3-N-Butylphthalide Confers Antiarrhythmic Features in Ischemia/Reperfusion Injury of Diabetic Heart by Targeting Mitochondria-Endoplasmic Reticulum Network and Inhibiting Oxidative Stress and Inflammation

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Summary

While 3-N-butylphthalide (NBP) has demonstrated notable cardioprotective effects, its precise role in mitigating myocardial arrhythmia following ischemia/reperfusion (IR) injury in diabetes remains unclear. This study aimed to explore the potential mechanisms through which NBP mitigates reperfusion-induced myocardial arrhythmia in diabetic rats, with a particular focus on mitochondrial function and biogenesis, endoplasmic reticulum (ER) stress, and oxidative/inflammatory responses. Sixty Sprague-Dawley rats were divided into non-diabetic and diabetic groups, subjected to *in-vivo* myocardial IR injury, and treated with NBP (100 mg/kg, intraperitoneally) through different modalities: preconditioning, postconditioning, or a combination of both. Electrocardiography (ECG) was employed to assess the incidence and severity of arrhythmia. Fluorometric, Western blotting and ELISA analyses were utilized to measure the mitochondrial, ER stress, and cellular outcomes. Treatment of non-diabetic rats with NBP in preconditioned, postconditioned, and combined approaches significantly reduced cardiotroponin-I and the frequency and severity of arrhythmias induced by IR injury. However, only the combined preconditioning plus postconditioning approach of NBP had protective and antiarrhythmic effects in diabetic rats, in an additive manner. Moreover, the NBP combined approach improved mitochondrial function and upregulated the expression of PGC-1α, Sirt1, and glutathione while concurrently downregulating ER stress and oxidative and pro-inflammatory-related proteins in diabetic rats. In conclusion, the combined approach of NBP treatment was effective in mitigating myocardial arrhythmia in diabetic rats. This approach coordinates interactions within the mitochondria-endoplasmic reticulum network and inhibits oxidative and inflammatory mediators, offering a promising strategy for managing myocardial arrhythmia in diabetic patients.

Key words

Myocardial Infarction • Mitochondria • Arrhythmia • Reperfusion • Diabetes • Ischemia

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Introduction

Myocardial ischemia/reperfusion (IR) injury is a condition where the heart muscle is temporarily deprived of oxygen and nutrients, and then blood flow is restored [1]. In clinical settings, when blood flow is reestablished during reperfusion (for example by angioplasty), it triggers a cascade of events that can exacerbate tissue injury, a phenomenon known as reperfusion injury, which adds to the ischemic-induced cell damage [1,2]. Myocardial IR injury, coupled with chronic diabetes, poses a significant threat to heart health, often culminating in reperfusion-induced lethal arrhythmias and other cardiac complications [2]. Myocardial arrhythmia stands as a critical complication in patients with diabetes enduring IR injury [3]. This complex interplay between physiological and

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pathophysiological processes has led researchers to explore the complexities of this challenging medical scenario.

In the context of chronic diabetes, as a known risk factor for cardiovascular complications, the heart is already under stress due to metabolic abnormalities and underlying cardiovascular complications [3]. The presence of diabetes amplifies the vulnerability of the heart to IR injury and reduces the potency of cardio-protective modalities [4]. Increased oxidative stress and chronic inflammation are the hallmarks of diabetes. When ischemia is followed by reperfusion, it triggers a robust pro-inflammatory cytokines release and harmful free radicals' production, which can disrupt the heart's electrical conduction system, potentially leading to arrhythmias [5]. In addition, the mitochondria-endoplasmic reticulum (Mito-ER) network, a complex and dynamic web of cellular structures, plays a pivotal role in regulating various cellular functions, including calcium signaling, energy production, and cell homeostasis in diabetic cardiomyocytes [6]. After the induction of ER stress, the phosphorylation of eukaryotic initiation factor-2α (eIF2α) enhances the expression of activating transcription factor-4 (ATF-4). Prolonged ATF-4 overexpression leads to the elevation of C/EBP homologous protein (CHOP), which is associated with promoting cell death. CHOP levels are typically low under normal conditions but rise during diabetes, increasing the likelihood of cell death [6-8]. Recent research suggests that dysregulation of the Mito-ER network following IR insults may affect cell death pathways and contribute to cardiac arrhythmias, particularly in diabetic hearts [7]. This dysregulation leads to the disruption of mitochondrial function and biogenesis, enhanced ER stress, increased oxidative stress and inflammation, and aberrant cellular signaling, creating a perfect storm of factors that elevate the risk of life-threatening cardiac arrhythmias during the critical reperfusion phase [7-9]. Investigating this intricate network is crucial for understanding the root causes of cardiac arrhythmias in IR settings. Understanding and addressing these intricate processes is essential in developing targeted therapeutic approaches to mitigate the adverse effects of IR injury and overcome the loss of cardioprotection in diabetic hearts.

3-N-butylphthalide (NBP) possesses multi-faceted therapeutic potential and has gained attention as a potential protective agent in the context of IR injury and diabetes [10-13]. This substance has demonstrated a noteworthy capacity to decrease oxidative stress, inflammation, and apoptosis, and it has already been confirmed to alleviate cardiac and cerebrovascular ischemic damage [10-12]. In the specific context of diabetic hearts, which are highly susceptible to IR-induced arrhythmias due to pre-existing cellular abnormalities, NBP's beneficial characteristics make it an ideal candidate [13,14]. Extensive prior research indicates that chronic diabetes reduces the effectiveness of cardioprotective treatments, leaving standard interventions insufficient to address IR complications in diabetic patients [15,16]. This highlights a need to enhance therapeutic efficacy to counter IR-induced damage in diabetic hearts, where existing strategies may struggle due to disrupted intracellular homeostasis. While administering protective drugs before ischemia (preconditioning) or at reperfusion onset (postconditioning) provides some protection against IR damage [2,15,16], combining these strategies in diabetic conditions could offer a dual-pronged approach to reinstating heart protection. Given its proven effectiveness in alleviating ischemic conditions, NBP emerges as a potential protective agent for this purpose. Preconditioning with NBP could prime the myocardium for better endurance against impending IR insult, while postconditioning with NBP could further mitigate damage during reperfusion. Thus, our study aims to investigate NBP's efficacy as a combined preconditioning and postconditioning strategy to enhance cardioprotection and alleviate IR-induced arrhythmias in diabetic hearts, examining its effects on the Mito-ER network, mitochondrial function, and oxidative and inflammatory responses.

