triggering several signaling cascades and protein kinases of cardiac cell death (Li et al., 2016). Therefore, cardioprotection of GBR might be related to the regulation of cell signaling pathways of survival and/or apoptosis. This hypothesize is become clear with the further our finding results, which has been demonstrated the cardioprotective effect of GBR via mediating on p38 MAPK, proapoptotic Bax and Bcl-2, and caspase-3 protein expression (Data not shown). Taken together, our findings may explain the potential mechanism on cardioprotective effect of GBR in cardiomyocytes against I/R injury.

Conclusion

This study provides novel evidence that GBR pretreatment effectively protects against I/R injury in H9c2 cardiomyocyte through attenuation of cell death and apoptosis, and maintenance of mitochondrial function. The underlying therapeutic activities are possibly associated with its bio-functional compounds. However, further studies are needed regarding the underlying mechanism on cardioprotective effects of GBR in simulated ischemic/reperfusion injury.

Materials And Methods

1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and trypsin-EDTA were purchased from Gibco®; Life Technologies Inc. FITC Annexin V Apoptosis Detection Kit I was obtained from BD Pharmingen, USA. MitoScreen (JC-1) and Via-probe (7-AAD staining) were purchased from BD Bioscience, USA. XF Cell Mito Stress Test kit was purchased from Seahorse Bioscience, USA.

2. The Extraction Of Bioactive Compound Of GBR

Germinated brown rice seeds were dried at 50 °C overnight. Germinated seeds were ground and stored at -20 °C and used for subsequent analyses. Germinated brown rice (GBR) was extracted with 80% ethanol (ratio 1:2) at room temperature for 72 hours and filtrated. The maceration process was repeated 2 times. Each filtrate was pooled and evaporated under reduced pressure to dryness. The extracts were further freeze-dried to produce GBR crude extracts. The crude extract was subsequently partition by hexane, dichloromethane, and ethyl acetate to give crude hexane, dichloromethane, ethyl acetate, and water extracts, respectively.

3. Cell Culture

The rat cardiac myoblast cell line (H9c2) was originated from embryonic BD1X rat cardiac tissue and purchased from American Type Cell Culture (ATCC-CRL1446). H9c2 cell line was routinely cultured as monolayer in Dulbecco's Modified Eagle's Medium (Gibco®) with 10% fetal bovine serum (FBS), amphotericin, penicillin G and streptomycin under pH 7.4 at 37 °C in humidified air containing 5% CO_2. Cell density of 70% confluence in passage number 5–10 was used for all experiments. The culture medium was changed with fresh warm medium in every 3 days.

4. Simulated Ischemia In H9c2 Cardiomyocyte Cell

In order to this study performed the ischemic injury *in vitro* study, we provided sodium dithionite to induce the simulated ischemic condition. The H9c2 cardiomyocytes were incubated in 2 ml of ischemic buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl2, 1.8 mM CaCl2 and 4.0 mM HEPES), followed by 20 mM 2deoxyglucose, 30% sodium lactate and 1 mM sodium dithionite at pH 6.3, in order to induce sI/R in 24-well plate. Ischemic induction was performed in the period of time for 40 minutes. After ischemic induction, the ischemic buffer was replaced and the cell were incubated with growth Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) for all experimental assessments.

5. Determination of cell death by 7-Aminoactinomycin D (7-AAD) viability staining

For cell death measurement, 7-AAD cell viability staining was used in this study based on specific binding to double-stranded DNA with high affinity. The H9c2 cardiomyocyte cells were placed in 24-well plate at a density of 2×10^5 cells per well and maintained in 2 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) for 24 hours. Then, the medium was replaced, following which they were treated with or without concentration of 5 µg/ml GBR and further incubated for 24 hours. The cells were induced sI/R condition for 40 minutes. After Ischemic induction, cultured media was collected and the cells were trypsinization. After centrifuged, the cells were collected at a concentration 1×10^5 cells/ml, and then stained with 20 µl of 7-AAD in 100 µl of PBS for 20 minutes in dark at room temperature. The percentage of 7-AAD-stained cells was optimized by Accuri C6 flow cytometer (BD Bioscience, USA) for the viability 7-AAD staining and presented as a bar graph.

