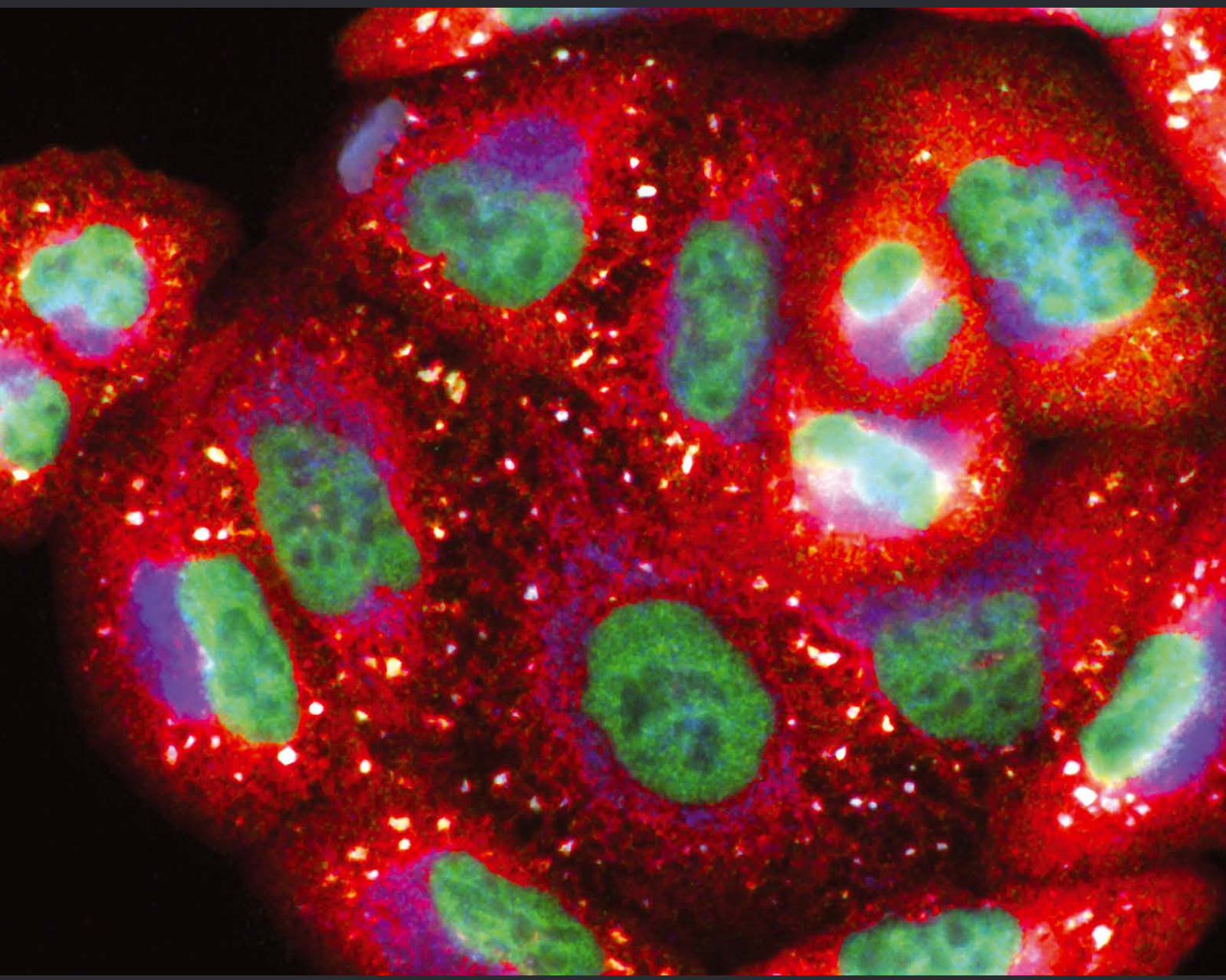
Oxidative Medicine and Cellular Longevity

Redox Signaling and Myocardial Cell Death: Molecular Mechanisms and Drug Targets

Guest Editors: Mohanraj Rajesh, Lu Cai, Partha Mukhopadhyay, and Srinivasan Vedantham



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Editorial Board

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Editorial

Redox Signaling and Myocardial Cell Death: Molecular Mechanisms and Drug Targets

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Cardiovascular diseases (CVD) are the major causes of mortality and morbidity in the world. Apoptotic cardiac cell death has been reported in myocardial tissues obtained from patients with congestive heart failure, myocardial infraction, arrhythmogenic right ventricular dysplasia, myocarditis, chemotherapy induced cardiomyopathies, diabetic cardiomyopathy, and so forth. Further, loss of functional capacity of the myocytes via apoptotic cell death accounts for the major cause of morbidity and mortality in the abovementioned heart diseases. Thus prevention of cardiomyocyte cell death [preserving functional myocardium] could profoundly improve the clinical outcome of the treatment for the aforementioned CVD. Despite significant progress made with clinical management of CVD, development of specific inhibitors to thwart the cardiomyocyte apoptosis is currently restricted due to the limited knowledge underlying the signaling process involved in this process. In addition, the factors triggering and mediating the apoptotic cell death in the myocardium are also murky. Therefore, it is imperative to understand risk factors, regulators, and biomarkers for apoptotic cell death which could aid in the development of therapeutic strategies to mend the injured myocardium.

Myocardial ischemia/reperfusion (I/R) injury is often encountered during various surgical interventions of CVD. Although the sterile inflammation plays a pivotal role in resolving the myocardial tissue injury, dysregulated inflammation process can alter the homeostasis process and perpetuate the tissue injury. Excessive generation of reactive oxygen species (ROS) due to mitochondrial dysfunction is thought to be the central player in myocardial injury. D. M. Muntean et al. have thoroughly reviewed the various sources of ROS arising from mitochondria and their physiological and pathological role during the myocardial ischemia/reperfusion (I/R) injury. Further, the authors also discussed the therapeutic strategies and mitochondria targeted antioxidants molecules being investigated in clinical trial targeting mitochondrial dysfunction associated with I/R injury. In a similar tone, G. A. Kurian et al. in their review article have discussed the central role of oxidative, reductive stress in the development of myocardial I/R injury, diabetic heart disease, and heart failure.

Stroke accounts for the major cause of mortality among subjects with increased risk for CVD such as in patients with diabetes, hypertension, and dyslipidemia. In an effort to develop newer therapeutic agent for the management of stroke, H. Hu et al. used an active component isosteviol derived from *Stevia rebaudiana* leaf and demonstrated the cerebral-vascular protective property in a rodent model of stroke. Further, Z. Fan et al. demonstrated that isosteviol could modulate the sarcK_{ATP} and mitoK_{ATP} channels in

isolated ventricular myocytes. J. Pálóczi et al. demonstrated that nitric oxide donor protects the cardiomyocytes derived from human embryonic stem cells against ischemia induced apoptosis.

Geriatric population is at increased risk of developing sepsis. F. Li et al. demonstrated the key role of translocation factor EB (TFEB) in regulating the lipopolysaccharide induced inflammation, oxidative stress, autophagy, and apoptosis in the aged heart and postulated TFEB as drug target to ameliorate the myocardial tissue injury in aged subjects. Several anticancer agents exhibit profound noxious effects on the heart and increase the mortality rates among cancer survivors. Therefore, myocardial toxicity imposed by these anticancer agents often restricts the clinical usage particularly among pediatric patients. In this direction, S. Ojha et al. have in depth reviewed the need for effective cardioprotective adjuvants that could circumvent the chemotherapeutic agent induced cardiotoxicity. Specific phytochemicals and their mechanistic actions in combating doxorubicin induced cardiotoxicity in various preclinical studies were discussed. Burn can inflict severe myocardial tissue injury. W. Cai et al. demonstrated the pivotal role of Notch signaling pathway in mitigating burn-induced myocardial tissue in a rodent model.

The articles published in this special issue have addressed some of the contentious issues that pertain to redox signaling and myocardial cell death in CVD. We hope that these articles could stimulate our continuing efforts to understand the molecular and cellular pathophysiological mechanisms and impairments that culminate in the cardiomyocyte death in CVD.

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Research Article

Exogenous Nitric Oxide Protects Human Embryonic Stem Cell-Derived Cardiomyocytes against Ischemia/Reperfusion Injury

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Background and Aims. Human embryonic stem cell- (hESC-) derived cardiomyocytes are one of the useful screening platforms of potential cardiocytoprotective molecules. However, little is known about the behavior of these cardiomyocytes in simulated ischemia/reperfusion conditions. In this study, we have tested the cytoprotective effect of an NO donor and the brain type natriuretic peptide (BNP) in a screening platform based first on differentiated embryonic bodies (EBs, 6 + 4 days) and then on more differentiated cardiomyocytes (6 + 24 days), both derived from hESCs. *Methods*. Both types of hESC-derived cells were exposed to 150 min simulated ischemia, followed by 120 min reperfusion. Cell viability was assessed by propidium iodide staining. The following treatments were applied during simulated ischemia in differentiated EBs: the NO-donor S-nitroso-N-acetylpenicillamine (SNAP) (10^{-7}, 10^{-6}, and 10^{-5} M), BNP (10^{-9}, 10^{-8}, and 10^{-7} M), and the nonspecific NO synthase inhibitor Nω-nitro-L-arginine (L-NNA, 10^{-5} M). *Results*. SNAP (10^{-6}, 10^{-5} M) significantly attenuated cell death in differentiated EBs. However, simulated ischemia/reperfusion-induced cell death was not affected by BNP or by L-NNA. In separate experiments, SNAP (10^{-6} M) also protected hESC-derived cardiomyocytes. *Conclusions*. We conclude that SNAP, but not BNP, protects differentiated EBs or cardiomyocytes derived from hESCs against simulated ischemia/reperfusion injury. The present screening platform is a useful tool for discovery of cardiocytoprotective molecules and their cellular mechanisms.

1. Introduction

Ischemic heart disease is the leading cause of mortality in the Western world; therefore, the development of cardioprotective therapies is currently a main focus of research. *In vitro* cardiac myocyte-based drug-screening platforms are widely used, especially at the early stage of the development of cardioprotective agents. However, these assays are based on cardiomyoblast cell lines or primary neonatal and adult cardiac myocytes [1] and thus have major limitations, including a low proliferation capacity, uncontrolled stress during cell isolation, low throughput, and poor predictability

of the assays towards *in vivo* efficacy [2]. Moreover, the relationship between drug responses from animal-derived primary cardiomyocytes and their human counterparts may be significantly different [3]. Human embryonic stem cells (hESCs) are capable of differentiating towards cardiac lineages [4]; therefore, hESCs provide a promising source of cardiomyocytes for *in vitro* drug screening [5, 6]. In addition, hESCs may also provide new tools for regenerative therapies [7–9].

Despite the encouraging results and the enormous potential of the hESC-derived cardiomyocytes, several complications need to be overcome regarding their therapeutic utilization, such as ethical problems, tumor formation, and immunoreactivity. Moreover, it has been demonstrated that the survival of implanted cells is enormously reduced after transplantation [10–12], with these cells undergoing a significant cell death within the first 24 hours [13]. A plausible reason for this effect is the unfavorable microenvironment the grafted cells face when injected into the ischemic host myocardium. Characterization of these cells in an ischemia/reperfusion test system thus would be important, since little is known about the ischemic tolerance of hESC-derived cardiomyocytes.

We have previously shown that the nitric oxide donor S-nitroso-n-acetylpenicillamine (SNAP) and the particulate guanylate cyclase activator B-type natriuretic peptide (BNP) exert a cytoprotective effect against simulated ischemia/reperfusion (SI/R) injury in primary neonatal rat cardiomyocytes [14]. More recently, the cytoprotective effect of SNAP has been shown in mouse embryonic stem cell- (mESC-) derived cardiomyocytes subjected to SI/R treatment [15]. This protection occurs via the activation of protein kinase G (PKG) and stimulation of its downstream signal transduction pathway, which leads to increased cell viability against SI/R injury [14–17]. However, this cytoprotective effect of SNAP and BNP against SI/R injury has not been explored as yet in human cardiomyocytes derived from hESCs.

Therefore, the aim of this present study was to test whether the nitric oxide donor SNAP and the particulate guanylate cyclase activator BNP can protect hESC-derived cardiomyocytes against SI/R injury.

2. Methods

2.1. Human Embryonic Stem Cell Culture. The CAG promoter driven eGFP expressing human HUES9 stem cell culture (Ethic license: Hungarian Committee of Human Reproduction; 31681-1/2004-1016EHR12534-0/2009-1016EHR; ES2HEART consortium) [18, 19] was dispersed by 0.5 mg/mL collagenase type IV (Gibco, Invitrogen; Carlsbad, CA, USA) dissolved in KnockOut™ Dulbecco's Modified Eagle Medium (Gibco). Subsequently, cells were maintained in cell suspension culture for 6 days in KnockOut Dulbecco's Modified Eagle Medium (Gibco), supplemented with 20% embryonic stem cell-qualified fetal bovine serum (Gibco), 1% nonessential amino acids, 1% L-glutamine (Gibco), and 0.2% beta-mercaptoethanol (Gibco). To allow clump formation, cell attachment was hampered by using polyhema (5 mg/mL, Sigma; St. Louis, MO, USA) coated surface. After 6 days, the formation of small clumps was observed which are designated as embryonic bodies (EBs).

2.2. Differentiation of EBs and Cardiomyocytes Derived from Human Embryonic Stem Cells. Six-day-old EBs were seeded onto gelatin-coated coverslips in 24-well plates. 5–10 EBs were plated into each well. Differentiation of EBs was supported by differentiating media containing Dulbecco's Modified Eagle Medium (Sigma) supplemented with 15% fetal bovine serum (Gibco). EBs were kept under normal conditions (at 37°C, in 95% air and 5% CO_2 gas mixture) for 4 days prior to SI/R experiments.

In separate experiments, hESC-derived EBs were maintained in differentiating medium for 24 days. At this stage of their differentiation, spontaneous contractions were observed as the sign of the formation of mature cardiac tissues, and these areas were designated as cardiomyocyte-rich region of EBs.

2.3. Real-Time PCR Analysis of Differentiated EBs. Cardiac-oriented differentiation of the cells was documented in differentiated EBs by real-time quantitative PCR analysis. Total RNA was isolated from cells using TRIzol™ reagent (Invitrogen; Carlsbad, CA, USA). Subsequently, cDNA samples were prepared from 1 μg total RNA using the Promega Reverse Transcription System Kit (Promega Corp.; Fitchburg, WI, USA). All these steps were performed according to the manufacturer's instructions. For real-time quantitative PCR, the following predeveloped TaqMan® assays were purchased from Applied Biosystems (Thermo Fisher Scientific; Waltham, MA, USA): octamer-binding transcription factor 4 (OCT4) and the divergent homeodomain protein NANOG as undifferentiated stem cell markers [20–22], BRACHYURY as mesoderm marker [23], the homeobox protein NKX2.5 and the zinc finger transcription factor GATA4 as early markers of cardiac differentiation [24, 25], and activated leukocyte cell adhesion molecule (CD166, ALCAM) as a marker of cardiomyocytes [26]. P0 ribosomal protein was used as endogenous control for quantification. Real-time PCR analyses were carried out using the StepOne™ Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. The fold changes of mRNA in experimental and control cells were determined using the $2^{-\Delta\Delta Ct}$ method. Relative mRNA levels were presented as mean ± SEM of 3 independent experiments.

2.4. Immunofluorescence. In order to test the specificity of CAG-driven eGFP expression during cardiac differentiation, immunostaining of cardiac troponin I (cTnI) was performed in 6 + 24-day-old adherent EBs. Samples were fixed with 4% paraformaldehyde in Dulbecco's modified phosphate buffered saline (D-PBS, Sigma) for 15 min at room temperature, followed by three washing steps with D-PBS. To block nonspecific antibody binding, samples were incubated in D-PBS containing 2 mg/mL bovine serum albumin, 1% fish gelatin, 5% goat serum, and 0.1% Triton-X 100 for 1 h at room temperature. The samples were then incubated with primary antibodies (monoclonal mouse anti-cTnI, Sigma)

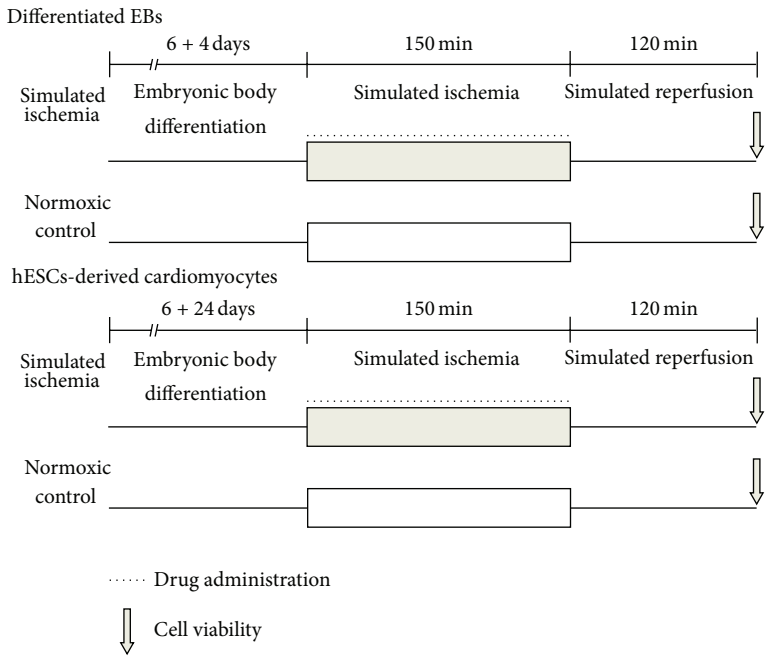

Figure 1: Experimental design of simulated ischemia (SI) and reperfusion (R). hESC-derived EBs (6 + 4 days of differentiation) and differentiated cardiomyocytes (6 + 24 days of differentiation) were exposed to 150 min SI, followed by 120 min R. Cell viability was assessed by propidium iodide staining. The following treatments were applied during SI in differentiated EBs (6 + 4 days of differentiation): the NO-donor S-nitroso-N-acetylpenicillamine (SNAP) (10^{-7}, 10^{-6}, and 10^{-5} M), BNP (10^{-9}, 10^{-8}, and 10^{-7} M), and the nonspecific nitric oxide (NO) synthase inhibitor Nω-nitro-L-arginine (L-NNA, 10^{-5} M). In case of the hESC-derived cardiomyocytes (6 + 24 days of differentiation), 10^{-6} M SNAP was applied during SI. Viability data were normalized to the cardiac specific CAG-driven eGFP fluorescence.

at the dilution of 1 : 500 for 1 h at room temperature. After washing with D-PBS, the cells were incubated with secondary antibodies (Alexa Fluor 568-conjugated goat anti-mouse antibody, Invitrogen) for 1 h at room temperature. Secondary antibodies were diluted in the blocking solution at 1 : 250. 4′,6-Diamidino-2-phenylindole·2HCl (DAPI, Invitrogen) was used for nuclear staining (10 μM, for 10 min in D-PBS). The stained samples were examined by an Olympus fluorescence microscope.

2.5. Experimental Groups. For cell viability experiments, hESC-derived cells were tested under normoxic condition or were subjected to SI (Figure 1). In normoxic conditions, the differentiating medium was replaced with a normoxic solution (in mM: NaCl 125, KCl 5.4, NaH_2PO_4 1.2, $MgCl_2$ 0.5, HEPES 20, glucose 15, taurine 5, $CaCl_2$ 1, creatine 2.5, BSA 0.1%, pH 7.4, and 310 mOsm/L) and cells were incubated in a normoxic incubator at 37°C for 2.5 h. Regarding ischemic conditions, the cells were subjected to SI by incubating them in hypoxic solution (in mM: NaCl 119, KCl 5.4, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 5, $MgCl_2$ 0.5, $CaCl_2$ 0.9, Na-lactate 20, BSA 0.1%, 310 mOsm/L, and pH = 6.4) and exposed to a constant flow of a mixture of 95% N_2 and 5% CO_2 for 2.5 hours at 37°C. Cells were subjected to the following treatments during SI: (1) untreated control; (2) SNAP (10^{-7}, 10^{-6}, and 10^{-5} M) (Sigma); (3) brain type natriuretic peptide-32 (BNP, 10^{-9}, 10^{-8}, and 10^{-7} M) (American Peptides); (4) NOS inhibitor N-nitro-L-arginine (L-NNA, 10^{-5} M) (Sigma). Concentrations of the compounds were selected according to our previous data [14, 15].

At the second set of experiments, cardiomyocytes derived from hESCs were subjected to the following treatments during SI: (1) untreated control; (2) SNAP (10^{-6} M).

Either normoxic or SI treatments were followed by 2 h reperfusion, when the previously applied solutions were replaced with differentiating medium, and the cells were maintained in a normoxic incubator, gassed with 95% air and 5% CO_2 at 37°C.

2.6. Cell Viability Measurements. At the first set of experiments with differentiated EBs derived from hESCs, after simulated reperfusion, cell viability was assessed by propidium iodide (PI) assay as described previously [15]. Briefly, the growth medium was removed, and the cells were washed with PBS twice and incubated with PI (50 μM, Sigma) for 7 minutes. Each experiment included a digitonin (10^{-4} M, Sigma) treated positive control. Then, PI solution was replaced with fresh PBS and fluorescence intensity of each well was detected by a fluorescent plate reader using 544 ex/610 em filters (FluoStar Optima, BMG Labtech, Thermo Fisher Scientific). PI intensity reflecting cell death was evaluated in the cardiomyocyte-rich region. Since the elevation of eGFP expression is associated with cardiac-oriented differentiation of this hESCs model, the evaluation of cardiomyocyte committed regions was performed manually on each plate by detecting eGFP expression driven by the CAG promoter

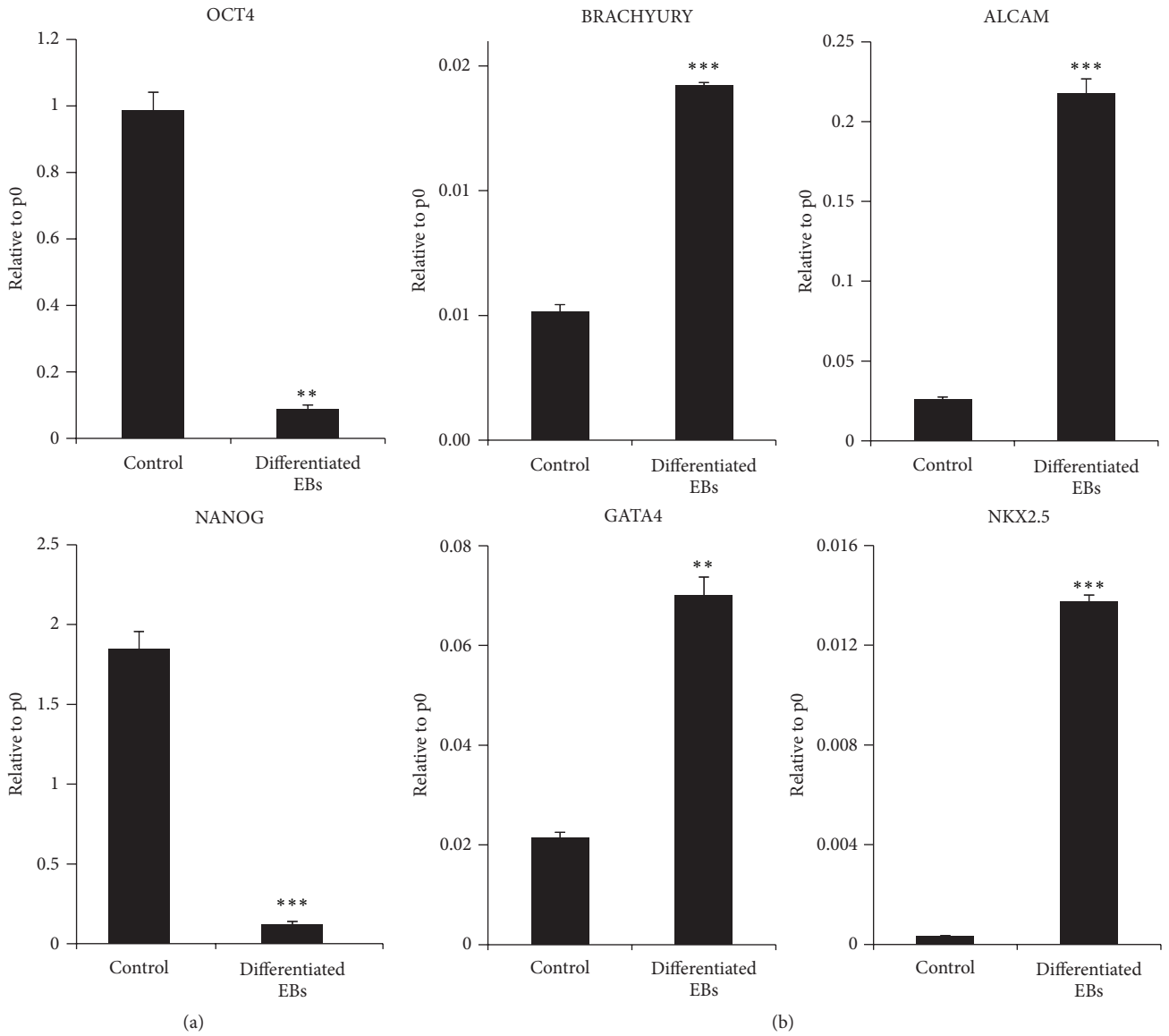

Figure 2: Real-time PCR analysis of differentiated EBs (6 + 4 days of differentiation). OCT4 and NANOG pluripotency markers were downregulated (a), whereas mesodermal (BRACHYURY) and early and later cardiac markers (NKX2.5, GATA4, and ALCAM) were upregulated (b) as compared to the undifferentiated human HUES9 embryonic stem cell line control. Data are expressed as mean ± SEM; $^{**}p < 0.01$, and $^{***}p < 0.001$, differentiated EBs versus undifferentiated control; Student t-test, $n = 2$.

(Supplemental Figure 1, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4298945). The cytoprotective effect of different compounds was compared to simulated ischemic control groups, where cell death was designated as 100 percent.

At the second set of experiments, cardiomyocytes derived from hESCs underwent SI/R procedure similar to other groups. Cell viability was assessed by the above-described method.

2.7. Statistical Analysis. Results are expressed as mean ± SEM. Unpaired t-test and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc tests were used to determine differences in mean values between the experimental groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Cardiac Differentiation of EBs. Real-time quantitative PCR analysis confirmed the cardiac-oriented development of cells in differentiated EBs. Both OCT4 and NANOG pluripotency markers were downregulated in the differentiated EBs as compared to the undifferentiated human HUES9 embryonic stem cell line control (Figure 2). Additionally, the expression of mesodermal (BRACHYURY) and early cardiac

Table 1: Cell death of both differentiated EBs (6 + 4 days of differentiation) and cardiomyocytes derived from hESCs (6 + 24 days of differentiation) exposed to normoxia or SI: representative results obtained from one plate. Data are expressed as mean ± SEM; $p < 0.05$ normoxia versus SI; unpaired t-test, $n = 5$-6 in both groups.

(a) The effect of SI/R on cell death of differentiated EBs derived from hESCs (6 + 4 days of differentiation)

Group	Mean RFU ± SEM	p value
Normoxia	1141 ± 69.83	$p = 0.0019$
SI	3624 ± 516.2	(unpaired t-test)

(b) The effect of SI/R on cell death of hESC-derived cardiomyocytes (6 + 24 days of differentiation)

Group	Mean RFU ± SEM	p value
Normoxia	65817 ± 10272	$p = 0.0027$
SI	137045 ± 17555	(unpaired t-test)

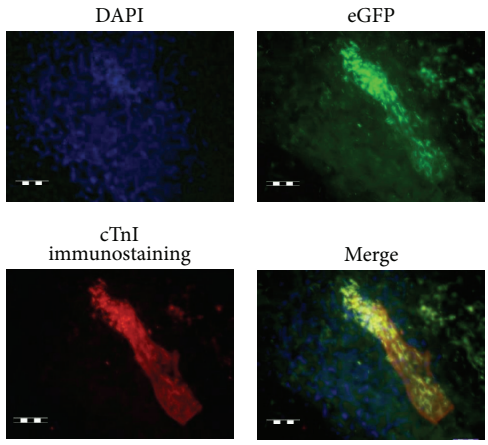

Figure 3: Colocalization of cTnI and enhanced CAG-driven eGFP signals in cardiomyocytes derived from hESCs (6 + 24 days of differentiation). Scale bar: 200 μm.

markers (NKX2.5 and GATA4) were elevated at this stage of differentiation. Moreover, the cardiac specific ALCAM expression was also upregulated as compared to the control.

The specificity of CAG-driven eGFP expression during cardiac differentiation was also documented. In later stage EBs (6 + 24 days), an enhanced eGFP expression was observed in cTnI positive cardiomyocytes derived from hESCs, as confirmed by the colocalization of both signals obtained by immunostaining of cTnI (Figure 3).

3.2. Cell Viability after SI/R. The cytoprotective effect of the NO-donor SNAP that activates soluble guanylate cyclase (sGC) was tested in the model of simulated ischemia (SI). Reperfusion-induced cell death was monitored in differentiated EBs as well as in cardiomyocytes derived from hESCs. We found that SI followed by reperfusion caused significantly higher cell death in differentiated EBs or cardiomyocytes than in time-matched controls kept under normoxic conditions (Table 1).

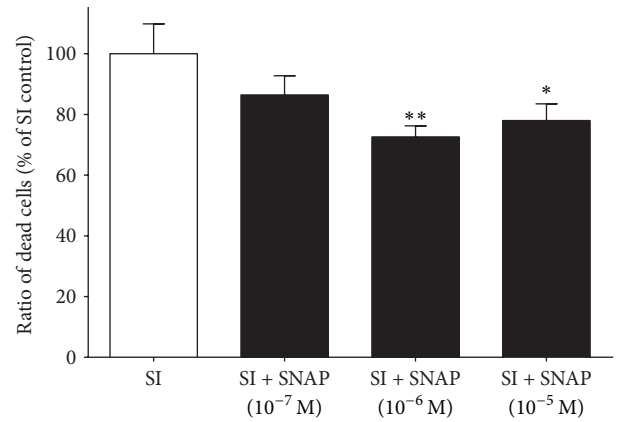

Figure 4: Effect of SNAP on cell viability of differentiated EBs derived from hESCs (6 + 4 days of differentiation). SNAP was applied during SI. Data are expressed as mean ± SEM; $^{*}p < 0.05$ SNAP treated versus SI control; $^{**}p < 0.01$ SNAP treated versus SI control; one-way ANOVA followed by Fischer LSD post hoc test, $n = 8$ in each group.

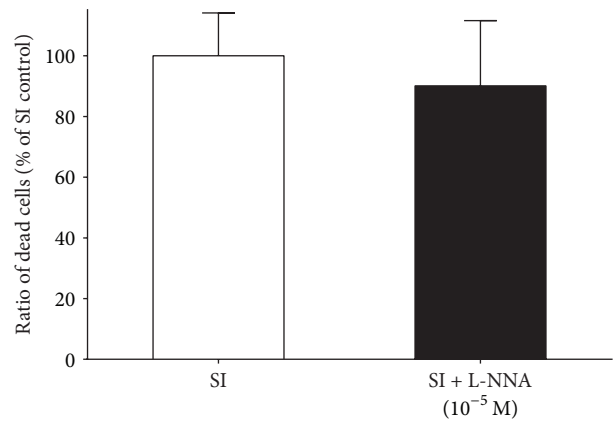

Figure 5: Effect of L-NNA on cell viability of differentiated EBs derived from hESCs (6 + 4 days of differentiation). L-NNA was applied during SI. Data are expressed as mean ± SEM; unpaired t-test, $n = 4$ in each group.

In differentiated EBs, cell death was significantly decreased by SNAP in a concentration-dependent manner when applied during the SI period (Figure 4).

The endogenous NO production was abolished by the administration of the nonselective NOS inhibitor L-NNA at 10^{-5} M concentration. The presence of L-NNA alone did not influence cell death after SI (Figure 5). BNP, an activator of particulate guanylate cyclase tested at 10^{-9}, 10^{-8}, and 10^{-7} M concentrations, did not influence cell death significantly (Figure 6).

In order to confirm the cytoprotective effect of SNAP, it was administered to hESC-derived cardiomyocytes. SNAP, as compared to vehicle, attenuated cell death in the hESC-derived cardiomyocytes at 10^{-6} M concentration (Figure 7).

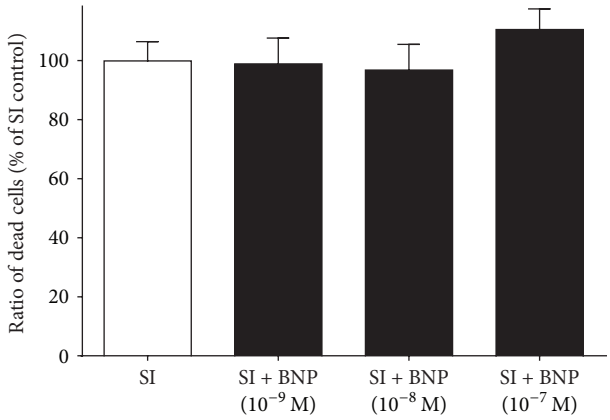

Figure 6: Effect of BNP on cell viability of differentiated EBs derived from hESCs (6 + 4 days of differentiation). BNP was applied during SI. Data are expressed as mean ± SEM, $n = 8$ in each group.

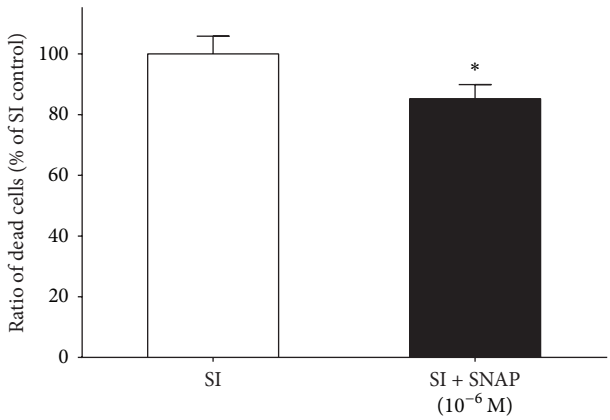

Figure 7: Effect of SNAP on cell viability of hESC-derived cardiomyocytes (6 + 24 days of differentiation). SNAP was applied during SI. Data are expressed as mean ± SEM; $^{*}p < 0.05$ SNAP treated versus SI control; one-way ANOVA followed by Fischer LSD post hoc test, $n = 8$ in each group.

4. Discussion

Here, we show for the first time that the NO-donor SNAP is able to provide cardiocytoprotective effect against SI/R-induced cell death in a model of differentiated human EBs as well as in contracting cardiomyocytes derived from hESCs. Moreover, this study is the first demonstration of a hESC-based drug-screening platform that is able to identify cardioprotective compounds against SI/R injury.

Currently used primary cardiomyocyte-based or cardiomyoblast cell line-based drug-screening platforms have many limitations for their utilization. The disadvantages of these assays strongly limit their applicability in preclinical research. Primary neonatal cardiomyocytes are widely used to test cardioprotective drugs; however, results may vary due to culture variability introduced by the isolation procedure or the limited proliferation [27]. Adult cardiomyocytes are suitable to study individual cells, and particularly the pharmacological properties of different ion channels can be examined using this model. However, such cells require special conditions during culturing, especially laminin coated surface, which is indispensable for proper attachment and cell survival [2]. Cell lines are preferable test systems for drug screening, however presenting several limitations. The H9c2 cardiomyoblast line shows different phenotype even from neonatal and adult cardiac myocytes, and additionally spontaneous electric activity and sarcomeric structure cannot be observed in them [28]. Since the translational value of hESC-based platforms for representing human conditions may be substantially higher than the abovementioned cell culture models, here we validated a hESC-derived cardiomyocyte-based drug-screening platform to test cardioprotective agents by using the well-known cardiocytoprotective NO-donor SNAP [14, 15, 29–32].

In our experiments, we found that the NO-donor SNAP increased the viability of differentiated EBs derived from hESCs, subjected to SI/R. This protective effect was dose-dependent, showing the same dose response characteristics as found in neonatal rat cardiomyocytes [14] exposed to SI/R. Our research had shown that NO exerts direct cardiocytoprotection via cGMP-PKG signaling pathway. Moreover, another study on normoxic, neonatal rat cardiac myocyte showed that SNAP caused significant necrosis at 1 mM concentration (no significant changes at 10 and 100 μM), but a bell-shaped effect on apoptosis was observed, that is, significant increase at 100 μM and no effect at 10 μM and 1 mM [33].