Materials and Methods

Animal handling

A total of sixty male Sprague-Dawley rats, weighing between 250-300 g, were procured and housed in a controlled environment with a 12-hour light-dark cycle. They were given unrestricted access to food and water. All procedures involving animal use adhered to the guidelines outlined in the "Guide for Care and Use of Laboratory Animals" (revised in 1996, United States National Institutes of Health Publication No. 85-23) and received approval from the Institutional Yunnan Animal Experiments Medical Research Ethics Committee (No. YN9940).

Induction of diabetes

Following a two-week adaptation period, II-type diabetes was induced through a high-fat diet (62 % calories from fat) and a single intraperitoneal injection of streptozotocin (35 mg/kg, citrated buffer; pH 4.5; Sigma Aldrich, USA) after four weeks of high-fat diet feeding. The blood glucose levels of the rats were assessed 72 hours after streptozotocin administration with a glucometer device, and those with levels equal to or greater than 16.7 mmol/L were classified as diabetic. To validate II-type diabetes modeling in rats, we utilized the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) test. This involved measuring fasting glucose and insulin levels in blood samples collected from the tail vein. Insulin levels were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The HOMA-IR was then calculated by multiplying fasting insulin (in μU/mL) by fasting glucose (in mmol/L) and dividing the product by 22.5. The diabetic regimen was maintained for eight weeks to induce the chronic features of diabetes.

Experimental design

Thirty healthy (non-diabetic) and thirty diabetic rats were randomly divided into corresponding five groups, each comprising six rats: sham-operated group (Sham), IR injury group (IR), IR plus NBP preconditioning group (IR+pre-NBP), IR plus NBP postconditioning group (IR+post-NBP), and IR plus NBP preconditioning and post-conditioning combined group (IR+pre/post-NBP). In the NBP preconditioning group, NBP (Sigma-Aldrich, USA) was intraperitoneally administered to diabetic rats at a dosage of 100 mg/kg/day [12] for 4 weeks before surgery. In the NBP postconditioning group, it was injected intraperitoneally at single dose of 150 mg/kg at the first minute of reperfusion. In the combined group, administration of NBP postconditioning occurred one day following the final NBP preconditioning administration. Rats in the sham and IR groups in both categories of diabetic and non-diabetic rats were given an equivalent volume of solvent (1% DMSO).

Sample size calculation

To determine the sample size, we employed an alpha level of significance at 0.05 and a power of 0.80 to ensure the study's capacity to detect statistically significant differences. Utilizing previous literature and anticipated effect sizes, a power analysis was conducted, indicating that a sample size of six rats per group would provide sufficient statistical power to detect significant inter-group differences.

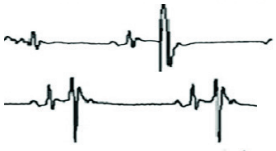
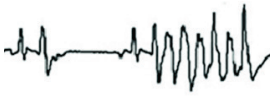
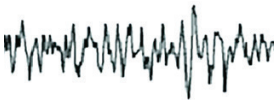
IR injury modeling

To establish the IR model, we adhered to a previously reported methodology [16]. In brief, rats were anesthetized with 1% sodium pentobarbital (40 mg/kg) and underwent a left thoracotomy to expose their hearts. The left anterior descending (LAD) coronary artery was occluded for 35 minutes, followed by 24 hours of reperfusion to induce the IR model. The sham group underwent an identical procedure but without LAD coronary artery ligation.

Electrocardiogram recording and analysis

Throughout the experiment, surface electro-cardiography (ECG) was employed to monitor heart rhythm. ECG recordings and interpretations were made during the initial 30 minutes of the ischemic phase and the subsequent 30 minutes of the reperfusion phase. The electrocardiograms were examined for the presence of premature ventricular complexes (PVC), ventricular tachycardia (VT), and fibrillation (VF) in line with the Lambeth convention for arrhythmia interpretation in animals. Additionally, the severity of arrhythmias was rated using a 5-degree scale as follows: 0 (no arrhythmia), 1 (ventricular premature beats), 2 (ventri-cular bigeminy or salvos), 3 (VT), and 4 (VF). A sample of arrhythmia in ECG recording is seen in Table 1.

Table 1. Samples of ECG Recording and Ventricular Arrhythmias.

Type of Arrhythmia	Sample Recording
Normal ECG	
Premature Ventricular Complex	
Ventricular Tachycardia	
Ventricular Fibrillation	

Measurement of injury marker cardio-troponin-I

After the reperfusion period, rats were anesthetized with 40 mg/kg sodium pentobarbital, and their hearts were exposed. Blood samples were collected directly from the heart and used to measure cardio-troponin-I (cTnI) levels using an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance of the samples was measured at 450 nm, and cTnI levels were expressed in U/L.

Measurement of oxidative stress and inflammatory markers

To assess the levels of oxidative stress, the cardiac levels of 8-isoprostane and reduced glutathione were examined. Cardiac TNF-α and IL-10 were also measured to determine the myocardial inflammatory responses. After 24 hours of reperfusion, the hearts were harvested, and the samples from the ischemic regions of the left ventricles (areas at risk) were homogenized in RIPA lysis buffer. The levels of oxidative stress and inflammatory markers were quantified using commercial ELISA kits following the manufacturer's instructions (MyBioSource, Inc., USA). The protein concentration of the samples was determined using the bicinchoninic acid (BCA) assay kit (Beyotime, China). The levels of markers in each sample were normalized based on the protein concentration in the respective sample.