6. Determination of cell apoptosis by AnnexinV/ Propidium Iodine (PI) assay

Cellular apoptosis was evaluated by Annexin V/ PI assay. H9c2 cells were seeded at a density of 2×10^5 cells per well in 24-well plate in 2 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). After 24 hours, the old medium was discarded, following by which they were pre-treated with or without concentrations of 5 µg/ml GBR for 24 hours. After 72 hours incubation, sI/R induction was performed as previous experiment. Briefly, cultured media in each well was collected and the cells were washed with PBS followed by trypsinization. Approximately, 1×10^5 cells/ml in each condition was collected and centrifuged. After that the cell pellets were mixed with 100 µl of 1X binding buffer and 3 µl of Annexin V solution and incubated in dark room for 10 minutes. Then, this cell was incubated with 2 µl

of PI solution for 5 minutes. Finally, cell apoptosis was determined by flow cytometry using Accuri C6 flow cytometer (BD Bioscience, USA). Percentage of total cell apoptosis was presented as a bar graph.

7. Determination Of Mitochondria Membrane Potential Alteration (ΔΨm)

Mitochondria membrane potential alteration was detected by JC-1 staining due to its specific formation in mitochondria. The qualitative data of mitochondrial membrane potential was assessed by fluorescence microscopy (Operetta CLS™ high content analysis system, PerkinElmer, USA) with JC-1 staining. H9c2 cardiomyocyte cells were cultured at a density of 1×10^4 cells per well in 96-wells plate in cultured medium. After 24 hours, cell were pre-treated with or without concentrations of 5 μg/ml GBR for 24 hours and exposed to sI/R for 40 minutes. 5% DMSO was used as a positive control of the experiment. Then cultured media was removed and cells were washed with PBS. After that, the cells were incubated with JC-1 working solution at 37 °C with 5% CO_2 incubator for 1 hour in dark. Then JC-1 dye was removed and the cells were washed with 1X of JC-1 assay buffer. Finally, the mitochondrial membrane potential changes were immediately analyzed by fluorescence microscopy using the Columbus image data storage and analysis system.

For further analysis of mitochondria membrane potential alteration, the quantitative measurement was performed by flow cytometry. Density of H9c2 cardiomyocyte cells at 2×10^5 cells/well were seeded into 24-well plate in cultured medium. The cells were harvested by trypsinization after treatment as previous experiment. At the day of experiment, cultured media was collected and H9c2 cells were washed and centrifuged. After that, approximately 1×10^5 cells/ml in each condition were collected and then were incubated with JC-1 working solution according to manufacturer's protocol. Finally, the quantitative analysis of red/green fluorescence intensity ratio was evaluated by using Accuri C6 flow cytometer (BD Bioscience, USA) by measuring in the FL1(530 nm) and FL2 (585 nm) channels for fluorescence compensation controls analysis.

8. Determination Of Mitochondrial Respiration

A mitochondrial respiratory study was conducted by measuring the oxygen consumption rate (OCR) that reflexed all key parameters of mitochondrial respiration. The H9c2 cardiomyocyte cells at density of 1.5×10^4 cells per assay were cultured in the XFp cell culture miniplate. Prior to the day of assay, cells were treated either concentrations of 5 μg/ml GBR for 24 hours or sI/R induction for 40 minutes. In the day of assay, cultured medium was discarded with XF assay medium-modified with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose and then incubated at 37 °C without CO_2 for 1 h. Then, the cells were serially exposed three times to 5 μM of oligomycin, 2 μM of FCCP and 0.5 μM of rotenone plus antimycin A, respectively, using the XF Cell Mito Stress Test kit (Seahorse Bioscience). The parameters of OCR were measured according to the manufacturer's protocol by using the Seahorse XFp Extracellular Flux analyzer

and software (Seahorse Bioscience, USA). All mitochondrial respiratory parameters were calculated and presented as a bar graph.