Similar results were presented by our group on mouse embryonic stem cell-derived cardiomyocytes, where NO had concentration-dependent direct cytoprotective action and soluble guanylate cyclase, PKG, and KATP channels play a role in the downstream pathway of SNAP-induced cytoprotection [15]. Hsieh et al. showed recently that short pretreatment with NO donor ($NaNO_2$) combined with hypoxia protects neonatal cardiac myocyte but not cardiac fibroblast from hypoxic injury, and apoptosis decreased in human ES-derived cardiac myocytes [34]. These results further suggest that NO donors may protect stem cells implanted into ischemic areas of the myocardium. Additionally, it has been shown that NO is able to facilitate ESC differentiation and cardiomyogenesis [35]. To investigate if endogenous NO affects ischemic tolerance of differentiated EBs derived from hESCs, a nonspecific NOS inhibitor, L-NNA, was given during SI. L-NNA did not affect cell viability after SI/R injury of differentiated EBs, indicating that ischemic tolerance of these cells is not altered by endogenous NO. However, local NO concentration largely depends on the ratio of NO and local superoxide production (see, for reviews, Ferdinandy and Schulz, 2003; Andreadou et al., 2015) [36, 37]. Bioavailability of NO also depends on NO synthase (NOS) expression, and previously it was shown that NOS expression has a developmental stage-dependent expression pattern in rat mouse embryos. Mouse EBs treated with NOS inhibitor were prone to less differentiate after embryonic age D-13. Here, we have shown, in human ES-derived cardiac myocytes, that NO has a direct cytoprotective effect in early and more differentiated stage.

Intracellular biosynthesis of cGMP can be catalyzed by both soluble (sGC) and particulate (pGC) guanylate cyclase.

The activation of the same intracellular signaling pathway was achieved by administration of BNP (activator of pGC) which is an effective cardioprotective peptide. We have previously shown that exogenously administered BNP reduced cell death after SI/R injury in neonatal rat cardiomyocyte cultures [14] and reduced infarct size at nM concentration range in rat hearts [38]. In agreement with our previous finding in a mouse ESC-derived cardiomyocyte model [15], the exogenously administered BNP was not protective against SI/R-induced cell death in differentiated EBs. The lack of protection may be attributed to the low expression of the NPR-A receptor (specific for BNP) during hESC differentiation [39]. In another study, BNP significantly increased the number of apoptotic neonatal cardiac myocytes subjected to mild hypoxic stimulus (3% O_2) in a concentration-dependent manner (0.01; 0.1; 1 μmol/L); however, it had no significant effect on the number of necrotic cells [40].

hESC differentiation with suspension EB method results in around 30% cardiac myocyte population [41]; however, the efficacy of cardiac differentiation may vary depending on stem cell line or experimental circumstances [7, 8, 42–44]. Here, in our study, we were able to selectively investigate cardiac myocyte population in the embryonic bodies by using eGFP overexpression, which enhances the specificity of screening platform.

In summary, here, we demonstrate for the first time that SNAP, but not BNP, protects differentiated human EBs or cardiomyocytes against SI/R injury. Our findings also suggest that hESC-derived differentiated EBs containing early cardiac committed cells may serve as a screening platform for the discovery of cardiocytoprotective molecules; additionally, the present platform is suitable for testing the cardiac myocyte population of the EBs.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Ágota Apáti and Balázs Sarkadi provided hESC-derived EBs. János Pálóczi and Zoltán V. Varga cultured EBs and performed SI/R experiments and viability measurements. Ágota Apáti and Kornélia Szebényi carried out the qRT-PCR experiments and immunostaining of hESC-derived cardiomyocytes. János Pálóczi, Zoltán V. Varga, and Kornélia Szebényi analyzed and interpreted the data, made the statistical analysis, and prepared the figures. János Pálóczi, Anikó Görbe, and Péter Ferdinandy drafted the paper. Balázs Sarkadi, Tamás Csont, Rosalinda Madonna, Raffaele De Caterina, and Thomas Eschenhagen participated in the study design and coordination and critically revised the paper. Anikó Görbe and Péter Ferdinandy conceived the study and coordinated and supervised it. All authors read and approved the final paper. Péter Ferdinandy and Anikó Görbe contributed equally to this work.

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References

[1] A. Hansen, A. Eder, M. Bönstrup et al., "Development of a drug screening platform based on engineered heart tissue," *Circulation Research*, vol. 107, no. 1, pp. 35–44, 2010.

[2] E. A. Woodcock and S. J. Matkovich, "Cardiomyocytes structure, function and associated pathologies," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 9, pp. 1746–1751, 2005.

[3] D. A. Terrar, C. M. Wilson, S. G. Graham, S. M. Bryant, and B. M. Heath, "Comparison of guinea-pig ventricular myocytes and dog Purkinje fibres for in vitro assessment of drug-induced delayed repolarization," *Journal of Pharmacological and Toxicological Methods*, vol. 56, no. 2, pp. 171–185, 2007.

[4] C. Xu, S. Police, N. Rao, and M. K. Carpenter, "Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells," *Circulation Research*, vol. 91, no. 6, pp. 501–508, 2002.

[5] S. E. Harding, N. N. Ali, M. Brito-Martins, and J. Gorelik, "The human embryonic stem cell-derived cardiomyocyte as a pharmacological model," *Pharmacology and Therapeutics*, vol. 113, no. 2, pp. 341–353, 2007.

[6] H. Liang, M. Matzkies, H. Schunkert et al., "Human and murine embryonic stem cell-derived cardiomyocytes serve together as a valuable model for drug safety screening," *Cellular Physiology and Biochemistry*, vol. 25, no. 4-5, pp. 459–466, 2010.

[7] O. Caspi, I. Huber, I. Kehat et al., "Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts," *Journal of the American College of Cardiology*, vol. 50, no. 19, pp. 1884–1893, 2007.

[8] L. W. van Laake, R. Passier, J. Monshouwer-Kloots et al., "Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction," *Stem Cell Research*, vol. 1, no. 1, pp. 9–24, 2007.

[9] W. Dai, L. J. Field, M. Rubart et al., "Survival and maturation of human embryonic stem cell-derived cardiomyocytes in rat hearts," *Journal of Molecular and Cellular Cardiology*, vol. 43, no. 4, pp. 504–516, 2007.

[10] R. J. Hassink, A. B. de La Rivière, C. L. Mummery, and P. A. Doevendans, "Transplantation of cells for cardiac repair," *Journal of the American College of Cardiology*, vol. 41, no. 5, pp. 711–717, 2003.

[11] D. K. Singla, "Embryonic stem cells in cardiac repair and regeneration," *Antioxidants and Redox Signaling*, vol. 11, no. 8, pp. 1857–1863, 2009.

[12] W.-H. Zimmermann and T. Eschenhagen, "Embryonic stem cells for cardiac muscle engineering," *Trends in Cardiovascular Medicine*, vol. 17, no. 4, pp. 134–140, 2007.

[13] H. Qiao, S. Surti, S. R. Choi et al., "Death and proliferation time course of stem cells transplanted in the myocardium," *Molecular Imaging and Biology*, vol. 11, no. 6, pp. 408–414, 2009.

[14] A. Gorbe, Z. Giricz, A. Szunyog et al., "Role of cGMP-PKG signaling in the protection of neonatal rat cardiac myocytes subjected to simulated ischemia/reoxygenation," *Basic Research in Cardiology*, vol. 105, no. 5, pp. 643–650, 2010.

[15] A. Görbe, Z. V. Varga, J. Pálóczi et al., "Cytoprotection by the NO-donor SNAP against ischemia/reoxygenation injury in mouse embryonic stem cell-derived cardiomyocytes," *Molecular Biotechnology*, vol. 56, no. 3, pp. 258–264, 2014.

[16] R. M. Bell and D. M. Yellon, "The contribution of endothelial nitric oxide synthase to early ischaemic preconditioning: the lowering of the preconditioning threshold. An investigation in eNOS knockout mice," *Cardiovascular Research*, vol. 52, no. 2, pp. 274–280, 2001.

[17] D. S. Burley and G. F. Baxter, "B-type natriuretic peptide at early reperfusion limits infarct size in the rat isolated heart," *Basic Research in Cardiology*, vol. 102, no. 6, pp. 529–541, 2007.

[18] T. I. Orbán, Á. Apáti, A. Németh et al., "Applying a'double-feature' promoter to identify cardiomyocytes differentiated from human embryonic stem cells following transposon-based gene delivery," *STEM CELLS*, vol. 27, no. 5, pp. 1077–1087, 2009.

[19] K. Szebényi, A. Péntek, Z. Erdei et al., "Efficient generation of human embryonic stem cell-derived cardiac progenitors based on tissue-specific enhanced green fluorescence protein expression," *Tissue Engineering—Part C: Methods*, vol. 21, no. 1, pp. 35–45, 2015.

[20] J. Nichols, B. Zevnik, K. Anastassiadis et al., "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4," *Cell*, vol. 95, no. 3, pp. 379–391, 1998.

[21] Y.-H. Loh, Q. Wu, J.-L. Chew et al., "The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells," *Nature Genetics*, vol. 38, no. 4, pp. 431–440, 2006.

[22] I. Chambers, D. Colby, M. Robertson et al., "Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells," *Cell*, vol. 113, no. 5, pp. 643–655, 2003.

[23] H. J. Fehling, G. Lacaud, A. Kubo et al., "Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation," *Development*, vol. 130, no. 17, pp. 4217–4227, 2003.

[24] F. A. Stennard, M. W. Costa, D. A. Elliott et al., "Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart," *Developmental Biology*, vol. 262, no. 2, pp. 206–224, 2003.

[25] V. L. F. Linhares, N. A. S. Almeida, D. C. Menezes et al., "Transcriptional regulation of the murine Connexin40 promoter by cardiac factors Nkx2-5, GATA4 and Tbx5," *Cardiovascular Research*, vol. 64, no. 3, pp. 402–411, 2004.

[26] H. Hirata, Y. Murakami, Y. Miyamoto et al., "ALCAM (CD166) is a surface marker for early murine cardiomyocytes," *Cells Tissues Organs*, vol. 184, no. 3-4, pp. 172–180, 2007.

[27] K. B. Walsh, T. C. Rich, and Z. J. Coffman, "Development of a high-throughput assay for monitoring cAMP levels in cardiac ventricular myocytes," *Journal of Cardiovascular Pharmacology*, vol. 53, no. 3, pp. 223–230, 2009.

[28] B. Ózsvári, L. G. Puskás, L. I. Nagy et al., "A cell-microelectronic sensing technique for the screening of cytoprotective compounds," *International Journal of Molecular Medicine*, vol. 25, no. 4, pp. 525–530, 2010.

[29] A. Nakano, G. S. Liu, G. Heusch, J. M. Downey, and M. V. Cohen, "Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning," *Journal of Molecular and Cellular Cardiology*, vol. 32, no. 7, pp. 1159–1167, 2000.

[30] J. Pálóczi, Z. V. Varga, Á. Apáti et al., "NO-donor SNAP protects human embryonic stem cell-derived cardiomyocytes against ischemia/reperfusion injury," in *Proceedings of the Cost Action BM1005 Gasotransmitters Meeting*, Conference Abstract, Messina, Italy, September 2015, http://real.mtak.hu/28715/.

[31] H. Takano, X.-L. Tang, Y. Qiu, Y. Guo, B. A. French, and R. Bolli, "Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism," *Circulation Research*, vol. 83, no. 1, pp. 73–84, 1998.

[32] Z. Xu, X. Ji, and P. G. Boysen, "Exogenous nitric oxide generates ROS and induces cardioprotection: involvement of PKG, mitochondrial KATP channels, and ERK," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 286, no. 4, pp. H1433–H1440, 2004.

[33] T. Uchiyama, H. Otani, T. Okada et al., "Nitric oxide induces caspase-dependent apoptosis and necrosis in neonatal rat cardiomyocytes," *Journal of Molecular and Cellular Cardiology*, vol. 34, no. 8, pp. 1049–1061, 2002.

[34] A. Hsieh, N. T. Feric, and M. Radisic, "Combined hypoxia and sodium nitrite pretreatment for cardiomyocyte protection in vitro," *Biotechnology Progress*, vol. 31, no. 2, pp. 482–492, 2015.

[35] S. Kanno, P. K. M. Kim, K. Sallam, J. Lei, T. R. Billiar, and L. L. Shears, "Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 33, pp. 12277–12281, 2004.

[36] P. Ferdinandy and R. Schulz, "Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning," *British Journal of Pharmacology*, vol. 138, no. 4, pp. 532–543, 2003.

[37] I. Andreadou, E. K. Iliodromitis, T. Rassaf, R. Schulz, A. Papapetropoulos, and P. Ferdinandy, "The role of gasotransmitters NO, H_2S and CO in myocardial ischaemia/reperfusion injury and cardioprotection by preconditioning, postconditioning and remote conditioning," *British Journal of Pharmacology*, vol. 172, no. 6, pp. 1587–1606, 2015.

[38] S. P. D'Souza, D. M. Yellon, C. Martin et al., "B-type natriuretic peptide limits infarct size in rat isolated hearts via KATP channel opening," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 284, no. 5, pp. H1592–H1600, 2003.

[39] E. M. Abdelalim and I. Tooyama, "BNP signaling is crucial for embryonic stem cell proliferation," *PLoS ONE*, vol. 4, no. 4, Article ID e5341, 2009.

[40] T.-N. Wang, Y.-K. Ge, J.-Y. Li, X.-H. Zeng, and X.-X. Zheng, "B-type natriuretic peptide enhances mild hypoxia-induced apoptotic cell death in cardiomyocytes," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 6, pp. 1084–1090, 2007.

[41] L. Zwi-Dantsis and L. Gepstein, "Induced pluripotent stem cells for cardiac repair," *Cellular and Molecular Life Sciences*, vol. 69, no. 19, pp. 3285–3299, 2012.

[42] R. Madonna, P. Ferdinandy, R. De Caterina, J. T. Willerson, and A. J. Marian, "Recent developments in cardiovascular stem cells," *Circulation Research*, vol. 115, no. 12, pp. e71–e78, 2014.

[43] R. Madonna, L. W. V. Laake, S. M. Davidson, F. B. Engel, D. J. Hausenloy, and S. Lecour, "Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure," *European Heart Journal*, Article ID ehw113, 2016.

[44] R. Madonna, F. B. Engel, S. M. Davidson et al., "Stem cell aging and age-related cardiovascular disease: perspectives of treatment by ex-vivo stem cell rejuvenation," *Current Drug Targets*, vol. 16, no. 8, pp. 780–785, 2015.

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Review Article

The Role of Oxidative Stress in Myocardial Ischemia and Reperfusion Injury and Remodeling: Revisited

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Oxidative and reductive stress are dual dynamic phases experienced by the cells undergoing adaptation towards endogenous or exogenous noxious stimulus. The former arises due to the imbalance between the reactive oxygen species production and antioxidant defenses, while the latter is due to the aberrant increase in the reducing equivalents. Mitochondrial malfunction is the common denominator arising from the aberrant functioning of the rheostat that maintains the homeostasis between oxidative and reductive stress. Recent experimental evidences suggest that the maladaptation during oxidative stress could play a pivotal role in the pathophysiology of major cardiovascular diseases such as myocardial infraction, atherosclerosis, and diabetic cardiovascular complications. In this review we have discussed the role of oxidative and reductive stress pathways in the pathogenesis of myocardial ischemia/reperfusion injury and diabetic cardiomyopathy (DCM). Furthermore, we have provided impetus for the development of subcellular organelle targeted antioxidant drug therapy for thwarting the deterioration of the failing myocardium in the aforementioned cardiovascular conditions.

1. Introduction

Cardiovascular diseases (CVD) generally denote disorders of the heart and blood vessels that include coronary heart disease, cerebrovascular disease, and other vascular conditions, and this accounts for the leading cause of death and disability in the world. Interestingly, four out of five CVD deaths are due to heart attack and stroke [1]. The primary approach in understanding the nature of the disease and improving the treatment is to retrospectively study the molecular and cellular signaling mechanisms. Myocardium experiences oxidative challenge in all forms of heart diseases and the oxidative modified molecules not only act as the determinant in the extent of injury but may be useful in the diagnostic and prognostic measures where they can serve as specific biomarkers. In general, cardiomyocytes possess firm defense mechanisms to counter the oxidative challenge via enzymatic and nonenzymatic molecules [2]. It is generally perceived that molecules which neutralize the free radicals generated in the tissues could be beneficial in ameliorating several pathologies, and hence this notion provides the foundation to develop these molecules as drug candidates in combating oxidative tissue mediated tissue injury.

However, a recent clinical study emphasized the significance and pathogenic consequences of proteotoxicity and proteinopathy in the failing human hearts. A homeostatic balance (proteostasis) between synthesis and degradation of defective proteins is crucial for sustaining the health of dynamically active cardiomyocytes [3]. Hence the accumulation of reducing molecules that resulted in the reductive stress (abnormal increase in reducing equivalents) could lead to dysfunction of the endoplasmic reticulum (an organelle involved in the proteins synthesis and folding) and proteotoxicity [3, 4]. Similarly, reactive oxygen species (ROS), a key player that induces oxidative stress, participate not only in the pathological roles in the heart diseases, but also in the physiological function that may regulate survival and demise of the cardiomyocytes [2]. Myocardial adaptation to oxidative/reductive stress is a crucial mechanism evolved for the survival of heart from different disorders. Herein we have discussed the current understanding regarding the proximal relationship between oxidative stress, reductive stress, and

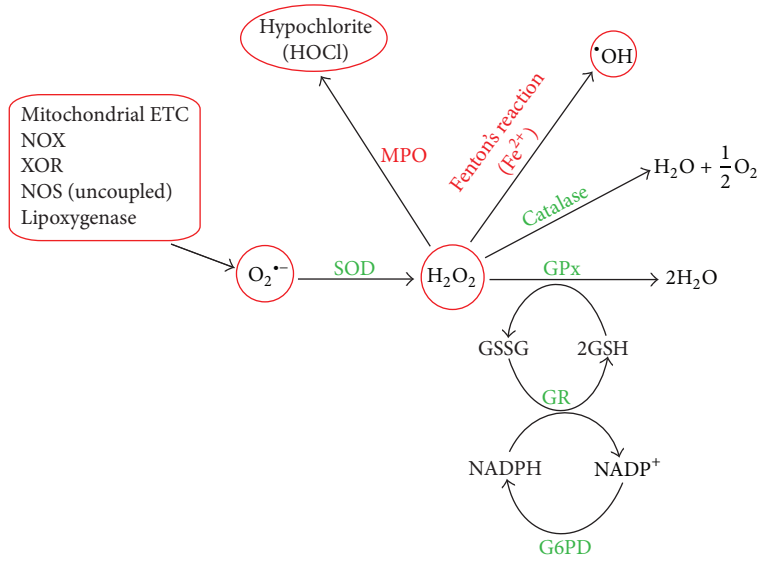

FIGURE 1: This scheme shows the sources for $O_2^{\bullet-}$ generation and its detoxification by endogenous antioxidants. $O_2^{\bullet-}$ is dismutated by SOD resulting in the generation of H_2O_2. Then H_2O_2 is detoxified via catalase or glutathione peroxidase (GPx) involving GSH. GSSG is recycled with the aid of glutathione reductase (GR). The reducing equivalents are recycled via glucose-6-phosphate dehydrogenase (G6PD). Myeloperoxidase (MPO) utilizes H_2O_2 as substrate to produce the powerful oxidant, HOCl, which damages the biomolecules such as lipids, proteins, and nucleic acids. Similarly hydroxyl radical ($^{\bullet}OH$) formed via Fenton's reaction also attacks the biomolecules.

the corresponding cellular adaptation process in the diseased heart, chiefly associated with ischemia/reperfusion injury and diabetic cardiomyopathy (DCM).

2. Oxidative Stress in Cardiovascular Diseases

The oxidant and antioxidant imbalance in the cardiomyocytes that favors the accumulation of oxidants, leading to cellular damage, constitutes oxidative stress [5]. Generally cells will initiate an adaptive system to protect them against the dangerous effects of oxidative stress, but when the oxidant concentration often exceeds the cell's adaptive capacity, the cell will experience exacerbated oxidative stress. There exist misnomers when referring to the terms such as oxidative stress and free radical damage, which are often interchanged. The term "oxidative stress" is used to describe the imbalances in redox couples such as reduced to oxidized glutathione (GSH/GSSG) or NADPH/$NADP^+$ ratios. While the term "free radical damage" denotes the alterations in the structure and function of the biomolecules such as proteins, lipids, and deoxyribonucleic acid (DNA). The exaggerated generation of reactive free radicals from different metabolites is generally inactivated in the cells by several endogenous antioxidants, and it utilizes the redox couples to regenerate the enzyme or assist in these enzymatic reactions. Thus, the terms "oxidative stress" and "free radical damage" are not synonymous in the precise sense, but their biological effects are interdependent.

Oxidative stress related to heart was evident in patients undergoing by-pass surgery, where oxidized glutathione (GSSG) accumulation was found to be negatively correlated with the functional recovery of the myocardium [6]. Currently, the ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) is used as a marker of oxidative stress, considering the fact that GSH, a tripeptide (γ-glutamyl-cysteinylglycine) containing free thiol group, is one of the most important scavengers of ROS in the heart [7, 8]. Furthermore, it can act as an antioxidant in association with glutathione peroxidase (GPx) catalyzed reaction by providing reducing equivalents and thereby helping in the reduction of lipid hydro peroxides (Figure 1). Commonly observed GSH/GSSG ratio in the mammalian heart is relatively high [9], with a 50–70% decrease observed under oxidative stress [10] conditions. It is a general notion that the oxidants are harmful, having no positive or regulatory roles in the biological processes. At low or moderate concentrations, oxidants can act as weapons for the host defense system and this process governs the growth and development of tissues. Thus, tissues have to maintain a tight balance between prooxidant and antioxidant level not only in the organ and tissues, but also in specific cell types. Moreover various cell organelles such as mitochondria, peroxisomes, and endoplasmic reticulum have their own localized antioxidant system. Recently, role of these antioxidant systems is being investigated for understanding the concept of redox signaling and homeostasis towards the pathogenesis of cardiovascular diseases [11].

Oxidative stress is more often associated with elevated levels of ROS or reactive nitrogen species (RNS) in the cellular and subcellular levels [5]. However, ROS/RNS in the suboptimal level can act as signaling molecules in maintaining the cardiovascular function [12]. On the other hand, increased ROS/RNS levels can induce pathology by damaging lipids, proteins, and DNA [13]. Thus, ROS depending on their concentration, the site of production, and the overall redox equilibrium of the cell will determine its biological action (beneficial or deleterious) in the tissues. Cardiovascular pathology

associated with oxidative stress is observed in several cardiac diseases like ischemia/reperfusion injury [14, 15], atherosclerosis [16], diabetic cardiomyopathy, and so forth [17].

3. ROS/RNS in Myocardial Ischemia Reperfusion Injury

ROS include radicals, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen (O^-), and hypochlorous acid (HClO). RNS includes nitroxyl anion (NO^-), nitrosonium cation (NO^+), higher oxides of nitrogen, peroxynitrite (NO_3^-), S-nitrosothiols, and dinitrosyl iron complexes [5]. Peroxynitrite is generated when $O_2^{\bullet -}$ reacts with $NO^{\bullet}$ and facilitates both oxidizing and nitrating reactions of the biomolecules [18, 19].

According to Wolff the ratio of oxygen delivery to oxygen consumption in the heart is around 1.6 to 1.8 times higher than other tissues, suggesting an excess consumption of oxygen in the myocardium [20]. Molecular oxygen when it accepts an electron will form $O_2^{\bullet -}$ and this univalent reduction can be mediated by either enzymatic (by the action of NADPH oxidase and xanthine oxidases) [21] or nonenzymatic reactions (with redox active compounds such as semiubiquinone of the mitochondrial electron transport chain) [22]. The resultant $O_2^{\bullet -}$ is converted to hydrogen peroxide (H_2O_2), either by the catalytic action of superoxide dismutase (SOD) or through the spontaneous dismutation [19]. Further interaction of H_2O_2 with Fe^{2+} will result in the formation of highly reactive hydroxyl radical ($OH^{\bullet}$) via Fenton's reaction (Figure 1). In addition myeloperoxidase (MPO), an important constituent of leukocytes (neutrophils and macrophages) utilizes H_2O_2 as their substrate for the generation of hypochlorite (HOCl) an potent oxidant that attacks biological macromolecules such as lipids, proteins, DNA, and glycoproteins [23].

ROS production and release during myocardial ischemia/reperfusion injury was confirmed by trapping these free radicals using nitrone DMPO [24] and alpha-phenyl N-tert-butyl nitrone spin trap probes [25] and measured by electron spin resonance spectrometer. Numerous preclinical [26, 27] and clinical studies [28] have demonstrated that treatment with antioxidants could render cardioprotection and therefore emphasize the involvement of ROS mediated stress injury in the pathology of myocardial ischemia/reperfusion injury (Table 1).

Among the well-known cellular sources of ROS, mitochondria have emerged as primary source due to their ability to sense the cellular oxygen levels. In fact, in the healthy myocardium, ROS is an unintended byproduct of mitochondrial respiration, where its concentration is tightly controlled to low steady state level by SOD [29]. However, in the ischemic heart, $NO^{\bullet}$, $O_2^{\bullet -}$, and NO_3^- formation are elevated after reperfusion [30]. The electron leakage from complexes I and III of the electron transport chain (ETC) is primarily responsible for $O_2^{\bullet -}$ generation in the mitochondria [31]. This in turn damages cardiolipin [32], the phospholipid component of the inner mitochondrial membrane resulting in the destabilization of ETC complexes and supercomplexes, ultimately leading to decreased ATP production and disorganizing the dimeric ADP/ATP carrier functional capacity [33]. Thus, ROS mediated mitochondrial dysfunction and the sequence of biochemical events following revascularization of ischemic area are the cardinal features of the myocardial ischemia/reperfusion injury.

Palmer et al. showed that the heart comprises two spatially distinct mitochondria, namely, interfibrillar (IFM) and subsarcolemmal mitochondria (SSM) [34]. We have demonstrated that IFM and SSM populations respond differently to the oxidative stress induced by myocardial ischemia/reperfusion [35]. This difference in the activities of IFM and SSM towards ROS may be partly due to their spatial location and the metabolic system that detoxifies the radical. It was shown that certain oxidants like H_2O_2 are capable of diffusing across the mitochondrial membrane into the cytoplasm [36], while $O_2^{\bullet -}$ generated in the mitochondria is unlikely to escape into the cytoplasm, indicating the need for localized antioxidant system in the mitochondrial subcompartments. In fact, recent studies have demonstrated that $O_2^{\bullet -}$ generation was higher in SSM when compared with IFM [37]. In this regard our studies also accede with these observations [38, 39]. Emerging evidences reinforce the concept that ROS could act as regulatory molecules and could protect the myocardium against myocardial ischemia/reperfusion injury [12]. Therefore considering the beneficial effect of ROS, it can be proposed that lower concentration of radicals in IFM during reperfusion phase may possibly activate redox signaling cascade, culminating in the cardioprotection. However, this hypothesis needs to be proven with carefully planned experiments.

4. Physiological and Pathological Role of Cardiac Redox Signaling

Redox sensitive modulation of different cardiac proteins such as receptors, transporters, phosphatases, transcription factors like hypoxia inducible factors (HIF), and nuclear factor-κB (NFκB) are well established in the setting of myocardial ischemia/reperfusion injury [40]. It was found that posttranslational modifications of these proteins by ROS/RNS are responsible for the development of CVD [41, 42]. In particular, the Cys-residues in the cardiac proteins undergo oxidative posttranslational modification by the formation of either cysteine thiols, hydroxylation, or nitration which may determine their structure and function [41]. The redox signaling is integral to the maintenance of cardiomyocyte homeostasis. The postnatal cardiomyocyte differentiation and proliferation depends on the redox activation of phosphatidyl inositol 3-kinase, Akt pathway that in turn modulates the β catenin signaling, whereby regulating the proper cardiac specification, progenitor expansion, and myocardial growth [43].

Bergmann et al. reported the gradual decrease in cardiomyocyte renewal from 1% at adolescent age to <0.3% in advanced age, indicating the ability of cardiomyocytes regeneration in the adult human hearts [44]. In fact, different studies in lower vertebrates [45], neonatal [46], and adult hearts showed that the main source of cardiomyocyte turnover is from the preexisting cardiomyocytes, rather than

Table 1: Clinical trials for evaluating the efficacy of antioxidant based pharmacotherapy in preventing the oxidative stress mediated myocardial tissue damage in cardiovascular diseases.

Drug	Number of subjects	Trial type	Key findings	Reference
N-acetylcysteine (NAC)	98	Double-blind, randomized clinical trial	NAC prevented early remodeling by reducing the level of MMP-2 and MMP-9	[121]
	52	Randomized clinical trial	NAC decreased pump-induced oxidative stress during cardiopulmonary bypass	[28]
Resveratrol	75	Triple-blinded, randomized, parallel, dose-response, and placebo-controlled trial	Resveratrol-rich grape supplement improved the inflammatory and fibrinolytic status in patients who were on statins for primary prevention of CVD	[122]
Rapeseeds	59	Randomized, double-blind, controlled, and crossover study	Intake of a stabilized rapeseed oil enriched in cardioprotective micronutrients prevented the risk of cardiovascular diseases by improving the cholesterol profile and reducing LDL oxidation	[123]
Flavonoids-epicatechin and quercetin	37	Randomized, double-blind, placebo-controlled, and crossover trial	Epicatechin contributed to the cardioprotective effects of cocoa and tea by improving insulin resistance	[124]
Pravastatin	10	Randomized clinical trial	Oral pravastatin reloading before nonemergent coronary artery bypass grafting (CABG) significantly attenuated postoperative inflammation and systemic NO/iNOS concentrations and reduced the myocardial injury	[125]
Magnesium	52	Randomized clinical trial	The extensive treatment of the patients with magnesium influences the cellular response to ischemia and thus induces cardioprotection against oxidative stress	[126]
Coenzyme Q_{10}	51	Randomized clinical trial	Coenzyme Q_{10} supplementation at 300 mg/day significantly enhances antioxidant enzymes activities and lowers inflammation in patients who have coronary artery disease during statin therapy	[127]
Silymarin	102	Randomized trial	The anti-inflammatory and antioxidant effects of silymarin treatment provided protection against reperfusion injury and inflammation after CABG surgery	[128]

from undifferentiated progenitor population [47]. In this direction, it is pertinent to note that thymosin $\beta 4$ (T $\beta 4$), a G-actin ensiling protein involved in plethora of biological functions, has been demonstrated to confer cardiac protection against ischemic insult and promote myocardial regeneration via recruiting HIF-1α and suppression of oxidative stress [48]. Moreover, redox signaling is involved not only in the physiological processes and homeostatic pathways, but also in the pathology like fibrosis (adverse cardiac remodeling), where it plays a role in cell metabolism to regulate growth and survival. S-Nitrosylation of the protein kinase B/Akt has been reported to be inactivated during insulin resistance and cardiac dysfunction [49]. Similarly, another study reported that reversible S-nitrosylation of complex I slows the reactivation of mitochondria during the early phase of the reperfusion, thereby reducing ROS production and limiting oxidative tissue damage [50]. Mitochondrial permeability transition pore (MPTP) modulation is associated with electron transport chain derived ROS mediated redox signaling and thereby influences the cellular mitochondrial function as arbitrator or savior of the cell. Perhaps this fact is more significant when considering the point that one of the major constituents of MPTP, namely, the adenine nucleotide translocase, is known to be affected by $ONOO^-$ [51]. MPTP is important for the maintenance of mitochondrial structure and function and myocyte differentiation [52]. Thus the redox signaling pathways orchestrated by the mitochondria play a pivotal role in the maintenance of cardiomyocyte structure and function in health and diseases.

Retrograde mitochondrial signaling, a pathway of communication from mitochondria to the nucleus, was reported with hypoxia where the cardiomyocytes gets adapted to the hypoxia through ROS released from mitochondria, resulting in the stabilization of HIF-1α and the stimulation of genes responsible for metabolic reprograming towards adapting to the low oxygen tension, and augmentation of collateral circulation via neoangiogenesis [53]. Since myocardium is enriched with mitochondria, ROS emerging from this organelle has been implicated as the key modulator of wide range of cardiomyocyte functions, such as oxygen sensing and mitophagy [54]. Another important physiological role of redox signaling in the myocardium is the regulation of vascular tone by $NO^\bullet$ or H_2O_2 [55]. H_2O_2 plays a key role in vascular function and homeostasis by modifying

the protein thiols where it induces cysteine dimerization (R–S–S–R) via the formation of the unstable intermediate sulfenic acid (R–SOH) [55]. Nitric oxide synthase (NOS) isoforms modulate the availability of $NO^{\bullet}$ levels in cells and tissues [56]. Under physiological oxidative stress, $NO^{\bullet}$ mediates S-nitrosylation of critical protein thiols and thus averts them from further oxidative modifications by ROS, thereby rendering cardioprotection [56].

The pathological role of cardiac redox signaling features contractile and energetic dysfunction, arrhythmia, transcriptional changes, and mitochondrial free radical release, leading to abnormal myocardial calcium homeostasis [57]. Oxidant mediated impairment of ryanodine receptor is associated with the activation of PKA/CaMKII, leading to the disruption of calcium homeostasis [58]. Furthermore, ROS derived from NADPH oxidase (NOX) activation in different cardiac pathologies such as ischemia/reperfusion injury activates cell stress-response signaling network that includes p38 mitogen activated protein kinases (MAPK) and c-Jun NH_2-terminal kinase (JNK) [59]. It is also well established that NOX may have a significant role in stress-induced conditioning such as in ischemic preconditioning, which mediates its cardioprotection by activating prosurvival protein kinases such as Akt and Erk1/2 [60]. The elevated myocardial $NO^{\bullet}$ production during ischemia/reperfusion injury has been associated with ventricular arrhythmia and increased infarct size via modulation of protein-S-nitrosylation [61].

Mitochondrial dysfunction is considered to be the prominent feature of myocardial ischemia/reperfusion injury as this cell organelle is the major contributor of ROS as well as the major target for ROS inflicted damage. During sustained mitochondrial dysfunction, the damaged mitochondria are eliminated by mitophagy. Failure of mitophagy can lead to the persistent loss of calcium homeostasis, excess production of ROS, impaired cellular energetics and ATP production, and culminating in the cell death [61]. Simultaneously, the dysfunctional mitochondria also generate signals to induce stress response such as induction of mitochondrial heat shock proteins to augment mitophagy and promote mitochondrial biogenesis [62].

5. Reductive Stress in Cardiovascular Diseases

The current approach to understand the cardiovascular pathology is confined to oxidative stress that may occur as a result of augmented ROS generation and/or reduced production of antioxidants. However, with the reversal of prooxidant to antioxidant status results in the accumulation of reducing equivalents and this will cause reductive stress, a notion initially demonstrated in mice expressing the human mutant αB-crystallin [63]. Thus, too much of oxidative radicals or reductive species will disrupt the normal physiological function of cells, which underscores the need to strike a balance between prooxidant and antioxidant concentration for proper cellular function. The concept of reductive stress as a potential contributor to heart failure development [64] and progression is further strengthened by the findings of Zhang and his coworkers [65], wherein they showed the development of cardiomyopathy is more profound in cardiac-specific overexpression of heat shock protein 27 (Hsp27) transgenic mice. The function of Hsp27 is primarily to render cardioprotection via its antioxidative functions. In addition, myocardial ischemia/reperfusion injury is reported to be associated with a hypoxic state that results in an increased $NADH/NAD^+$ ratio, leading to a reductive cytosolic environment [66].

The aforementioned phenotypic changes that occur during myocardial ischemia/reperfusion injury could be coupled with mitochondrial dysfunction where GSH-mediated reductive stress in mitochondria is corroborated by decreased expression of redox biosensors, mitochondrial reduction-oxidation proteins, or the oxidation of mitochondrial thioredoxin. Under physiological conditions, GSH has a relatively low redox potential (−240 mV at pH 7.0) and at high intracellular levels (1–13 mm) making it a primary determinant of the cellular redox environment [67]. The pathophysiology of reductive stress varies between cell types as their subcellular compartments have different redox requirements, primarily driven by the reduced (GSH) and oxidized glutathione (GSSG) redox couple [68] and also based on the functional requirement of the organ/tissue. The GSH/GSSG ratio ranges from 30 : 1 to 100 : 1 in the cytosol which results in a redox potential of −290 mV [69]. In an oxidized environment the ratio of redox couple changes in order to achieve a potential difference between −170 and −185 mV. However, in mitochondria, the GSH/GSSG ratios are 20 : 1 to 40 : 1 making the redox potential difference of −250 mV to −280 mV at pH 7.8. Recently, Korge et al. showed that reductive stress can even influence the release of ROS by modulating reduced glutathione reductase (GR) and thioredoxin (Trx), which can donate electrons to O_2 when the supply of their natural electron acceptors (GSSG for GR and oxidized Trx for Trx) is limited or electron transport to acceptors is inhibited. The above phenotypic changes result in the impairment of ROS-scavenging capacities by GSH/GPx/GR and Prx/Trx/TrxR2 systems [68].

During reductive stress, the electron acceptors in the mitochondria are expected to be reduced and some redox sensitive proteins can donate their electrons to O_2, leading to ROS production. Similarly, the redox regulatory system includes thioredoxin (Trx) and glutaredoxin (Grx) in the cytosol and mitochondria play an important role in providing reducing equivalent for DNA synthesis, maintaining cellular thiol-redox homeostasis, protection against oxidative stress, governing protein folding, and regulation of cell growth/apoptosis [70]. Alterations in GSH homeostasis affect the cellular redox status by disturbing Trx and Grx balance, which accounts for the possible reductive stress, as evidenced in the reperfused heart [68].