Mitochondrial function

The method for assessing mitochondrial function involved the isolation of mitochondrial content from left ventricular samples taken from the areas at risk. The samples were homogenized in a mitochondrial isolation buffer containing a protease inhibitor (Sigma Aldrich, USA) and centrifuged at 10,000 g for ten minutes. The mitochondrial fraction was then obtained by re-centrifuging the resulting pellets at 21,000 g. Total protein in the samples was measured using a BCA kit. To determine the levels of mitochondrial ROS, the supernatant was mixed with 2 µM dichloro-dihydrofluorescein diacetate (DCFD) dye and incubated at 37 °C for 30 minutes. The solution was then measured for absorbance at 480 nm and 530 nm, using fluorometry. Additionally, the JC-1 assay kit (Sigma Aldrich, USA) was used to assess changes in mitochondrial membrane potential. We mixed 2 µl of cationic carbocyanine JC-1 dye with 100 µl of mitochondrial supernatant, incubated it in the dark at 37 °C for 30 minutes, and calculated the extent of mitochondrial membrane depolarization by analyzing the red-to-green ratio of JC-1 fluorescence intensities in each sample. The fluorescence intensities of red and green emissions were measured at 530 nm and 590 nm for the former and 480 nm and 530 nm for the latter. A decrease in the ratio of fluorescent aggregates to monomers indicated depolarization, while an increase suggested hyperpolarization. The red-to-green ratio of JC-1 fluorescence intensities was used to assess changes in mitochondrial membrane potential.

Western blotting

To analyze the expression levels of ER stress proteins including C/EBP homologous protein (CHOP) and eukaryotic translation initiation factor 2 alpha (eIF-2α), and mitochondrial biogenesis protein including phosphorylated Sirtuin 1 (Sirt1) and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), Western blotting was employed. Equal quantities of protein extracted from the areas at risk of left ventricles were separated via SDS-PAGE and transferred to PVDF membranes. The blocked membranes were probed with primary antibodies (1:1500, all from Cell Signaling Technology, USA) targeting the aforementioned proteins and β-actin (used as a loading control), followed by incubation with secondary antibodies conjugated to horseradish peroxidase. The protein bands were visualized using an enhanced chemiluminescence kit, and the ratio of their intensity to β-actin intensity was analyzed.

Statistical analysis

All data are presented as the mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's post-hoc test. A p-value less than 0.05 was considered statistically significant.

Results

Confirmation of II-Type diabetes induction, and results outline

Table 2 presents the fasting glucose levels (in mmol/l), insulin levels (in µU/ml), and HOMA-IR values for both healthy (non-diabetic) and diabetic rats. It is evident that the blood glucose and insulin levels, as well as the insulin resistance value (HOMA-IR), were significantly higher in diabetic rats compared to healthy rats ($p<0.001$). This confirms the successful development

of II-type diabetes in the experimental model.

It is important to highlight as the results outline that during the initial phase of the study, we noted that administering the NBP alone did not result in significant cardioprotective effects in diabetic rats. However, when given in both pre and post modalities, it exhibited notable

Table 2. Fasting Blood Glucose, Insulin and HOMA-IR Test Values in Healthy and Diabetic Rats.

Animal Groups	Fasting Glucose Levels (mmol/L)	Insulin Level (μU/mL)	HOMA-IR Values
Healthy (non-diabetics)	5.49±0.28	8.33±1.97	2.04±0.52
Diabetics	23.35±2.73***	15.83±2.11***	16.35±2.34***

HOMA-IR: Homeostatic Model Assessment for Insulin Resistance. *** p<0.001 vs. Healthy group.

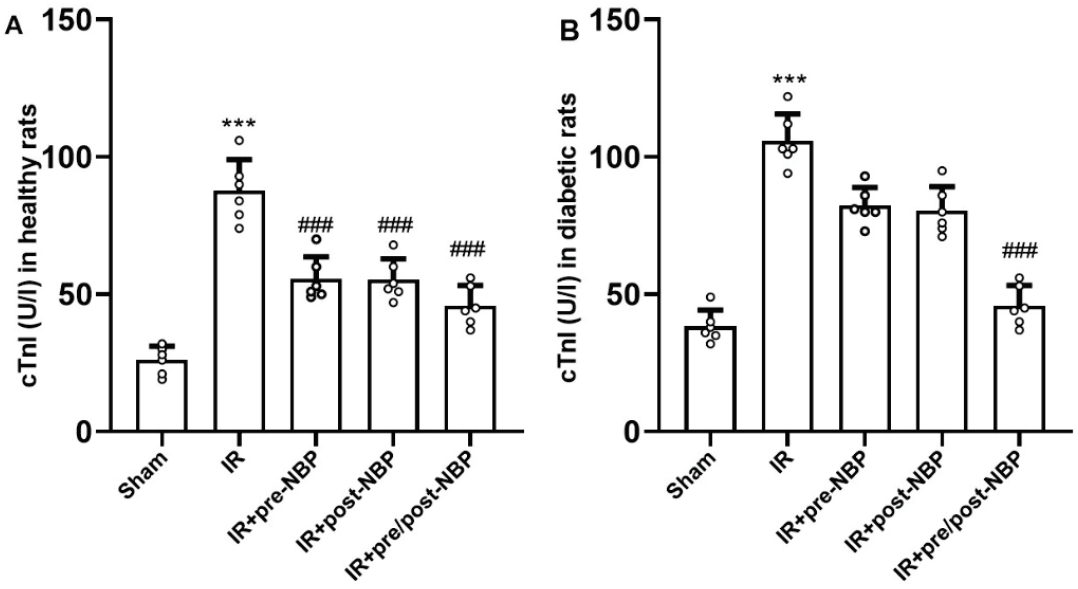


Fig. 1. Effect of 3-N-butylphthalide on Myocardial Injury Marker, cTnI in healthy (**A**) and diabetic (**B**) rats. Data are presented as mean ± SD. (*** p<0.001 vs. Sham group; ### p<0.001 vs. IR group). Abbreviations: cTnI: cardiotroponin I; IR: ischemia/reperfusion; pre: preconditioning; post: postconditioning; NBP: 3-N-butylphthalide.