9. Statistical Analysis

All data are expressed as the mean ± standard error of mean (SEM) at least three independent experiments. Comparisons between different groups were performed by one-way analysis of variance (ANOVA) followed by post hoc analysis with Tukey's multiple comparison test using GraphPad Prism 8 software. Statistical significance was set at $P < 0.05$.

Abbreviations

Germinated Brown Rice: GBR; Ischemic Heart Disease: IHD; Myocardial Infarction: MI; Ischemia/Reperfusion: I/R; mitochondrial Permeability Transition Pores: MPTPs; Gamma-aminobutyric acid: GABA; Dulbecco's modified Eagle's medium: DMEM; Fetal Bovine Serum: FBS; 7-Aminoactinomycin D: 7-AAD; Mitochondria membrane potential: ΔΨm; Oxygen consumption rate: OCR; Nitric oxide: NO; Interleukin-6: IL-6; Tumor necrosis factor-α: TNF-α; Interleukin-1 beta: IL-1β; Extracellular signal-regulated kinases: ERK; c-Jun N-terminal kinases: JNK; nuclear factor-kappa B: NF-κB; Reactive oxygen species: ROS

Declarations

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Author's contributions

All authors contributed the relevant work; Soontaree Petchdee (SP): Principle investigator, designed the study conception, drafted and revised the manuscript. Wichit Suthammarak (WS) provided critical revision of the article. Kanokwan Demeekul (KD) performed the experiments and wrote the manuscript with support from SP and WS.

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no potential competing interests.