One of the important consequences of impaired equilibrium between prooxidants and antioxidant is the opening of MPTP. MPTP's nonselective permeabilization of the inner mitochondrial membrane commenced by the combined forces comprising calcium overload, adenine nucleotide depletion, and oxidative stress, leading to apoptotic cell death during myocardial ischemia/reperfusion injury [71]. Mitochondrial membrane potential regulated by the redox couple will determine the opening and closing of MPTP. Reductive stress mediated cardiac pathology is linked with functional

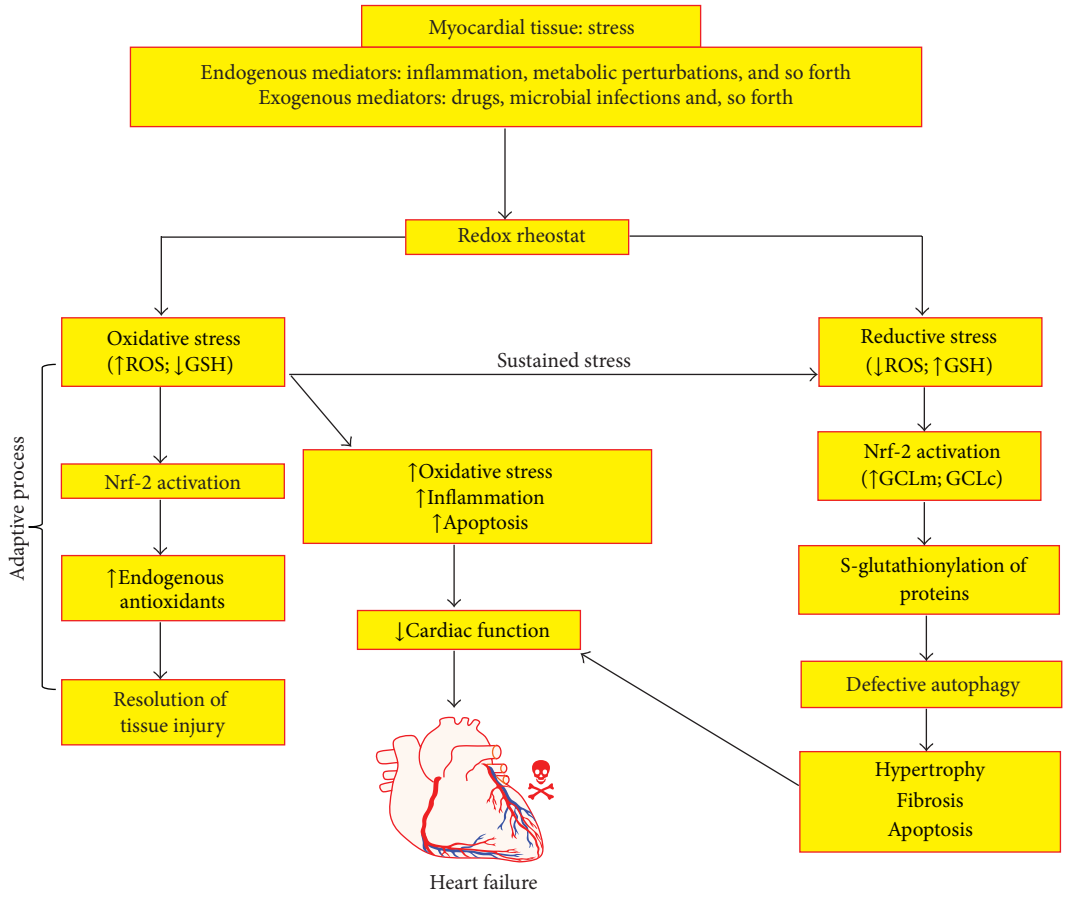

Figure 2: This schematic illustration describes the central role of reductive stress in modulating the myocardial tissue injury. Under normal oxidative stress condition, activation of Nrf-2 results in augmenting endogenous defense system, which aids in the resolution of the tissue injury. However, during sustained oxidative stress condition, Nrf-2 is profoundly activated, which results in the production of increased reducing equivalents such as GSH, which then indulges in posttranslational modification of critical proteins in cardiomyocyte function, whereby affecting their structure and function. These phenotypic events culminate in defective autophagy and drive the cardiomyocytes to become hypertrophic, producing extracellular matrix and committing suicide (apoptosis). All these phenotypic events collectively contribute to the pathogenesis of heart failure. Glutamate-cysteine ligase modifier subunit (GCLm); glutamate-cysteine ligase catalytic subunit (GCLc).

status of cardiac mitochondria and its redox status. There are limited reports available regarding the status of mitochondrial Trx and GRx in IFM and SSM [72, 73]. Therefore, it is imperative to note that the subcellular concentration of redox couple will not only determine the efficacy of the cellular antioxidant system to counter the ROS attack, but also decide the fate of the mitochondrial function. To date, no evidence is available to suggest the differential redox status existing among IFM and SSM that can be helpful in deciding why IFM is more stable than SSM during ischemia/reperfusion injury. The role of reductive stress in pathophysiology of myocardial dysfunction is illustrated in Figure 2.

6. Myocardial Cellular Stress Response and Oxidative Stress

Hearts exhibit a remarkable adaptive response to the physiological and pathological changes in order to maintain contractility. Cardiac dysfunction will occur when the compensatory responses are not sustainable. Under the circumstances of oxidative stress, cells will respond by activating certain signal transduction pathways that promotes their survival and if the cellular strategy failed to accomplish its goal, this will activate cell death signaling pathways. Such cellular prosurvival activities include the production of heat shock proteins, unfolded protein, and DNA damage responses [74]. Previous studies have reported the production of protective heat shock protein during ischemia/reperfusion injury [75] and different conditioning techniques which include preconditioning [76] and postconditioning [77]. One major pathway in cellular death is apoptosis that involves the release of cytochrome *C* from mitochondria that in turn binds to a protein known as Apaf-1 (apoptotic protease activating factor 1) and triggers its oligomerisation and subsequent execution of programmed cell death [2].

Heat shock proteins (Hsp) produced in the low stress level are shown to inhibit the proapoptotic pathway; in particular, Hsp27 binds to cytochrome *C* and prevents its binding to Apaf-1 [78]. Similarly, Hsp90 binds to Apaf-1 and prevents its binding to cytochrome *C*, while Hsp70 prevents

oligomerised Apaf-1 from recruiting pro-caspase-9, thereby imparting cardioprotection. The above observations are further supported by another study, wherein cardiac-specific overexpression of Hsp70 conferred cardioprotection against myocardial ischemia/reperfusion injury [79, 80]. Mitochondria is considered to be the hub for cellular redox processes and a number of mitochondrial stress signals that emerge participate in cell-to-cell communication and stimulate cellular adaptations to exogenous or endogenous stimulus. The oxidative stress in the cells is perceived by mitochondria and initiates release of stress signals that lead to membrane depolarization, alterations in adenine nucleotide levels, ROS production, Ca^{2+} fluxes, and permeability transition pore opening [81]. When a perturbation in the mitochondrial oxidative phosphorylation occurs, it results in the enhanced rate of ROS generation. This process not only reduces the cellular energetic process (decreased ATP production), but also results in greater propensity for generation of ROS. Collectively, the above homeostatic mechanisms are crucial in the determination of cardiomyocyte recovery response during myocardial ischemia/reperfusion injury.

7. Oxidative/Reductive Stress in Cardiac Remodeling and Diabetic Cardiomyopathy

The hallmark pathological characteristics of diabetic cardiomyopathy (DCM) include myocardial hypertrophy [82] and fibrosis [83]. In addition, in diabetic milieu, the metabolic disturbances result in impaired calcium handling, lipotoxicity (increased production of ceramide, etc.) mitochondrial dysfunction, oxidative stress, and altered insulin sensitivity in the cardiomyocytes [84–86]. Furthermore, in subjects with diabetes, increased accumulation of advanced glycated end products (AGEs) in the plasma/serum is reflective of the secondary effect of myocardial collagen cross linkages leading to myocardial stiffness and impaired cardiac relaxation, indicating myocardial remodeling (fibrosis) [87]. A series of pathological events initiated by ROS in diabetic cardiomyopathy revolves around the mitochondrial dysfunction and its impaired functional activities like irregular calcium handling capacity and defective oxidative phosphorylation [88, 89].

Interestingly, human enzyme Aldose reductase (AR), an aldo-keto reductase involved in the development of diabetic cardiovascular complications, has also been implicated in the myocardial tissue injury during ischemia/reperfusion studies, owing to the enhanced generation of ROS [90–92]. In addition, the ROS catalyzes the oxidation of cysteine residues to sulfenic acid which in turn increases the activity of AR [93]. Recent reports have demonstrated the glucose flux via the polyol pathway which can occur during ischemic condition, even in the absence of diabetes [94, 95]. In fact, other studies also reported the increase in AR and succinate dehydrogenase (SDH) activates in the aged hearts [96, 97]. Surprisingly, subsequent studies suggested that mitochondria could play a pivotal role in ischemia/reperfusion injury by opening the MPTP. This in turn is associated with the fact that AR causes increased oxidative stress and depletes GSH, thereby leading to the intracellular accumulation of H_2O_2 and its defective dismutation [98]. Another mechanism which postulates the role of AR in ischemia/reperfusion injury is the decreased phosphorylation of cardiac glycogen synthase kinase-3β which impairs normal mitochondrial function as well as the functional recovery of heart during the stressed condition [99]. In addition, overactivation of polyol pathway comprising of AR and SDH has also been shown to impair the function of critical proteins such as sacro/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and ryanodine receptor (RyR), whose major role is involved in the regulation of cardiac contractility. This phenomenon has also been implicated in the pathogenesis of myocardial ischemia/reperfusion injury [100]. Further, it was confirmed that the contribution of polyol pathway to ischemia/reperfusion injury was due to the accumulation of Fe^{2+} that exacerbates oxidative stress, which leads to the increased lipid peroxidation [101]. On the contrary, few studies suggest that the increase in AR activity during ischemia was a result of augmented $NO^{\bullet}$ and protein kinase C (PKC) signaling pathways that plays a cardioprotective role [100, 102]. However, this notion needs to be confirmed with rigorous studies.

8. Cardiac Remodeling and Ischemia/Reperfusion Injury

Cardiac remodeling comprises of both gross anatomical changes that alter shape, size, and function and cellular modifications like changes in the gene expression and cellular and interstitial and cytoskeletal reorganization [103]. The above-mentioned features may be described as physiological or pathological, mainly to adapt to the diverse cellular stress conditions. For instance, the initial cardiovascular response in higher altitude will be tachycardia with constant stroke volume, but with a slight increase in blood pressure to adapt to the lower partial pressure of oxygen [104]. Similarly, hypertrophy is considered to be the pathological adaptive responses, especially in myocardial infarction, cardiomyopathy, valvular disease, and ischemia/reperfusion injury. Increasing evidence highlights the role of ROS and RNS in the maladaptation of heart during the pathology, through redox signaling process [105]. At least one-quarter of patients who experienced myocardial infarction may develop cardiac remodeling and subsequent heart failure. As a result of an ischemic insult, the number of cardiomyocytes deceases and the surviving myocytes become elongated or hypertrophied in a compensatory process to maintain stroke volume after the loss of contractile tissue. The thickness of the ventricular wall also increases. Similar to cardiomyocytes, other resident cells of the myocardial tissue, such as fibroblast and endothelial cells are also activated by ischemic insult resulting in increased collagen synthesis and fibrosis, thereby contributing to the myocardial remodeling. In general, progression to heart failure is a determinant of the way in which the ventricles counteract the factors that influence the malfunction. ROS is considered to be a major factor in regulation of myocardial remodeling in a number of ways, such that (A) it can act as a signaling molecule in the development of compensatory hypertrophy [106], (B) it may activate matrix metalloproteases (MMPs) that reconfigure the extracellular

Table 2: Evidence for the amelioration of left ventricle (LV) remodeling by dietary antioxidants and other drugs in preclinical studies.

Antioxidant	Principal findings	Reference
Celiprolol	The β-1 blocker at 100 mg/kg that prevented hypoxia induced LV remodeling is in mice, by increasing eNOS	[129]
Fluvastatin	20 mg/kg reduced infarct size and improved the hemodynamics in a rat model of MI	[130]
Pranidipine	The Ca^{2+} channel antagonist at 3 mg/kg improved systolic and diastolic function accompanied by suppressed abnormal gene expression after MI in rats	[131]
Hydrogen sulfide	Exerts antioxidant effects on left ventricular remodeling in rat model of passive smoking via PI3K/Akt-dependent activation of Nrf2 signaling	[132]
Captopril	In patients with anterior MI, it improved left ventricular remodeling and prevented its enlargement, better than digitalis	[133]
Indacaterol + metoprolol	Indacaterol, a new ultra-long-acting β2-adrenoceptor agonist at 0.3 mg/kg reversed cardiac remodeling and its effects in combination with metoprolol 100 mg/kg, a selective β1-adrenoceptor antagonist in a rat model of heart failure, by reducing cAMP and cardiac GPCR kinase-2 expression	[134]
Vildagliptin	In type 2 DM rats subjected to MI, at 10 mg/kg, the DPP-4 inhibitor restored the autophagy in noninfarcted region and increased survival rate	[135]
Sinapic acid (SA)	SA protected cardiomyocytes and perfused heart from revascularization injury induced oxidative stress by increasing eNOS expression	[136]

matrix [107], and (C) it may account for the loss of myocytes via apoptosis or other cell death mechanisms.

Myocardial infarcted heart exhibits ventricular remodeling and the whole process is divided into an early phase (within 72 hours) and a late phase (beyond 72 hours). Left ventricular remodeling is characterized by reorganization of the extracellular matrix, disfiguration of the geometry, interstitial inflammation, fibrosis, extensive ventricular dilatation, and deterioration in cardiac function, resulting in progressive heart failure [108]. In fact, recent studies have postulated that peroxynitrite could protect against myocardial ischemia/reperfusion injury [109, 110]. However this discrepancy needs to be clarified with further experiments. Nonetheless, several studies have suggested that various small molecules conferred cardioprotection against myocardial ischemia/reperfusion injury via mitigating ROS generation, left ventricular hypertrophy, and myocardial fibrosis (Table 2) [111]. However, these contradictory observations involving the role of free radicals in cardiac remodeling provide sufficient evidence for its both beneficial and pathological roles in the development of cardiovascular diseases.

Cellular and systemic response to myocardial ischemia/reperfusion with respect to $NO^{\bullet}$ is considered to be modulatory in nature, as pathological flux may overcome the protective role of $NO^{\bullet}$ [112, 113]. Excess $NO^{\bullet}$ level in the myocardium could be detrimental if it combines $O_2^{\bullet-}$ to form peroxynitrite radical that can initiate the formation of several other reactive free radicals and aid in myocardial tissue injury [113]. Moreover, peroxynitrite radical can alter the protein function by forming S-nitrosylation and S-glutathiolation, when it combines with sulfhydryl group containing molecules [114, 115]. S-Nitrosylation of cysteine residues as posttranslational modification influence cardiac function includes receptors, enzymes, ion channels, transcription factors, and structural proteins [116]. All these studies discussed herein reveal the fact that a rigid regulatory mechanism is vital in keeping a check on oxidative stress adaptive processes and this could be an apparent prerequisite for a normal cardiac function. In this line of observations, several studies suggest that delineating the molecular mechanisms purported to reverse the cardiac remodeling might serve as tenable therapeutic target in the management of DCM and ischemia/reperfusion injury [117]. The central role of ROS in the pathogenesis of DCM and ischemia/reperfusion injury is schematically illustrated in Figure 3.

9. Failure of Antioxidant Based Clinical Trials

Several preclinical studies have indicated the potential cardioprotective actions of antioxidants. However, human clinical studies have failed to establish the cardioprotective activity of antioxidant treatments in reducing infarct size. Further, it did not decrease the risk of mortality rates or retarded the deteriorating myocardial function [118]. This discrepancy perhaps could be due to number of factors that contribute to the failure of clinical trials such as (I) inadequate knowledge of antioxidant pharmacological actions in clinical subjects, (II) insufficient dose response studies, (III) the presence of interfering drugs which could affect the pharmacokinetics of antioxidants, and finally (V) the lack of bonafide biomarkers and clinical end points to evaluate the efficacy of antioxidants against cardiovascular diseases. Most importantly, poor sample size and lack of reproducible studies in different populations across the world impedes our knowledge in unraveling the definitive outcome of antioxidants treatment efficacy in combating cardiovascular diseases.

In spite of these limitations, still the prevalent opinion is that antioxidants can in part delay the inevitable rather than completely preventing the occurrence of myocardial infarction. Despite these setbacks, the focus has now shifted towards targeting mitochondria with the selective antioxidants, since this subcellular organelle is unequivocally

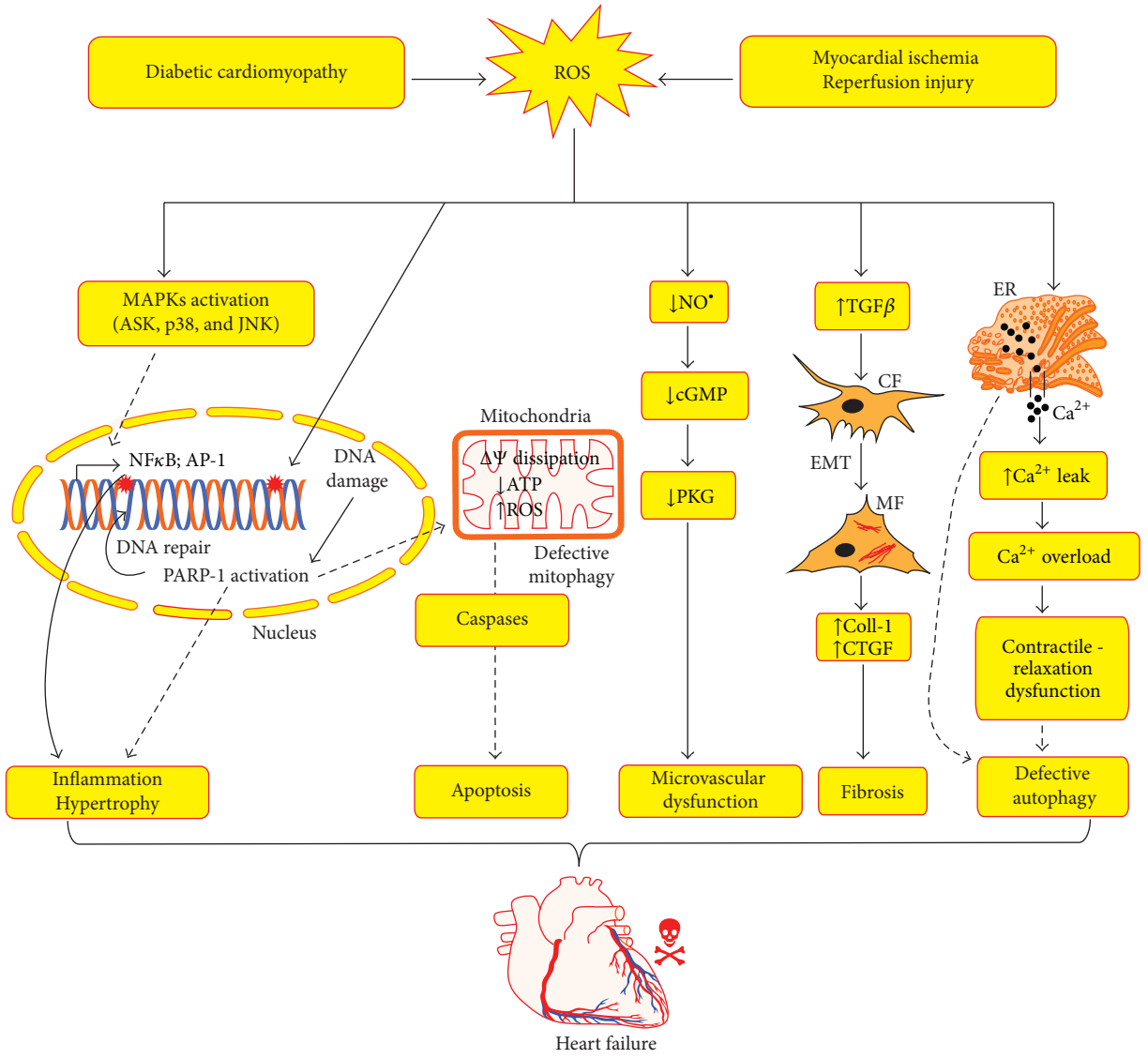

Figure 3: This scheme depicts the role of ROS in the pathophysiology of diabetic cardiomyopathy and myocardial ischemia/reperfusion injury. ROS triggers the activation of MAPKs in the cardiomyocytes, which results in the activation of transcription factors such as NFκB, AP-1. This results in the expression of proinflammatory cytokines and prohypertrophy proteins. Further, ROS directly induces DNA damage and activates poly (ADP-ribose) polymerase (PARP-1) in the nucleus, whereby it mends the damaged DNA. Overactivation of PARP-1 results in depletion of ATP, MPTP opening, mitochondrial dysfunction, and initiation of apoptotic cell death pathways. Next, ROS depletes $NO^{\bullet}$ in the cardiac microvasculatures and promotes endothelial dysfunction via $ONOO^{-}$ generation. Further ROS induces myocardial fibrosis via activation of profibrotic mediators such as TGFβ and priming the epithelial mesenchymal transition (EMT) process of cardiac fibroblasts (CF) differentiation to myofibroblasts (MF), which produces the extracellular matrix. In addition, ROS also perturbs the calcium handling capacity of the cardiomyocytes and interferes in the autophagy process. All these phenotypic events modulated by ROS orchestrates in the development of cardiac failure. Collagen-1 (Coll-1); connective tissue growth factor (CTGF); cyclic GMP (cGMP); protein kinase G (PKG).

involved in the pathophysiology of cardiovascular diseases. In this direction, preclinical studies have strongly shown the proof of concept and evidence that mitochondria targeting antioxidants are in fact effective in preventing the deleterious effects of myocardial ischemia/reperfusion injury and in other rodent models of cardiovascular diseases [119, 120]. However, we need to ascertain if these approaches are successful in the human clinical trials.

10. Conclusion

There is a general notion that ROS are always deleterious and hence it should be scavenged. Nevertheless, this is not the case in all the scenarios. A homeostatic balance (proteostasis) between synthesis and degradation of defective proteins is crucial to maintain proper health of the dynamically and metabolically active cardiomyocytes. This balance

depends not only on oxidative stress but also with reductive stress in the myocardium and subsequent posttranslational modification of vital and sensitive cardiac proteins that are involved in the basic function of contractile myocytes. Hence the drugs that need to be developed to treat the cardiovascular pathologies like ischemia/reperfusion injury and DCM should be able to modulate both oxidative and reductive stress pathways. Nevertheless, a series of mitochondria targeted antioxidants developed recently provides further impetus to delve further to characterize their pharmacokinetics and pharmacodynamic properties and progress with the pharmaceutical development. In this direction, if we achieve the fruition in clinical trials, these agents would set a milestone in the drug development and establish a new paradigm in the treatment or management of debilitating cardiovascular diseases.

Competing Interests

There are no competing interests to disclose.

Authors' Contributions

Gino A. Kurian, Srinivasan Vedantham, and Mohanraj Rajesh conceived the idea for drafting the review. Gino A. Kurian and Rashmi Rajagopal collected the literature and drafted the paper. Srinivasan Vedantham contributed to the discussion. Mohanraj Rajesh compiled the literature and revised and edited the paper. All authors have read the paper and agreed to the contents.

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References

[1] D. Mozaffarian, E. J. Benjamin, A. S. Go et al., "Heart disease and stroke statistics-2015 update : a report from the American Heart Association," *Circulation*, vol. 131, no. 4, pp. e29–e39, 2015.

[2] C. X. C. Santos, N. Anilkumar, M. Zhang, A. C. Brewer, and A. M. Shah, "Redox signaling in cardiac myocytes," *Free Radical Biology and Medicine*, vol. 50, no. 7, pp. 777–793, 2011.

[3] M. S. Willis and C. Patterson, "Proteotoxicity and cardiac dysfunction—Alzheimer's disease of the heart?" *The New England Journal of Medicine*, vol. 368, no. 5, pp. 455–464, 2013.

[4] E. S. Christians and I. J. Benjamin, "Proteostasis and REDOX state in the heart," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 302, no. 1, pp. H24–H37, 2012.

[5] J. Navarro-Yepes, M. Burns, A. Anandhan et al., "Oxidative stress, redox signaling, and autophagy: cell death versus survival," *Antioxidants and Redox Signaling*, vol. 21, no. 1, pp. 66–85, 2014.

[6] R. Ferrari, O. Alfieri, S. Curello et al., "Occurrence of oxidative stress during reperfusion of the human heart," *Circulation*, vol. 81, no. 1, pp. 201–211, 1990.

[7] O. Zitka, S. Skalickova, J. Gumulec et al., "Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients," *Oncology Letters*, vol. 4, no. 6, pp. 1247–1253, 2012.

[8] C. Kupatt, R. Hinkel, J. Horstkotte et al., "Selective retroinfusion of GSH and cariporide attenuates myocardial ischemia-reperfusion injury in a preclinical pig model," *Cardiovascular Research*, vol. 61, no. 3, pp. 530–537, 2004.

[9] P. L. Wendell, "Measurement of oxidized glutathione and total glutathione in the perfused rat heart," *Biochemical Journal*, vol. 117, no. 4, pp. 661–665, 1970.

[10] M. A. Aon, S. Cortassa, C. Maack, and B. O'Rourke, "Sequential opening of mitochondrial ion channels as a function of glutathione redox thiol status," *Journal of Biological Chemistry*, vol. 282, no. 30, pp. 21889–21900, 2007.

[11] P. Das, K. K. Nutan, S. L. Singla-Pareek, and A. Pareek, "Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles," *Frontiers in Environmental Science*, vol. 2, article 70, 2015.

[12] C. Penna, D. Mancardi, R. Rastaldo, and P. Pagliaro, "Cardioprotection: a radical view: free radicals in pre and postconditioning," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1787, no. 7, pp. 781–793, 2009.

[13] H. Wiseman and B. Halliwell, "Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer," *Biochemical Journal*, vol. 313, no. 1, pp. 17–29, 1996.

[14] E. Roche and D. Romero-Alvira, "Role of oxidative stress in gene expression: myocardial and cerebral ischemia, cancer and other diseases," *Medicina Clinica*, vol. 104, no. 12, pp. 468–476, 1995.

[15] P. Venditti, P. Masullo, and S. Di Meo, "Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress," *Cellular and Molecular Life Sciences*, vol. 58, no. 10, pp. 1528–1537, 2001.

[16] J. F. Keaney Jr. and J. A. Vita, "Atherosclerosis, oxidative stress, and antioxidant protection in endothelium-derived relaxing factor action," *Progress in Cardiovascular Diseases*, vol. 38, no. 2, pp. 129–154, 1995.

[17] R. A. Thandavarayan, V. V. Giridharan, K. Watanabe, and T. Konishi, "Diabetic cardiomyopathy and oxidative stress: role of antioxidants," *Cardiovascular and Hematological Agents in Medicinal Chemistry*, vol. 9, no. 4, pp. 225–230, 2011.

[18] D. F. Stowe and A. K. S. Camara, "Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function," *Antioxidants & Redox Signaling*, vol. 11, no. 6, pp. 1373–1414, 2009.

[19] A. K. S. Camara, E. J. Lesnefsky, and D. F. Stowe, "Potential therapeutic benefits of strategies directed to mitochondria," *Antioxidants and Redox Signaling*, vol. 13, no. 3, pp. 279–347, 2010.

[20] C. B. Wolff, "Normal cardiac output, oxygen delivery and oxygen extraction," *Advances in Experimental Medicine and Biology*, vol. 599, pp. 169–182, 2007.

[21] B. Gopalakrishnan, K. M. Nash, M. Velayutham, and F. A. Villamena, "Detection of nitric oxide and superoxide radical anion by electron paramagnetic resonance spectroscopy from cells using spin traps," *Journal of Visualized Experiments*, no. 66, article e2810, 2012.

[22] S. Raha, G. E. McEachern, A. T. Myint, and B. H. Robinson, "Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase," *Free Radical Biology and Medicine*, vol. 29, no. 2, pp. 170–180, 2000.

[23] E. Ho, K. Karimi Galougahi, C.-C. Liu, R. Bhindi, and G. A. Figtree, "Biological markers of oxidative stress: applications to cardiovascular research and practice," *Redox Biology*, vol. 1, no. 1, pp. 483–491, 2013.

[24] C. M. Arroyo, J. H. Kramer, B. F. Dickens, and W. B. Weglicki, "Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO," *FEBS Letters*, vol. 221, no. 1, pp. 101–104, 1987.

[25] K. Carvajal, M. El Hafidi, and G. Baños, "Myocardial damage due to ischemia and reperfusion in hypertriglyceridemic and hypertensive rats: participation of free radicals and calcium overload," *Journal of Hypertension*, vol. 17, no. 11, pp. 1607–1616, 1999.

[26] G. A. Kurian and J. Paddikkala, "Administration of aqueous extract of Desmodium gangeticum (L) root protects rat heart against ischemic reperfusion injury induced oxidative stress," *Indian Journal of Experimental Biology*, vol. 47, no. 2, pp. 129–135, 2009.

[27] G. A. Kurian, S. Suryanarayanan, A. Raman, and J. Padikkala, "Antioxidant effects of ethyl acetate extract of *Desmodium gangeticum* root on myocardial ischemia reperfusion injury in rat hearts," *Chinese Medicine*, vol. 5, article 3, 2010.

[28] G. A. Kurian and J. Paddikkala, "N-acetylcysteine and magnesium improve biochemical abnormalities associated with myocardial ischaemic reperfusion in South Indian patients undergoing coronary artery bypass grafting: a comparative analysis," *Singapore Medical Journal*, vol. 51, no. 5, pp. 381–388, 2010.

[29] F. J. Giordano, "Oxygen, oxidative stress, hypoxia, and heart failure," *The Journal of Clinical Investigation*, vol. 115, no. 3, pp. 500–508, 2005.

[30] B. R. Lucchesi, "Myocardial ischemia, reperfusion and free radical injury," *The American Journal of Cardiology*, vol. 65, no. 19, pp. 14–23, 1990.

[31] C. Tanaka-Esposito, Q. Chen, and E. J. Lesnefsky, "Blockade of electron transport before ischemia protects mitochondria and decreases myocardial injury during reperfusion in aged rat hearts," *Translational Research*, vol. 160, no. 3, pp. 207–216, 2012.

[32] A. K. Gadicherla, D. F. Stowe, W. E. Antholine, M. Yang, and A. K. S. Camara, "Damage to mitochondrial complex i during cardiac ischemia reperfusion injury is reduced indirectly by anti-anginal drug ranolazine," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1817, no. 3, pp. 419–429, 2012.

[33] G. Paradies, G. Petrosillo, M. Pistolese, and F. M. Ruggiero, "Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage," *Gene*, vol. 286, no. 1, pp. 135–141, 2002.

[34] J. W. Palmer, B. Tandler, and C. L. Hoppel, "Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle," *Journal of Biological Chemistry*, vol. 252, no. 23, pp. 8731–8739, 1977.

[35] S. A. Banu, S. Ravindran, and G. A. Kurian, "Hydrogen sulfide post-conditioning preserves interfibrillar mitochondria of rat heart during ischemia reperfusion injury," *Cell Stress and Chaperones*, 2016.

[36] B. Chance, H. Sies, and A. Boveris, "Hydroperoxide metabolism in mammalian organs," *Physiological Reviews*, vol. 59, no. 3, pp. 527–605, 1979.

[37] C. Crochemore, M. Mekki, C. Corbière et al., "Subsarcolemmal and interfibrillar mitochondria display distinct superoxide production profiles," *Free Radical Research*, vol. 49, no. 3, pp. 331–337, 2015.

[38] G. A. Kurian, E. Berenshtein, A. Saada, and M. Chevion, "Rat cardiac mitochondrial sub-populations show distinct features of oxidative phosphorylation during ischemia, reperfusion and ischemic preconditioning," *Cellular Physiology and Biochemistry*, vol. 30, no. 1, pp. 83–94, 2012.

[39] G. A. Kurian, E. Berenshtein, O. Kakhlon, and M. Chevion, "Energy status determines the distinct biochemical and physiological behavior of interfibrillar and sub-sarcolemmal mitochondria," *Biochemical and Biophysical Research Communications*, vol. 428, no. 3, pp. 376–382, 2012.

[40] A. Lejay, F. Fang, R. John et al., "Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus," *Journal of Molecular and Cellular Cardiology*, vol. 91, pp. 11–22, 2016.

[41] H. S. Chung, S.-B. Wang, V. Venkatraman, C. I. Murray, and J. E. Van Eyk, "Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system," *Circulation Research*, vol. 112, no. 2, pp. 382–392, 2013.

[42] H. S. Chung, C. I. Murray, V. Venkatraman et al., "Dual labeling biotin switch assay to reduce bias derived from different cysteine subpopulations: a method to maximize s-nitrosylation detection," *Circulation Research*, vol. 117, no. 10, pp. 846–857, 2015.

[43] M. Talkhabi, N. Aghdami, and H. Baharvand, "Human cardiomyocyte generation from pluripotent stem cells: a state-of-art," *Life Sciences*, vol. 145, pp. 98–113, 2016.

[44] O. Bergmann, S. Zdunek, A. Felker et al., "Dynamics of cell generation and turnover in the human heart," *Cell*, vol. 161, no. 7, pp. 1566–1575, 2015.

[45] C. Jopling, E. Sleep, M. Raya, M. Martí, A. Raya, and J. C. I. Belmonte, "Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation," *Nature*, vol. 464, no. 7288, pp. 606–609, 2010.

[46] E. R. Porrello, A. I. Mahmoud, E. Simpson et al., "Transient regenerative potential of the neonatal mouse heart," *Science*, vol. 331, no. 6020, pp. 1078–1080, 2011.

[47] O. Bergmann, R. D. Bhardwaj, S. Bernard et al., "Evidence for cardiomyocyte renewal in humans," *Science*, vol. 324, no. 5923, pp. 98–102, 2009.

[48] J.-O. Jo, S.-R. Kim, M.-K. Bae et al., "Thymosin β4 induces the expression of vascular endothelial growth factor (VEGF) in a hypoxia-inducible factor (HIF)-1α-dependent manner," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1803, no. 11, pp. 1244–1251, 2010.

[49] T. Yasukawa, E. Tokunaga, H. Ota, H. Sugita, J. A. J. Martyn, and M. Kaneki, "S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance," *The Journal of Biological Chemistry*, vol. 280, no. 9, pp. 7511–7518, 2005.

[50] E. T. Chouchani, C. Methner, S. M. Nadtochiy et al., "Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I," *Nature Medicine*, vol. 19, no. 6, pp. 753–759, 2013.

[51] H. L. A. Vieira, A.-S. Belzacq, D. Haouzi et al., "The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite,

and 4-hydroxynonenal," *Oncogene*, vol. 20, no. 32, pp. 4305–4316, 2001.

[52] J. R. Hom, R. A. Quintanilla, D. L. Hoffman et al., "The permeability transition pore controls cardiac mitochondrial maturation and myocyte differentiation," *Developmental Cell*, vol. 21, no. 3, pp. 469–478, 2011.

[53] N. S. Chandel, "Mitochondria as signaling organelles," *BMC Biology*, vol. 12, article 34, 2014.

[54] L. M. Buja and R. S. Vander Heide, "Pathobiology of ischemic heart disease: past, present and future," *Cardiovascular Pathology*, vol. 25, no. 3, pp. 214–220, 2016.

[55] R. Bretón-Romero and S. Lamas, "Hydrogen peroxide signaling in vascular endothelial cells," *Redox Biology*, vol. 2, no. 1, pp. 529–534, 2014.

[56] M. Y. Lee and K. K. Griendling, "Redox signaling, vascular function, and hypertension," *Antioxidants & Redox Signaling*, vol. 10, no. 6, pp. 1045–1059, 2008.

[57] N. R. Madamanchi and M. S. Runge, "Redox signaling in cardiovascular health and disease," *Free Radical Biology and Medicine*, vol. 61, pp. 473–501, 2013.

[58] A. S. Johnston, S. E. Lehnart, and J. R. Burgoyne, "Ca^{2+} signaling in the myocardium by (redox) regulation of PKA/CaMKII," *Frontiers in Pharmacology*, vol. 6, article 166, 2015.

[59] Y.-S. Zhang, L. He, B. Liu et al., "A novel pathway of NADPH oxidase/vascular peroxidase 1 in mediating oxidative injury following ischemia-reperfusion," *Basic Research in Cardiology*, vol. 107, no. 3, article 266, 2012.

[60] V. Braunersreuther, F. Montecucco, M. Asrih et al., "Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 64, pp. 99–107, 2013.

[61] I. Novak, "Mitophagy: a complex mechanism of mitochondrial removal," *Antioxidants & Redox Signaling*, vol. 17, no. 5, pp. 794–802, 2012.

[62] G. W. Dorn, R. B. Vega, and D. P. Kelly, "Mitochondrial biogenesis and dynamics in the developing and diseased heart," *Genes & Development*, vol. 29, no. 19, pp. 1981–1991, 2015.

[63] N. S. Rajasekaran, P. Connell, E. S. Christians et al., "Human α B-crystallin mutation causes oxido-reductive stress and protein aggregation cardiomyopathy in mice," *Cell*, vol. 130, no. 3, pp. 427–439, 2007.