cardioprotection and exerted a more pronounced impact. In nondiabetic rats, concurrent administration of NBP in pre and post modalities yielded effects similar to single administration. Subsequently, to investigate the potential mechanisms underlying NBP's action in concurrent administration under diabetic conditions, we progressed to the second phase of the study, where we focused on measuring molecular and biochemical parameters in diabetic rats.

NBP attenuates myocardial injury induced by IR injury in healthy and diabetic rats

To validate the protective role of NBP against IR injury in healthy non-diabetic and diabetic rats, we assessed myocardial injury by measuring the serum levels of cTnI. Our findings revealed a significant elevation in cTnI levels after IR injury both in non-diabetic and diabetic rats ($p<0.001$). The administration of NBP, as alone preconditioning (Pre), alone postconditioning (Post), or in combination (Pre plus Post) approaches in non-diabetic rats significantly lowered the elevated cTnI levels as compared with the non-diabetic IR group ($p<0.01$) (Fig. 1A). However, only the combination approach of NBP significantly reduced cTnI levels in diabetic rats when compared to the diabetic IR group ($p<0.01$). Administration of NBP in individual Pre or Post approaches had no significant effect on this cardiac injury marker (Fig. 1B).

NBP mitigates myocardial arrhythmia induced by IR injury in healthy and diabetic rats

To assess the impact of NBP on myocardial arrhythmia in healthy non-diabetic and diabetic rats subjected to I/R injury, we conducted an ECG analysis following reperfusion. Our results demonstrated a substantial increase in the number of PVCs, VT, and VF as well as the arrhythmia scoring in both non-diabetic and diabetic rats following the induction of IR injury, in comparison to the corresponding sham-operated groups ($p<0.001$). Remarkably, the administration of NBP, as alone Pre, alone Post, or combination Pre and Post approaches significantly reduced the occurrence and severity of myocardial arrhythmias in non-diabetic rats when compared to the non-diabetic IR group ($p<0.01$) (Fig. 2A-D). However, the alone Pre and alone Post approaches did not exert significant antiarrhythmic effects in diabetic rats. The antiarrhythmic feature of NBP in diabetic rats was achieved when this compound was administered in a combined manner, in comparison to the diabetic IR group ($p<0.05$) (Fig. 3A-D). The impact of the NBP combined approach in diabetic rats was additive and substantial enough to be deemed a reliable cardioprotective effect, while there was no additive effect following the combination of Pre and Post approaches in non-diabetic rats (Fig. 2, 3).

NBP enhances mitochondrial function and biogenesis following IR injury in diabetic rats

In the quest to elucidate the underlying mechanisms of NBP's protective effects in a combination approach against IR injury-induced arrhythmia in diabetic rats, we delved into the assessment of cardiac mitochondrial function and the expression of key signaling molecules implicated in ER and mitochondrial biogenesis pathways. Firstly, compared to healthy rats, the cardiac mitochondria were dysfunctional in diabetic rats as indicated by mitochondrial depolarization and increased ROS production ($p<0.05$) (Fig. 4 A, B). Similarly, the expression level of PGC-1α and p-Sirt1 proteins was lower in the diabetic versus non-diabetic group ($p<0.05$) (Fig. 4C, D). The induction of IR injury in diabetic rats led to further depolarization of the mitochondrial membrane and augmented mitochondrial ROS production when compared with the diabetic group without IR ($p<0.05$). Administration of NBP in alone approaches of Pre and Post did not show any mitoprotective features in diabetic rats experiencing myocardial IR injury. However, the combination of Pre and Post approaches of NBP significantly upregulated the expression of PGC-1α and p-Sirt1 ($p<0.01$) and reduced mitochondrial membrane depolarization and ROS production ($p<0.001$) in diabetic rats as compared with diabetic IR, diabetic IR+pre-NBP, and diabetic IR+psot-NBP groups.

NBP suppresses the ER stress induced by IR injury in diabetic rats

Similar to prior studies, our results indicated a notable increase in the expression of ER stress proteins CHOP and p-eIF2α in the hearts of diabetic rats in comparison to healthy rats ($p<0.05$) (Fig. 5A, B). The levels of the expression of these proteins were further increased in diabetic rats following myocardial IR induction ($p<0.05$). Significantly, treatment with NBP resulted in a marked reduction in the levels of these markers only in the combined application of Pre and Post approaches when compared to the diabetic IR group ($p<0.01$). NBP pre-treatment or post-treatment strategies failed to inhibit the IR-induced upregulation of ER stress proteins in diabetic rats. Also, there was a significant difference between the effects of the combined approach with those of individual approaches ($p<0.05$). This highlights the potential of NBP to alleviate ER stress in diabetic hearts during IR injury in an additive manner, mirroring the effects observed on mitochondrial parameters.