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Figures

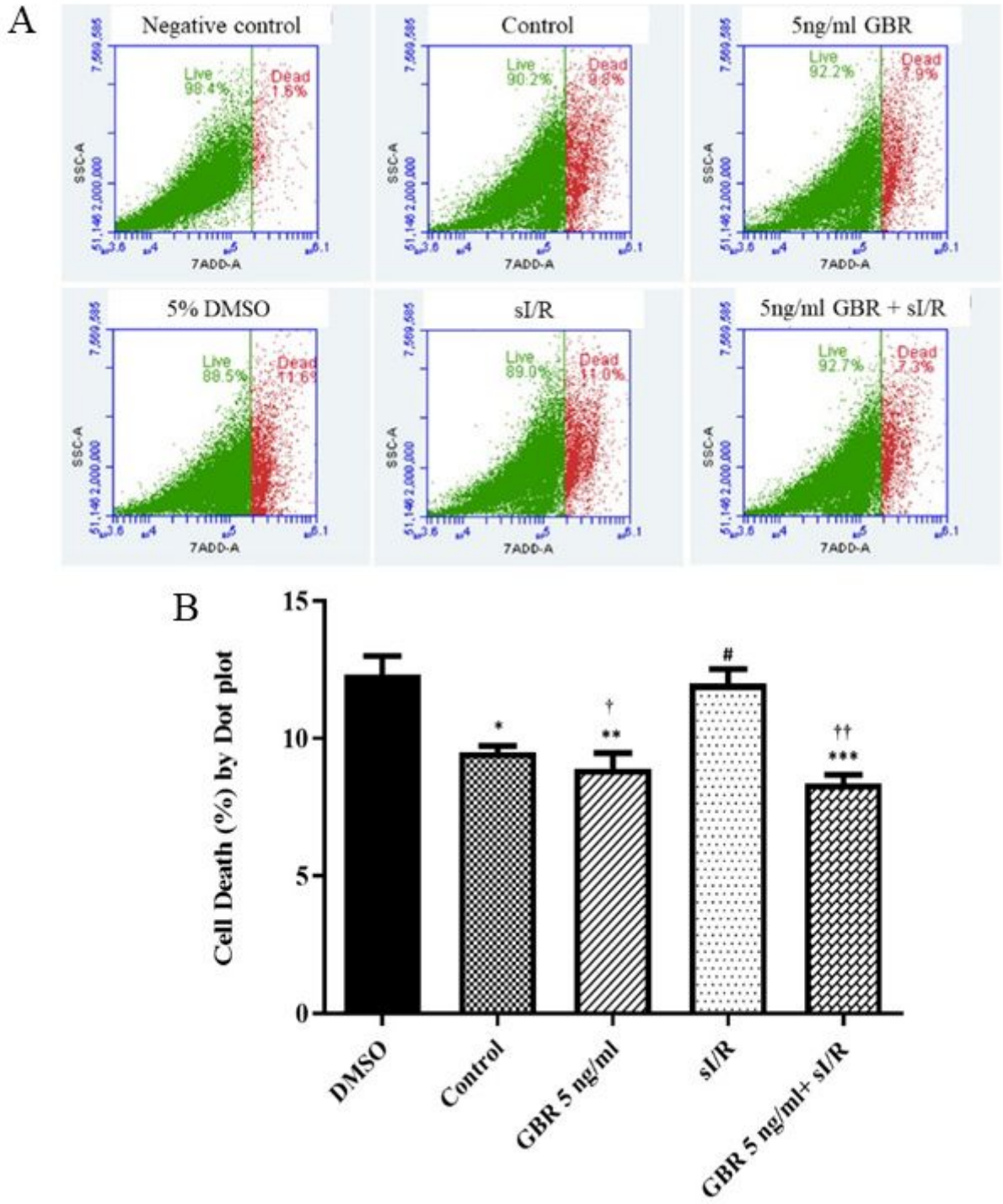

Figure 1

The protective property of GBR against sI/R in H9c2 cardiomyocyte. H9c2 cardiomyocytes were incubated with or without GBR for 12 hours and then exposed to sI/R for 40 minutes. (A) Flow cytometer plot for 7-AAD staining. (B) The percentage of 7-AAD-stained cells was analyzed by flow cytometry. All data was analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The mean ± SEM of six individual experiments are shown. *P<0.01, **P<0.001, ***P<0.0001 significant different from DMSO; # P < 0.05 significant different from control; † P < 0.01, †† P < 0.001 significant different from sI/R