[64] A. C. Brewer, S. B. Mustafi, T. V. A. Murray, N. S. Rajasekaran, and I. J. Benjamin, "Reductive stress linked to small HSPs, G6PD, and Nrf2 pathways in heart disease," *Antioxidants and Redox Signaling*, vol. 18, no. 9, pp. 1114–1127, 2013.

[65] X. Zhang, X. Min, C. Li et al., "Involvement of reductive stress in the cardiomyopathy in transgenic mice with cardiac-specific overexpression of heat shock protein 27," *Hypertension*, vol. 55, no. 6, pp. 1412–1417, 2010.

[66] R. Natarajan, F. N. Salloum, B. J. Fisher, R. C. Kukreja, and A. A. Fowler III, "Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing attenuates myocardial ischemia reperfusion injury," *Circulation Research*, vol. 98, no. 1, pp. 133–140, 2006.

[67] J. Hu, L. Dong, and C. E. Outten, "The redox environment in the mitochondrial intermembrane space is maintained separately from the cytosol and matrix," *The Journal of Biological Chemistry*, vol. 283, no. 43, pp. 29126–29134, 2008.

[68] P. Korge, G. Calmettes, and J. N. Weiss, "Increased reactive oxygen species production during reductive stress: the roles of mitochondrial glutathione and thioredoxin reductases," *Biochimica et Biophysica Acta*, vol. 1847, no. 6-7, pp. 514–525, 2015.

[69] H. Zhang, P. Limphong, J. Pieper et al., "Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity," *The FASEB Journal*, vol. 26, no. 4, pp. 1442–1451, 2012.

[70] M. Narasimhan and N. S. Rajasekaran, "Reductive potential—a savior turns stressor in protein aggregation cardiomyopathy," *Biochimica et Biophysica Acta*, vol. 1852, no. 1, pp. 53–60, 2015.

[71] G. Morciano, C. Giorgi, M. Bonora et al., "Molecular identity of the mitochondrial permeability transition pore and its role in ischemia-reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 78, pp. 142–153, 2015.

[72] X.-H. Gao, S. Qanungo, H. V. Pai et al., "Aging-dependent changes in rat heart mitochondrial glutaredoxins-implications for redox regulation," *Redox Biology*, vol. 1, no. 1, pp. 586–598, 2013.

[73] E. L. Holmuhamedov, A. Oberlin, K. Short, A. Terzic, and A. Jahangir, "Cardiac subsarcolemmal and interfibrillar mitochondria display distinct responsiveness to protection by diazoxide," *PLoS ONE*, vol. 7, no. 9, Article ID e44667, 2012.

[74] S. Fulda, A. M. Gorman, O. Hori, and A. Samali, "Cellular stress responses: cell survival and cell death," *International Journal of Cell Biology*, vol. 2010, Article ID 214074, 23 pages, 2010.

[75] B. Zingarelli, P. W. Hake, M. O'Connor et al., "Differential regulation of activator protein-1 and heat shock factor-1 in myocardial ischemia and reperfusion injury: role of poly(ADP-ribose) polymerase-1," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 286, no. 4, pp. H1408–H1415, 2004.

[76] M. Chello, P. Mastroroberto, G. Patti, A. D'Ambrosio, G. Di Sciascio, and E. Covino, "Intermittent warm blood cardioplegia induces the expression of heat shock protein-72 by ischemic myocardial preconditioning," *Cardiovascular Surgery*, vol. 11, no. 5, pp. 367–374, 2003.

[77] G.-Q. Zhong, R.-H. Tu, Z.-Y. Zeng et al., "Novel functional role of heat shock protein 90 in protein kinase C-mediated ischemic postconditioning," *Journal of Surgical Research*, vol. 189, no. 2, pp. 198–206, 2014.

[78] D. Lindholm and U. Arumäe, "Cell differentiation: reciprocal regulation of Apaf-1 and the inhibitor of apoptosis proteins," *The Journal of Cell Biology*, vol. 167, no. 2, pp. 193–195, 2004.

[79] J. Jayakumar, K. Suzuki, M. Khan et al., "Gene therapy for myocardial protection: transfection of donor hearts with heat shock protein 70 gene protects cardiac function against ischemia-reperfusion injury," *Circulation*, vol. 102, no. 19, supplement 3, pp. III302–III306, 2000.

[80] K. Suzuki, Y. Sawa, Y. Kaneda, H. Ichihara, R. Shirakura, and H. Matsuda, "In vivo gene transfection with heat shock protein 70 enhances myocardial tolerance to ischemia-reperfusion injury in rat," *Journal of Cardiology*, vol. 31, no. 3, pp. 187–188, 1998.

[81] J. A. Barbour and N. Turner, "Mitochondrial stress signaling promotes cellular adaptations," *International Journal of Cell Biology*, vol. 2014, Article ID 156020, 12 pages, 2014.

[82] T. Masuda, S. Muto, G. Fujisawa et al., "Heart angiotensin II-induced cardiomyocyte hypertrophy suppresses coronary angiogenesis and progresses diabetic cardiomyopathy," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 302, no. 9, pp. H1871–H1883, 2012.

[83] J. Asbun and F. J. Villarreal, "The pathogenesis of myocardial fibrosis in the setting of diabetic cardiomyopathy," *Journal of the American College of Cardiology*, vol. 47, no. 4, pp. 693–700, 2006.

[84] M. Rajesh, P. Mukhopadhyay, S. Bátkai et al., "Xanthine oxidase inhibitor allopurinol attenuates the development of diabetic cardiomyopathy," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 8, pp. 2330–2341, 2009.

[85] M. Rajesh, P. Mukhopadhyay, S. Btkai et al., "Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy," *Journal of the American College of Cardiology*, vol. 56, no. 25, pp. 2115–2125, 2010.

[86] M. Rajesh, S. Bátkai, M. Kechrid et al., "Cannabinoid 1 receptor promotes cardiac dysfunction, oxidative stress, inflammation, and fibrosis in diabetic cardiomyopathy," *Diabetes*, vol. 61, no. 3, pp. 716–727, 2012.

[87] S. Raposeiras-Roubín, C. Barreiro-Pardal, E. Álvarez, and J. R. González Juanatey, "Is glycated hemoglobin an accurate enough predictor of subclinical myocardial injury or a simple precursor of advanced glycation end products?" *Journal of the American College of Cardiology*, vol. 60, no. 2, pp. 166–167, 2012.

[88] Z. V. Varga, Z. Giricz, L. Liaudet, G. Haskó, P. Ferdinandy, and P. Pacher, "Interplay of oxidative, nitrosative/nitrative stress, inflammation, cell death and autophagy in diabetic cardiomyopathy," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1852, no. 2, pp. 232–242, 2015.

[89] Q. Liu, S. Wang, and L. Cai, "Diabetic cardiomyopathy and its mechanisms: role of oxidative stress and damage," *Journal of Diabetes Investigation*, vol. 5, no. 6, pp. 623–634, 2014.

[90] S. Vedantham, D. Thiagarajan, R. Ananthakrishnan et al., "Aldose reductase drives hyperacetylation of EGR-1 in hyperglycemia and consequent upregulation of proinflammatory and prothrombotic signals," *Diabetes*, vol. 63, no. 2, pp. 761–774, 2014.

[91] M. Abdillahi, R. Ananthakrishnan, S. Vedantham et al., "Aldose reductase modulates cardiac glycogen synthase kinase-3β phosphorylation during ischemia-reperfusion," *American Journal of Physiology*, vol. 303, no. 3, pp. H297–H308, 2012.

[92] V. Srinivasan, A. Radha, S. Ann Marie, and R. Ravichandran, "Aldose reductase, oxidative stress and diabetic cardiovascular complications," *Cardiovascular & Hematological Agents in Medicinal Chemistry*, vol. 10, no. 3, pp. 234–240, 2012.

[93] S. D. Funk, A. Yurdagul Jr., and A. W. Orr, "Hyperglycemia and endothelial dysfunction in atherosclerosis: lessons from type 1 diabetes," *International Journal of Vascular Medicine*, vol. 2012, Article ID 569654, 19 pages, 2012.

[94] K. Kaiserova, S. Srivastava, J. D. Hoetker et al., "Redox activation of aldose reductase in the ischemic heart," *The Journal of Biological Chemistry*, vol. 281, no. 22, pp. 15110–15120, 2006.

[95] Y. C. Hwang, M. Kaneko, S. Bakr et al., "Central role for aldose reductase pathway in myocardial ischemic injury," *FASEB Journal*, vol. 18, no. 11, pp. 1192–1199, 2004.

[96] Y. C. Hwang, S. Sato, J.-Y. Tsai et al., "Aldose reductase activation is a key component of myocardial response to ischemia," *The FASEB Journal*, vol. 16, no. 2, pp. 243–245, 2002.

[97] R. Ananthakrishnan, Q. Li, T. Gomes, A. M. Schmidt, and R. Ramasamy, "Aldose reductase pathway contributes to vulnerability of aging myocardium to ischemic injury," *Experimental Gerontology*, vol. 46, no. 9, pp. 762–767, 2011.

[98] N.-H. Son, R. Ananthakrishnan, S. Yu et al., "Cardiomyocyte aldose reductase causes heart failure and impairs recovery from ischemia," *PLoS ONE*, vol. 7, no. 9, Article ID e46549, 2012.

[99] W. H. Tang, S. Wu, T. M. Wong, S. K. Chung, and S. S. M. Chung, "Polyol pathway mediates iron-induced oxidative injury in ischemic-reperfused rat heart," *Free Radical Biology and Medicine*, vol. 45, no. 5, pp. 602–610, 2008.

[100] K. Shinmura, R. Bolli, S.-Q. Liu et al., "Aldose reductase is an obligatory mediator of the late phase of ischemic preconditioning," *Circulation Research*, vol. 91, no. 3, pp. 240–246, 2002.

[101] W. H. Tang, G. M. Kravtsov, M. Sauert et al., "Polyol pathway impairs the function of SERCA and RyR in ischemic-reperfused rat hearts by increasing oxidative modifications of these proteins," *Journal of Molecular and Cellular Cardiology*, vol. 49, no. 1, pp. 58–69, 2010.

[102] R. Ananthakrishnan, M. Kaneko, Y. C. Hwang et al., "Aldose reductase mediates myocardial ischemia-reperfusion injury in part by opening mitochondrial permeability transition pore," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 2, pp. H333–H341, 2009.

[103] J. James and J. Robbins, "Molecular remodeling of cardiac contractile function," *American Journal of Physiology*, vol. 273, no. 5, part 2, pp. H2105–H2118, 1997.

[104] G. K. Savard, N.-H. Areskog, and B. Saltin, "Cardiovascular response to exercise in humans following acclimatization to extreme altitude," *Acta Physiologica Scandinavica*, vol. 154, no. 4, pp. 499–509, 1995.

[105] I. Afanas'ev, "ROS and RNS signaling in heart disorders: could antioxidant treatment be successful?" *Oxidative Medicine and Cellular Longevity*, vol. 2011, Article ID 293769, 13 pages, 2011.

[106] M. Ozeki, H. Nagasu, M. Satoh et al., "Reactive oxygen species mediate compensatory glomerular hypertrophy in rat uninephrectomized kidney," *Journal of Physiological Sciences*, vol. 59, no. 5, pp. 397–404, 2009.

[107] S. Givvimani, C. Munjal, R. Gargoum et al., "Hydrogen sulfide mitigates transition from compensatory hypertrophy to heart failure," *Journal of Applied Physiology*, vol. 110, no. 4, pp. 1093–1100, 2011.

[108] J. G. Travers, F. A. Kamal, J. Robbins, K. E. Yutzey, and B. C. Blaxall, "Cardiac fibrosis: the fibroblast awakens," *Circulation Research*, vol. 118, no. 6, pp. 1021–1040, 2016.

[109] S. Gorressen, M. Stern, A. M. van de Sandt et al., "Circulating NOS3 modulates left ventricular remodeling following reperfused myocardial infarction," *PLoS ONE*, vol. 10, no. 4, Article ID e0120961, 2015.

[110] J. Li, N. Loukili, N. Rosenblatt-Velin et al., "Peroxynitrite is a key mediator of the cardioprotection afforded by ischemic postconditioning in vivo," *PLoS ONE*, vol. 8, no. 7, Article ID e70331, 2013.

[111] C. M. Sag, C. X. C. Santos, and A. M. Shah, "Redox regulation of cardiac hypertrophy," *Journal of Molecular and Cellular Cardiology*, vol. 73, pp. 103–111, 2014.

[112] R. Schulz, M. Kelm, and G. Heusch, "Nitric oxide in myocardial ischemia/reperfusion injury," *Cardiovascular Research*, vol. 61, no. 3, pp. 402–413, 2004.

[113] P. Mukhopadhyay, M. Rajesh, S. Bátkai et al., "Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 5, pp. H1466–H1483, 2009.

[114] Y. Yang, X. Jin, and C. Jiang, "S-glutathionylation of ion channels: insights into the regulation of channel functions, thiol modification crosstalk, and mechanosensing," *Antioxidants & Redox Signaling*, vol. 20, no. 6, pp. 937–951, 2014.

[115] P. Pacher and C. Szabó, "Role of peroxynitrite in the pathogenesis of cardiovascular complications of diabetes," *Current Opinion in Pharmacology*, vol. 6, no. 2, pp. 136–141, 2006.

[116] D. R. Gonzalez, A. Treuer, Q.-A. Sun, J. S. Stamler, and J. M. Hare, "S-nitrosylation of cardiac ion channels," *Journal of Cardiovascular Pharmacology*, vol. 54, no. 3, pp. 188–195, 2009.

[117] N. Koitabashi and D. A. Kass, "Reverse remodeling in heart failure-mechanisms and therapeutic opportunities," *Nature Reviews Cardiology*, vol. 9, no. 3, pp. 147–157, 2012.

[118] V. R. Pell, E. T. Chouchani, M. P. Murphy, P. S. Brookes, and T. Krieg, "Moving forwards by blocking back-flow: the Yin and yang of MI therapy," *Circulation Research*, vol. 118, no. 5, pp. 898–906, 2016.

[119] A. O. Oyewole and M. A. Birch-Machin, "Mitochondria-targeted antioxidants," *The FASEB Journal*, vol. 29, no. 12, pp. 4766–4771, 2015.

[120] J. W. Walters, D. Amos, K. Ray, and N. Santanam, "Mitochondrial redox status as a target for cardiovascular disease," *Current Opinion in Pharmacology*, vol. 27, pp. 50–55, 2016.

[121] A. H. Talasaz, H. Khalili, F. Fahimi et al., "Effects of *N*-acetylcysteine on the cardiac remodeling biomarkers and major adverse events following acute myocardial infarction: a randomized clinical trial," *American Journal of Cardiovascular Drugs*, vol. 14, no. 1, pp. 51–61, 2014.

[122] J. Tomé-Carneiro, M. Gonzálvez, M. Larrosa et al., "One-year consumption of a grape nutraceutical containing resveratrol improves the inflammatory and fibrinolytic status of patients in primary prevention of cardiovascular disease," *American Journal of Cardiology*, vol. 110, no. 3, pp. 356–363, 2012.

[123] C. Gladine, N. Combe, C. Vaysse et al., "Optimized rapeseed oil enriched with healthy micronutrients: a relevant nutritional approach to prevent cardiovascular diseases. Results of the Optim'Oils randomized intervention trial," *Journal of Nutritional Biochemistry*, vol. 24, no. 3, pp. 544–549, 2013.

[124] J. I. Dower, J. M. Geleijnse, L. Gijsbers, C. Schalkwijk, D. Kromhout, and P. C. Hollman, "Supplementation of the pure flavonoids epicatechin and quercetin affects some biomarkers of endothelial dysfunction and inflammation in (pre)hypertensive adults: a randomized double-blind, placebo-controlled, crossover trial," *Journal of Nutrition*, vol. 145, no. 7, pp. 1459–1463, 2015.

[125] M. Castaño, J. M. González-Santos, J. López et al., "Effect of preoperative oral pravastatin reload in systemic inflammatory response and myocardial damage after coronary artery bypass grafting. A pilot double-blind placebo-controlled study," *Journal of Cardiovascular Surgery*, vol. 56, no. 4, pp. 617–629, 2015.

[126] G. A. Kurian, M. Phil, and J. Paddikkala, "Antioxidant status of South Indian patients undergoing coronary artery bypass graft surgery: a role of intra operative magnesium supplementation," *International Journal of Cardiology*, vol. 128, no. 1, pp. 139–141, 2008.

[127] J. S. Petrofsky, M. Laymon, H. Lee et al., "CoQ10 and endothelial function in Asians from Korea compared to Asians born in the United States and US born Caucasians," *Medical Science Monitor*, vol. 19, no. 1, pp. 339–346, 2013.

[128] T. Altaei, "Protective effect of silymarin during coronary artery bypass grafting surgery," *Experimental and Clinical Cardiology*, vol. 17, no. 1, pp. 34–38, 2012.

[129] S. Nishioka, T. Yoshioka, A. Nomura et al., "Celiprolol reduces oxidative stress and attenuates left ventricular remodeling induced by hypoxic stress in mice," *Hypertension Research*, vol. 36, no. 11, pp. 934–939, 2013.

[130] Z.-H. Zhao, J. Shan, M.-X. Xiang, G. Xu, G.-S. Fu, and X.-F. Bao, "Influence of fluvastatin on left ventricular remodeling after myocardial infarction in rats," *Zhejiang Da Xue Xue Bao Yi Xue Ban*, vol. 34, no. 5, pp. 447–464, 2005.

[131] K. Takeuchi, T. Omura, M. Yoshiyama et al., "Long-acting calcium channel antagonist pranidipine prevents ventricular remodeling after myocardial infarction in rats," *Heart and Vessels*, vol. 14, no. 3, pp. 111–119, 1999.

[132] X. Zhou, L. Zhao, J. Mao, J. Huang, and J. Chen, "Antioxidant effects of hydrogen sulfide on left ventricular remodeling in smoking rats are mediated via PI3K/Akt-dependent activation of Nrf2," *Toxicological Sciences*, vol. 144, no. 1, pp. 197–203, 2015.

[133] D. Bonaduce, M. Petretta, P. Arrichiello et al., "Effects of captopril treatment on left ventricular remodeling and function after anterior myocardial infarction: comparison with digitalis," *Journal of the American College of Cardiology*, vol. 19, no. 4, pp. 858–863, 1992.

[134] B. Rinaldi, M. Donniacuo, L. Sodano et al., "Effects of chronic treatment with the new ultra-long-acting β2 -adrenoceptor agonist indacaterol alone or in combination with the β1 -adrenoceptor blocker metoprolol on cardiac remodelling," *British Journal of Pharmacology*, vol. 172, no. 14, pp. 3627–3637, 2015.

[135] H. Murase, A. Kuno, T. Miki et al., "Inhibition of DPP-4 reduces acute mortality after myocardial infarction with restoration of autophagic response in type 2 diabetic rats," *Cardiovascular Diabetology*, vol. 14, no. 1, article 103, 2015.

[136] T. Silambarasan, J. Manivannan, M. K. Priya, N. Suganya, S. Chatterjee, and B. Raja, "Sinapic acid protects heart against ischemia/reperfusion injury and H9c2 cardiomyoblast cells against oxidative stress," *Biochemical and Biophysical Research Communications*, vol. 456, no. 4, pp. 853–859, 2015.

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Review Article

Cardioprotective Potentials of Plant-Derived Small Molecules against Doxorubicin Associated Cardiotoxicity

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Doxorubicin (DOX) is a potent and widely used anthracycline antibiotic for the treatment of several malignancies. Unfortunately, the clinical utility of DOX is often restricted due to the elicitation of organ toxicity. Particularly, the increased risk for the development of dilated cardiomyopathy by DOX among the cancer survivors warrants major attention from the physicians as well as researchers to develop adjuvant agents to neutralize the noxious effects of DOX on the healthy myocardium. Despite these pitfalls, the use of traditional cytotoxic drugs continues to be the mainstay treatment for several types of cancer. Recently, phytochemicals have gained attention for their anticancer, chemopreventive, and cardioprotective activities. The ideal cardioprotective agents should not compromise the clinical efficacy of DOX and should be devoid of cumulative or irreversible toxicity on the naïve tissues. Furthermore, adjuvants possessing synergistic anticancer activity and quelling of chemoresistance would significantly enhance the clinical utility in combating DOX-induced cardiotoxicity. The present review renders an overview of cardioprotective effects of plant-derived small molecules and their purported mechanisms against DOX-induced cardiotoxicity. Phytochemicals serve as the reservoirs of pharmacophore which can be utilized as templates for developing safe and potential novel cardioprotective agents in combating DOX-induced cardiotoxicity.

1. Introduction

Doxorubicin (DOX) is a potent and widely used anthracycline antibiotic for the treatment of cancers. However, the major impeding issue pertaining to the clinical application of DOX is related to its ability to induce untoward toxicity to the healthy tissues [1]. The occurrence of fatal cardiotoxicity in pediatric as well as in adult patients is characterized by an irreversible cardiomyopathy which compromises the clinical utility of DOX and accounts for the major cause of the chemotherapy related morbidity and mortality [1]. In spite of introducing several less toxic derivatives of DOX, elicitation of cardiotoxicity still remains the major concern [2]. However, with the advent of newer class of monoclonal antibodies revolutionized cancer chemotherapy, still this approach is burdened with myriad adverse effects [3]. Thus, the use of traditional cytotoxic drugs continues to be a preferred mode for the treatment of cancer.

To limit the DOX-induced cardiotoxicity, several molecules, such as beta blockers, angiotensin receptor blockers, amifostine, dexrazoxane, Mesna (2-mercaptoethane sulfonate Na), leucovorin, and erythropoietin, have been evaluated as cardioprotective adjuvants in preclinical studies [4]. Recently, dexrazoxane, when subjected to clinical trial against combating DOX-induced cardiotoxicity, exhibited marked cardioprotection and did not compromise the anticancer activity of DOX [5]. Similarly, carvedilol (beta blocker) has also been demonstrated to confer protection against DOX-induced cardiotoxicity in human subjects [6]. However, large-scale clinical applications of these adjuvants

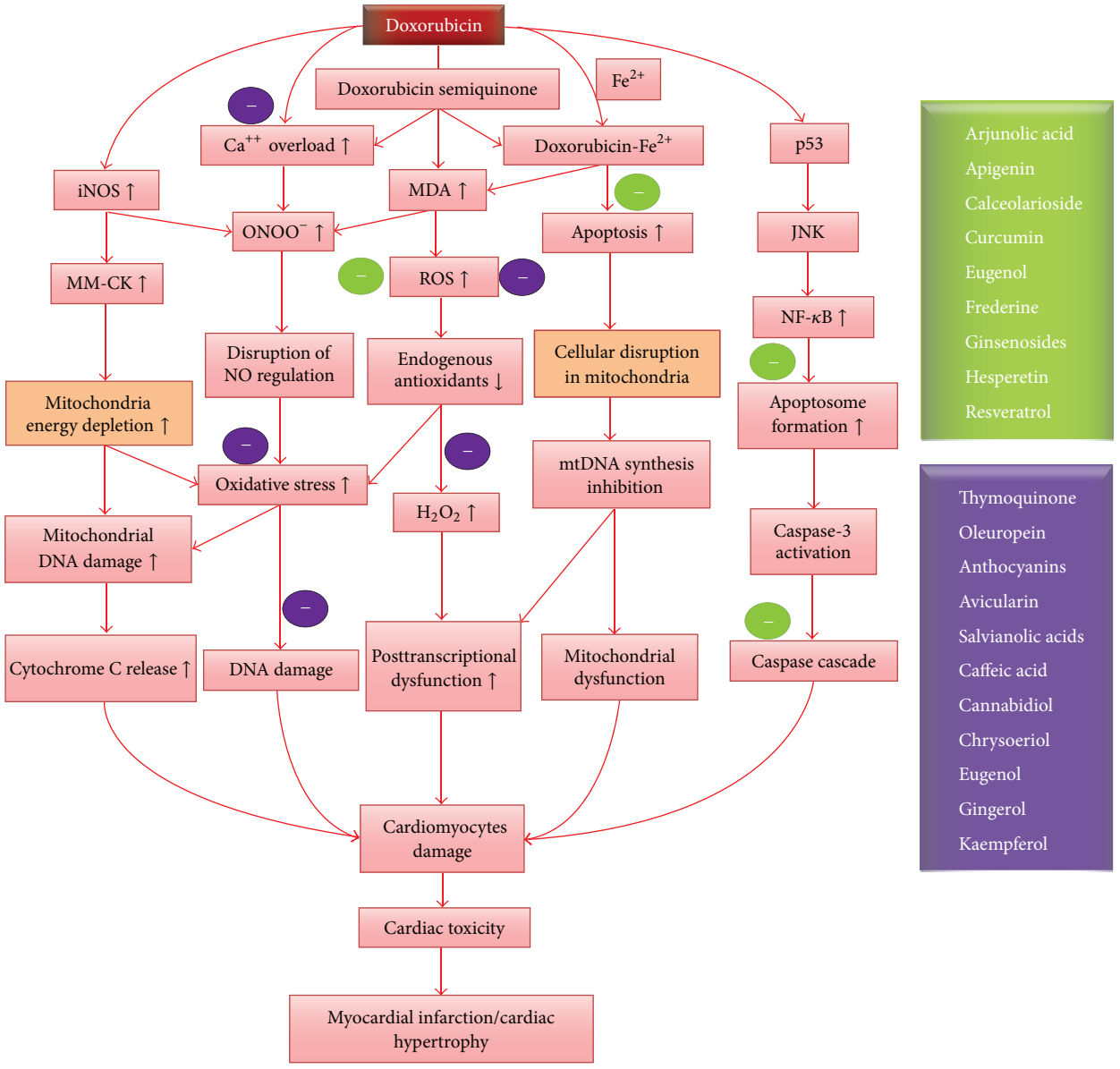

Figure 1: This scheme shows the pathways involved in the elicitation of DOX-induced adverse effects in the myocardium and its attenuation by phytochemicals.

are yet to be established in human subjects. The substantial burden arising from cancer and cardiotoxicity and their interrelationship in imposing morbidity and mortality has emerged as the major driving factor for the academia and pharmaceutical industry to devise and develop strategies that can simultaneously provide long-term cardioprotection from DOX-associated cardiotoxicity without compromising the efficacy of cancer chemotherapy [7]. Since the origin of the human civilization, plants and herbs have been traditionally used in the treatment of various diseases and ailments [8]. In this direction, the prospect of harnessing the potentials of plant-derived small molecules (phytochemicals) appears to provide rich dividends, since phytochemicals have been extensively studied in the preclinical studies and shown to possess anti-inflammatory, antioxidant, and anticancer activities. Phytochemicals are the natural constituents of herbs and plants. Moreover, anticancer drug paclitaxel and antimalarial agent artemisinin are phytochemicals originally extracted from plants; however, these agents are now chemically synthesized [9, 10].

In this review, we have systematically presented the evidence wherein the phytochemicals were investigated for cardioprotective effects against DOX and the relevant mechanisms. Several mechanisms have been postulated for the development of DOX-induced cardiotoxicity. However, oxidative stress driven inflammation, apoptosis, and myocardial remodeling have emerged to be the key players in DOX-induced cardiotoxicity. In-depth description of pathophysiology of DOX-induced cardiotoxicity has been reviewed elsewhere [11, 12]; however, to provide clarity to our discussion, we have provided a simplified scheme for the pathomechanisms in Figure 1.

2. Phytochemicals Limiting DOX-Induced Cardiotoxicity

In this section, we systematically described the phytochemicals investigated for their cardioprotective activity against DOX-induced toxic effects in the myocardium. Phytochemicals investigated in the animal model of DOX-induced cardiotoxicity are listed in Table 1 and the molecules investigated in cell culture models (*in vitro*) are presented in Table 2.

2.1. Arjunolic Acid. Arjunolic acid is a chiral triterpenoid saponin, isolated from *Terminalia arjuna*. Arjunolic acid treatment to adult rat cardiomyocytes in the presence of DOX attenuated caspase-dependent apoptotic signaling by ameliorating proapoptotic p53, p38, and JNK-MAPKs and mitochondrial pathways leading to apoptosis. Furthermore, arjunolic acid when administered to rats significantly inhibited DOX-induced myocardial toxicity by mitigating oxidative stress and apoptotic pathways [13].

2.2. Anthocyanins. Anthocyanins are a group of polyphenolic compounds which are abundantly found in common fruits and vegetables. The effect of six anthocyanidins (cyanidin chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride, peonidin chloride, and petunidin chloride) and seven anthocyanins (cyanidin 3-O-β-galactopyranoside chloride, cyanidin 3-O-β-glucopyranoside chloride, delphinidin 3-O-β-glucopyranoside chloride, malvidin 3-O-β-glucopyranoside chloride, pelargonidin 3-O-β-glucopyranoside chloride, peonidin 3-O-β-glucopyranoside chloride, and petunidin 3-O-β-glucopyranoside chloride) was investigated against DOX-induced cardiotoxicity in H9c2 cardiomyoblasts. All these anthocyanidins improved the cell viability via quenching of reactive oxygen species (ROS) [14]. Delphinidin was found to confer protection against DOX and etoposide inhibition of topoisomerase II, thus warranting careful scrutiny against the use of these agents in combating DOX-induced cardiotoxicity.

2.3. Apigenin. Apigenin is a flavone type flavonoid predominantly found in flowers of chamomile. It is also present in edible plants such as fruits (oranges, apples, cherries, and grape fruits) and vegetables (onions, celery, parsley, broccoli, pepper, barley, and tomatoes) [15]. Apigenin treatment to adult rat cardiomyocytes significantly improved cell viability in the presence of DOX and the mechanism appeared to involve quenching of ROS, mitigation of lipid peroxidation, and myocyte necrosis [16]. However, to date, no studies have demonstrated apigenin efficacy *in vivo*.

2.4. Avicularin. Avicularin, chemically a biflavonoid and a quercetin glycoside, is isolated from the leaves of a flowering plant, *Malus hupehensis*, popularly known as Chinese crabapple, Hupeh crab, or tea crabapple [17]. The protective effects of avicularin against DOX-induced cardiotoxicity were demonstrated in H9c2 cells and the mechanism purported was via its antioxidant property [17]. However, the cardioprotective effects of avicularin are yet to be confirmed *in vivo*.

2.5. Berberine. Berberine is an alkaloid identified in the roots and bark of the *Berberis* species [18]. For the first time, its protective effects on DOX-induced cardiotoxicity in mice were studied by Lv et al. [19]. Berberine was found to reduce mortality, improve body weight and cardiac function, and restore ECG changes in DOX-treated rats. Furthermore, in neonatal rat cardiomyocytes, berberine inhibited DOX-induced apoptosis via counteracting ROS induced p53 activation, mitochondrial dissipation, executioner caspase activation, and activation of AMPK [19]. Moreover, berberine has also been shown to inhibit DOX-induced cardiotoxicity via mitigation of biotransformation of DOX, thereby limiting the bioavailability of doxorubicinol (a major alcohol metabolite of DOX) in the cytoplasm of rat hearts [20].

2.6. Baicalein. Baicalein is a flavonoid derived from the roots of *Scutellaria baicalensis* [21]. By using cultured chick cardiomyocytes *in vitro*, it was shown that baicalein attenuated DOX-induced ROS activation, proapoptotic MAPK, and apoptosis [22]. Recently, in a murine model of DOX-induced cardiotoxicity, baicalein significantly mitigated cardiac injury via augmenting nuclear factor E2-related factor-2 (Nrf2), antioxidant defense, blunting of nitrative stress, inflammation, and apoptosis [23].

2.7. Caffeic Acid Phenethyl Ester (CAPE). Caffeic acid phenethyl ester is an active component of propolis which is the major hive product of bees and is known to be rich in flavonoids [24]. CAPE has been demonstrated to attenuate DOX-induced cardiotoxicity via attenuation of ROS generation and apoptosis. Furthermore, it also improved cardiac function as assessed by hemodynamic measurements and preserved the myocardial structure [25].

2.8. Calceolarioside. Calceolarioside is a phenylpropanoid glycoside isolated from *Calceolaria hypericina* known to possess antiplatelet and anticancer activities [26]. Calceolarioside attenuated DOX-induced cardiotoxicity in H9c2 cells via upregulation of antioxidant enzymes and suppression of apoptosis [27]. However, these effects are yet to be confirmed in animal models.

2.9. Cannabidiol. Cannabidiol (CBD) is a major nonpsychoactive constituent of the plant *Cannabis sativa*, popularly known as Marijuana and used for recreational as well as medicinal purposes [28]. In a chronic model of DOX-induced cardiotoxicity in rats, CBD has been demonstrated to suppress myocardial toxicity via attenuating oxidative stress, inflammation, and cell death pathways [29]. Recently, Hao et al. demonstrated that CBD attenuated DOX-induced cardiotoxicity via augmenting mitochondrial biogenesis and blunting of oxidative and nitrative stress and apoptosis [30]. It is also pertinent to note that CBD also exerts several cardioprotective actions against diabetic cardiovascular complications [31] and it has been approved in Canada and

Table 1: Phytochemicals investigated for cardioprotective activity against DOX-induced cardiotoxicity in *in vivo* studies.

Phytochemical	DOX-induced cardiomyopathy-animal model (acute or chronic)	Cardiac function determined (yes/no)	References
Arjunolic acid	Chronic	Yes	[13]
Berberine	Chronic	Yes	[20]
Berberine	Acute	Yes	[19]
Baicalein	Chronic	No	[23]
Caffeic acid phenethyl ester	Acute	Yes	[25]
Cannabidiol	Acute	Yes	[30]
Cannabidiol	Chronic	No	[29]
Carotenoids	Acute	No	[34]
Eugenol	Acute	Yes	[51]
Gingerol	Chronic	No	[56]
23-Hydroxybetulinic acid	Chronic	Yes	[60]
Hesperetin	Chronic	No	[64]
Hesperidin	Acute	No	[62]
Isorhamnetin	Chronic	No	[66]
Indole-3-carbinol	Chronic	No	[67]
Kaempferol	Chronic	No	[69]
Lycopene	Acute	No	[70]
Lycopene	Acute	No	[71]
Lycopene	Acute	Yes	[72]
Mangiferin	Acute	No	[79]
Mangiferin	Acute	No	[78]
Naringenin	Acute	No	[79]
Naringenin	Acute	No	[80]
Ocotillol	Acute	No	[86]
Ocotillol	Chronic	No	[86]
Hydroxytyrosol	Chronic	No	[65]
Tetrandrine	Chronic	Yes	[140]
Periplogenin	Chronic	No	[92]
p-coumaric acid	Acute	No	[82]
Procyanidins	Chronic	Yes	[100]
Robinin	Acute	No	[114]
Thymoquinone	Acute	No	[137]
Thymoquinone	Acute	No	[138]
Silibinin	Chronic	Yes	[126]
Sesamin	Acute	No	[124]
Sesamol	Chronic	No	[125]
Tetrahydroxystilbene glucoside	Acute	No	[135]
Oleuropein	Acute	No	[87]
Oleuropein	Acute	No	[88]
Oleuropein	Chronic	Yes	[89]
Frederine	Acute	Yes	[52]

Table 1: Continued.

Phytochemical	DOX-induced cardiomyopathy-animal model (acute or chronic)	Cardiac function determined (yes/no)	References
Visnagin	Acute	Yes	[146]
Visnagin	Chronic	Yes	[146]
Schisandrin B	Acute	Yes	[121]
Schisandrin B	Chronic	Yes	[122]
Salvianolic acid A	Acute	Yes	[117]
Tanshinone IIA	Chronic	Yes	[133]
Oleuropein	Acute	No	[87]
Oleuropein	Acute	No	[88]
Oleuropein	Chronic	Yes	[89]
Frederine	Acute	Yes	[52]
Visnagin	Acute	Yes	[146]
Visnagin	Chronic	Yes	[146]
Schisandrin B	Acute	Yes	[121]
Schisandrin B	Chronic	Yes	[122]
Salvianolic acid A	Acute	Yes	[117]
Tanshinone IIA	Chronic	Yes	[133]

Europe for the management of pain associated with multiple sclerosis [32].

2.10. Carotenoids. Carotenoids are the organic pigments and constitute a large group of more than 600 compounds found in plants, which impart color to the leaves and fruits [33]. These are produced from 8 isoprene molecules and contain 40 carbon atoms and are also known as tetraterpenoids with polyisoprenoid structure having a long conjugated double bond system forming the backbone of the molecule, which may be terminated by cyclic end groups that contain oxygen-bearing substitutes. The electron-rich conjugated system of the polyene is believed to afford antioxidant and free radical scavenging activity and these pharmacological effects were attributed to health benefits offered by carotenoids [33]. Recently, the cardioprotective efficacy of carotenoids was demonstrated in DOX-induced tumor-bearing mice [34]. Specifically, carotenoids were found to improve the antioxidant defense and preserve the myocardial membranes, reflected as reduced leakage of myocyte injury marker enzymes without compromising DOX activity on the tumor growth inhibition [34].

2.11. Chrysin. Chrysin is a flavone class of flavonoid and one of the most important bioactive constituents of different fruits, vegetables, and mushrooms [35]. Recently, chrysin cardioprotective effect against DOX-induced acute cardiotoxicity in rats was demonstrated by Mantawy et al. [36]. Chrysin was found to improve antioxidant defense, attenuate oxidative/nitrative stress, and suppress the generation of inflammatory mediators [36].