NBP suppresses the oxidative stress and inflammatory responses induced by IR injury in diabetic rats

Furthermore, we probed the involvement of oxidative stress and inflammatory responses in the antiarrhythmic effect of NBP in myocardial IR injury diabetic rats. Similar to mitochondrial and ER markers, chronic diabetes significantly amplified the cardiac oxidative stress, as indicated by elevated oxidant 8-isoprostane and decreased antioxidant glutathione, and the cardiac inflammatory responses, as indicated by elevation of pro-inflammatory cytokine TNF-α and reduction of anti-inflammatory cytokine IL-10 ($p<0.05$) (Fig. 6A-D). Induction of IR injury in the hearts of diabetic rats exacerbates these alterations of oxidative and inflammatory settings ($p<0.05$). Although none of the NBP preconditioning or NBP postconditioning approaches in diabetic rats considerably reduced the cardiac oxidative stress and inflammatory responses, the combined approach of NBP treatment (Pre plus Post) significantly increased cardiac glutathione and IL-10 levels while reducing 8-isoprostane and TNF-α levels ($p<0.001$). These findings suggest that when NBP is

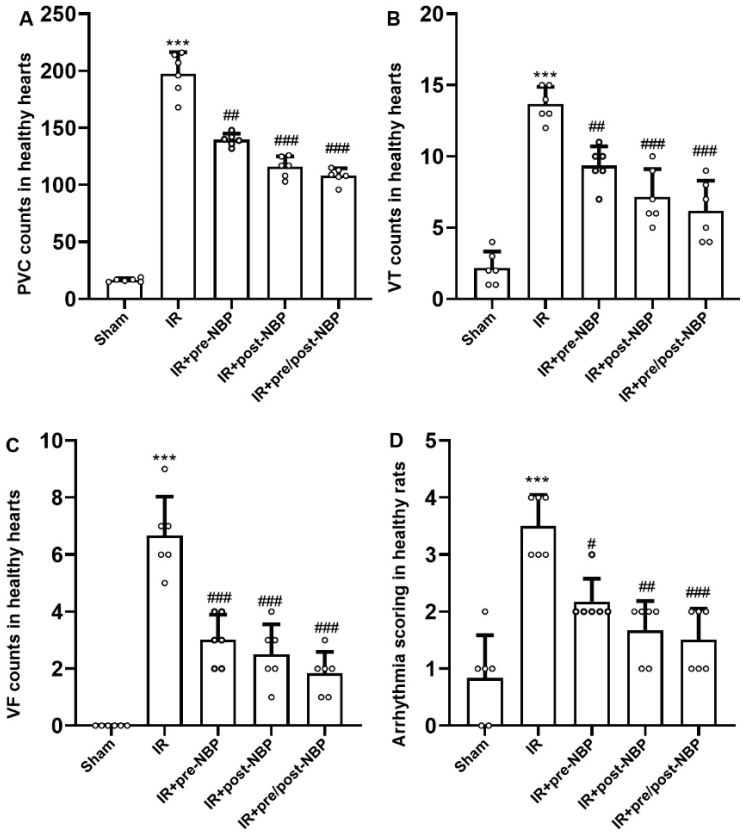


Fig. 2. Effect of 3-N-butylphthalide on Ventricular Arrhythmias in Healthy Rats.

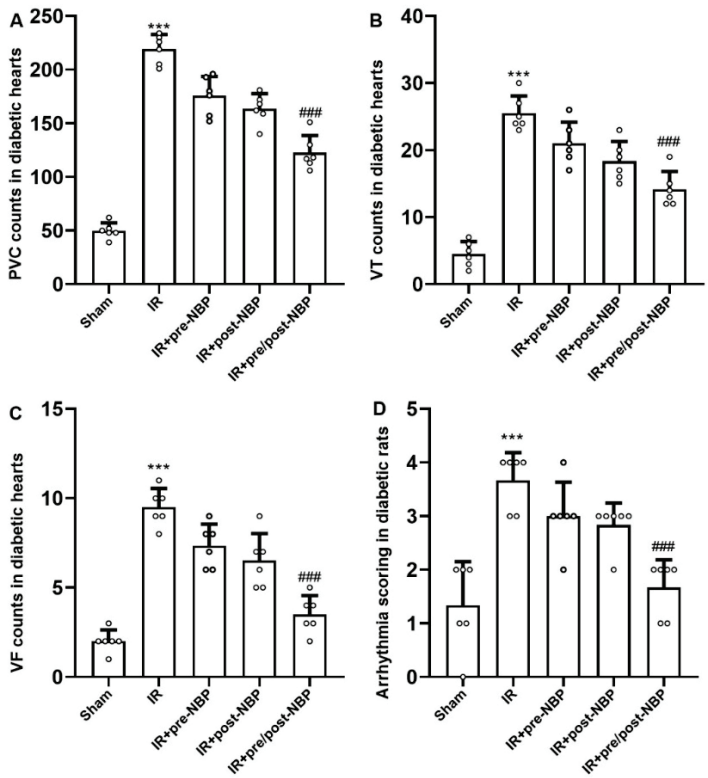


Fig. 3. Effect of 3-N-butylphthalide on Ventricular Arrhythmias in diabetic Rats. (**A**) premature ventricular complexes (PVC) counts; (**B**) ventricular tachycardia (VT) counts; (**C**) ventricular fibrillation (VF) counts; and (**D**) arrhythmia scoring. Data are presented as mean ± SD. (*** $p<0.001$ vs. Sham group; ### $p<0.001$ vs. IR group). Abbreviations: IR: ischemia/reperfusion; pre: preconditioning; post: postconditioning; NBP: 3-N-butylphthalide.