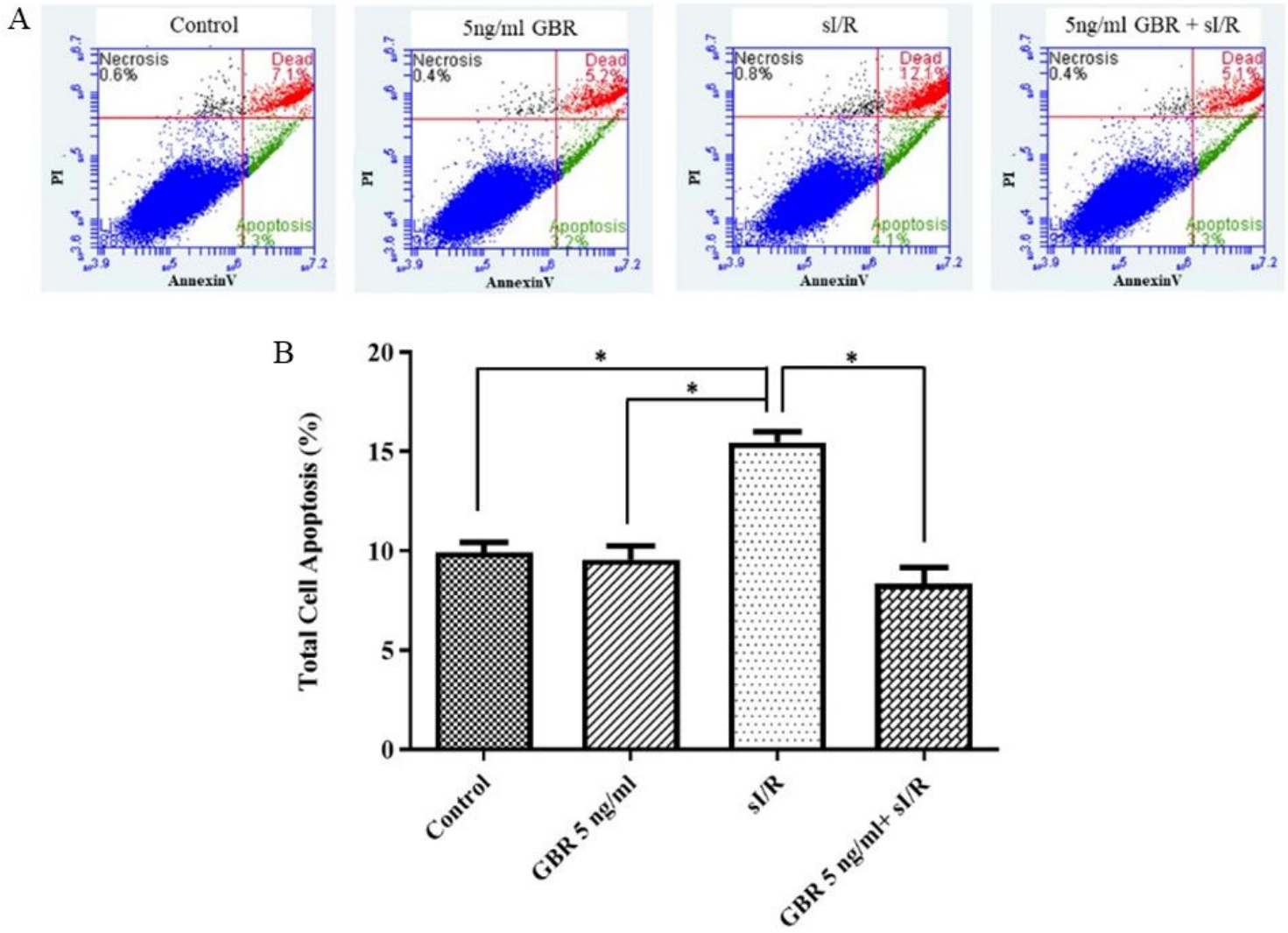

Figure 2

Anti-apoptotic effect of GBR on H9c2 cells during sI/R induction. (A) Flow cytometer dot plot of AnnexinV/PI staining. (B) The percentage of total cell apoptosis in H9c2 cardiomyocytes was analyzed by flow cytometry. All data was analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The values are expressed as the mean ± SEM of six independent experiments. *P<0.0001 significant different from sI/R.