2.12. Catechins. Epigallocatechin-3-gallate (EGCG) accounts for 50–80% of catechins in green tea and represents about 200–300 mg in a brewed cup of green tea. Convincing data are available to demonstrate that catechins possess potent antioxidant, anti-inflammatory, immunomodulatory, cardioprotective, and anticancer activities [37]. Green tea leaf extract supplementation in cultured rat cardiomyocytes showed its ability to protect the cells against DOX-induced decreased H9c2 cells viability, via quenching of ROS generation [38]. EGCG *in vitro* has been demonstrated to protect cardiomyocytes of neonatal rat hearts from DOX-induced cytotoxicity by attenuating ROS production, apoptosis, and increasing activities and protein expression of endogenous antioxidant enzymes [39]. In another study, EGCG treatment to rat cardiomyocytes significantly attenuated DOX-induced ROS generation and alterations in myocyte contractile dynamics via modulation of proteins involved in calcium handling system [40]. In addition, EGCG also elicited cardioprotective effects on a chronic model of DOX-induced cardiotoxicity via attenuation of oxidative stress and apoptosis pathways [41].

It has been reported that supplementation of EGCG in the diet increases the activities of P-450 family of reductase, augments the bioavailability of DOX, and could predispose the subject to increased risk of cardiotoxicity [42]. Hence, further studies are warranted to investigate and extend the cardioprotective benefits of ECGG, without untoward perpetuation of DOX-induced cardiotoxicity.

2.13. Chrysoeriol. Chrysoeriol is a flavone compound isolated from the leaves of *Digitalis purpurea*, popularly known as Foxglove and well reputed for its cardioprotective actions

Table 2: Phytochemicals exhibiting cytoprotection in the *in vitro* models of DOX-induced cardiotoxicity.

Phytochemical	Concentration of the phytochemical	Cell culture model	DOX dose and time of incubation	References
Arjunolic acid	100 μg/mL	Neonatal rat cardiomyocytes	1 μM for 12 h	[13]
Apigenin	25–100 μM	Rat heart cardiomyocytes	100 μM for 8 h	[16]
Avicularin	10–80 μM	H9c2 cells	20 μM for 24 h	[17]
Berberine	0.06, 0.25, 1.0, and 4.0 μM	Neonatal rat cardiomyocytes and MCF-7 cells	1 μM for 2 h	[19]
Baicalein	25 μM	Chick embryo cardiomyocytes and MCF-7 cells	1, 10, 50, or 100 μM for 24 h	[22]
Calceolarioside	40 μM	H9c2 cells	1, 2, or 5 μM for 30 h	[27]
23-Hydroxybetulinic acid	0.2, 2, and 20 μM	H9c2 cells	5 μM for 18 h	[60]
Isorhamnetin	3.125 to 25 μg/mL	MCF-7, HepG2, and Hep2 cells	1 μM for 36 h	[66]
Kaempferol	5 to 50 μM	H9c2 cells	1 μM for 24 h	[69]
Morin hydrate	0.17 mM	ECV304 and HepG2 cells	6 mM for 12 h	[77]
Naringenin-7-O-glucoside	10–80 μM	H9c2 cells	10 μM for 24 h	[83]
Osthole	10–40 μM	Neonatal rat cardiomyocytes	1 μmol for 24 h	[90]
Luteolin-7-*O*-β-D-glucopyranoside	5, 10, and 20 μM	H9c2 cells	20 μM for 24 h	[75]
Luteolin-7-*O*-β-D-glucopyranoside	5–80 μM	H9c2 cells	10 μM for 24 h	[76]
Vincristine	10–30 μM	Adult mouse cardiomyocytes	15 and 20 μg/mL for 24 h	[144]
Sulforaphane	2.5 μM	H9c2 cells	5 μg/mL for 16–18 h	[129]
C-Phycocyanin	10 μM	Adult rat cardiomyocytes	10 μM for 4, 24, and 48 h	[94]
Plantainoside D	1–20 μg/mL	H9c2 cells	1, 2, and 4 μM for 30 h	[93]
Sesamol	12.5–50 μM	H9c2 cells	1 μM for 30 min	[125]
Tetrahydroxystilbene glucoside	3–300 μM	Neonatal rat cardiomyocytes	1 μmol/L for 24 h	[143]
Chrysoeriol	20 μg/mL	H9c2 cells	1 μM for 24 h	[43]
Visnagin	20 μM	Neonatal rat and zebrafish cardiomyocytes, cardiac HL-1 cells	100 μM for 48 h	[146]
Z-Guggulsterone	1–30 μM	H9C2 cells	1 μM for 24 h	[143]
Tanshinone IIA	0.1, 0.3, 1, and 3 μM	Neonatal rat cardiomyocytes	1 μM for 24 h	[134]
Tanshinone IIA	1.6–40 μM	H9c2 cells	1 μM for 24 h	[133]
Tanshinone IIA	0.5, 1, and 2 μmol/L	Neonatal rat cardiomyocytes	1 μmol/L for 24 h	[132]
Sodium tanshinone IIA sulphonate	0.05–0.5 mM	Mice heart mitochondria	0.2 mmol for 10 min	[131]
Anthocyanidins and anthocyanins	0–100 μM	H9c2 cells and MCF-7 cells	1 μM for 24 h	[14]

Table 2: Continued.

Phytochemical	Concentration of the phytochemical	Cell culture model	DOX dose and time of incubation	References
Caffeic, chlorogenic, and rosmarinic acid	100 and 200 μM	Rat heart microsomes and mitochondria	100 μM for 8 h	[116]

ECV304 cells: human umbilical vein endothelial cells; HepG2 cells: human hepatocellular carcinoma cells; MCF-7 cells: human breast carcinoma; H9c2 cells: rat ventricular cardiomyoblast cells.

[43]. Chrysoeriol has been found to reduce cell death and attenuate ROS generated oxidative stress and lipid peroxidation in DOX-induced cardiotoxicity in H9c2 cardiomyoblasts without affecting antitumor activity of DOX [43]. For an evidence based approach, additional studies on its cardioprotective efficacies are warranted.

2.14. Curcumin. Curcumin is a phenolic yellow pigment constituent found in the rhizomatous parts of *Curcuma longa* (turmeric) [44]. Several *in vitro* and *in vivo* studies have demonstrated curcumin cardioprotective actions against DOX-induced myocardial toxicity. The key mechanisms postulated for curcumin cardioprotective activity include diminution of oxidative stress, inflammation, and associated cell death pathways [45–48]. Although curcumin has been reported to elicit several beneficial effects in various preclinical studies, the bioavailability is yet to be established in human subjects. Therefore, derivatives of curcumin are being perused to increase its bioavailability and, in this direction, a recent report suggests that the nanoparticle of curcumin could ameliorate DOX-induced cardiotoxicity [49].

2.15. Eugenol. Eugenol is the active component of essential oil isolated from *Syzygium aromaticum*, popularly known as clove, which is one of the common ingredients of spice mixtures [50]. Eugenol treatment was shown to significantly improve antioxidant defense mechanisms, decrease lipid peroxidation, and attenuate abnormal Ca^{2+} transients in the cardiomyocytes along with inhibition of apoptosis in rat hearts following acute DOX administration. Eugenol also preserved the myocardium and restored hemodynamics along with preserved histology [51].

2.16. Frederine. 7-Monohydroxyethylrutoside (monoHER2, frederine) is a synthetic flavonoid, and it significantly inhibited DOX-induced myocardial toxicity, via suppression of oxidative stress and apoptosis in a chronic murine model [52, 53]. In addition, monoHER2 did not interfere with DOX anticancer activity *in vitro* and *in vivo* [54]. Considering these observations, monoHER2 could be further developed for this clinical application as cardioprotective adjuvant.

2.17. Gingerol. Gingerol is the pungent phenolic constituent of *Zingiber officinalis* (ginger) [55]. In a chronic model of DOX-induced cardiomyopathy, gingerol inhibited DOX-induced myocardial ROS generation, inflammation via attenuation of NF-κB activation, and downregulation of soluble receptor for advanced glycation end products (sRAGE). In addition, gingerol also inhibited myocardial apoptosis via mitigating caspase-3 activities [56].

2.18. Ginsenosides. Ginsenosides are the saponin constituents of *Panax notoginseng*, popularly known as ginseng in traditional Chinese medicine [57]. Ginsenosides have been classified into protopanaxatriols (Rg1, Rh1, and PPT) and protopanaxadiols (Rg3, Rh2, and PPD). Of the ginsenosides, protopanaxadiols such as ginsenoside Rb1, ginsenoside Rh2, and compound K have been shown to exhibit anticancer and anti-inflammatory activities [57].

Ginsenoside Rh2 (Rh2) has been shown to elicit cardioprotective effects against DOX-induced cardiotoxicity in H9c2 cell line, as well as *in vivo* in an acute mouse and chronic rat model of DOX-induced cardiomyopathy. Rh2 enhanced cell viability of H9c2 cells and ameliorated DOX-induced release of the CK-MB, LDH. Furthermore, Rh2 ameliorated DOX-induced myocardial toxicity in mouse and rats via suppressing oxidative stress and improved the indices of cardiac function as determined by ECG [58].

2.19. Diosgenin. Diosgenin is a steroid saponin found abundantly in several plants including *Solanum* and *Dioscorea* species and *Costus speciosus*. In a chronic model of DOX-induced cardiomyopathy, diosgenin elicited cardioprotective effects, via activation of prosurvival kinase, protein kinase A (PKA), diminution of p38-MAPK, caspase-3 activities, and generation of free radicals along with attenuation of inflammatory mediators. Mechanistically, it was found to improve myocardial fibrosis and increase the cardiac levels of cGMP via modulation of phosphodiesterase-5 activity [59].

2.20. Hydroxybetulinic Acid. 23-Hydroxybetulinic acid is isolated from *Pulsatilla chinensis*. In a chronic murine model of DOX-induced cardiomyopathy, 23-hydroxybetulinic acid significantly improved the survival of the animals and inhibited apoptosis mainly via inhibition of DOX metabolism in the mitochondria. Similar results were also obtained in H9c2 cells [60].

2.21. Hesperidin. Hesperidin is a bioflavonoid abundantly found in vegetables and citrus fruits such as oranges, lemons,

and grapefruits [61]. Citrus flavonoids have been shown to reduce risk of cardiovascular diseases prominently due to their antioxidant and anti-inflammatory effects involving numerous cell signaling pathways [61]. It has been found to improve antioxidant status, inhibit lipid peroxidation, and reduce myocardial enzyme leakage by salvaging myocardium in DOX-induced cardiotoxicity in acute toxicity model conducted rats [62]. Additionally, it sensitized cancer cells to DOX-induced apoptosis and showed synergism in inhibiting P-gp and multidrug resistance, thus appearing to be effective as an adjunct to enhance the efficacy and attenuate the resistance to DOX during chemotherapy [63]. Thus, the potential of citrus flavonoids as cochemotherapeutic and cardioprotective agents is encouraging but further studies are warranted for conclusive evidence in cancer as well as chemotherapy associated cardiotoxicity. In addition, hesperetin (aglycone) derivative of hesperidin also ameliorated chronic DOX treatment associated cardiotoxicity in rats, via attenuation of p38-MAPK, caspase-3, and NF-κB activation and oxidative stress in the myocardial tissues [64].

2.22. Hydroxytyrosol. Hydroxytyrosol is a polyphenolic constituent in *Olea europaea*, popularly known as olive oil, which is widely used in food and medicine [65]. Hydroxytyrosol has been shown to improve cardiac function by maintaining homeostasis at mitochondrial level, by preserving mitochondrial electron transport chain complexes I–IV and inhibiting apoptosis-inducing factor, and oxidative stress markers in chronic DOX-induced cardiotoxicity in rats harboring breast cancer [65]. Furthermore, hydroxytyrosol did not compromise the DOX antitumor activity against the implanted tumor cells and also improved the survival of the animals [65].

2.23. Isorhamnetin. Isorhamnetin is a flavonol aglycone abundantly found in several medicinal plants, such as *Hippophae rhamnoides* (sea buckthorn) [66]. Isorhamnetin significantly conferred cardioprotection in a chronic model of DOX-induced cardiotoxicity. The mechanism of cardioprotection involves suppression of oxidative stress and activation of mitochondrial apoptotic pathway and mitogen-activated protein kinase pathways, suggesting antioxidant mediated cardioprotective mechanism. Similar results were obtained in H9c2 cells [66]. Furthermore, it also synergistically improved the DOX anticancer activity in tumor cell lines [66].

2.24. Indole-3-carbinol. Indole-3-carbinol is a natural indole compound predominantly found in cruciferous vegetables [67]. In a chronic DOX-infusion associated cardiotoxicity murine model, indole-3-carbinol was found to reduce solid Ehrlich tumor size and volume, augment antioxidant defense, and inhibit lipid peroxidation, leading to stabilization of cell membrane and reduced leakage of myocyte injury marker enzymes. It was also found to decrease sphingosine kinase 1 (SphK1) activity and inflammatory mediators along with mitigating histological perturbations and modulating cell death mediators [67].

2.25. Kaempferol. Kaempferol is one of the most common dietary flavonoids and it is well studied for its antiapoptotic, cardioprotective, antioxidative, anti-inflammatory, chemopreventive, and anticancer properties as well as modulation of chemoresistance [68]. The cardioprotective effects of kaempferol against DOX-induced cardiotoxicity in rats were demonstrated using a chronic model. Kaempferol counteracted cardiotoxicity by inhibiting p53 expression in mitochondrion-dependent apoptotic signaling and ERK-dependent mitogen-activated protein kinase pathway following binding to the promoter region of the Bax proapoptotic gene. It also effectively suppressed DOX-induced extracellular signal-regulated kinase (ERK1/2) activation but had no effect on p38 and JNK [69].

2.26. Lycopene. Lycopene is a carotenoid and nonprovitamin A found abundantly in *Lycopersicum esculentum* (tomatoes) and known to impart color to tomatoes [33]. Karimi et al., in a very early study, demonstrated the protective effect of tomato extract and lycopene on acute DOX-induced cardiotoxicity in mice [70]. Tomato extract and lycopene prevented rise in myocyte injury marker enzyme, CK-MB, in serum and ameliorated cardiomyocytes injury evidenced by histopathological examination. Furthermore, Yilmaz et al. studied the protective role of lycopene in DOX-induced heart and kidney toxicities using biochemical and histopathological assessments and reported that lycopene has potential to inhibit lipid peroxidation and improve antioxidants evidenced by reduced lipid peroxides and improved GSH in both the heart and the kidneys. The protective effect was further substantiated by histopathological changes. The authors concluded that treatment with lycopene might prevent cardiac and renal toxicities in rats [71]. However, the results were not further substantiated by functional improvement as lycopene did not prevent left ventricular systolic dysfunction induced by DOX [72]. However, it suppressed DOX-induced myocyte damage without preventing interstitial collagen accumulation increase [72].

Lycopene supplementation also increased lycopene absorption in heart, liver, and plasma and, in another study, the same group of authors showed that there was no depletion of lycopene from myocardium of lycopene-supplemented rats treated with DOX and that higher antioxidant capacity in myocardium and less oxidative cleavage of lycopene in intestinal mucosa of DOX-treated rats suggest an antioxidant role of DOX rather than acting as a prooxidant [73]. The authors further showed that tomato-oleoresin enhances the chemotherapeutic effect of DOX. It maintained lycopene levels in heart and protected against cardiac oxidative DNA damage induced by DOX in rats [73]. The lycopene protected the heart against DOX associated cardiotoxicity by several mechanisms including the quenching of singlet oxygen, peroxy radicals, reaction with free radicals, restoring levels of vitamin E and vitamin C, reducing DNA damage, restoring cellular antioxidants, and preventing depletion of glutathione. Several studies showed the potential role of lycopene in the prevention of side effects of antineoplastic drugs in cell culture and animal models [74]. These results

suggest that tomato extract and lycopene inhibit DOX cardiotoxicity and collectively might serve as a novel combination chemotherapeutic agent with DOX to limit free radical-mediated organ injury. However, further studies are required to investigate the role of lycopene in mitigating the side effects of chemotherapy in human subjects.

2.27. Luteolin-7-O-β-D-glucopyranoside. Luteolin-7-O-β-D-glucopyranoside is a flavonoid isolated from the plant *Dracocephalum tanguticum* that is widely used in Chinese and Tibetan traditional medicine [75]. The cytoprotective activities were demonstrated on DOX-induced cytotoxicity in H9c2 cardiomyocytes. Among several isolated compounds, luteolin-7-O-β-D-glucopyranoside was found to show antioxidant effect and a potent cytoprotective activity against DOX-induced toxicity as evidenced by decreased death of H9c2 cells, reduced myocyte injury marker enzymes, and reduced intracellular concentration of ROS and Ca^{2+} [75]. Recently, Yao et al. also demonstrated protective effects of luteolin-7-O-glucoside on DOX cytotoxicity in H9c2 cells [76]. It was found to improve cell viability and ameliorate ROS generation and mitochondrial depolarization. Furthermore, it enhanced the expression of prosurvival kinases and diminished ROS generation [76].

2.28. Morin Hydrate. Morin hydrate is a biflavonoid commonly found in fruits such as guava, fig, almond, grapes, and apple and vegetables such as onion, seed weeds, and several other members of Moraceae family [77]. It has been observed to enhance antioxidant defense against oxidative stress in human umbilical vein endothelial cells (ECV304) and HepG2 cells and minimize DOX toxicity in ECV304 and primary mouse cardiomyocytes [77]. However, *in vivo* studies regarding morin efficacy in combating DOX-induced cardiotoxicity are still lacking.

2.29. Mangiferin. Mangiferin is a xanthonoid structure with C-glucosyl linkage and polyhydroxy component found in many plant species; however, *Mangifera indica* (mango tree) is the major source [78]. Using a chronic model of cardiomyopathy in rats, mangiferin has been shown to exert cardioprotective action against DOX-induced cardiotoxicity by inhibition of proinflammatory mediators and proapoptotic genes and regulating calcium homeostasis modulating proteins [79].

2.30. Naringin. Naringin is a flavanone glycoside abundantly found in citrus fruits such as lemon, oranges, and grapefruits and in tomatoes. It has been documented to possess numerous biological properties such as antioxidant, anti-inflammatory, and antiapoptotic activities [80]. In an acute model of DOX-induced cardiotoxicity, naringin treatment improved antioxidant defense and inhibited lipid peroxidation along with histopathological preservation, thereby reducing leakage of myocyte marker enzymes. It also decreased the levels of inflammatory mediators and restored the mitochondrial complexes (I–IV) activities in the heart tissues along with histopathological salvage [80]. The studies indicate cardioprotective effects; however, further clinical research is required to provide significant insights into the mechanisms underlying the effects of naringin on human subjects.

2.31. Naringenin and Its Derivatives. Naringenin is a flavanone commonly found in citrus fruits such as grapefruit, orange, and lemon [80]. Arafa et al. demonstrated that naringenin elicited antioxidant mediated protection against DOX-induced cardiac toxicity in Swiss albino rats [81]. Recently, the combination of p-coumaric acid and naringenin was found to be superior in exerting antioxidant mediated cardioprotection against DOX-induced cardiotoxicity in rats [82]. Additionally, naringenin enhanced antitumor effect of DOX by selectively modulating drug efflux pathways; that is, it inhibited the activity of multidrug resistance-associated protein and did not affect the *in vivo* pharmacokinetics of intravenously administered DOX [80].

Naringenin-7-O-glucoside is a flavanone glycoside isolated from *Dracocephalum rupestre*. It has been demonstrated to protect against DOX-induced cardiotoxicity in H9c2 cells [83]. Particularly, naringenin-7-O-glucoside improved cell viability, prevented the release of myocyte injury marker enzymes LDH and CK, and augmented antioxidant defense [84]. Furthermore, naringenin-7-O-glucoside was observed to enhance NAD(P)H: quinone oxidoreductase (NQO1) and ERK activation and Nrf2 protein levels in DOX stressed H9c2 cells. These phenotypic changes brought about by naringenin-7-O-glucoside are attributed to the induction of antioxidant defense and attenuation of cell death pathways [85]. However, *in vivo* studies are yet to be performed.

2.32. Ocotillol. Ocotillol is an aglycone derivative of pseudoginsenoside-F11, which is devoid of sugar moiety and is found in American ginseng, *Panax quinquefolius*. Fu et al. reported that ocotillol enhances survival rate of animals in both acute and chronic models of DOX-induced cardiotoxicity. Ocotillol prevented depletion of glutathione and lipid peroxidation along with restoration of myocyte injury marker enzymes following preservation of cardiomyocytes cell membrane. Furthermore, ocotillol also improved cardiac function and hence was suggested as an adjuvant for counteracting DOX-induced cardiotoxicity [86].

2.33. Oleuropein. Oleuropein is a phenolic constituent of *Olea europaea* (olive oil) [87]. Andreadou et al. have shown the protective effect of oleuropein against DOX-induced acute cardiotoxicity in rats. Oleuropein was found to restore the myocardial necrosis marker enzyme levels and attenuation of oxidative stress and apoptosis [88]. Moreover, the same group of authors in a separate study demonstrated that oleuropein treatment aids the compensation of distressed energy metabolic pathways mechanistically by restoration of metabolites to the normal levels as DOX generated free radicals nonenzymatically convert pyruvate to acetate and alpha-ketoglutarate to succinate [87]. The cardioprotective role of oleuropein in chronic DOX-induced

cardiomyopathy has also been demonstrated [89]. Particularly, oleuropein treatment significantly suppressed DOX-induced oxidative/nitrative stress, augmented prosurvival kinases, and improved the cardiac functions [89].

2.34. Osthole. Osthole is a coumarin compound found in several medicinal plants such as *Cnidium monnieri* and *Angelica pubescens* [90]. In rat neonatal cardiomyocytes, osthole significantly improved the survival of the cells by abrogating apoptosis, wherein the mechanism appeared to involve the suppression of mitochondrial pathway of apoptosis triggered by DOX [90]. However, *in vivo* studies have not been conducted, and it is warranted to recapitulate the *in vitro* cardioprotective actions of osthole.

2.35. p-coumaric Acid. p-coumaric acid is a phenolic acid that serves as a precursor of other phenolic compounds and is found in plants such as peanut, tea, and coffee [82]. p-coumaric acid has been shown to attenuate oxidative stress and inhibit myocyte injury in DOX-induced myocardial injury in rats [91]. In a separate study, p-coumaric acid in combination with naringenin showed cardioprotection by augmentation of antioxidant defense against DOX-induced cardiotoxicity in rats [82].

2.36. Periplogenin. Periplogenin is a cardenolide isolated from *Aegle marmelos*, commonly known as Bael in the traditional Indian system of medicine [92]. Periplogenin has been shown to decrease lipid peroxide levels, improve antioxidant defense, and salvage cardiomyocytes in a chronic model of DOX-induced cardiotoxicity in rats [92].

2.37. Plantainoside D. Plantainoside D is an iridoid glucoside isolated from *Picrorhiza scrophulariiflora* (Picrorhiza). The chemopreventive and antioxidant activities encouraged evaluating the cardioprotective effect of plantainoside D against DOX-induced apoptosis in H9c2 cells. Plantainoside D was found to inhibit oxidative stress and proinflammatory cytokines expression and attenuate apoptosis in H9c2 cardiomyoblasts [93].

2.38. Phycocyanin. C-Phycocyanin is a biliprotein found in *Spirulina platensis*, blue-green algae. Phycocyanin has been found to protect against DOX-induced oxidative stress and apoptosis in adult rat cardiomyocytes as evidenced by reduced ROS formation, DNA fragmentation, and attenuation of Bax as well as release of cytochrome C and increase in the activity of caspase-3 [94]. However, these *in vitro* findings are yet to be confirmed in rodent model of DOX-induced cardiomyopathy.

2.39. Proanthocyanidin and Derivatives. Proanthocyanidins are a mixture of structurally and functionally diverse chemicals which are predominantly found in grape seed and show high bioavailability and protect the organs from toxic chemicals used to induce diseases in *in vitro* and *in vivo* studies [95, 96]. Ray et al. reported the bioavailability and protective property of grape seed proanthocyanidin and a novel IH636 grape seed proanthocyanidin extract against DOX-induced cardiotoxicity as well as multiorgan protection in mice [97]. Bagchi et al. further reported that IH636 proanthocyanidin extract afforded protection was superior to vitamin C, vitamin E, and beta-carotene and demonstrated significant cytotoxicity towards human breast, lung, and gastric adenocarcinoma cells, while enhancing the growth and viability of normal cells in both *in vitro* and *in vivo* studies [98]. In another study, proanthocyanidins enhanced DOX-induced antitumor effect and reversed drug resistance and mechanisms attributed partially to the promotion of DOX-induced apoptosis through elevation of intracellular DOX, Ca^{2+}, and Mg^{2+} concentration and reduction of pH value and mitochondrial membrane potential in DOX-resistant K562/DOX cells [99]. Furthermore, proanthocyanidin strongly enhanced the antitumor activity of DOX and ameliorated chronic DOX-induced myocardial oxidative stress and immunosuppression in tumor-bearing mice [99]. In addition, grape seed proanthocyanidin also showed antioxidant mediated cardioprotection against both high and low dose DOX-induced cumulative chronic cardiotoxicities in rats [100].

2.40. Resveratrol. Resveratrol, a natural phytoalexin, is commonly found in *Vitis vinifera* (grapes) [101]. Resveratrol induced antioxidants and phase 2 enzymes in the H9c2 cells, accompanied by increased resistance to oxidative and electrophilic cell injury [102]. Additionally, there was no significant effect of resveratrol on NADPH-cytochrome P-450 reductase (P-450 reductase), which plays an important role in the metabolism of many endogenous compounds and xenobiotics including DOX. The enzyme P-450 reductase activates them to their more toxic metabolites via one electron reduction which triggers free radical cascade. In some cases, however, such transformation is essential to produce therapeutic effect of anticancer drugs [42].

In DOX-induced cardiotoxicity in rats, resveratrol has been shown to ameliorate the severity of cardiac dysfunction and prevent oxidant stress responses [103, 104]. Furthermore, resveratrol was found to confer cardioprotection and reduce cardiac fibrosis in acute as well as chronic *in vivo* models of DOX-induced cardiomyopathy in rats. Mechanistically, resveratrol has been demonstrated to protect against DOX-induced oxidative stress through changes in mitochondrial function, specifically the Sirt1 pathway, leading to cardiac cell survival [105]. Resveratrol attenuated DOX-induced cardiomyocyte apoptosis in mice via upregulation of Sirt1-mediated p53 deacetylation and activation of Sirt1, a NAD^+-dependent deacetylase, resulting in improved mitochondrial function, which culminates in activation of the transcription factors which coordinate expression of key antioxidant proteins by binding to the antioxidant response elements that regulate cell survival [106]. The overexpression of Sirt1 inhibited cell apoptosis by suppression of p38-MAPK phosphorylation and caspase-3 activation along with amelioration of ROS generation and prevented DOX-induced functional loss in DOX-induced cardiomyocyte injury [106]. DOX induces autophagy in cardiomyocytes which is a degradation system

for eukaryotic cells to turn over organelles and long-lived proteins, thereby maintaining cellular homeostasis. Thus, aberrant autophagy activity impairs basal cardiac structure and function, making animals more sensitive to stress-induced heart failure. The ability of resveratrol to inhibit autophagy is mediated by inhibition of p70S6 kinase 1 (S6 K1) that is essential for resveratrol to suppress DOX-induced autophagy and cytotoxic effects [107].

DOX inhibits AMP-activated protein kinase (AMPK), resulting in Sirt1 dysfunction and p53 accumulation in mouse embryonic fibroblasts, and pharmacological activation of AMPK by resveratrol has been shown to alleviate the side effects of DOX in H9c2 cells [108]. Furthermore, resveratrol has been shown to confer cardioprotection in DOX-induced cardiomyocyte apoptosis in nude mice by induction of heme oxygenase-1 (HO-1) mediated mechanisms [109, 110]. Resveratrol was also reported to aid the differentiation of adipose-derived mesenchymal stem cells to cardiomyocytes and protected against noxious effects of DOX to the myocardium [111]. Furthermore, resveratrol supplement along with exercise training was found to be more effective in preventing DOX-induced LV remodeling associated with the reduction of DOX-induced oxidative stress [112]. In spite of these advances made in preclinical studies, resveratrol bioavailability is seldom established in human subjects and this warrants further approaches to extend its beneficial effects to mankind [113].

2.41. Robinin. Robinin is a flavonoid glycoside isolated from leaves of *Vigna unguiculata*, a dietary plant used in traditional cuisine in India [114]. Treatment with robinin was found to improve endogenous antioxidants, reduce ROS generation, and inhibit lipid peroxidation and proinflammatory mediators such as cyclooxygenase (COX2) and lipoxygenase (LOX15) along with restoring myocyte injury marker enzymes. The improvement in the level of transforming growth factor-β1 (TGF-β1), Smad2, murine double minute (Mdm2), Smad3, cyclin-dependent kinase inhibitor 2A, Smad4, and Smad7 in addition to favorable modulation of p53, Bcl-2, and Bax revealed the cardioprotective mechanism of robinin in combating DOX-induced cardiotoxicity [114].

2.42. Rosmarinic Acid. Rosmarinic acid is an ester of caffeic acid abundantly found in numerous plants, being most common in Boraginaceae and Lamiaceae families [115]. Rosmarinic acid showed remarkable cytoprotection against DOX toxicity in neonatal rat cardiomyocytes and DOX-induced lipid peroxidation of heart membranes, mitochondria, and microsomes and effects were found to be comparable to dexrazoxane [116]. Furthermore, it inhibited DOX-induced oxidative stress and apoptosis in H9c2 cardiomyoblasts by improving cell viability, inhibiting the production of ROS, and activation of prosurvival kinases [115]. However, *in vivo* cardioprotective actions against DOX-induced cardiotoxicity are hitherto unknown.

2.43. Salvianolic Acids. Salvianolic acids especially salvianolic acid A and salvianolic acid B are the most abundant water-soluble compounds extracted from *Salvia miltiorrhiza* (Danshen or red sage) [117]. Salvianolic acid A has been shown to protect against DOX-induced mitochondrial toxicity *in vitro* in rat cardiomyocytes due to its antioxidant action, without antagonizing effect on the antitumor activity of DOX [118]. The protective effects of salvianolic acid were reconfirmed *in vivo* in another study, against DOX cardiotoxicity in mice [117], via abrogation of oxidative stress and inflammation.

2.44. Schisandrin B. Schisandrin B, a dibenzocyclooctadiene lignin, is isolated from the fruit of *Schisandra chinensis*. It has been shown to salvage cardiomyocytes, via conferring antioxidant defense by restoring glutathione flux in an acute animal model of DOX-induced cardiotoxicity [119]. Furthermore, it also mitigated DOX-induced cardiotoxicity in rabbits [120]. Recently, cardioprotective effects of schisandrin B against DOX-induced cardiotoxicity were reconfirmed and the mechanism of protection was evidenced by amelioration of proinflammatory cytokines, lipid peroxidation, DNA damage, apoptosis, and MAPK activation in the myocardial tissues [121, 122].

2.45. Salidroside. Salidroside, a phenylethanoid glycoside, has been isolated from the roots of *Rhodiola rosea* (Roseroot). Wang et al. demonstrated that treatment of salidroside to either H9c2 cells or mice stressed with acute DOX administration conferred cardioprotective effects. The mechanism was defined to involve antioxidant and suppression of proapoptotic mediators [123]. The cardioprotective effects of salidroside were reconfirmed in a placebo controlled clinical trial wherein sixty patients with breast cancer receiving epirubicin were given salidroside (600 mg/day) or placebo starting 1 week before chemotherapy and assessed at baseline and 7 days after each new epirubicin dose of 100 mg/m^2. Decline in strain rate peak was observed at an epirubicin dose of 200 mg/m^2, with no significant differences between salidroside and placebo. At increasing cumulative doses of epirubicin, the strain rate normalized only with salidroside, showing a significant difference in comparison with placebo at epirubicin doses of 300 mg/m^2. The authors concluded that salidroside may provide protection against chemotherapy-induced early left ventricular regional systolic dysfunction in patients with breast cancer. Based on preclinical and clinical data, salidroside needs to be investigated further in a larger population for advocating salidroside as an adjuvant to thwart the DOX-induced cardiotoxicity.

2.46. Sesamin. Sesamin is a major lignin obtained from seeds of *Sesamum indicum*. Sesamin was observed to increase the endogenous antioxidant enzymes and prevent mitochondrial damage via activation of Sirt1 in an acute model of DOX-induced cardiotoxicity [124].

2.47. Sesamol. Sesamol is a phenolic constituent of oil obtained from seeds of *Sesamum indicum* and is used commonly as an edible oil. The cardioprotective effects of

sesamol were confirmed in an *in vivo* study, wherein sesamol mitigated cumulative DOX-induced cardiomyopathy in rats [125]. Sesamol improved antioxidant defense status, reduced myocyte injury marker enzymes released from cardiomyocytes, and inhibited lipid hydroperoxide. The salvage of tissues evidenced by biochemical and histopathological studies demonstrated cardioprotective effects of sesamol [125]. However, further mechanistic studies should be carried out investigating the effect on DOX efficacy and pharmacokinetic interaction.

2.48. Silibinin. Silibinin, a flavonolignan, is an active component of *Silybum marianum* (milk thistle), popularly known as silymarin and known to constitute 50–70% of the silymarin extract. The cardioprotective effect exerted by silymarin, silibinin, dehydrosilibinin, and silychristin was comparable to that of dexrazoxane, while silydianin exerted the best protective effect [126].

2.49. Sulforaphane. Sulforaphane is an organosulfur compound found in a significant amount in cruciferous vegetables, especially in broccoli (*Brassica oleracea*) [127]. The cardioprotective effects of sulforaphane were first demonstrated in H9c2 rat myoblasts as evidenced by reduced number of apoptotic cells along with decreased expression of proapoptotic proteins such as Bax, caspase-3, and cytochrome C [128]. It also reduced ROS generation and restored mitochondrial membrane potential [128]. Moreover, the cardioprotective effects of sulforaphane were found to be mediated by the activation of the Kelch-like ECH-associated protein 1 (Keap1)/NF-E2-related factor-2 (Nrf2)/antioxidant-responsive element (ARE) pathway, which in turn mediates the induction of HO-1 [129].

2.50. Tanshinone IIA and Derivatives. Tanshinones are the group of bioactive compounds isolated from *Salvia miltiorrhiza* (Danshen), a Chinese medicinal plant reputed for the management of cardiovascular diseases in particular, angina pectoris, atherosclerosis, myocardial infarction, and ischemic-reperfusion injury [130]. Sodium tanshinone IIA sulfonate, a water-soluble derivative of tanshinone IIA, was demonstrated to be beneficial in reducing DOX-induced cardiotoxicity in mice hearts and in cultured cardiomyocytes [131]. Treatment with sodium tanshinone IIA sulfonate prevented decrease in body weight and reduced myocardial lipid peroxidation in mice along with improved activities of endogenous antioxidant enzymes. In addition, the antioxidative mechanism was also supported by *in vitro* experiments showing that sodium tanshinone IIA sulphonate scavenged DOX semiquinone free radical in heart homogenate and inhibited DOX-induced mitochondrial lipid peroxidation and swelling [131].

Furthermore, another study demonstrated the beneficial effect of tanshinone IIA on decreasing DOX-induced apoptosis in neonatal rat cardiomyocytes and underlying molecular mechanisms [132]. Tanshinone IIA ameliorated apoptosis and ROS generation induced by DOX in a dose-dependent manner. It was further supported by the inhibition of DOX-mediated reduction of the ratio of Bcl-2/Bax [132]. Furthermore, a separate study also recapitulated that tanshinone IIA significantly inhibited DOX-induced toxic effects in H9c2 cells as well as in animal models of cardiotoxicity [133]. In this study, tanshinone IIA was shown to improve cell viability and ameliorate apoptosis of DOX-induced cytotoxicity in H9c2 cells. Furthermore, the cardioprotective effects of tanshinone IIA sodium sulfonate were confirmed by decreased ST interval and QRS interval in ECG; improved histological appearance of myocardium; increased myocardial tensile strength; and decreased fibrosis [133]. Recently, Hong et al. evaluated the protective effect of tanshinone IIA on DOX-induced cardiomyocyte apoptosis and explored its intracellular mechanisms using primary cultured neonatal rat cardiomyocytes. Tanshinone IIA was found to inhibit DOX-induced reactive oxygen species generation, reduce the expression of caspase-3 and cytochrome C, and increase BcL-x(L) expression, resulting in protecting cardiomyocytes from DOX-induced apoptosis. In addition, tanshinone IIA also enhanced Akt phosphorylation in cardiomyocytes and inhibited apoptosis [134].

2.51. Tetrahydroxystilbene Glucoside. Tetrahydroxystilbene glucoside is one of the active components extracted from *Polygonum multiflorum* (knot grass). For the first time, Zhang et al. demonstrated its protective effect on neonate rat cardiomyocytes and on an acute mouse model of DOX-induced cardiotoxicity [135]. In the mouse model, it was shown to inhibit lipid peroxidation accompanying improved glutathione, reduced animal mortality, preserved histopathological changes, and restored levels of myocyte injury marker enzymes. In the *in vitro* study, it prevented DOX-induced loss of mitochondrial membrane potential, caspase-3 activation, and upregulation of Bax protein expression along with upregulation of Bcl-2 and inhibited ROS generation. It was also observed to inhibit DOX-induced increases in intracellular Ca^{2+} and apoptosis of cardiomyocytes in a concentration-dependent manner [135].