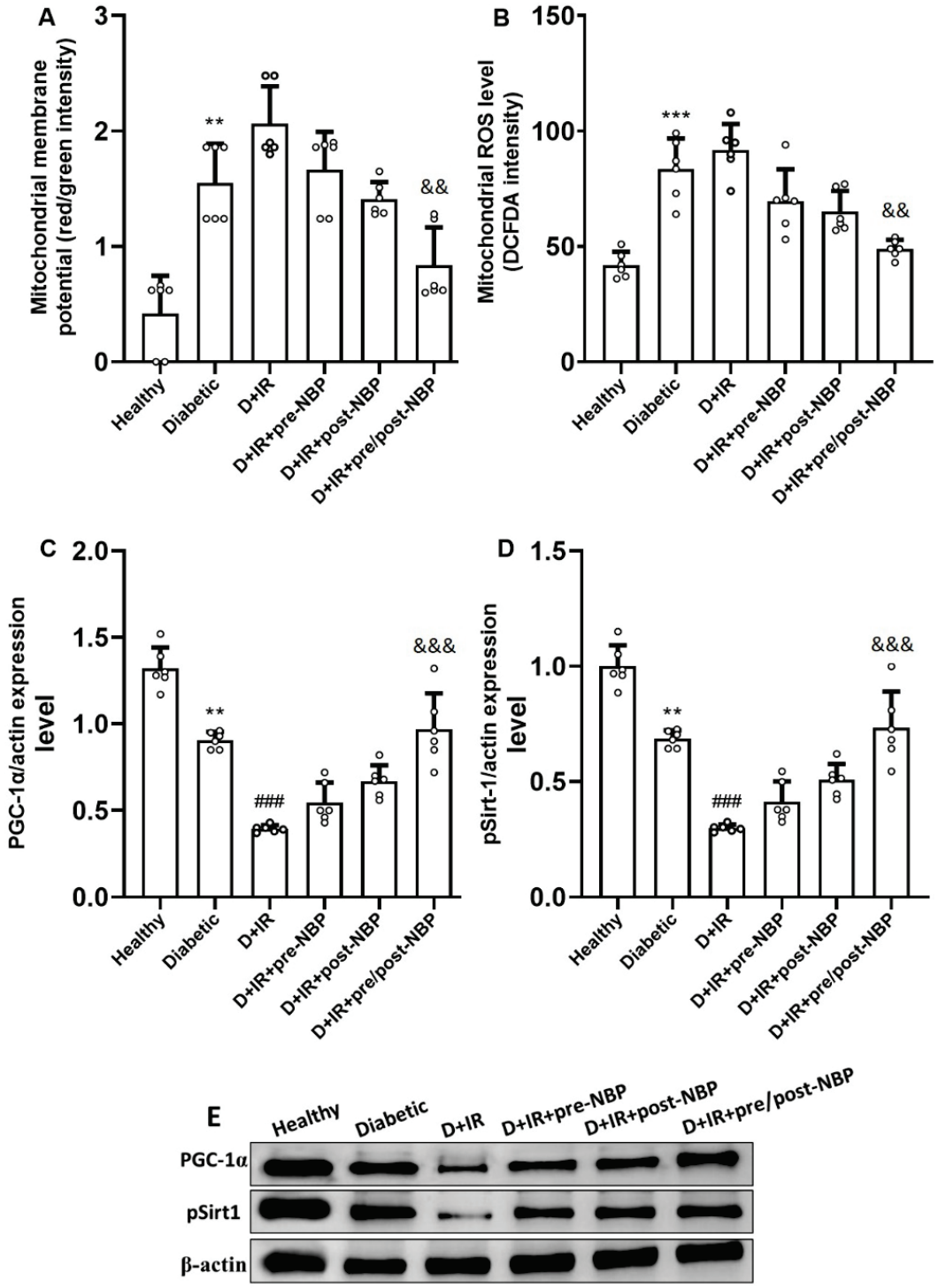


Fig. 4. Effect of 3-N-butylphthalide on Mitochondrial Function and Biogenesis in Diabetic Rats. (**A**) mitochondrial membrane potential changes; (**B**) mitochondrial ROS levels; (**C**) PGC-1α protein expression levels; (**D**) phosphorylated Sirt-1 (pSirt-1) protein expression levels; and (**E**) immunoblotting bands of proteins. Data are presented as mean ± SD. (** p<0.01, *** p<0.001 vs. Healthy group; ## p<0.01, ### p<0.001 vs. Diabetic group; && p<0.01, &&& p<0.001 vs. D+IR group). Abbreviations: IR: ischemia/reperfusion; pre: preconditioning; post: postconditioning; NBP: 3-N-butylphthalide; D: diabetic.

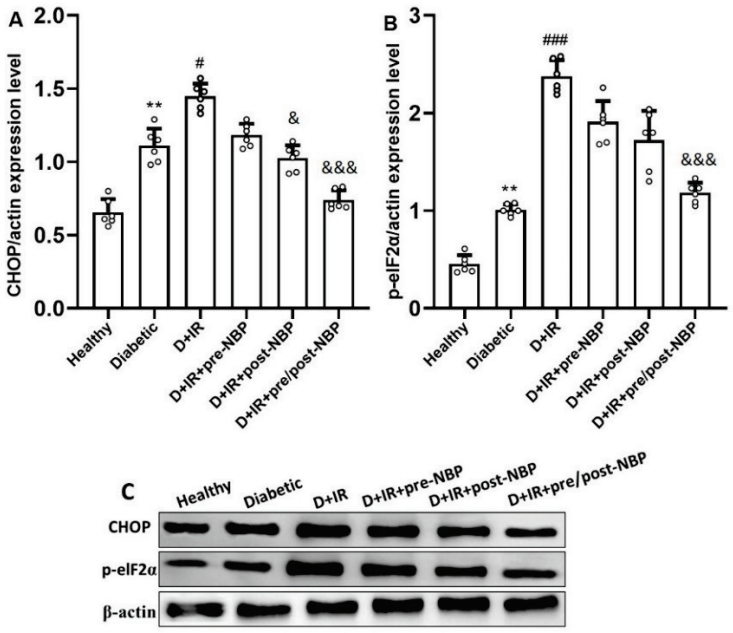


Fig. 5. Effect of 3-N-butylphthalide on Endoplasmic Reticulum Stress Proteins Expression in Diabetic Rats. (**A**) CHOP protein expression levels; (**B**) phosphorylated eIF2α (p-eIF2α) protein expression levels; and (**C**) immunoblotting bands of proteins. Data are presented as mean ± SD. (** p<0.01 vs. Healthy group; # p<0.05, ### p<0.001 vs. Diabetic group; & p<0.05, &&& p<0.001 vs. D+IR group). Abbreviations: IR: ischemia/reperfusion; pre: preconditioning; post: postconditioning; NBP: 3-N-butylphthalide; D: diabetic.