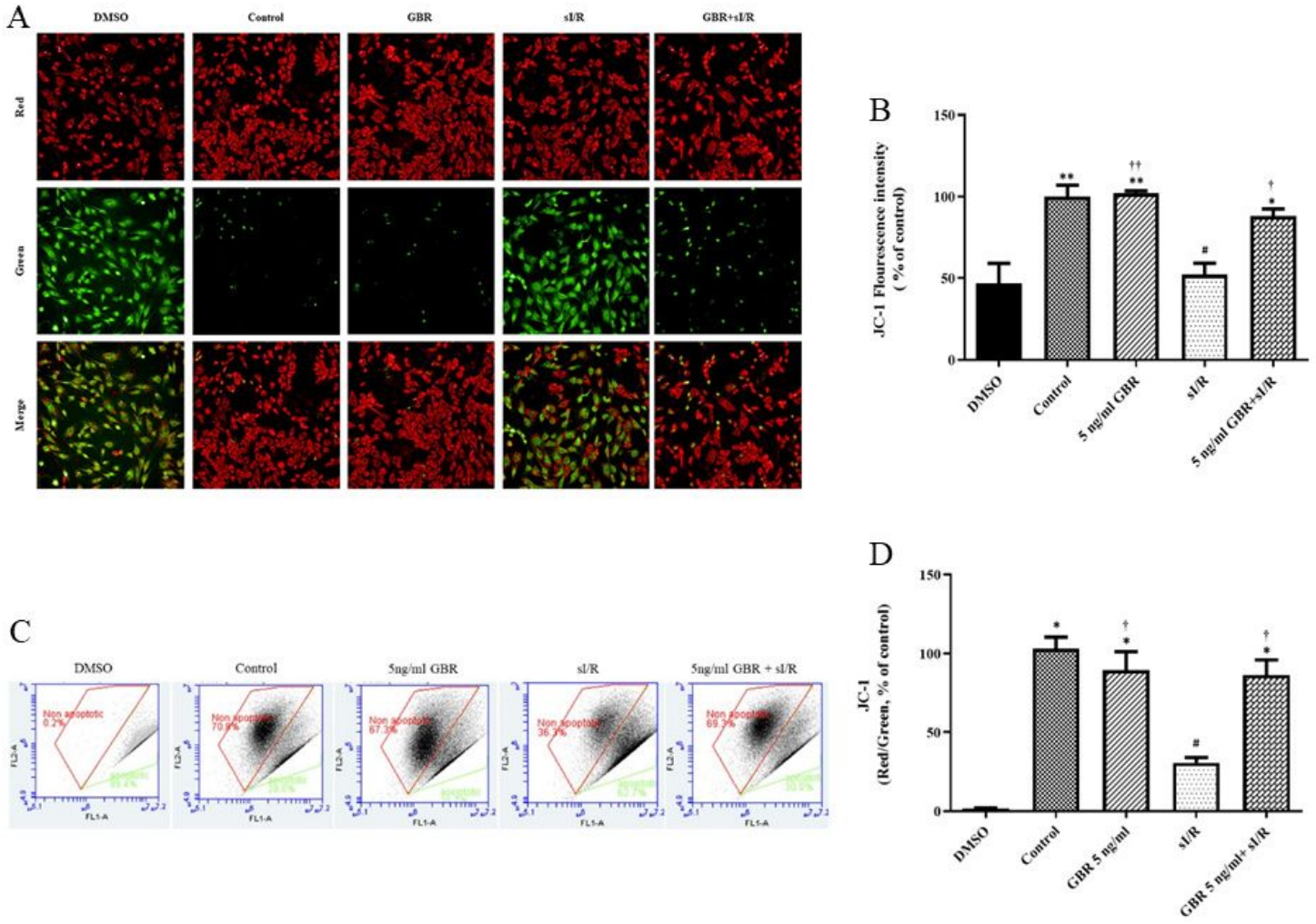

Figure 3

Protective effect of GBR on mitochondrial transmembrane potential disruption against sI/R induction. H9c2 cardiomyocytes were incubated with or without GBR for 12 hours and then exposed to sI/R for 40 minutes. (A) Qualitative analysis of mitochondria membrane potential was assessed by fluorescence microscopy with JC-1 staining. (B) JC-1 Fluorescence intensity was calculated using Columbus image data storage and analysis system. (C) Scatter diagram of JC-1 staining by flow cytometer. (D) Quantitative analysis of red/green ratio. All values were analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The representative images were collected in three separated images per experiment from at least three independent experiments and all values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001 significant different from DMSO; #P<0.01, ##P<0.0001 significant different from control; † P < 0.05 †† P < 0.01, †††P < 0.0001 significant different from sI/R.