2.52. Thymoquinone. Thymoquinone is the main active constituent of the volatile oil of *Nigella sativa* Linn., popularly known as black seed, used for culinary and medicinal purposes [136]. Thymoquinone suppressed DOX-induced cardiotoxicity in an acute murine model of cardiomyopathy, without compromising antitumor activity of DOX [137]. Furthermore, thymoquinone also circumvented DOX-mediated cardiotoxicity in acute model, wherein the key mechanism was postulated to involve antioxidant pathways [138]. Finally, thymoquinone synergistically increased DOX activity in several cancer cell lines and prevented DOX-induced toxicity in H9c2 cells [139].

2.53. Tetrandrine. Tetrandrine is a bisbenzylisoquinoline alkaloid isolated from the dried root of *Stephania tetrandra*. In a chronic model of DOX-induced cardiomyopathy, tetrandrine significantly inhibited myocardial apoptosis via quenching of ROS and restoration of mitochondrial capacity. These beneficial effects were corroborated with improved

indices of cardiac function [140]. It is pertinent to note that tetrandrine had a negligible effect in DOX pharmacokinetics properties in rodents, suggesting that tetrandrine might be a suitable candidate to be developed as cardioprotective adjuvant [141].

2.54. Z-Guggulsterone. Guggulsterone is a major active component of *Commiphora mukul,* popularly known as Guggul and reputed for its antihyperlipidemic and cardioprotective effects in Ayurvedic medicine [142]. Wang et al. demonstrated the protective activity of guggulsterone against DOX-induced cytotoxicity in H9c2 cells. It was found to improve cell viability, morphology, and cytotoxicity and cellular antioxidants along with inhibition of apoptosis by altering activity of PARP, caspase-3, cytochrome C release, and Bcl-2 proteins and reducing the activation of NF-κB [143].

2.55. Vincristine. Vincristine is an alkaloidal constituent isolated from *Catharanthus roseus* (Madagascar periwinkle), also known as *Vinca rosea*. Recently, its potential to prevent DOX-induced cardiomyocyte death and related mechanisms has been reported in adult mouse cardiac myocytes [144]. Vincristine treatment to cardiomyocytes in the presence of DOX increased the cell viability. This was concordant with decreased PARP and caspase-3 activities and increased activation of prosurvival kinase Akt and diminished MAPK pathways [144]. However, the precise cardioprotective effects *in vivo* are yet to be demonstrated.

2.56. Visnagin. Visnagin is an active constituent isolated from fruit extracts of *Ammi visnaga* known as toothpick weed and used in traditional Chinese medicine for cardiovascular diseases [145]. Visnagin was recently shown to be cardioprotective in a zebrafish model of DOX-induced cardiomyopathy that recapitulates the cardiomyocyte apoptosis and contractility similar to those observed in humans [146]. Visnagin was found to rescue the cardiac performance and circulatory defects caused by DOX in zebrafish. It also attenuated DOX-induced apoptosis in cultured cardiomyocytes and *in vivo* in zebrafish and mouse hearts along with improved cardiac contractility in DOX-treated mice. Additionally, it did not interfere with DOX efficacy in several cultured tumor lines or in zebrafish and mouse xenograft models. Visnagin was observed to bind mitochondrial malate dehydrogenase (MDH2), a key enzyme in the tricarboxylic acid cycle that contributed to cardioprotection [146].

3. Concluding Remarks and Future Perspectives

From the analysis of the literature, it is evident that several phytochemicals exhibited cardioprotective effects *in vitro* and *in vivo* against DOX-induced cardiotoxicity. The key pathways modulated by phytochemicals in cardiomyocytes include oxidative stress, inflammation, and cell death pathways, as demonstrated in Figure 1. Majority of the phytochemicals were demonstrated to elicit cardioprotective activity in preclinical studies. However, they have not been translated for clinical utility in human subjects. The major impediment to the development of phytochemical based cardioprotective adjuvants pertains to their negligible pharmacokinetic actions in human subjects. Particularly, the poor or lack of bioavailability in human subjects retards the enthusiasm for further pharmaceutical development [113, 147].

In order to improve the bioavailability of phytochemicals, various synthetic derivatives have been pursued [44]. Although significant strides have been taken in delineating the pathomechanisms for DOX-induced cardiotoxicity, still we do not have bona fide clinical biomarker to predict early changes in the myocardium of patients who received DOX treatment [148]. Therefore, it is of paramount significance to devise a biomarker to predict the DOX-induced cardiotoxicity, because most patients (cancer survivors) exhibit dilated cardiomyopathy several years after exposure to DOX [148]. Furthermore, from Table 1, it is evident that there is discrepancy regarding the employment of appropriate models in studying phytochemicals protective effects in nullifying DOX-induced cardiotoxicity. Therefore, future studies addressing the phytochemicals protective effects against DOX cardiotoxicity should utilize the physiologically relevant cumulative (chronic) dosage regimen in rodents. In addition, future studies should obligatorily investigate the noninterference of phytochemicals against DOX anticancer activities in orthotropic tumor-bearing mouse models.

In sum, to exploit the true potentials of plant-derived compounds for drug development, significant intellectual and financial contributions are warranted from academia and pharmaceutical industry. Unfortunately, the major impediment in this direction is the lack of proper intellectual property rights protection that could protect the financial viability of the drug development projects based on phytochemicals for the treatment of cardiomyopathy. This caveat coupled with other pharmacodynamics and pharmacokinetic lapses pertaining to the phytochemicals precludes the attention of major pharmaceutical companies in their portfolio investments toward the drug development. However, academic research should be directed to develop phytochemicals derived small molecules with significant bioavailability in human subjects. Perhaps this approach could be envisaged for translational application in combating DOX-induced cardiotoxicity.

Competing Interests

The authors disclose no competing interests.

Authors' Contributions

Shreesh Ojha, Hasan Al Taee, Sameer Goyal, and Umesh B. Mahajan researched the literature. Shreesh Ojha, Sameer Goyal, and Umesh B. Mahajan drafted the paper. Chandrgouda R. Patil and D. S. Arya edited the paper. Mohanraj Rajesh researched the literature and wrote and edited the paper. All authors read the contents of the paper and approved the same.

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References

[1] T. Simunek, M. Sterba, O. Popelova, M. Adamcova, R. Hrdina, and V. Gersl, "Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron," *Pharmacological Reports*, vol. 61, no. 1, pp. 154–171, 2009.

[2] N. Yamaguchi, T. Fujii, S. Aoi, P. S. Kozuch, G. N. Hortobagyi, and R. H. Blum, "Comparison of cardiac events associated with liposomal doxorubicin, epirubicin and doxorubicin in breast cancer: a Bayesian network meta-analysis," *European Journal of Cancer*, vol. 51, no. 16, pp. 2314–2320, 2015.

[3] T. V. Jerjian, A. E. Glode, L. A. Thompson, and C. L. O'Bryant, "Antibody-drug conjugates: a clinical pharmacy perspective on an emerging cancer therapy," *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, vol. 36, no. 1, pp. 99–116, 2016.

[4] A. R. Lehenbauer Ludke, A. A.-R. S. Al-Shudiefat, S. Dhingra, D. S. Jassal, and P. K. Singal, "A concise description of cardioprotective strategies in doxorubicin-induced cardiotoxicity," *Canadian Journal of Physiology and Pharmacology*, vol. 87, no. 10, pp. 756–763, 2009.

[5] B. L. Asselin, M. Devidas, L. Chen et al. et al., "Cardioprotection and safety of dexrazoxane in patients treated for newly diagnosed t-cell acute lymphoblastic leukemia or advanced-stage lymphoblastic non-hodgkin lymphoma: a report of the children's oncology group randomized trial pediatric oncology group 9404," *Journal of Clinical Oncology*, vol. 34, no. 8, pp. 854–862, 2016.

[6] A. Tashakori Beheshti, H. Mostafavi Toroghi, G. Hosseini, A. Zarifian, F. Homaei Shandiz, and A. Fazlinezhad, "Carvedilol administration can prevent doxorubicin-induced cardiotoxicity: a double-blind randomized trial," *Cardiology*, vol. 134, no. 1, pp. 47–53, 2016.

[7] S. E. Lipshultz, V. I. Franco, T. L. Miller, S. D. Colan, and S. E. Sallan, "Cardiovascular disease in adult survivors of childhood cancer," *Annual Review of Medicine*, vol. 66, pp. 161–176, 2015.

[8] C.-J. Weng and G.-C. Yen, "Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: phenolic acids, monophenol, polyphenol, and their derivatives," *Cancer Treatment Reviews*, vol. 38, no. 1, pp. 76–87, 2012.

[9] G. Wen, X.-X. Qu, D. Wang et al., "Recent advances in design, synthesis and bioactivity of paclitaxel-mimics," *Fitoterapia*, vol. 110, pp. 26–37, 2016.

[10] W. Wang and Y.-S. Liang, "Artemisinin: a wonder drug from Chinese natural medicines," *Chinese Journal of Natural Medicines*, vol. 14, no. 1, pp. 5–6, 2016.

[11] P. Angsutararux, S. Luanpitpong, and S. Issaragrisil, "Chemotherapy-induced cardiotoxicity: overview of the roles of oxidative stress," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 795602, 13 pages, 2015.

[12] Z. V. Varga, P. Ferdinandy, L. Liaudet, and P. Pacher, "Drug-induced mitochondrial dysfunction and cardiotoxicity," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 309, no. 9, pp. H1453–H1467, 2015.

[13] J. Ghosh, J. Das, P. Manna, and P. C. Sil, "The protective role of arjunolic acid against doxorubicin induced intracellular ROS dependent JNK-p38 and p53-mediated cardiac apoptosis," *Biomaterials*, vol. 32, no. 21, pp. 4857–4866, 2011.

[14] E. H. Choi, H.-J. Chang, J. Y. Cho, and H. S. Chun, "Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities," *Food and Chemical Toxicology*, vol. 45, no. 10, pp. 1873–1881, 2007.

[15] M. Saeed, O. Kadioglu, H. Khalid, Y. Sugimoto, and T. Efferth, "Activity of the dietary flavonoid, apigenin, against multidrug-resistant tumor cells as determined by pharmacogenomics and molecular docking," *Journal of Nutritional Biochemistry*, vol. 26, no. 1, pp. 44–56, 2015.

[16] J. Psotová, Š. Chlopčíkova, P. Miketová, J. Hrbác, and V. Šimánek, "Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part III. Apigenin, baicalelin, kaempherol, luteolin and quercetin," *Phytotherapy Research*, vol. 18, no. 7, pp. 516–521, 2004.

[17] S.-Q. Wang, X.-F. Zhu, X.-N. Wang, T. Shen, F. Xiang, and H.-X. Lou, "Flavonoids from *Malus hupehensis* and their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells," *Phytochemistry*, vol. 87, pp. 119–125, 2013.

[18] Z. Li, Y.-N. Geng, J.-D. Jiang, and W.-J. Kong, "Antioxidant and anti-inflammatory activities of berberine in the treatment of diabetes mellitus," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 289264, 12 pages, 2014.

[19] X. Lv, X. Yu, Y. Wang et al., "Berberine inhibits doxorubicin-triggered cardiomyocyte apoptosis via attenuating mitochondrial dysfunction and increasing Bcl-2 expression," *PLoS ONE*, vol. 7, no. 10, Article ID e47351, 2012.

[20] G. Hao, Y. Yu, B. Gu, Y. Xing, and M. Xue, "Protective effects of berberine against doxorubicin-induced cardiotoxicity in rats by inhibiting metabolism of doxorubicin," *Xenobiotica*, vol. 45, no. 11, pp. 1024–1029, 2015.

[21] E. Ciesielska, A. Gwardys, and D. Metodiewa, "Anticancer, antiradical and antioxidative actions of novel Antoksyd S and its major components, baicalin and baicalein," *Anticancer Research*, vol. 22, no. 5, pp. 2885–2892, 2002.

[22] W.-T. Chang, J. Li, H.-H. Haung et al., "Baicalein protects against doxorubicin-induced cardiotoxicity by attenuation of mitochondrial oxidant injury and JNK activation," *Journal of Cellular Biochemistry*, vol. 112, no. 10, pp. 2873–2881, 2011.

[23] B. D. Sahu, J. M. Kumar, M. Kuncha, R. M. Borkar, R. Srinivas, and R. Sistla, "Baicalein alleviates doxorubicin-induced cardiotoxicity via suppression of myocardial oxidative stress and apoptosis in mice," *Life Sciences*, vol. 144, pp. 8–18, 2016.

[24] P. Zhang, Y. Tang, N.-G. Li, Y. Zhu, and J.-A. Duan, "Bioactivity and chemical synthesis of caffeic acid phenethyl ester and its derivatives," *Molecules*, vol. 19, no. 10, pp. 16458–16476, 2014.

[25] E. Fadillioglu, E. Oztas, H. Erdogan et al., "Protective effects of caffeic acid phenethyl ester on doxorubicin-induced cardiotoxicity in rats," *Journal of Applied Toxicology*, vol. 24, no. 1, pp. 47–52, 2004.

[26] J. G. Sena Filho, S. L. Nimmo, H. S. Xavier, J. M. Barbosa-Filho, and R. H. Cichewicz, "Phenylethanoid and lignan glycosides from polar extracts of *Lantana*, a genus of verbenaceous plants widely used in traditional herbal therapies," *Journal of Natural Products*, vol. 72, no. 7, pp. 1344–1347, 2009.

[27] D.-S. Kim, H.-R. Kim, E.-R. Woo et al., "Protective effect of calceolarioside on adriamycin-induced cardiomyocyte toxicity,"

European Journal of Pharmacology, vol. 541, no. 1-2, pp. 24–32, 2006.

[28] R. Durst, H. Danenberg, R. Gallily et al., "Cannabidiol, a nonpsychoactive Cannabis constituent, protects against myocardial ischemic reperfusion injury," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 6, pp. H3602–H3607, 2007.

[29] A. A. Fouad, W. H. Albuali, A. S. Al-Mulhim, and I. Jresat, "Cardioprotective effect of cannabidiol in rats exposed to doxorubicin toxicity," *Environmental Toxicology and Pharmacology*, vol. 36, no. 2, pp. 347–357, 2013.

[30] E. Hao, P. Mukhopadhyay, Z. Cao et al., "Cannabidiol protects against doxorubicin-induced cardiomyopathy by modulating mitochondrial function and biogenesis," *Molecular Medicine*, vol. 21, pp. 38–45, 2015.

[31] M. Rajesh, P. Mukhopadhyay, S. Btkai et al., "Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy," *Journal of the American College of Cardiology*, vol. 56, no. 25, pp. 2115–2125, 2010.

[32] B. Horvth, P. Mukhopadhyay, G. Hask, and P. Pacher, "The endocannabinoid system and plant-derived cannabinoids in diabetes and diabetic complications," *American Journal of Pathology*, vol. 180, no. 2, pp. 432–442, 2012.

[33] M. Pennant, M. Steur, C. Moore, A. Butterworth, and L. Johnson, "Comparative validity of vitamin C and carotenoids as indicators of fruit and vegetable intake: a systematic review and meta-analysis of randomised controlled trials," *British Journal of Nutrition*, vol. 114, no. 9, pp. 1331–1340, 2015.

[34] R. Indu, T. S. Azhar, A. Nair, and C. K. K. Nair, "Amelioration of doxorubicin induced cardio-and hepato-toxicity by carotenoids," *Journal of Cancer Research and Therapeutics*, vol. 10, no. 1, pp. 62–67, 2014.

[35] S. F. Nabavi, N. Braidy, S. Habtemariam et al., "Neuroprotective effects of chrysin: from chemistry to medicine," *Neurochemistry International*, vol. 90, pp. 224–231, 2015.

[36] E. M. Mantawy, W. M. El-Bakly, A. Esmat, A. M. Badr, and E. El-Demerdash, "Chrysin alleviates acute doxorubicin cardiotoxicity in rats via suppression of oxidative stress, inflammation and apoptosis," *European Journal of Pharmacology*, vol. 728, no. 1, pp. 107–118, 2014.

[37] H.-S. Kim, M. J. Quon, and J.-A. Kim, "New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate," *Redox Biology*, vol. 2, pp. 187–195, 2014.

[38] S. Hrelia, A. Bordoni, C. Angeloni et al., "Green tea extracts can counteract the modification of fatty acid composition induced by doxorubicin in cultured cardiomyocytes," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 66, no. 5-6, pp. 519–524, 2002.

[39] W. Li, S. Nie, M. Xie, Y. Chen, C. Li, and H. Zhang, "A major green tea component, (−)-epigallocatechin-3-gallate, ameliorates doxorubicin-mediated cardiotoxicity in cardiomyocytes of neonatal rats," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 16, pp. 8977–8982, 2010.

[40] J. Zheng, H. C. M. Lee, M. M. bin Sattar, Y. Huang, and J.-S. Bian, "Cardioprotective effects of epigallocatechin-3-gallate against doxorubicin-induced cardiomyocyte injury," *European Journal of Pharmacology*, vol. 652, no. 1–3, pp. 82–88, 2011.

[41] N. M. Saeed, R. N. El-Naga, W. M. El-Bakly, H. M. Abdel-Rahman, R. A. Salah Eldin, and E. El-Demerdash, "Epigallocatechin-3-gallate pretreatment attenuates doxorubicin-induced cardiotoxicity in rats: a mechanistic study," *Biochemical Pharmacology*, vol. 95, no. 3, pp. 145–155, 2015.

[42] J. Dudka, J. Jodynis-Liebert, E. Korobowicz et al., "Activity of NADPH-cytochrome P-450 reductase of the human heart, liver and lungs in the presence of (-)-epigallocatechin gallate, quercetin and resveratrol: an *in vitro* study," *Basic & Clinical Pharmacology & Toxicology*, vol. 97, no. 2, pp. 74–79, 2005.

[43] Z. Liu, X.-D. Song, Y. Xin et al., "Protective effect of chrysoeriol against doxorubicin-induced cardiotoxicity in vitro," *Chinese Medical Journal*, vol. 122, no. 21, pp. 2652–2656, 2009.

[44] S. Prasad, A. K. Tyagi, and B. B. Aggarwal, "Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice," *Cancer Research and Treatment*, vol. 46, no. 1, pp. 2–18, 2014.

[45] N. Venkatesan, "Curcumin attenuation of acute adriamycin myocardial toxicity in rats," *British Journal of Pharmacology*, vol. 124, no. 3, pp. 425–427, 1998.

[46] A. Dayton, K. Selvendiran, S. Meduru et al., "Amelioration of doxorubicin-induced cardiotoxicity by an anticancer-antioxidant dual-function compound, HO-3867," *Journal of Pharmacology and Experimental Therapeutics*, vol. 339, no. 2, pp. 350–357, 2011.

[47] L. Hosseinzadeh, J. Behravan, F. Mosaffa, G. Bahrami, A. Bahrami, and G. Karimi, "Curcumin potentiates doxorubicin-induced apoptosis in H9c2 cardiac muscle cells through generation of reactive oxygen species," *Food and Chemical Toxicology*, vol. 49, no. 5, pp. 1102–1109, 2011.

[48] A. V. Swamy, S. Gulliaya, A. Thippeswamy, B. C. Koti, and D. V. Manjula, "Cardioprotective effect of curcumin against doxorubicin-induced myocardial toxicity in albino rats," *Indian Journal of Pharmacology*, vol. 44, no. 1, pp. 73–77, 2012.

[49] D. Pramanik, N. R. Campbell, S. Das et al., "A composite polymer nanoparticle overcomes multidrug resistance and ameliorates doxorubicin-associated cardiomyopathy," *Oncotarget*, vol. 3, no. 6, pp. 640–650, 2012.

[50] K. Pramod, S. H. Ansari, and J. Ali, "Eugenol: a natural compound with versatile pharmacological actions," *Natural Product Communications*, vol. 5, no. 12, pp. 1999–2006, 2010.

[51] A. A. Fouad and M. T. Yacoubi, "Mechanisms underlying the protective effect of eugenol in rats with acute doxorubicin cardiotoxicity," *Archives of Pharmacal Research*, vol. 34, no. 5, pp. 821–828, 2011.

[52] F. A. A. van Acker, E. Boven, K. Kramer, G. R. M. M. Haenen, A. Bast, and W. J. F. van der Vijgh, "Frederine, a new and promising protector against doxorubicin-induced cardiotoxicity," *Clinical Cancer Research*, vol. 7, no. 5, pp. 1378–1384, 2001.

[53] F. A. A. van Acker, S. A. B. E. van Acker, K. Kramer, G. R. M. M. Haenen, A. Bast, and W. J. F. van der Vijgh, "7-Monohydroxyethylrutoside protects against chronic doxorubicin-induced cardiotoxicity when administered only once per week," *Clinical Cancer Research*, vol. 6, no. 4, pp. 1337–1341, 2000.

[54] S. A. B. E. van Acker, E. Boven, K. Kuiper et al., "Monohydroxyethylrutoside, a dose-dependent cardioprotective agent, does not affect the antitumor activity of doxorubicin," *Clinical Cancer Research*, vol. 3, no. 10, pp. 1747–1754, 1997.

[55] A. A. Oyagbemi, A. B. Saba, and O. I. Azeez, "Molecular targets of [6]-gingerol: its potential roles in cancer chemoprevention," *Biofactors*, vol. 36, no. 3, pp. 169–178, 2010.

[56] W. M. El-Bakly, M. L. Louka, A. M. El-Halawany, and M. F. Schaalan, "6-Gingerol ameliorated doxorubicin-induced cardiotoxicity: role of nuclear factor kappa B and protein glycation," *Cancer Chemotherapy and Pharmacology*, vol. 70, no. 6, pp. 833–841, 2012.

[57] S. F. Nabavi, A. Sureda, S. Habtemariam, and S. M. Nabavi, "Ginsenoside Rd and ischemic stroke; a short review of literatures," *Journal of Ginseng Research*, vol. 39, no. 4, pp. 299–303, 2015.

[58] H. Wang, P. Yu, H. Gou et al., "Cardioprotective effects of 20(S)-ginsenoside Rh2 against doxorubicin-induced cardiotoxicity *in vitro* and *in vivo*," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 506214, 8 pages, 2012.

[59] C.-T. Chen, Z.-H. Wang, C.-C. Hsu, H.-H. Lin, and J.-H. Chen, "In vivo protective effects of diosgenin against doxorubicin-induced cardiotoxicity," *Nutrients*, vol. 7, no. 6, pp. 4938–4954, 2015.

[60] F. Zhou, G. Hao, J. Zhang et al., "Protective effect of 23-hydroxybetulinic acid on doxorubicin-induced cardiotoxicity: a correlation with the inhibition of carbonyl reductase-mediated metabolism," *British Journal of Pharmacology*, vol. 172, no. 23, pp. 5690–5703, 2015.

[61] A. Roohbakhsh, H. Parhiz, F. Soltani, R. Rezaee, and M. Iranshahi, "Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases," *Life Sciences*, vol. 124, pp. 64–74, 2015.

[62] I. T. Abdel-Raheem and A. A. Abdel-Ghany, "Hesperidin alleviates doxorubicin-induced cardiotoxicity in rats," *Journal of the Egyptian National Cancer Institute*, vol. 21, no. 2, pp. 175–184, 2009.

[63] N. F. Khedr and R. M. Khalil, "Effect of hesperidin on mice bearing Ehrlich solid carcinoma maintained on doxorubicin," *Tumor Biology*, vol. 36, no. 12, pp. 9267–9275, 2015.

[64] P. P. Trivedi, S. Kushwaha, D. N. Tripathi, and G. B. Jena, "Cardioprotective effects of hesperetin against doxorubicin-induced oxidative stress and DNA damage in rat," *Cardiovascular Toxicology*, vol. 11, no. 3, pp. 215–225, 2011.

[65] S. Granados-Principal, N. El-Azem, R. Pamplona et al., "Hydroxytyrosol ameliorates oxidative stress and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer," *Biochemical Pharmacology*, vol. 90, no. 1, pp. 25–33, 2014.

[66] J. Sun, G. Sun, X. Meng et al., "Isorhamnetin protects against doxorubicin-induced cardiotoxicity in vivo and in vitro," *PLoS ONE*, vol. 8, no. 5, Article ID e64526, 2013.

[67] A. A. Adwas, A. A. Elkhoely, A. M. Kabel, M. N. Abdel-Rahman, and A. A. Eissa, "Anti-cancer and cardioprotective effects of indol-3-carbinol in doxorubicin-treated mice," *Journal of Infection and Chemotherapy*, vol. 22, no. 1, pp. 36–43, 2016.

[68] P. Souček, E. Kondrová, J. Heřmánek et al., "New model system for testing effects of flavonoids on doxorubicin-related formation of hydroxyl radicals," *Anti-Cancer Drugs*, vol. 22, no. 2, pp. 176–184, 2011.

[69] J. Xiao, G.-B. Sun, B. Sun et al., "Kaempferol protects against doxorubicin-induced cardiotoxicity *in vivo* and *in vitro*," *Toxicology*, vol. 292, no. 1, pp. 53–62, 2012.

[70] G. Karimi, M. Ramezani, and A. Abdi, "Protective effects of lycopene and tomato extract against doxorubicin-induced cardiotoxicity," *Phytotherapy Research*, vol. 19, no. 10, pp. 912–914, 2005.

[71] S. Yilmaz, A. Atessahin, E. Sahna, I. Karahan, and S. Ozer, "Protective effect of lycopene on adriamycin-induced cardiotoxicity and nephrotoxicity," *Toxicology*, vol. 218, no. 2-3, pp. 164–171, 2006.

[72] A. L. A. Ferreira, D. M. F. Salvadori, M. C. M. O. Nascimento et al., "Tomato-oleoresin supplement prevents doxorubicin-induced cardiac myocyte oxidative DNA damage in rats," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 631, no. 1, pp. 26–35, 2007.

[73] A. L. A. Ferreira, K.-J. Yeum, L. S. Matsubara et al., "Doxorubicin as an antioxidant: maintenance of myocardial levels of lycopene under doxorubicin treatment," *Free Radical Biology and Medicine*, vol. 43, no. 5, pp. 740–751, 2007.

[74] K. Sahin, N. Sahin, and O. Kucuk, "Lycopene and chemotherapy toxicity," *Nutrition and Cancer*, vol. 62, no. 7, pp. 988–995, 2010.

[75] S.-Q. Wang, X.-Z. Han, X. Li, D.-M. Ren, X.-N. Wang, and H.-X. Lou, "Flavonoids from *Dracocephalum tanguticum* and their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells," *Bioorganic & Medicinal Chemistry Letters*, vol. 20, no. 22, pp. 6411–6415, 2010.

[76] H. Yao, Z. Shang, P. Wang et al., "Protection of luteolin-7-*O*-glucoside against doxorubicin-induced injury through PTEN/Akt and ERK pathway in H9c2 cells," *Cardiovascular Toxicology*, vol. 16, no. 2, pp. 101–110, 2016.

[77] L. D. S. Kok, Y. P. Wong, T. W. Wu, H. C. Chan, T. T. Kwok, and K. P. Fung, "Morin hydrate: a potential antioxidant in minimizing the free-radicals- mediated damage to cardiovascular cells by anti-tumor drugs," *Life Sciences*, vol. 67, no. 1, pp. 91–99, 2000.

[78] W. Arozal, F. D. Suyatna, V. Juniantito et al., "The effects of mangiferin (Mangifera indica L) in doxorubicin-induced cardiotoxicity in rats," *Drug Research*, vol. 65, no. 11, pp. 574–580, 2015.

[79] F. D. Agustini, W. Arozal, M. Louisa et al., "Cardioprotection mechanism of mangiferin on doxorubicin-induced rats: focus on intracellular calcium regulation," *Pharmaceutical Biology*, In press.

[80] H.-S. Park, J. U.-H. Oh, J. H. Lee, and Y.-J. Lee, "Minor effects of the citrus flavonoids naringin, naringenin and quercetin, on the pharmacokinetics of doxorubicin in rats," *Pharmazie*, vol. 66, no. 6, pp. 424–429, 2011.

[81] H. M. Arafa, M. F. Abd-Ellah, and H. F. Hafez, "Abatement by naringenin of doxorubicin-induced cardiac toxicity in rats," *Journal of the Egyptian National Cancer Institute*, vol. 17, no. 4, pp. 291–300, 2005.

[82] S. S. Shiromwar and V. R. Chidrawar, "Combined effects of p-coumaric acid and naringenin against doxorubicin-induced cardiotoxicity in rats," *Pharmacognosy Research*, vol. 3, no. 3, pp. 214–219, 2011.

[83] X. Han, J. Pan, D. Ren, Y. Cheng, P. Fan, and H. Lou, "Naringenin-7-O-glucoside protects against doxorubicin-induced toxicity in H9c2 cardiomyocytes by induction of endogenous antioxidant enzymes," *Food and Chemical Toxicology*, vol. 46, no. 9, pp. 3140–3146, 2008.

[84] X. Han, D. Ren, P. Fan, T. Shen, and H. Lou, "Protective effects of naringenin-7-*O*-glucoside on doxorubicin-induced apoptosis in H9C2 cells," *European Journal of Pharmacology*, vol. 581, no. 1-2, pp. 47–53, 2008.

[85] X. Han, S. Gao, Y. Cheng et al., "Protective effect of naringenin-7-O-glucoside against oxidative stress induced by doxorubicin in H9c2 cardiomyocytes," *BioScience Trends*, vol. 6, no. 1, pp. 19–25, 2012.

[86] X. Fu, L. Kong, M. Tang et al., "Protective effect of ocotillol against doxorubicin-induced acute and chronic cardiac injury," *Molecular Medicine Reports*, vol. 9, no. 1, pp. 360–364, 2014.

[87] I. Andreadou, M. Papaefthimiou, A. Zira et al., "Metabonomic identification of novel biomarkers in doxorubicin cardiotoxicity and protective effect of the natural antioxidant oleuropein," *NMR in Biomedicine*, vol. 22, no. 6, pp. 585–592, 2009.

[88] I. Andreadou, F. Sigala, E. K. Iliodromitis et al., "Acute doxorubicin cardiotoxicity is successfully treated with the phytochemical oleuropein through suppression of oxidative and nitrosative stress," *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 3, pp. 549–558, 2007.

[89] I. Andreadou, E. Mikros, K. Ioannidis et al., "Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism," *Journal of Molecular and Cellular Cardiology*, vol. 69, pp. 4–16, 2014.

[90] H. Xu, Y. Han, M. Zhang, M. Yan, and C. Gao, "Protective role of Osthole on myocardial cell apoptosis induced by doxorubicin in rats," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 9, pp. 10816–10823, 2015.

[91] M. H. Abdel-Wahab, M. A. El-Mahdy, M. F. Abd-Ellah, G. K. Helal, F. Khalifa, and F. M. A. Hamada, "Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart," *Pharmacological Research*, vol. 48, no. 5, pp. 461–465, 2003.

[92] S. Panda and A. Kar, "Periplogenin-3-O- -D-Glucopyranosyl - (1 → 6)- -D-glucopyaranosyl- -(1 → 4) -D-cymaropyranoside, isolated from Aegle marmelos protects doxorubicin induced cardiovascular problems and hepatotoxicity in rats," *Cardiovascular Therapeutics*, vol. 27, no. 2, pp. 108–116, 2009.

[93] D.-S. Kim, E.-R. Woo, S.-W. Chae et al., "Plantainoside D protects adriamycin-induced apoptosis in H9c2 cardiac muscle cells via the inhibition of ROS generation and NF-κB activation," *Life Sciences*, vol. 80, no. 4, pp. 314–323, 2007.

[94] M. Khan, S. Varadharaj, J. C. Shobha et al., "C-phycocyanin ameliorates doxorubicin-induced oxidative stress and apoptosis in adult rat cardiomyocytes," *Journal of Cardiovascular Pharmacology*, vol. 47, no. 1, pp. 9–20, 2006.

[95] A. Maimoona, I. Naeem, Z. Saddiqe, and K. Jameel, "A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 261–277, 2011.

[96] N. A. E. Boghdady, "Antioxidant and antiapoptotic effects of proanthocyanidin and ginkgo biloba extract against doxorubicin-induced cardiac injury in rats," *Cell Biochemistry and Function*, vol. 31, no. 4, pp. 344–351, 2013.

[97] S. D. Ray, D. Patel, V. Wong, and D. Bagchi, "In vivo protection of DNA damage associated apoptotic and necrotic cell deaths during acetaminophen-induced nephrotoxicity, amiodarone-induced lung toxicity and doxorubicin-induced cardiotoxicity by a novel IH636 grape seed proanthocyanidin extract," *Research Communications in Molecular Pathology and Pharmacology*, vol. 107, no. 1-2, pp. 137–166, 2000.

[98] D. Bagchi, S. D. Ray, M. Bagchi, H. G. Preuss, and S. J. Stohs, "Mechanistic pathways of antioxidant cytoprotection by a novel IH636 grape seed proanthocyanidin extract," *Indian Journal of Experimental Biology*, vol. 40, no. 6, pp. 717–726, 2002.

[99] X.-Y. Zhang, D.-C. Bai, Y.-J. Wu, W.-G. Li, and N.-F. Liu, "Proanthocyanidin from grape seeds enhances anti-tumor effect of doxorubicin both in vitro and in vivo," *Pharmazie*, vol. 60, no. 7, pp. 533–538, 2005.

[100] E. Demirkaya, A. Avci, V. Kesik et al., "Cardioprotective roles of aged garlic extract, grape seed proanthocyanidin, and hazelnut on doxorubicin-induced cardiotoxicity," *Canadian Journal of Physiology and Pharmacology*, vol. 87, no. 8, pp. 633–640, 2009.

[101] C. Cal, H. Garban, A. Jazirehi, C. Yeh, Y. Mizutani, and B. Bonavida, "Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities," *Current Medicinal Chemistry—Anti-Cancer Agents*, vol. 3, no. 2, pp. 77–93, 2003.

[102] Z. Cao and Y. Li, "Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury," *European Journal of Pharmacology*, vol. 489, no. 1-2, pp. 39–48, 2004.

[103] E. Tatlidede, Ö. Şehirli, A. Velioğlu-Öğün et al., "Resveratrol treatment protects against doxorubicin-induced cardiotoxicity by alleviating oxidative damage," *Free Radical Research*, vol. 43, no. 3, pp. 195–205, 2009.

[104] G. Y. Wang, Y. M. Wang, L. N. Zhang et al., "Effect of resveratrol on heart function of rats with adriamycin-induced heart failure," *Zhongguo Zhong Yao Za Zhi*, vol. 32, no. 15, pp. 1563–1565, 2007.

[105] E. D. Brookins Danz, J. Skramsted, N. Henry, J. A. Bennett, and R. S. Keller, "Resveratrol prevents doxorubicin cardiotoxicity through mitochondrial stabilization and the Sirt1 pathway," *Free Radical Biology and Medicine*, vol. 46, no. 12, pp. 1589–1597, 2009.

[106] C. Zhang, Y. Feng, S. Qu et al., "Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in mice through SIRT1-mediated deacetylation of p53," *Cardiovascular Research*, vol. 90, no. 3, pp. 538–545, 2011.

[107] X. Xu, K. Chen, S. Kobayashi, D. Timm, and Q. Liang, "Resveratrol attenuates doxorubicin-induced cardiomyocyte death via inhibition of p70 S6 kinase 1-mediated autophagy," *Journal of Pharmacology and Experimental Therapeutics*, vol. 341, no. 1, pp. 183–195, 2012.

[108] S. Wang, P. Song, and M.-H. Zou, "Inhibition of AMP-activated protein kinase α (AMPKα) by doxorubicin accentuates genotoxic stress and cell death in mouse embryonic fibroblasts and cardiomyocytes: role of p53 and SIRT1," *The Journal of Biological Chemistry*, vol. 287, no. 11, pp. 8001–8012, 2012.

[109] C.-Y. Chen, J.-H. Jang, M.-H. Li, and Y.-J. Surh, "Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells," *Biochemical and Biophysical Research Communications*, vol. 331, no. 4, pp. 993–1000, 2005.

[110] J. Gu, Z.-P. Song, D.-M. Gui, W. Hu, Y.-G. Chen, and D.-D. Zhang, "Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in lymphoma nude mice by heme oxygenase-1 induction," *Cardiovascular Toxicology*, vol. 12, no. 4, pp. 341–349, 2012.

[111] F. A. Pinarli, N. N. Turan, F. G. Pinarli et al., "Resveratrol and adipose-derived mesenchymal stem cells are effective in the prevention and treatment of doxorubicin cardiotoxicity in rats," *Pediatric Hematology and Oncology*, vol. 30, no. 3, pp. 226–238, 2013.

[112] V. W. Dolinsky, K. J. Rogan, M. M. Sung et al., "Both aerobic exercise and resveratrol supplementation attenuate doxorubicin-induced cardiac injury in mice," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 305, no. 2, pp. E243–E253, 2013.

[113] M. G. Novelle, D. Wahl, C. Diéguez, M. Bernier, and R. de Cabo, "Resveratrol supplementation: where are we now and where should we go?" *Ageing Research Reviews*, vol. 21, 15 pages, 2015.