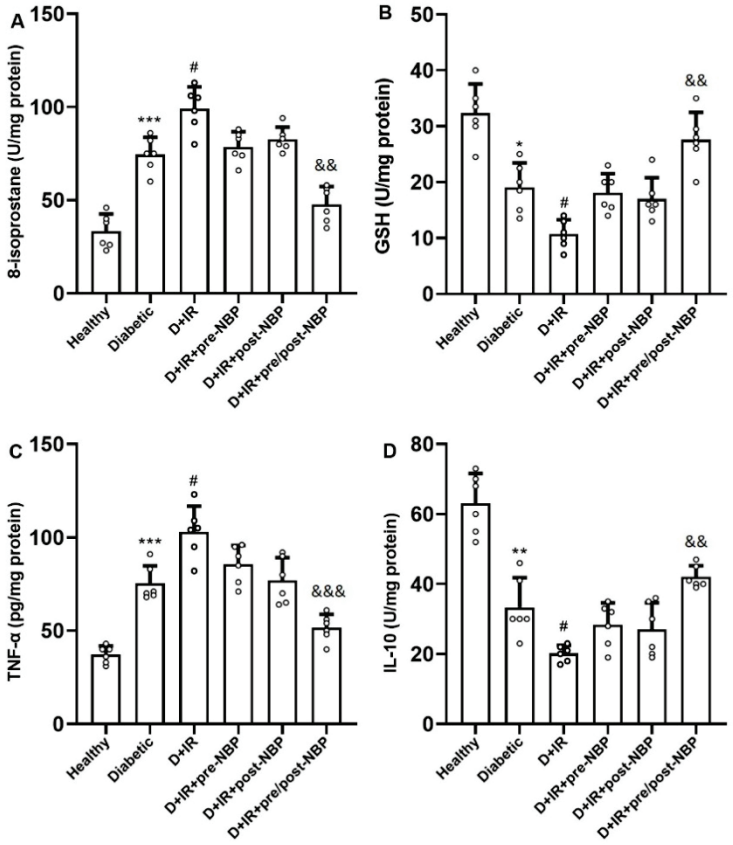


Fig. 6. Effect of 3-N-butylphthalide on Oxidative Stress and Inflammatory Cytokines in Diabetic Rats. (**A**) 8-isoprostane levels; (**B**) glutathione (GSH) levels; (**C**) TNF-α levels; and (**D**) IL-10 levels. Data are presented as mean ± SD. (* p<0.05, ** p<0.01, *** p<0.001 vs. Healthy group; # p<0.05 vs. Diabetic group; && p<0.01, &&& p<0.001 vs. D+IR group). Abbreviations: IR: ischemia/reperfusion; pre: preconditioning; post: postconditioning; NBP: 3-N-butylphthalide; D: diabetic.

administered concurrently in preconditioning and postconditioning modalities, it can produce substantial anti-oxidative and anti-inflammatory impacts in diabetic hearts subjected to IR injury.

Discussion

The results of this study demonstrate that all three strategies with NBP, preconditioning alone, postconditioning alone, and their combined approach, equally reduced ventricular arrhythmia and the severity of IR injury in healthy non-diabetic rat hearts. However, when administered individually in diabetic rats as either preconditioning or postconditioning, NBP was unable to provide complete protection. It was the combined strategy that proved to be most effective in ensuring comprehensive cardioprotection in diabetic heart. High levels of inflammation and oxidative stress in diabetes had a significant impact on the ineffectiveness of monotherapies in diabetes. Notably, the combined strategy in the diabetic rats was associated with reduced oxidative stress markers and inflammatory cytokines, accompanying cardiac protection, and antiarrhythmic effects. Additionally, indicators of mitochondrial dysfunction and ER stress were more pronounced in diabetic rats than in healthy rats. Monotherapies (Pre alone or Post alone approaches) couldn't significantly mitigate these impairments in diabetic rats. However, the combination therapy (Pre plus Post approach) improved mitochondrial function, increased the expression of proteins involved in mitochondrial biogenesis and notably reduced the expression of ER stress-related proteins. Thus, the antiarrhythmic effects of NBP in diabetic hearts, when administered in a combination of preconditioning and postconditioning, are associated with the modification of the Mito-ER network interaction.

Maintaining normal cellular homeostasis, ion channel activity, survival proteins, and electrolyte balance across the membranes is critical for the normal function of cellular structures such as mitochondria and ER [17]. In chronic diabetes, however, cellular homeostasis is disrupted due to cellular stress conditions, exacerbated inflammatory and oxidative responses, and disturbances in the function of vital mediators and ion channels [18]. The exacerbation of these events following IR injury in the myocardium alters cellular conditions, setting the stage for reperfusion-induced arrhythmias and reduced cardiac function. In such conditions, interventions that effectively manage IR-induced cardiac damage in animals without diabetes-related comorbidities prove insufficient, as they cannot effectively activate their cellular targets [15]. In our study, it was observed that both preconditioning and postconditioning with NBP reduced cardiac arrhythmias following reperfusion in healthy non-diabetic rats. However, these protective effects were not observed in diabetic hearts. Based on the above findings, it can be concluded that the single approach of drug administration, either before or after ischemia in diabetic hearts, while showing anti-diabetic effects, is not sufficient to advance the mechanisms of cardiac protection against IR injury. Therefore, to enhance the effectiveness of NBP, a combined regimen was introduced, involving both preconditioning and postconditioning. The effect of combined therapy in non-diabetic hearts did not significantly differ from the effects of the individual therapies, indicating a lack of synergistic or additive effects in hearts without diabetes-related risk factors, which is consistent with previous reports [19,20]. However, in diabetic hearts, the combined therapy significantly reduced the levels of cardiac injury marker compared to the IR group and even the alone Pre and alone Post groups. This combined approach played a pivotal role in significantly preventing ventricular arrhythmias. These beneficial effects of NBP, coupled with the reduction in oxidative stress, inflammatory markers, and the improvement of Mito-ER network interaction, indicate that the use of combined NBP therapy in the diabetic heart shows significant additive effects in mitigating the reperfusion-induced ventricular arrhythmias, leading to a protective phenotype in the diabetic heart. Long-standing diabetes disturbs the ionic and electrolyte balance within cells, increasing the likelihood of arrhythmias during reperfusion. Applying therapeutic intervention prior to ischemia improves these conditions somewhat, but falls short of fully correcting damages from ischemia and reperfusion and effectively controlling arrhythmias. Yet, implementing therapeutic intervention at the start of reperfusion enhances the benefits of prior intervention and adds to the safeguarding of the heart. Preconditioning with NBP can diminish diabetes-induced cardiomyocyte abnormalities and prepare the myocardium to better withstand the impending IR insult, while simultaneously, postconditioning with NBP can further reduce the damage during reperfusion.