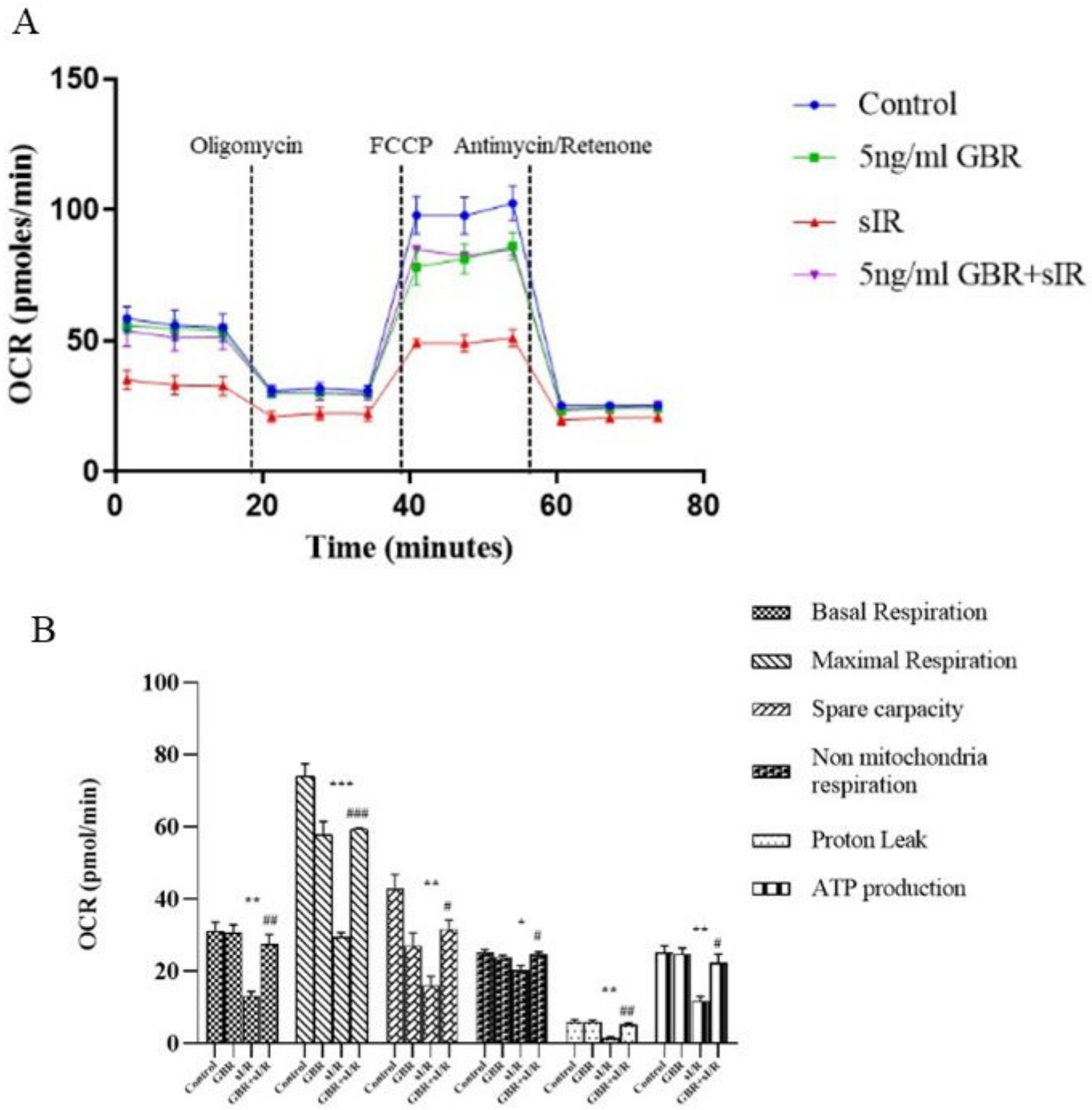

Figure 4

Effect of GBR on mitochondrial respiration against sI/R induction. (A) Time period for measurement of oxygen consumption rate (OCR) was shown using Seahorse XFp analyzer, followed by the serial injection of oligomycin (5 μM), FCCP (2 μM) and rotenone (0.5 μM) plus antimycin (0.5 μM). (B) Representative mitochondrial respiration parameters, including basal respiration, maximal respiration, spare capacity, non-mitochondrial respiration, proton leak, and ATP production were evaluated. All values were analyzed by One-way ANOVA with Tukey's Multiple Comparison Test. The values are expressed as the mean ± SEM

of three independent experiments. *P<0.05, **P<0.01, *** P<0.0001 significant different from control; #P< 0.05, ## P< 0.01, ###P< 0.001 significant different from sl/R.