[114] P. A. Janeesh and A. Abraham, "Robinin modulates doxorubicin-induced cardiac apoptosis by TGF-Î²1 signaling pathway in Sprague Dawley rats," *Biomedicine & Pharmacotherapy*, vol. 68, no. 8, pp. 989–998, 2014.

[115] D.-S. Kim, H.-R. Kim, E.-R. Woo, S.-T. Hong, H.-J. Chae, and S.-W. Chae, "Inhibitory effects of rosmarinic acid on adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting reactive oxygen species and the activations of c-Jun N-terminal kinase and extracellular signal-regulated kinase," *Biochemical Pharmacology*, vol. 70, no. 7, pp. 1066–1078, 2005.

[116] Š. Chlopčíková, J. Psotová, P. Miketová, J. Soušek, V. Lichnovský, and V. Šimánek, "Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes part II. Caffeic, chlorogenic and rosmarinic acids," *Phytotherapy Research*, vol. 18, no. 5, pp. 408–413, 2004.

[117] B. Jiang, L. Zhang, M. Li et al., "Salvianolic acids prevent acute doxorubicin cardiotoxicity in mice through suppression of oxidative stress," *Food and Chemical Toxicology*, vol. 46, no. 5, pp. 1510–1515, 2008.

[118] T.-J. Lin, G.-T. Liu, Y. Liu, and G.-Z. Xu, "Protection by salvianolic acid A against adriamycin toxicity on rat heart mitochondria," *Free Radical Biology and Medicine*, vol. 12, no. 5, pp. 347–351, 1992.

[119] L. Li, Q. Pan, W. Han, Z. Liu, L. Li, and X. Hu, "Schisandrin B prevents doxorubicin-induced cardiotoxicity via enhancing glutathione redox cycling," *Clinical Cancer Research*, vol. 13, no. 22, pp. 6753–6760, 2007.

[120] F. Che, Y. Liu, and C. Xu, "Prevention and treatment of doxorubicin-induced cardiotoxicity by dexrazoxane and schisandrin B in rabbits," *International Journal of Toxicology*, vol. 30, no. 6, pp. 681–689, 2011.

[121] R. A. Thandavarayan, V. V. Giridharan, S. Arumugam et al., "Schisandrin b prevents doxorubicin induced cardiac dysfunction by modulation of DNA damage, oxidative stress and inflammation through inhibition of mapk/p53 signaling," *PLoS ONE*, vol. 10, no. 3, Article ID e0119214, 2015.

[122] Y. Xu, Z. Liu, J. Sun et al., "Schisandrin B prevents doxorubicin-induced chronic cardiotoxicity and enhances its anticancer activity in vivo," *PLoS ONE*, vol. 6, no. 12, Article ID e28335, 2011.

[123] X.-L. Wang, X. Wang, L.-L. Xiong et al., "Salidroside improves doxorubicin-induced cardiac dysfunction by suppression of excessive oxidative stress and cardiomyocyte apoptosis," *Journal of Cardiovascular Pharmacology*, vol. 62, no. 6, pp. 512–523, 2013.

[124] S. Su, Q. Li, Y. liu et al., "Sesamin ameliorates doxorubicin-induced cardiotoxicity: involvement of Sirt1 and Mn-SOD pathway," *Toxicology Letters*, vol. 224, no. 2, pp. 257–263, 2014.

[125] A. Chennuru and M. T. S. Saleem, "Antioxidant, lipid lowering, and membrane stabilization effect of sesamol against doxorubicin-induced cardiomyopathy in experimental rats," *BioMed Research International*, vol. 2013, Article ID 934239, 5 pages, 2013.

[126] A. Raskovic, N. Stilinovic, J. Kolarovic, V. Vasovic, S. Vukmirovic, and M. Mikov, "The protective effects of silymarin against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats," *Molecules*, vol. 16, no. 10, pp. 8601–8613, 2011.

[127] C. A. Houghton, R. G. Fassett, and J. S. Coombes, "Sulforaphane: translational research from laboratory bench to clinic," *Nutrition Reviews*, vol. 71, no. 11, pp. 709–726, 2013.

[128] B. Li, D. S. Kim, R. K. Yadav, H. R. Kim, and H. J. Chae, "Sulforaphane prevents doxorubicin-induced oxidative stress and cell death in rat H9c2 cells," *International Journal of Molecular Medicine*, vol. 36, no. 1, pp. 53–64, 2015.

[129] P. Singh, R. Sharma, K. McElhanon, C. D. Allen, J. K. Megyesi, and H. Bene, "Sulforaphane protects the heart from doxorubicin-induced toxicity," *Free Radical Biology and Medicine*, vol. 86, pp. 90–101, 2015.

[130] H.-L. Tian, T. Yu, N.-N. Xu et al., "A novel compound modified from tanshinone inhibits tumor growth in vivo via activation of the intrinsic apoptotic pathway," *Cancer Letters*, vol. 297, no. 1, pp. 18–30, 2010.

[131] G.-Y. Zhou, B.-L. Zhao, J.-W. Hou, G.-E. Ma, and W.-J. Xin, "Protective effects of sodium tanshinone IIA sulphonate against adriamycin-induced lipid peroxidation in mice hearts in vivo and in vitro," *Pharmacological Research*, vol. 40, no. 6, pp. 487–491, 1999.

[132] J. Gao, G. Yang, R. Pi et al., "Tanshinone IIA protects neonatal rat cardiomyocytes from adriamycin-induced apoptosis," *Translational Research*, vol. 151, no. 2, pp. 79–87, 2008.

[133] B. Jiang, L. Zhang, Y. Wang et al., "Tanshinone IIA sodium sulfonate protects against cardiotoxicity induced by doxorubicin in vitro and in vivo," *Food and Chemical Toxicology*, vol. 47, no. 7, pp. 1538–1544, 2009.

[134] H.-J. Hong, J.-C. Liu, P.-Y. Chen, J.-J. Chen, P. Chan, and T.-H. Cheng, "Tanshinone IIA prevents doxorubicin-induced cardiomyocyte apoptosis through Akt-dependent pathway," *International Journal of Cardiology*, vol. 157, no. 2, pp. 174–179, 2012.

[135] S.-H. Zhang, W.-Q. Wang, and J.-L. Wang, "Protective effect of tetrahydroxystilbene glucoside on cardiotoxicity induced by doxorubicin in vitro and in vivo," *Acta Pharmacologica Sinica*, vol. 30, no. 11, pp. 1479–1487, 2009.

[136] C. C. Woo, S. Y. Loo, V. Gee et al., "Anticancer activity of thymoquinone in breast cancer cells: possible involvement of PPAR-γ pathway," *Biochemical Pharmacology*, vol. 82, no. 5, pp. 464–475, 2011.

[137] O. A. Al-Shabanah, O. A. Badary, M. N. Nagi, N. M. Al-Gharably, A. C. Al-Rikabi, and A. M. Al-Bekairi, "Thymoquinone protects against doxorubicin-induced cardiotoxicity without compromising its antitumor activity," *Journal of Experimental and Clinical Cancer Research*, vol. 17, no. 2, pp. 193–198, 1998.

[138] M. N. Nagi and M. A. Mansour, "Protective effect of thymoquinone against doxorubicin-induced cardiotoxicity in rats: a possible mechanism of protection," *Pharmacological Research*, vol. 41, no. 3, pp. 283–289, 2000.

[139] R. K. Brown, G. Wilson, M. A. Tucci, and H. A. Benghuzzi, "The effects of thymoquinone and Doxorubicin on leukemia and cardiomyocyte cell lines," *Biomedical Sciences Instrumentation*, vol. 50, pp. 391–396, 2014.

[140] M. Xu, L. Sheng, X. Zhu, S. Zeng, D. Chi, and G.-J. Zhang, "Protective effect of tetrandrine on doxorubicin-induced cardiotoxicity in rats," *Tumori*, vol. 96, no. 3, pp. 460–464, 2010.

[141] C.-L. Dai, H.-Y. Xiong, L.-F. Tang et al., "Tetrandrine achieved plasma concentrations capable of reversing MDR in vitro and had no apparent effect on doxorubicin pharmacokinetics in mice," *Cancer Chemotherapy and Pharmacology*, vol. 60, no. 5, pp. 741–750, 2007.

[142] R. Deng, "Therapeutic effects of guggul and its constituent guggulsterone: cardiovascular benefits," *Cardiovascular Drug Reviews*, vol. 25, no. 4, pp. 375–390, 2007.

[143] W.-C. Wang, Y.-H. Uen, M.-L. Chang et al., "Protective effect of guggulsterone against cardiomyocyte injury induced by doxorubicin in vitro," *BMC Complementary and Alternative Medicine*, vol. 12, no. 1, article 138, 10 pages, 2012.

[144] K. Chatterjee, J. Zhang, R. Tao, N. Honbo, and J. S. Karliner, "Vincristine attenuates doxorubicin cardiotoxicity," *Biochemical*

and Biophysical Research Communications, vol. 373, no. 4, pp. 555–560, 2008.

[145] L. Xi, "Visnagin—a new protectant against doxorubicin cardiotoxicity? Inhibition of mitochondrial malate dehydrogenase 2 (MDH2) and beyond," *Annals of Translational Medicine*, vol. 4, no. 4, p. 65, 2016.

[146] Y. Liu, A. Asnani, L. Zou et al., "Visnagin protects against doxorubicin-induced cardiomyopathy through modulation of mitochondrial malate dehydrogenase," *Science Translational Medicine*, vol. 6, no. 266, Article ID 266ra170, 2014.

[147] P. Anand, A. B. Kunnumakkara, R. A. Newman, and B. B. Aggarwal, "Bioavailability of curcumin: problems and promises," *Molecular Pharmaceutics*, vol. 4, no. 6, pp. 807–818, 2007.

[148] M. Airoldi, D. Amadori, S. Barni et al., "Clinical activity and cardiac tolerability of non-pegylated liposomal doxorubicin in breast cancer: a synthetic review," *Tumori*, vol. 97, no. 6, pp. 690–692, 2011.

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Review Article

The Role of Mitochondrial Reactive Oxygen Species in Cardiovascular Injury and Protective Strategies

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Ischaemia/reperfusion (I/R) injury of the heart represents a major health burden mainly associated with acute coronary syndromes. While timely coronary reperfusion has become the established routine therapy in patients with ST-elevation myocardial infarction, the restoration of blood flow into the previously ischaemic area is always accompanied by myocardial injury. The central mechanism involved in this phenomenon is represented by the excessive generation of reactive oxygen species (ROS). Besides their harmful role when highly generated during early reperfusion, minimal ROS formation during ischaemia and/or at reperfusion is critical for the redox signaling of cardioprotection. In the past decades, mitochondria have emerged as the major source of ROS as well as a critical target for cardioprotective strategies at reperfusion. Mitochondria dysfunction associated with I/R myocardial injury is further described and ultimately analyzed with respect to its role as source of both deleterious and beneficial ROS. Furthermore, the contribution of ROS in the highly investigated field of conditioning strategies is analyzed. In the end, the vascular sources of mitochondria-derived ROS are briefly reviewed.

1. Introduction

Ischaemia/reperfusion (I/R) injury of the heart represents a major health burden mainly associated with acute coronary syndromes. Each year, myocardial infarction (MI) is responsible for the death of millions of persons and, more importantly, due to the aging of the population, represents the first cause of chronic heart failure worldwide [1]. Thus, it is not surprising that it has been predicted, already 10 years ago, that more than 40% of US population will suffer from heart failure as end stage of cardiovascular pathologies by the year 2030 [2].

Timely coronary reperfusion by either thrombolysis or primary coronary artery angioplasty has become the established routine therapy in patients with ST-elevation MI (STEMI) which effectively decreases infarct size and reduces mortality [3]. Paradoxically, restoration of the blood flow into an ischaemic area is always accompanied by myocardial injury [4]. In fact, several distinctive pathophysiological changes have been systematically associated with revascularization. These changes, collectively denominated "reperfusion injury," comprise both (i) *reversible (sublethal)* events such as *reperfusion-induced arrhythmias* and *myocardial stunning* (prolonged but fully reversible contractile dysfunction) and (ii) *irreversible (lethal)* ones, namely, the *accelerated necrosis* in tissue that has been already irreversibly injured (the "oxygen paradox") [5], the induction of *microvascular obstruction* (responsible for the no-reflow phenomenon), and the *lethal reperfusion injury* (death of cardiomyocytes that were potentially viable at the end of the ischaemic event, that

is, prior to reperfusion) (reviewed in [6–8]). Although the existence of this last major event was at the time a matter of debate [9, 10], substantial experimental evidence supported, firstly, the fact that irreversible reperfusion injury (through necrosis, apoptosis, and autophagy) exists [11] and, secondly, the concept that early reperfusion represents a window of opportunity for the delivery of adjunctive therapies capable of preventing cardiomyocyte death [12–14].

During the past four decades, a tremendous research effort was put forward to elucidate the pathophysiology of I/R injury and identify strategies that are able to provide cardioprotection at reperfusion, that is, to enhance the amount of myocardium salvaged by timely restoration of the blood flow [6, 7, 14, 15]. In this respect, the mitochondrion is the organelle that has been unanimously indicated as the major culprit responsible for the development of cardiomyocyte death [11, 16–19] and, also, the primary target in protecting the heart against the deleterious effects of reperfusion injury [20–23].

Among the main mechanisms that underlie mitochondrial dysfunction and, ultimately, cardiomyocyte death in the setting of I/R injury, namely, calcium dysregulation, ATP depletion, release of proapoptotic proteins, and oxidative stress, the last issue, that is, excessive formation of reactive oxygen species (ROS) with the subsequent damage of cell constituents, plays a central role as it is able to trigger and/or potentiate each of the other mentioned mechanisms [24].

However, in neither field has the well-known principle stated by Paracelsus "*dosis sola venenum facit*" ("*the dose alone makes the poison*") been more true as in the case of redox biology. Indeed, while increased oxygen radical production is the central mechanism involved in postischaemic myocardial injury, minimal ROS formation is critical for the redox signaling of cardioprotection (reviewed in [25–31]).

Basic cardiovascular research has witnessed the discovery of a myriad of ways to protect the heart/cardiomyocytes in various experimental models of I/R injury. Despite the fact that clinical application of these strategies has been thus far limited [32], the development of specific molecules targeting mitochondria of living cells for therapeutic gain is a rapidly evolving field and a number of drugs have already entered clinical testing [21, 33–36].

Mitochondria dysfunction associated with postischaemic myocardial injury is further described and ultimately analyzed with respect to its role as source of both deleterious and beneficial reactive oxygen species in the setting of I/R injury and for cardioprotective signaling, respectively. Last but not least, the potential vascular sources of mitochondria-derived ROS are briefly reviewed.

2. Mitochondria Dysfunction in Ischaemia/Reperfusion Injury: Historical Perspective

Mitochondria occupy a fixed fractional volume (~21% of the total heart mass) in mammalians and are strategically placed in the vicinity of myofibrils to ensure the delivery of a huge amount of ATP (ten times the cardiac mass) which is largely generated via oxidative phosphorylation and required for the myocardial contraction that occurs within a wide workload range [37]. Since the heart is strictly dependent on aerobic metabolism, it is not surprising that cardiac pathology is intimately intricated with mitochondrial impairment in the setting of myocardial I/R injury. Moreover, heart is primarily a postmitotic organ and, therefore, the death of cardiomyocytes is the major phenomenon that underlies this organ pathology.

Most of our research knowledge regarding the structural and biochemical changes elicited by experimental ischaemia and reperfusion comes from the pioneering studies started in the late 60s by Robert Jennings. In his seminal papers, he provided a clear definition of *lethal ischaemic injury* as being "the ischaemic injury of sufficient severity and duration that the involved cells will continue to degenerate and become necrotic despite reoxygenation by reperfusion of arterial blood" [38, 39]. This definition points to the *gradual* pattern of the process, in which irreversibly injured ischaemic cardiomyocytes will ultimately progress to the loss of membrane integrity and necrotic cell death. At the end of prolonged ischaemic episodes (e.g., 40 min in dogs), mitochondria in irreversibly injured cells contain one or more small (80–150 μm) amorphous matrix densities, being ascribed as "the most reliable indicator of irreversibility" [40, 41]. Interestingly, these dense matrix deposits (consisting primarily of lipids and little calcium) were considered a characteristic feature not only of ischaemia-related irreversible injury of the heart but also of drug and toxic-induced injury in liver and kidney [41]. Besides the gradual pattern of progression to death, when reperfused *in vivo* after prolonged periods of ischaemia, cardiomyocytes undergo an *abrupt* irreversible injury characterized by hypercontracture and a *rapid* increase in permeability of the sarcolemmal membrane responsible for the release of intracellular enzymes [42], observations relevant for the phenomenon of *lethal reperfusion injury*. Mitochondria in these cells showed diffuse swelling and accumulated a second type of matrix densities, distinct from the amorphous ones already present at the time of reperfusion; these granular dense bodies contained a large amount of calcium precipitated as an initially undefined form of calcium phosphate [40, 41]. As noticed in a critical review, the most important finding of the classical studies was that the hallmark events of irreversible injury, that is, hypercontracture and calcium overload, *required functional (coupled) mitochondria* in order to occur [43]. Indeed, hypercontracture of sarcomeres into contraction bands [44] and mitochondrial accumulation of calcium as well as the enzymes release were all lessened by inhibiting mitochondrial respiration with the subsequent decrease in ATP synthesis (reviewed in [45, 46]). Accordingly, it was evident already almost 4 decades ago that restoration of mitochondrial function in myocardial cells after severe ischaemia was the major culprit for the double-edged sword effect of reperfusion, since ATP production via oxidative phosphorylation was mandatory for the recovery of cardiomyocytes but appeared to also contribute to the postischaemic cell death. Despite the thoroughly performed experiments, it should be mentioned that Jennings and Ganote refrained themselves from affirming a causal relationship

between the "observed changes in mitochondrial structure and function and the death of the myocardial cell" due to the technical limitations at that time [40].

Similarly, these authors acknowledged the role of excessive production of oxygen free radicals at reperfusion and their toxic effects on both myocardium and vasculature; however, they admitted only the possibility that in the case of irreversibly injured myocytes "free radicals might accelerate the degradation of dead cells, but not kill any cells which were otherwise viable" [42]. Moreover, 15 years ago, both the existence and, more importantly, the clinical relevance of the reperfusion injury were strongly questioned [47].

The disrupted mitochondrial electron system has been already identified by the mid-70s as a potential source of oxyradicals (in particular superoxide) in the setting of I/R injury; the process is further contributed by a decrease in the free radical scavenging capacity due to the loss of mitochondrial reduced superoxide dismutase and reduced glutathione (reviewed in [48]). The uncontrolled reactivation of mitochondria upon oxygenation with subsequent ROS generation has also been considered responsible for the peroxidation of cardiac lipids, increased sarcolemmal permeability, and enzyme release; the events have been prevented in the presence of either superoxide dismutase or reduced glutathione administered at the end of hypoxia and during reoxygenation [49].

Indeed, as Halliwell mentioned, it was the time when "*the field of free radicals and antioxidants was simple: free radicals are bad, antioxidants must be good*" [50]. The huge amount of research carried in the past decades in the field provided a progressive change of the paradigm, namely, that "*too many oxidants are bad, but some may be good*" [44]. Undoubtedly, free radicals account for the harmful effects (oxidative attack of proteins, lipids, and DNA) only when rapidly generated in elevated concentrations (e.g., during the postischaemic reperfusion) whereas in low or moderate amounts they act as signaling molecules with a critical role in the regulation of several fundamental physiological and adaptive processes (including cardioprotection).

3. Mitochondria as Sources of Harmful ROS

3.1. Oxidative Stress: Old and New Definitions. Oxidative stress has been classically defined as the spatiotemporal, quantitative imbalance between increased ROS formation (prooxidant stress) and decreased ROS removal (antioxidant defense) that is responsible for cellular damage [51, 52]. It has to be mentioned that the term does not refer only to the overproduction of "true" free radicals (molecules containing one or more unpaired electrons), such as superoxide anion (the primary ROS) and hydroxyl radical, but also to the increased generation of highly reactive nonradical derivatives, mainly hydrogen peroxide, peroxynitrite, and singlet oxygen [53].

However, ten years ago Jones proposed a new definition of stress as being "*an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage*," pointing to the crucial role of disrupting the ROS-mediated signal transduction [54]. Moreover, Jones challenged the free radical dependent-oxidative stress theory by postulating the radical-free "*redox hypothesis*" according to which oxidative stress occurs via the disruption of thiol pathways due to aberrant generation of nonradical oxidants in distinct subcellular compartments [55]. In this respect, the term has been recently redefined in that oxidative stress should be perceived rather as a subcellular deleterious event than as a global threat to the whole cell [53]. In any case, regardless of the theory, mitochondria are the organelles that lie at the heart of redox biology being at the same time the sources of harmful and beneficial ROS and the main targets for oxidation.

3.2. Mitochondrial Sources of Harmful ROS. Mitochondria consume about 98% of the inhaled oxygen in order to produce the energy required to sustain life [56]. The increased efficiency of the oxidative phosphorylation in eukaryote cells comes at a price of mitochondrial generation of ROS; thus, ROS production and lethal reperfusion injury appear to be both a sort of "necessary evil" [57, 58].

Mitochondria have been conventionally recognized as the major cellular source for ROS production. Indeed, several expert research groups have systematically studied along the years the mitochondrial origins of ROS; in this respect, the reader is referred to several comprehensive reviews of the topic [59–71]. Moreover, it has to be mentioned that an unbiased estimation of the contribution of mitochondrial sources to oxidative stress in living cells requires not only multiple ROS reporter molecules but also parallel assessment of parameters that may induce artifacts as well as testing conditions that could interfere with the mitochondrial generation of oxidants [72]. What is unequivocally established so far is that ROS can be generated *in vitro* as either an *accidental* or an *obligatory* by-product of mitochondrial metabolism [73]. The former case is best exemplified by a dysfunctional electron transport system (ETS) (due to toxic or stress-related inhibition, mutational damage, and elevation of mitochondrial membrane potential due to metabolic causes) whereas the latter is best exemplified by the increased activity/expression or assembly failure of enzymes with defined metabolic roles [51, 57, 73]. More recently, the group of Koopman suggested that local ROS and/or reactive nitrogen species (RNS) involved are short-term regulation of mitochondrial morphology (fusion and fission) and function via nontranscriptional pathways [74].

There is plethora of experimental evidence supporting the roles of complexes I and III of the ETC as major generators of superoxide, the primary ROS (reviewed in [26, 59–61, 64–66, 84] and summarized in Table 1). Interestingly, Forkink et al. have recently suggested that the increase in ROS levels is not surpassing the capacity of the antioxidant systems within the cells [153]. In this respect, they demonstrated that chronic inhibition of CI and CIII in HEK293 cells (i) stimulated oxidation of the ROS sensor hydroethidine, (ii) increased cytosolic (but not mitochondrial) H_2O_2 levels, and (iii) was not associated with oxidative stress or cell death [153].

In the past years, several studies have also demonstrated the role of complex II defect in $^{\bullet}O_2^-$ overproduction (Table 1).

Table 1: Mitochondrial sources of ROS generation.

ROS sources	Experimental model: references
Inner membrane	
CI (NADH dehydrogenase): *inner side*	(i) Bovine hearts: [75–83] (ii) Rat heart: [80, 84–86] (iii) Rat brain: [60, 86, 87] (iv) Rat lung: [88] (v) Rat liver: [84] (vi) Rat skeletal muscle: [84, 89–91] (vii) Cell cultures: [79] (viii) Human brain: [87]
CII (succinate dehydrogenase): *inner side*	(i) Rat heart: [92, 93] (ii) Bovine heart: [94–96] (iii) Rat brain: [97] (iv) Rat skeletal muscle: [98] (v) Yeast: [99] (vi) *E. coli*: [100]
CIII (ubiquinol-cytochrome c reductase): *inner and outer side*	(i) Bovine heart: [95, 96, 101, 102] (ii) Rat heart: [84, 103, 104] (iii) Rat liver: [84, 105] (iv) Rat brain: [104] (v) Rat skeletal muscle: [84] (vi) Mouse skeletal muscle: [106] (vii) *R. capsulatus* strains: [107]
Hyperphosphorylation of CIV (cytochrome *c* oxidase)	(i) Rabbit hearts and mouse monocyte macrophages: [108]
Glycerophosphate dehydrogenase (a.k.a. glycerol-3-phosphate dehydrogenase, a.k.a. mGPDH): *outer side*	(i) Mouse heart, brain, and kidney: [109] (ii) Hamster brown adipose tissue: [110] (iii) *Drosophila*: [111]
Dihydroorotate dehydrogenase (DHO): *outer side*	(i) Rat brain & liver: [112] (ii) Rat skeletal muscle: [113] (iii) Rat tissues (skeletal muscle, liver, GI tract, etc.): [114] (iv) Cell lines: [115] (v) Human skin and kidney: [114]
Intermembrane space	
$p66^{Shc}$ (growth factor adaptor Shc)	(i) Mouse liver: [116] (ii) Mouse aorta: [117]
Matrix	
Aconitase (mitochondrial- (m-) aconitase)	(i) Bovine heart: [118]
Alpha-ketoglutarate dehydrogenase complex (KGDHC, a.k.a. 2-oxoglutarate dehydrogenase)	(i) Bovine heart: [119] (ii) Mouse brain: [120]
Outer membrane	
Cytochrome *b*5 reductase	(i) Human brain tissue: [121]
Monoamine oxidases (MAO-A and MAO-B)	(i) Rat brain: [122] (ii) Rat hearts: [123] (iii) Mouse liver, kidney, and heart: [124–126] (iv) Mouse aorta: [127] (v) Rat aorta: [128] (vi) Cell line: [129] (vii) Human atrial samples: [130]

Among these, the groups of Quinlan et al. [98] and Siebels and Dröse [94] have studied ROS generation at complex II in artificial conditions, such as a low concentration of succinate and inhibition of respiratory chain downstream to CII [94, 98]. Finally, ROS generation by complex IV was demonstrated to be rather relevant in pathological conditions by Prabu et al. since hyperphosphorylation of complex IV on ischaemic hearts increases the electron leakage and, therefore, the $^{\bullet}O_2^-$ production [108].

In addition to the ETS, several other mitochondrial sites (see Table 1) can be also responsible for ROS production in a tissue-specific manner and dependent on the experimental conditions [64]. Moreover, one of the most pertinent observations has been recently formulated by Andreyev et al.; these authors acknowledged the fact that, in line with the observer effect postulated in quantum physics, directly assessing ROS production using the conventional systems is not possible without changing the process [57].

3.3. Mitochondrial ROS Generation in Ischaemic/Reperfused Heart. Physiological, low concentrations of mitochondrial ROS are considered to exert beneficial effects on cardiovascular function [97]. Accordingly, a tight redox control is responsible for cardiomyocyte differentiation and excitation-contraction coupling [154, 155]. On the contrary, ROS overproduction is responsible for the so-called phenomenon "ROS-induced ROS release" [70, 156] or the "kindling radicals" concept [157, 158], which postulates that (extra)mitochondrial ROS trigger mitochondrial ROS production, with a pathological impact on (1) cardiac cells via the cellular bioenergetic decline which leads to the impairment of excitation-contraction coupling, arrhythmias, cardiac hypertrophy, apoptosis, necrosis, and fibrosis [71]; (2) endothelial cells, with 2 major effects: (i) the inflammatory vascular reaction involved in the pathogenesis of atherosclerosis, hypertension, and diabetes [159] *via* the activation of Ca^{2+}-activated potassium channel (KCa channel) coupled with intracellular signaling of PKG-1α activation in the smooth muscle cells [160, 161] and (ii) the coronary collateral growth inhibition [162] *via* the coronary dilation mediated by the activation of voltage-dependent potassium channels (Kv channels) and thiol redox-dependent signaling [163, 164].

In the setting of I/R injury the contribution of mitochondria-derived ROS to oxidative stress is particularly true for the metabolically active organs, such as heart and

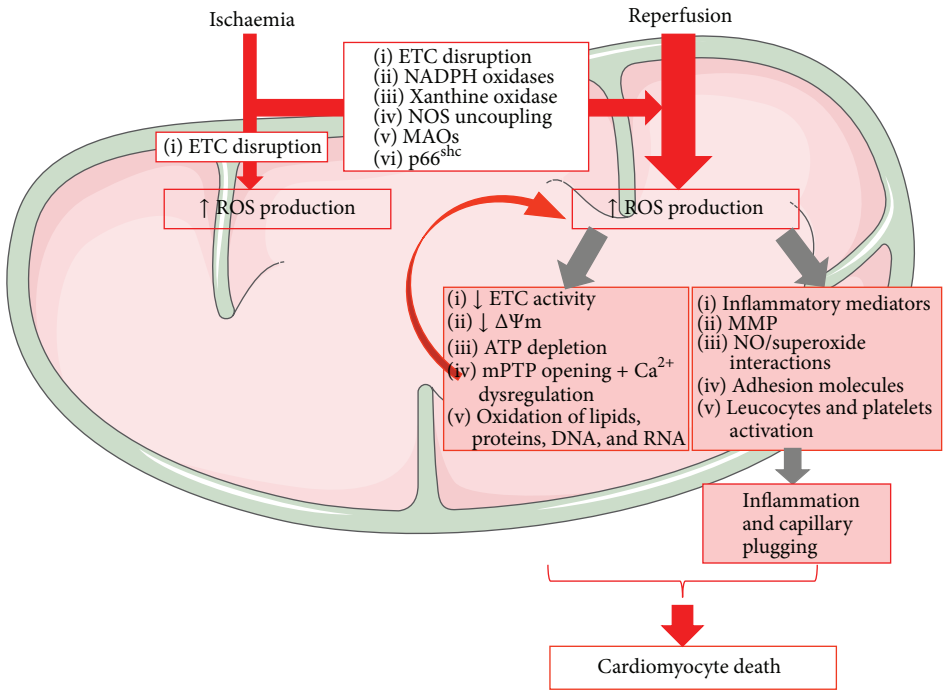

Figure 1: Mitochondrial ROS contribution to I/R injury. Cellular hypoxia secondary to ischaemia results in disruption of ETC activity in the IMM (inner mitochondrial membrane) with subsequent ROS production. Increased activity of MAOs, NADPH oxidase, and $p66^{shc}$; conformational changes of xanthine oxidase; and/or NO synthase uncoupling further amplify ROS production upon reoxygenation. Increased mitochondrial ROS damages mtDNA and RNA with ETC impairment. Dysfunctional ETC will amplify ROS generation, leading to a vicious cycle of mitochondrial cumulative damage, decreased mitochondrial membrane potential ($\Delta\psi_m$) and respiration, mPTP opening with cellular swelling and Ca^{2+} dysregulation, and oxidation of lipids and proteins. Postischaemic ROS generation also stimulates an inflammatory response, with the release of chemical mediators and expression of adhesion molecules by endothelial cells and leukocytes. ROS-dependent activation of MMPs (matrix metalloproteinases) is also responsible for the functional impairment of several proteins and receptors. The inflammatory response and the activation of leucocytes and platelets trigger the narrowing of capillaries during reperfusion, accelerating the progression towards cardiomyocyte death. (Illustration realized thanks to Servier Medical Art.)

brain [26]. Both hyperoxia (at reperfusion) and, also (albeit counterintuitively), hypoxia (during the ischaemic period) are able to trigger ROS production (Figure 1, [26, 68, 165]). During ischaemia, cardiomyocytes become hypoxic and the mitochondrial ETC complexes are highly reduced; the reaction of the electrons leaking from the respiratory complexes with residual oxygen will generate the superoxide anion. At reperfusion, hyperoxygenation will be associated with marked superoxide and superoxide-downstream ROS (mainly, hydrogen peroxide, peroxynitrite, and the hydroxyl radical) both due to electron leakage and due to a decrease in the detoxification capacity of mitochondria (Figure 1, [71]). An important consequence due to myocardial I/R is the change in the mitochondrial balance $NO/^{\bullet}O_2^-$, with an increased NO production, subsequent excess of $ONOO^-$ synthesis, and an increase of the related protein tyrosine nitration [166]. An enzyme with high susceptibility to oxidative stress is aconitase, whose activity is clearly impaired during myocardial I/R, followed by the increase of hydroxyl radicals release [167], an observation which could be suggestive for using the oxidative inactivation of mitochondrial aconitase activity as an additional marker of myocardial infarction [71].

The major contributor to ROS overproduction during I/R is related to the oxidative impairment of mitochondrial complex I along with a corresponding decrease in NADH-linked state 3 oxygen consumption and enhanced NADH-linked ROS production, respectively [168, 169], and reviewed in [23, 71]. During reperfusion, the NADH-ferricyanide reductase activity (the enzymatic activity of NADH dehydrogenase) is restored, which partially explains the $^{\bullet}O_2^-$ production during reperfusion, since NADH dehydrogenase is one of the major sources for $^{\bullet}O_2^-$ generation at complex I. Impaired complex I activity during reperfusion might be also responsible for ROS-induced damage of mitochondrial cardiolipin and respiratory supercomplexes that further increases the electron leakage at complex I and induces a vicious cycle of oxidative stress that ultimately leads to mitochondrial dysfunction [169, 170]. Lastly, an important protein tyrosine nitration of complex I in the postischaemic heart was demonstrated with a subsequent inactivation of complex I [166].

The involvement of complex II in ROS production in ischaemic hearts is unclear, despite the fact that diazoxide or atpenin A5 (specific complex II inhibitors) has been proven to exert cardioprotective effects by activating mitochondrial ATP-sensitive potassium (mK_{ATP}) channels [71].

Table 2: Potential mechanisms responsible for the decrease in ROS generation.

Site of action	Mechanism
(1) UCP2 or UCP3 overexpression [131–133]	Reduced mitochondrial ROS production *via* mitochondrial uncoupling with subsequent $\Delta\psi$ depolarization
(2) Brief transient mPTP opening [134]	Reduced ROS production and/or release into the cytosol *via* a reversible $\Delta\psi$ depolarization *Observation: a prolonged mPTP opening triggers apoptosis and cell death* [135, 136]
(3) Recruitment of hexokinase (HK) at the mitochondrial outer membrane [137]	Increased coupled respiration with subsequent reduced electron leak and ROS production
(4) Glutathionylation of CII and CV [92, 138, 139]	Decreased activity of CII and CV
(5) Glutathionylation of the 51-kDa (NDUFV1) and 75-kDa (NDUFS1) CI subunits [79, 81, 140, 141]	Decreased activity of CI *Observation: however, CI inactivation is not necessarily linked to reduced ROS production since Taylor and collaborators demonstrated that glutathionylation of CI was associated to increased superoxide production* [142]
(6) Reduction of electrons input [143, 144]	Lowered cellular glucose uptake and stimulation of pyruvate conversion to lactate with secretion of the latter into the extracellular environment
(7) Mild uncoupling [145, 146] and inhibition of succinate dehydrogenase [147] *via* the action of potassium channel openers	Inhibition of CI with subsequent reduction of H_2O_2 release into the cytosol

Complex III is also considered an important source for mitochondrial ROS production in ischaemic hearts [171], due to increased lipid peroxidation of cardiolipin required for complex III activity [172] and increased protein tyrosine nitration [166]. The electron leakage at complex III was associated with pharmacological preconditioning by diazoxide (the classic mitoK_{ATP} channel opener) *via* the inhibition of complex II with and transient generation of signaling ROS at complex III [173].

Lastly, complex IV-mediated ROS production can also be enhanced in ischaemic hearts *via* the activation of mitochondrial protein kinase A (PKA) which increased hyperphosphorylation of complex IV [108, 161]. Moreover, Spear et al. demonstrated that PKA-mediated depression of complex IV activity was reversed by blocking β1-adrenergic receptor activation during I/R, with a subsequent reduction of the myocardial injury [167].

Another recently investigated mitochondrial source for ROS generation is represented by monoamine oxidases (MAOs), two isoforms, MAO-A and MAO-B, located on the outer mitochondrial membrane. These FAD-containing dehydrogenases catalyze the electron transfer from the biogenic amines to O_2 and constantly generate hydrogen peroxide (H_2O_2) as by-product. MAO-derived H_2O_2 is the primary signaling molecule when generated in minute amounts and becomes harmful when highly generated during conditions associated with oxidative stress [73]. Accordingly, in settings of postischaemic reperfusion or heart failure, the increased activity of MAO-A isoform significantly contributed to the aggravation of myocardial injury [174–177] and progression towards the maladaptive left ventricle hypertrophy and remodeling, respectively [125, 178]. These studies have unequivocally demonstrated the role of MAO-A in cardiac pathology; however, recent experimental data also reported the presence and contribution of MAO-B isoform to oxidative stress in the murine cardiovascular system [126, 128, 179]. We have recently demonstrated that both MAOs isoforms are expressed in atrial appendages harvested from patients with cardiovascular pathology (i.e., valvular disease and coronary heart disease), with the predominance of the MAO-B isoform (Duicu et al., in press). To date there is only one study in the literature showing that an increased activity of MAO-B is responsible for the induction of mitochondrial dysfunction and cardiac structural/functional alterations in mice with experimentally induced heart failure [126].