Recently, it has been documented that disrupting the Mito-ER network balance plays a key role in the

pathogenesis of diabetes, as well as in the adverse consequences of ischemic heart injury [6-7]. Dysfunction of this network leads to the initiation and exacerbation of inflammatory responses, oxidative imbalances, and apoptosis, ultimately determining the fate of the heart during reperfusion by modifying cellular physiological conditions and promoting cardiac protection, especially in chronic diabetes [17,21]. In this study, NBP-postconditioning of diabetic rats pre-treated with NBP significantly upregulated the expression of mitochondrial biogenesis proteins, such as PGC1α and SIRT1, and reduced the expression of ER stress-related proteins, including elf-2α and CHOP. Similarly, in experiments conducted both *in vivo* and *in vitro*, it has been indicated that NBP demonstrates a protective impact on heart tissue subjected to ischemic injury by influencing and managing mitochondrial function and biogenesis [22]. The *in vivo* and *in vitro* experiments have suggested that NBP exerted a cardioprotective effect on cardiac ischemic injury *via* the regulation of mitochondrial function and biogenesis. Subsequently, key indicators of mitochondrial function, including mitochondrial membrane potential and the production of ROS, were improved. Furthermore, cellular changes resulting from the dysfunctional Mito-ER network, namely, inflammatory responses and oxidative stress in cardiomyocytes, were positively modified, leading to the manifestation of its antiarrhythmic effects of NBP. The improvement in mitochondrial function enhances electrolyte balance and facilitates better mitochondrial respiration, thus enabling the normal operation of ion pumps in the cell membrane, mitochondrial membrane, and ER [6,23]. Subsequently, the accumulation of calcium and sodium ions within the cell during early reperfusion is reduced, bringing cardiac pacemaker activity closer to physiological conditions and preventing excessive arrhythmias [24,25]. Therefore, modifying the Mito-ER network interaction through NBP combined administration before and after ischemia leads to the amelioration of these hierarchical events, making the protective effects of the drug more effective in diabetic hearts.

As the limitations of the study, it remains uncertain whether NBP directly affects mitochondrial and ER functions or if it accomplishes this task through the mediation of other survival and protective mediators. Future studies should investigate the NBP's impact on upstream mechanisms, such as the PI3K/Akt or JAK2/STAT3 pathways, autophagy, and more [26-28]. On the other hand, we aimed to increase the translatability of the results of basic research to the clinical setting by involving diabetic rats in our study to simulate the conditions of human patients suffering from myocardial infarction, primarily those with diabetes. Nevertheless, other underlying comorbidities, such as hypertension, hyperlipidemia, and aging, also inhibit the efficacy of cardioprotective interventions [15]. Hence, it is crucial to verify the efficiency of the NBP combined method (Pre plus Post) in diabetic hearts alongside these concurrent comorbidities, while also evaluating the long-term outcomes in addressing myocardial arrhythmia. Diabetic patients exhibit a heightened vulnerability to coronary ischemic injuries and consequent arrhythmias and the management of reperfusion-induced arrhythmias holds significant clinical importance in this population. But, the study's findings may not fully generalize to all diabetic populations, considering variations in patient demographics, comorbidities, and treatment regimens [29]. Clinical studies involving human subjects are warranted to validate the efficacy and safety of the BP combined approach before its adoption in clinical settings. Additionally, further investigation into how NBP may differ in efficacy between male and female subjects could provide valuable insights about its potential sex-dependent effects but was not addressed in our current research. Finally, it is worth noting that in cases of diabetes with shorter duration and acute I-type models, the levels of antioxidants typically increase as a compensatory response. However, this trend is not observed in chronic and II-type diabetes in our study, where the levels of glutathione enzyme was reduced. This decline is attributed to a combination of factors including prolonged oxidative stress, impaired enzyme synthesis, and increased enzyme utilization [30].

Conclusion

In conclusion, the antiarrhythmic effects of individual NBP preconditioning or postconditioning strategies were lost in diabetic IR hearts. Still, these strategies did not display synergistic or additive effects in the IR hearts of healthy rats. However, the NBP combined preconditioning and postconditioning strategy significantly produced additive effects in diabetic hearts, leading to the inhibition of reperfusion-induced ventricular arrhythmias. In addition, the inflammatory and oxidative responses, along with mitochondrial function and biogenesis and the function of the Mito-ER network in diabetic hearts, were impaired, and these

impairments were corrected by combined NBP administration. This correction was associated with the protective and antiarrhythmic effects of NBP. Therefore, improving the Mito-ER network interaction as a leading factor in modifying intracellular events through NBP combined prescription positions it as a potential candidate for human studies.

Conflict of Interest

There is no conflict of interest.

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