3.4. Antioxidant Strategies in Ischaemia/Reperfusion Injury. The mitochondrial antioxidant system is a network of high complexity (the reader is referred to several comprehensive reviews [51, 57, 143]) and comprises 3 major categories: (1) the first one includes superoxide dismutase 2 (MnSOD) and catalase, which exert their ROS neutralizing activity independent of the reducing equivalents; (2) the second one includes peroxiredoxins 3 and 5 (Prx3 and Prx5, located in the mitochondrial matrix), which depend on thioredoxin (Trx) and thioredoxin reductase (TRx2) for their regeneration; (3) the third one includes glutathione peroxidases 1 and 4 (GPX1 and GPX4) and glutaredoxins, which depend on GSH and glutathione reductase (GR) to regenerate GSH [57]. The last two categories of ROS scavengers depend on NADPH, which in turn is regenerated by 3 mitochondrial matrix enzymes: isocitrate dehydrogenase ($NADP^+$-linked), malic enzyme, and transhydrogenase [60]. The individual contribution of these enzymes to mitochondrial NADPH regeneration is far from being elucidated [180].

The mechanisms that potentially could underlie the mitigation of ROS generation are listed in Table 2.

Therapeutic antioxidant approaches against the I/R myocardial injury have been disappointingly ineffective [181–184] or even harmful [53], most probably because the applied strategies were not able to distinguish between deleterious and beneficial ROS generation [29] and these differences between animal and human pathological models [185]. However, recent data have proven an enhanced therapeutic efficiency of novel synthetic antioxidants in ameliorating the I/R-linked oxidative stress with a subsequent cardioprotective effect. Such antioxidants include NO-based and vitamin E (MitoVit-E) molecules which are able to sequester antioxidants in mitochondria and Alda 1, a small molecule activator of aldehyde dehydrogenase-2, a mitochondrial enzyme that detoxifies aldehydes involved in myocardial I/R (reviewed in [29]). In bovine aortic endothelial cells exposed to oxidative stress, MitoVit-E significantly decreased ROS production and apoptosis [186], yet it was not neuroprotective in striatal medium-spiny neurons subjected to acute perinatal hypoxic–ischaemic brain injury [187]. An important disadvantage of MitoVit-E is that its scavenging activity is not regenerated [188]. At variance, mitoquinone (MitoQ) containing the antioxidant coenzyme Q (quinone) is regenerated by ETC after detoxifying ROS and was proven to inhibit mitochondrial oxidative stress in rodent models of I/R [189]. Another synthetic mitochondrial scavenger is the plastoquinone SkQ1, which used in a lower concentration than that of MitoQ was also able to reduce the infarct size and arrhythmias in rats subjected to I/R [190].

Of a particular promise for the inhibition of deleterious ROS induced by I/R injury might be the gene therapy approaches as demonstrated so far by two recent studies that used target upregulation of mitochondrial antioxidant enzymes like MnSOD or matrix peroxiredoxins [191] or overexpressed prosurvival molecules such as aldehyde dehydrogenase-2 microRNAs [192].

4. Mitochondria as Sources of Beneficial ROS

Until the 80s, I/R injury was considered a black or white phenomenon, the cardiomyocytes being provided with no more than two options—recovery or death. However, starting with the 80s, it became apparent that myocardial cells exposed to a variety of insults, including ischaemia, have an innate ability to mount several cardioprotective responses and an inherent program for survival. In the early days of myocardial I/R research, it was found that while the reintroduction of oxygen through reperfusion was essential for recovery, this also caused a burst of free radicals finally leading to myocardial injury [193]. Based on the concept that ROS only have deleterious effects, the administration of free radical scavengers was thought to be an appropriate solution in this situation. Several studies reported protective effects of this strategy, which unfortunately were surprisingly not supported by other independent laboratories [194]. Nowadays, it is largely accepted that some ROS represent intracellular mediators in physiological processes like vasodilation, cell growth, and angiogenesis and redox signaling is an important determinant of epigenetic and genetic regulation of cellular function. It has now become abundantly clear that, in cardioprotection against I/R injury, ROS present a delicate beneficial to deleterious switch [28] and this cross talk to and from mitochondria [157] might be favorable since the inhibition of a single source of ROS partially or even completely abrogated the oxidative stress [127].

The most powerful intrinsic mechanism of cardioprotection is represented by ischaemic preconditioning (IPC) which has been reported to cause adaptation to ischaemia in, virtually, all experimental settings from cell cultures to mammals. This strategy was first established by Murry et al. in 1986 [195] and describes the ability of brief periods of nonlethal ischaemia alternated with reperfusion to protect the heart from a subsequent prolonged lethal or "index" ischaemia. In 2003, the term postconditioning (PostC) was coined by the group of Vinten-Johansen to define a series of brief mechanical interruptions of reperfusion that were early applied within the first 3 minutes of reperfusion, elicited an anti-infarct protection comparable to the one induced by IPC [196]. The introduction of this appealing term as a novel strategy to limit lethal reperfusion injury, even if criticized by some authors who considered it as a form of modified reperfusion or compared it to "an old wine in a new bottle" [197], has the huge merit of resuscitating the concept that more myocardium can be salvaged by adding adjunct therapies to the early reperfusion. IPC and PostC require direct intervention on the heart, which may be challenging in some clinical situations. Remote ischaemic conditioning (RIC) was developed as a procedure performed by applying brief cycles of nonlethal I/R in a vascular territory remote from the heart. Although it is similarly cardioprotective to IPC and PostC, the fact that it is implemented at a distance from the organ of interest constitutes an evident advantage [198]. All these strategies represent endogenous self-defense mechanisms that are dependent on ROS generation. The identification of the most relevant sources of ROS and the threshold at which they lose their potentially protective effect and become damaging to cellular function and integrity still represents an unmet need in the field of cardioprotection [27].

4.1. Ischaemic Preconditioning. To date, no other strategy aimed at reducing I/R injury has proven itself to be more effective than IPC and thousands of paper tackled the mechanisms underlying its protective effect with numerous signaling molecules being identified as participating in the signal transduction sequence [199]. Among these, the generation of sublethal amounts of ROS during the short cycles of ischaemia and/or reperfusion has been consistently reported to be the trigger of IPC, possibly through the oxidation of protective cytosolic kinases [200]. The direct consequence of minute ROS generation prior to the prolonged ischaemia was the triggering of a "ROS-induced ROS decrease" response during the postischaemic reperfusion in every species tested. The observation that the deleterious burst of ROS upon reperfusion is reduced when IPC is applied has been demonstrated 20 years ago [201] and is still valid until today [202].

In addition, administration of exogenous ROS induces a protective effect similar to IPC [203], whereas antioxidants decrease or abolish cardioprotection [204, 205].

Mitochondria have emerged as the major source of ROS generation within the cardiac myocytes in the setting of preconditioning [206]. The PKCε activated by IPC induces the stimulation of mitochondrial K_{ATP} channels causing a slight increase in H_2O_2 production which eventually leads to the inhibition of the mitochondrial permeability transition pore (mPTP) [207], seemingly the final effector of IPC [208]. Within this cascade of events, there might be a direct interaction between ROS and mPTP components or the sublethal oxidative stress can set in motion signaling pathways that decrease mitochondrial susceptibility to mPTP opening [27]. Also, a small level of ROS can be generated through a brief opening of the mPTP that may play an important role in cardioprotection [209]. In line with this observation, it has been reported that the inhibition of CyP-D results in abolition of ROS formation and of IPC-related cardioprotection, respectively [210].

In addition, it may be that all forms of cardiomyocyte stress lead to ROS signaling and that this could represent a mechanism for gaining ischaemic tolerance, as data suggests that hyperthermic preconditioning is reliant on ROS production [211].

An important source of mitochondrial H_2O_2 generation is the activation of MAO-A (as discussed in Section 3) during reperfusion with the occurrence of apoptosis in isolated cardiac myocytes; indeed, in lower concentrations, H_2O_2 was found to be partly responsible for the cardioprotective effect of IPC [212]. Supporting this claim, MAO inhibition in the settings of IPC has been reported to abolish cardioprotection (Di Lisa, unpublished observations, cited by [213]). However, in a recent study in isolated rat hearts subjected to a preconditioning protocol, we have demonstrated that bracketing the IPC episodes with MAO inhibitors did not interfere with the antinecrotic protection but potentiated the postischaemic functional recovery [214].

4.2. Ischaemic Postconditioning. Even though IPC is indeed the most efficient strategy, postconditioning (PostC) also proved to afford cardioprotection, although slightly less so than the former in terms of decreasing the infarct size. Penna et al. were the first to notice that ROS are needed to trigger PostC-related protection too [215]. In line with this observation, other studies have proven that PostC-mediated protection was abolished in the presence of ROS scavengers at the beginning of reperfusion [215, 216].

Cardioprotection is also possibly mediated by the prevention of mPTP opening by acidosis during the PostC cycles, while the intermittent bursts of oxygen throughout the brief I/R episodes allow mitochondria to produce just enough ROS in a moment when other enzymes, responsible for the generation of massive quantities of free radicals, are not yet reactivated. The consequent activation of the PKC pathway leads to the sensitization of adenosine receptors, signaling via the RISK pathway [199], and, finally, the prevention of mPTP formation even after the pH returned to normal [213]. Although all the mentioned evidence supports the idea that PostC-related cardioprotection is dependent on redox signaling, it is also apparent that the type, concentration, and the sources of ROS may be key factors in triggering protection at the time of reperfusion [213]. Contrary to the limited clinical applicability of IPC, PostC applied to humans in the cardiac catheterization laboratory has provided encouraging results by two clinical studies [217, 218], whereas others found no cardioprotective effects [219, 220]. Such discrepancies might be the result of different inclusion/exclusion criteria and the differences in the PostC chosen protocols. Thereby, the results of the DANAMI-3 trial (NCT01435408) designed to investigate postconditioning in STEMI patients are awaited this year with real interest [221].

4.3. Remote Ischaemic Conditioning. Remote ischaemic conditioning (RIC) was firstly described in 1993 by Przyklenk et al. [222] who noted that brief episodes of I/R applied in one region of the heart are protective for remote virgin myocardium in a separate myocardial territory. The mechanisms behind RIC are very complex and occur in three interrelated stages: (1) the I/R stimulus induces the synthesis of protective factors in the remote organ; (2) the protective signal is transmitted through a complex neurohumoral interaction to the target organ; (3) the events taking place in the target organ result in the protective effect.

Presumably, the signaling pathways activated in the remote and the target organ, respectively, are similar to those described in IPC and PostC [198]. Again, ROS production is part of the signaling cascade involved. Once the cardioprotective signal resulting from the ischaemic remote organ reaches the heart, it binds to G-protein cell surface coupled receptors which activate intracellular kinases like PKC-ε and other signaling molecules such as ROS and the mitochondrial K_{ATP} channel [223]. It has been demonstrated that IPC and remote ischaemic preconditioning (RIPC) both rely on free radicals to induce cardioprotection, as *N*-2-mercaptopropionyl glycine, an antioxidant, is capable of completely blocking the beneficial effect of RIC when ischaemia is induced by infrarenal occlusion of the rat aorta [224]. Also, in a model of RIPC obtained by occlusion of the mouse femoral artery, the ischaemia applied in the remote organ induced *S*-nitrosation of mitochondrial complex I in cardiomyocytes, which resulted in a reduction of ROS (i.e., H_2O_2) in the reperfused myocardium at risk [225].

As in the case of PostC, RIPC has been successfully translated to humans and recent pilot studies showed that it is able to improve clinical outcome and prognosis (excellently reviewed by [221]). In order to confirm these proof-of-concept studies, two multicentre trials of RIPC are currently ongoing, namely, NCT01857414 (CONDI II trial) and NCT02342522 (ERIC-PPCI trial).

Despite the successful results in animal models (reviewed in [30]) and in several pilot human studies (see above), the results of the cardioprotective trials targeting mitochondria have been rather disappointing (Table 3).

Table 3: Cardioprotective strategies targeting mitochondria in clinical trials.

Trial	Strategy	Results
NCT01502774 (CIRCUS trial)	A bolus injection of CsA administered at the onset of myocardial reperfusion in patients with anterior ST-segment-elevation MI (STEMI)	Worsened heart failure during the initial hospitalization, rehospitalization for heart failure, and adverse left ventricular remodeling at 1 year in 59.0% of the 395 patients randomized to cyclosporine and 58.1% of the 396 individuals randomized to placebo [148]
NCT01374321 (MITOCARE trial)	I.v. bolus administration of TRO40303 (an inhibitor of mPTP opening) in STEMI patients undergoing primary PCI (percutaneous coronary intervention)	TRO40303 did not show any protective effects as compared to placebo in preventing reperfusion injury in STEMI patients treated with primary PCI [149]
NCT01572909 (EMBRACE STEMI trial)	MTP-131 (a cell-permeable peptide that preserves the integrity of cardiolipin, enhances mitochondrial energetics, and improves myocyte survival during reperfusion in animal models) administration for 1 h among first-time anterior STEMI subjects undergoing primary PCI for a proximal or mid left anterior descending (LAD) artery occlusion	Administration of MTP-131 was not associated with a significant reduction in infarct size or clinical outcomes [150]
NCT01584453 (NITRITE-AMI trial)	Intracoronary injection of nitrite during primary PCI in STEI patients	The phase II showed that intracoronary nitrite infusion did not change the infarct size. Yet, in a subgroup of patients with TIMI flow ≤1, nitrite reduced infarct size and MACE and improved myocardial salvage index indicating a follow-up with the phase III of the clinical trial [151]
NCT01388504 (NIAMI trial)	Intravenous sodium nitrite administration immediately prior to PCI in patients with acute STEMI	Myocardial infarct size did not differ between nitrite and placebo groups. There were no significant differences in plasma troponin I and CK area under the curve, left ventricular volumes, and ejection fraction measured at 6–8 days and at 6 months and final infarct size measured at 6 months [152]

5. Mitochondria-Derived ROS and Endothelial Dysfunction

The mitochondrial content in the endothelial cells is rather poor as compared to other cells, for example, 2–6% of the rat cell volume versus 28% in hepatocytes or 32% in cardiomyocytes [226, 227]. At variance from cardiomyocytes, endothelial mitochondrial content and energy requirements are relatively reduced, glycolysis being the main source of ATP production [228]. Nowadays these organelles are considered major players in both cell signaling and vascular disease [188]. Moreover, mitochondrial cellular distribution represents a key factor for its function. In this view, the group of Gutterman demonstrated in endothelial cells isolated from human coronary arterioles that mitochondria are anchored to the cytoskeleton being thus responsible for ROS release in response to cell deformation by shear stress [229]. Another relevant study sustaining this theory demonstrated that pulmonary artery exposed to hypoxia induced a retrograde mitochondrial movement requiring microtubules and the microtubule motor protein dynein, changes that lead to a perinuclear clustering of mitochondria [230]; moreover, this mitochondrial redistribution was associated with ROS accumulation in the nucleus, which was further reduced by nocodazole which destabilized the microtubules and, thus, suppressed the perinuclear clustering of mitochondria [230].

In recent years, an increasing attention has been payed to the alterations of mitochondrial fusion and fission, due to their harmful consequences on cellular bioenergetics and endothelial dysfunction in the settings of cardiovascular disorders [74, 231, 232].

A wealth of clinical and experimental studies unequivocally demonstrated that endothelial dysfunction represents a central event in the pathogenesis of cardiovascular diseases (recently reviewed in [233]). Risk factors, such as aging, hypercholesterolemia, hyperglycemia, smoking, infections, and hypoxia, alter the mitochondrial membrane potential ($\Delta\psi_m$), with a subsequent contribution to excessive mitochondrial ROS production [233]. If the membrane is depolarized, complexes I and III show an increased activity in order to restore membrane potential, thus leading to ROS generation [226]. Metabolic disease states associated with high nutrient availability and low ATP demand are characterized by membrane hyperpolarization which also results in excessive ROS [227]. The consequential modifications of mitochondrial components affect the mtDNA, proteins, and lipids which in turn will stimulate ROS production, creating thus a vicious cycle that promotes vascular disease [234, 235]. Moreover, the mtDNA damage is responsible for the alteration of the ETS components expression, leading to an increased ROS production [236].

Apart from complexes I and III of the respiratory chain, another important source of mitochondria-derived ROS in endothelial cells is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX-4), which is the most highly expressed Nox family member in the endothelial layer of vasculature, being localized in many intracellular compartments, including mitochondria [237, 238]. Endothelial cells and basal ROS production, mainly H_2O_2 (rather than $^{\bullet}O_2^-$) as stated by [239], require NOX-4 and its homolog NOX-2 [238]. More recently, it was suggested that NOX-4 has rather a preventive function, since it protected the vasculature during ischaemic or inflammatory stress [240]. Thus, the contribution of NOX-4 to ROS signaling, angiogenesis, oxidative stress, endothelial dysfunction, and inflammation processes is far from being fully elucidated [240–244].

Another mitochondrial source of ROS is the growth factor adapter protein $p66^{shc}$. In physiological conditions, $p66^{shc}$ is included in a high-molecular-weight inhibitory protein complex located in the mitochondrial matrix or even in the cytoplasm. Circumstances associated with proapoptotic signals, such as hypoxia, activate $p66^{shc}$ which migrates in the mitochondrial intermembrane space, where, through the oxidation of cytochrome c, it generates H_2O_2 [116]. Moreover, $p66^{shc}$ can become active via phosphorylation by protein kinase C in conditions associated with hyperglycemia, contributing thus to diabetic endothelial dysfunction [117, 245]. $p66^{shc}$ deletion in models of vascular injury has yielded beneficial effects [117, 246], supporting thus the statement about its implication in oxidative stress [227].

Mitochondrial ATP-sensitive potassium channel ($mitoK_{ATP}$) represents a regulator of mitochondrial free oxygen radicals. Although it has not been the focus of interest in vascular dysfunction so far, its activation seems to protect cultured endothelial cells from ischaemic cell death and to maintain vasodilating capacity in Langendorff-perfused guinea-pig hearts suffering from I/R injury [247, 248]. Furthermore, glibenclamide, a nonselective K_{ATP} channel blocker, abolished the IPC-induced preservation of endothelium-dependent dilation in the human forearm, while the $mitoK_{ATP}$ opener diazoxide mimicked the IPC protection [249, 250]. These potassium channels also demonstrated the ROS-induced ROS release theory, since ROS produced by another cellular structure acted by opening $mitoK_{ATP}$, stimulating the generation of mitochondrial ROS [157, 251]. It might be, thus, reasonable to assume that the inhibition of these channels might be protective. As in the case of cardiomyocytes, the phenomenon of ROS-induced ROS release can equally contribute to the pathogenesis of endothelial dysfunction [252].

Lastly, H_2O_2 generated by MAO-A in vascular smooth muscle cells contributes to serotonin-induced vasoconstriction [253]. Although it was clearly demonstrated that endothelial cells express MAOs [254], the exact role of these enzymes in modulation of endothelial function has not been fully characterized. Recently, we have described the role of MAOs as mediators of endothelial dysfunction in two murine models of acute (induced with lipopolysaccharide, LPS) and chronic (induced with angiotensin II, AII) [127] oxidative stress or after the induction of experimental diabetes [128]. Both isoforms increased the expression of vascular MAOs with subsequent high H_2O_2 generation. This mechanism was deemed responsible for the induction of oxidative stress, altered level of cGMP with a central role in NO-mediated signaling, and a consecutive impairment of aortic rings relaxation [127]. It is important to note that all changes induced by MAOs activation were reversed by the MAO-A and MAO-B inhibitors [127], proving thus the contribution of mitochondria-derived ROS to endothelial dysfunction.

Reperfusion following ischaemia is associated with an increased endothelial generation of ROS and endothelin and a reduced availability of nitric oxide. This latter event promotes neutrophil adhesion to the vascular endothelium and platelet aggregation, which, coupled with the effect of endothelin, will eventually lead to vasoconstriction which is responsible for the no-reflow phenomenon; the adhesion of neutrophils will further enhance ROS release from the endothelium and neutrophils [255]. ROS production represents, thus, an important path in mitochondrial signaling [227], which explains the huge interest in the elucidation of their sources and regulatory mechanisms.

Obviously, the nature of mitochondrial ROS signaling in endothelial cells is still a matter of debate. It may be that the significance of mitochondrial ROS in endothelial cell signaling varies according to vascular bed and risk factor burden [256]. Accordingly, there is evidence that dilation of human cardiac arterioles depends on ETC-derived ROS [229] and that antioxidant therapy blunted the dilation of the healthy human brachial artery [257]. On the other hand, in coronary arteries of atherosclerotic patients, H_2O_2 scavengers

improved endothelium-dependent dilation [258], an effect also noticed in diabetic freshly isolated arterioles treated with mitochondria-targeted antioxidants [259].

In conclusion, targeting vascular ROS definitely represents an important research direction in order to alleviate endothelial dysfunction [260].

6. Conclusion

Despite the unequivocal beneficial effects of reperfusion in ceasing the progression of irreversible damage, it is largely accepted nowadays that (i) reperfusion is a double-edged sword as it is able to induce *per se* the myocardial lethal reperfusion injury which paradoxically alleviates the beneficial effects of revascularization and (ii) there is an unmet need and a strong interest in developing clinically effective cardioprotective interventions, which are able to further reduce infarct size in association with revascularization procedures. Mitochondrial dysfunction and the resulting oxidative stress are central in the pathogenesis of I/R injury and the drugs that can antagonize cardiomyocyte death by modulating mitochondrial function have started to be systematically investigated in clinical setting. Indeed, novel antioxidant compounds selectively targeting mitochondria appear to be an effective strategy to protect the heart against the deleterious effects of both ischaemic and reperfusion injury, two sides of the same coin. However, since the signaling mechanisms mediating I/R-induced mitochondrial dysfunction are diverse, a combination of pharmacological compounds or coadministration of drugs acting simultaneously on distinct targets should be envisaged.

Competing Interests

The authors disclose that there are no competing interests.

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References

[1] A. S. Go, D. Mozaffarian, V. L. Roger et al., "Heart disease and stroke statistics—2014 update: a report from the American Heart Association," *Circulation*, vol. 129, no. 3, pp. e28–e292, 2014.

[2] C. D. Mathers and D. Loncar, "Projections of global mortality and burden of disease from 2002 to 2030," *PLoS Medicine*, vol. 3, no. 11, article e442, 2002.

[3] G. M. Fröhlich, P. Meier, S. K. White, D. M. Yellon, and D. J. Hausenloy, "Myocardial reperfusion injury: looking beyond primary PCI," *European Heart Journal*, vol. 34, no. 23, pp. 1714–1722, 2013.

[4] E. Braunwald and R. A. Kloner, "Myocardial reperfusion: a double-edged sword?" *The Journal of Clinical Investigation*, vol. 76, no. 5, pp. 1713–1719, 1985.

[5] D. J. Hearse, "Ischemia, reperfusion, and the determinants of tissue injury," *Cardiovascular Drugs and Therapy*, vol. 4, supplement 4, pp. 767–776, 1990.

[6] S. R. J. Maxwell and G. Y. H. Lip, "Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options," *International Journal of Cardiology*, vol. 58, no. 2, pp. 95–117, 1997.

[7] D. M. Yellon and D. J. Hausenloy, "Myocardial reperfusion injury," *The New England Journal of Medicine*, vol. 357, no. 11, pp. 1074–1135, 2007.

[8] R. B. Jennings, "Historical perspective on the pathology of myocardial ischemia/reperfusion injury," *Circulation Research*, vol. 113, no. 4, pp. 428–438, 2013.

[9] R. A. Kloner, "Does reperfusion injury exist in humans?" *Journal of the American College of Cardiology*, vol. 21, no. 2, pp. 537–545, 1993.

[10] G. Heusch and R. Schulz, "Little evidence for lethal reperfusion injury to cardiomyocytes," *Journal of Thrombosis and Thrombolysis*, vol. 4, no. 1, pp. 111–112, 1997.

[11] S. B. Ong and A. B. Gustafsson, "New roles for mitochondria in cell death in the reperfused myocardium," *Cardiovascular Research*, vol. 94, no. 2, pp. 190–196, 2012.

[12] R. J. Diaz and G. J. Wilson, "Modifying the first minute of reperfusion: potential for myocardial salvage," *Cardiovascular Research*, vol. 62, no. 1, pp. 4–6, 2004.

[13] H. M. Piper, Y. Abdallah, and C. Schäfer, "The first minutes of reperfusion: a window of opportunity for cardioprotection," *Cardiovascular Research*, vol. 61, no. 3, pp. 365–371, 2004.

[14] D. J. Hausenloy and D. M. Yellon, "Myocardial ischemia-reperfusion injury: a neglected therapeutic target," *The Journal of Clinical Investigation*, vol. 123, no. 1, pp. 92–100, 2013.

[15] D. Garcia-Dorado, A. Rodríguez-Sinovas, M. Ruiz-Meana, and J. Inserte, "Protection against myocardial ischemia-reperfusion injury in clinical practice," *Revista Española de Cardiología*, vol. 67, no. 5, pp. 394–404, 2014.

[16] F. Di Lisa, "Mitochondrial contribution in the progression of cardiac ischemic injury," *IUBMB Life*, vol. 52, no. 3–5, pp. 255–261, 2002.

[17] A. L. Moens, M. J. Claeys, J. P. Timmermans, and C. J. Vrints, "Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process," *International Journal of Cardiology*, vol. 100, no. 2, pp. 179–190, 2005.

[18] D. N. Silachev, E. Y. Plotnikov, I. B. Pevzner et al., "The mitochondrion as a key regulator of ischaemic tolerance and injury," *Heart Lung and Circulation*, vol. 23, no. 10, pp. 897–904, 2014.

[19] A. P. Halestrap and A. P. Richardson, "The mitochondrial permeability transition: a current perspective on its identity and role in ischaemia/reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 78, pp. 129–141, 2015.

[20] P. Bernardi and F. Di Lisa, "The mitochondrial permeability transition pore: molecular nature and role as a target in cardioprotection," *Journal of Molecular and Cellular Cardiology*, vol. 78, pp. 100–106, 2015.

[21] D. Morin, T. Hauet, M. Spedding, and J.-P. Tillement, "Mitochondria as target for antiischemic drugs," *Advanced Drug Delivery Reviews*, vol. 49, no. 1-2, pp. 151–174, 2001.

[22] A. M. Walters, G. A. Porter Jr., and P. S. Brookes, "Mitochondria as a drug target in ischemic heart disease and cardiomyopathy," *Circulation Research*, vol. 111, no. 9, pp. 1222–1236, 2012.

[23] A. K. Camara, M. Bienengraeber, and D. F. Stowe, "Mitochondrial approaches to protect against cardiac ischemia and reperfusion injury," *Frontiers in Physiology*, vol. 2, article 13, 2011.

[24] J. L. Zweier and M. A. H. Talukder, "The role of oxidants and free radicals in reperfusion injury," *Cardiovascular Research*, vol. 70, no. 2, pp. 181–190, 2006.

[25] F. Di Lisa and P. Bernardi, "Mitochondria and ischemia-reperfusion injury of the heart: fixing a hole," *Cardiovascular Research*, vol. 70, no. 2, pp. 191–199, 2006.

[26] D. N. Granger and P. R. Kvietys, "Reperfusion injury and reactive oxygen species: the evolution of a concept," *Redox Biology*, vol. 6, pp. 524–551, 2015.

[27] F. Di Lisa, M. Canton, A. Carpi et al., "Mitochondrial injury and protection in ischemic pre-and postconditioning," *Antioxidants & Redox Signaling*, vol. 14, no. 5, pp. 881–891, 2011.

[28] F. Tullio, C. Angotti, M.-G. Perrelli, C. Penna, and P. Pagliaro, "Redox balance and cardioprotection," *Basic Research in Cardiology*, vol. 108, no. 6, article 392, 2013.

[29] T. Kalogeris, Y. Bao, and R. J. Korthuis, "Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning," *Redox Biology*, vol. 2, no. 1, pp. 702–714, 2014.

[30] O. M. Duicu, D. Angoulvant, and D. M. Muntean, "Cardioprotection against myocardial reperfusion injury: successes, failures, and perspectives," *Canadian Journal of Physiology and Pharmacology*, vol. 91, no. 8, pp. 657–662, 2013.

[31] F. Altamirano, Z. V. Wang, and J. A. Hill, "Cardioprotection in ischaemia-reperfusion injury: novel mechanisms and clinical translation," *Journal of Physiology*, vol. 593, no. 17, pp. 3773–3788, 2015.

[32] H. A. Cabrera-Fuentes, C. Alba-Alba, J. Aragones et al., "Meeting report from the 2nd International Symposium on New Frontiers in Cardiovascular Research. Protecting the cardiovascular system from ischemia: between bench and bedside," *Basic Research in Cardiology*, vol. 111, article 7, 2016.

[33] J. S. Armstrong, "Mitochondria-directed therapeutics," *Antioxidants & Redox Signaling*, vol. 10, no. 3, pp. 575–578, 2008.

[34] R. A. Smith, R. C. Hartley, H. M. Cocheme, and M. P. Murphy, "Mitochondrial pharmacology," *Trends in Pharmacological Sciences*, vol. 33, no. 6, pp. 341–352, 2012.

[35] C. Reily, T. Mitchell, B. K. Chacko, G. A. Benavides, M. P. Murphy, and V. M. Darley-Usmar, "Mitochondrially targeted compounds and their impact on cellular bioenergetics," *Redox Biology*, vol. 1, no. 1, pp. 86–93, 2013.

[36] J. P. G. Sluijter, G. Condorelli, S. M. Davidson et al., "Novel therapeutic strategies for cardioprotection," *Pharmacology & Therapeutics*, vol. 144, no. 1, pp. 60–70, 2014.

[37] R. S. Balaban, "Perspectives on: SGP symposium on mitochondrial physiology and medicine: metabolic homeostasis of the heart," *The Journal of General Physiology*, vol. 139, no. 6, pp. 407–414, 2012.

[38] R. B. Jennings and W. B. Wartman, "Production of an area of homogeneous myocardial infarction in the dog," *AMA Archives of Pathology*, vol. 63, no. 6, pp. 580–585, 1957.

[39] R. B. Jennings, C. E. Ganote, and K. A. Reimer, "Ischemic tissue injury," *The American Journal of Pathology*, vol. 81, no. 1, pp. 179–198, 1975.

[40] R. B. Jennings and C. E. Ganote, "Mitochondrial structure and function in acute myocardial ischemic injury," *Circulation Research*, vol. 38, supplement 1, no. 5, pp. 80–91, 1976.

[41] A. C. Shen and R. B. Jennings, "Myocardial calcium and magnesium in acute ischemic injury," *The American Journal of Pathology*, vol. 67, no. 3, pp. 417–440, 1972.

[42] V. J. Richard, C. E. Murry, R. B. Jennings, and K. A. Reimer, "Oxygen-derived free radicals and postischemic myocardial reperfusion: therapeutic implications," *Fundamental & Clinical Pharmacology*, vol. 4, no. 1, pp. 85–103, 1990.

[43] F. Di Lisa, R. Menabò, M. Canton, and V. Petronilli, "The role of mitochondria in the salvage and the injury of the ischemic myocardium," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1366, no. 1-2, pp. 69–78, 1998.

[44] B. Halliwell, "Free radicals and antioxidants: updating a personal view," *Nutrition Reviews*, vol. 70, no. 5, pp. 257–265, 2012.

[45] C. E. Ganote and J. P. Kaltenbach, "Oxygen-induced enzyme release: early events and a proposed mechanism," *Journal of Molecular and Cellular Cardiology*, vol. 11, no. 4, pp. 389–406, 1979.

[46] R. S. Vander Heide, J. P. Angelo, R. A. Altschuld, and C. E. Ganote, "Energy dependence of contraction band formation in perfused hearts and isolated adult myocytes," *The American Journal of Pathology*, vol. 125, no. 1, pp. 55–68, 1986.

[47] R. A. Kloner, K. Przyklenk, and P. Whittaker, "Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues," *Circulation*, vol. 80, no. 5, pp. 1115–1127, 1989.

[48] J. M. McCord, "Free radicals and myocardial ischemia: overview and outlook," *Free Radical Biology and Medicine*, vol. 4, no. 1, pp. 9–14, 1988.

[49] Y. Gauduel, P. Menasche, and M. Duvelleroy, "Enzyme release and mitochondrial activity in reoxygenated cardiac muscle: relationship with oxygen-induced lipid peroxidation," *General Physiology and Biophysics*, vol. 8, no. 4, pp. 327–340, 1989.

[50] B. Halliwell, "The wanderings of a free radical," *Free Radical Biology & Medicine*, vol. 46, no. 5, pp. 531–542, 2009.

[51] A. Y. Andreyev, Y. E. Kushnareva, and A. A. Starkov, "Mitochondrial metabolism of reactive oxygen species," *Biochemistry (Moscow)*, vol. 70, no. 2, pp. 200–214, 2005.

[52] W. Dröge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.

[53] H. H. Schmidt, R. Stocker, C. Vollbracht et al., "Antioxidants in translational medicine," *Antioxidants & Redox Signaling*, vol. 23, no. 14, pp. 1130–1143, 2015.

[54] D. P. Jones, "Redefining oxidative stress," *Antioxidants & Redox Signaling*, vol. 8, no. 9-10, pp. 1865–1879, 2006.

[55] D. P. Jones, "Radical-free biology of oxidative stress," *American Journal of Physiology—Cell Physiology*, vol. 295, no. 4, pp. C849–C868, 2008.

[56] M. R. Duchen, "Mitochondria in health and disease: perspectives on a new mitochondrial biology," *Molecular Aspects of Medicine*, vol. 25, no. 4, pp. 365–451, 2004.

[57] A. Y. Andreyev, Y. E. Kushnareva, A. N. Murphy, and A. A. Starkov, "Mitochondrial ROS metabolism: 10 years later," *Biochemistry*, vol. 80, no. 5, pp. 517–531, 2015.

[58] B. Ibanez, V. Fuster, J. Jiménez-Borreguero, and J. J. Badimon, "Lethal myocardial reperfusion injury: a necessary evil?" *International Journal of Cardiology*, vol. 151, no. 1, pp. 3–11, 2011.

[59] J. F. Turrens, "Mitochondrial formation of reactive oxygen species," *The Journal of Physiology*, vol. 552, part 2, pp. 335–344, 2003.

[60] Y. Liu, G. Fiskum, and D. Schubert, "Generation of reactive oxygen species by the mitochondrial electron transport chain," *Journal of Neurochemistry*, vol. 80, no. 5, pp. 780–787, 2002.

[61] Q. Chen, E. J. Vazquez, S. Moghaddas, C. L. Hoppel, and E. J. Lesnefsky, "Production of reactive oxygen species by mitochondria: central role of complex III," *The Journal of Biological Chemistry*, vol. 278, no. 38, pp. 36027–36031, 2003.

[62] V. Adam-Vizi and C. Chinopoulos, "Bioenergetics and the formation of mitochondrial reactive oxygen species," *Trends in Pharmacological Sciences*, vol. 27, no. 12, pp. 639–645, 2006.

[63] S. Orrenius, "Reactive oxygen species in mitochondria-mediated cell death," *Drug Metabolism Reviews*, vol. 39, no. 2-3, pp. 443–455, 2007.

[64] E. B. Tahara, F. D. T. Navarete, and A. J. Kowaltowski, "Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation," *Free Radical Biology & Medicine*, vol. 46, no. 9, pp. 1283–1297, 2009.

[65] A. J. Lambert and M. D. Brand, "Reactive oxygen species production by mitochondria," *Methods in Molecular Biology*, vol. 554, pp. 165–181, 2009.

[66] M. P. Murphy, "How mitochondria produce reactive oxygen species," *The Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.

[67] D. F. Stowe and A. K. Camara, "Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function," *Antioxidants & Redox Signaling*, vol. 11, no. 6, pp. 1373–1414, 2009.

[68] T. Finkel, "Signal transduction by mitochondrial oxidants," *The Journal of Biological Chemistry*, vol. 287, no. 7, pp. 4434–4440, 2012.

[69] V. G. Grivennikova and A. D. Vinogradov, "Mitochondrial production of reactive oxygen species," *Biochemistry*, vol. 78, no. 13, pp. 1490–1511, 2013.

[70] D. B. Zorov, M. Juhaszova, and S. J. Sollott, "Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release," *Physiological Reviews*, vol. 94, no. 3, pp. 909–950, 2014.

[71] Y. R. Chen and J. L. Zweier, "Cardiac mitochondria and reactive oxygen species generation," *Circulation Research*, vol. 114, no. 3, pp. 524–537, 2014.

[72] M. Forkink, J. A. M. Smeitink, R. Brock, P. H. G. M. Willems, and W. J. H. Koopman, "Detection and manipulation of mitochondrial reactive oxygen species in mammalian cells," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1797, no. 6-7, pp. 1034–1044, 2010.

[73] N. Kaludercic, J. Mialet-Perez, N. Paolocci, A. Parini, and F. Di Lisa, "Monoamine oxidases as sources of oxidants in the heart," *Journal of Molecular and Cellular Cardiology*, vol. 73, pp. 34–42, 2014.

[74] P. H. G. M. Willems, R. Rossignol, C. E. J. Dieteren, M. P. Murphy, and W. J. H. Koopman, "Redox homeostasis and mitochondrial dynamics," *Cell Metabolism*, vol. 22, no. 2, pp. 207–218, 2015.

[75] J. F. Turrens and A. Boveris, "Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria," *The Biochemical Journal*, vol. 191, no. 2, pp. 421–427, 1980.

[76] L. Kussmaul and J. Hirst, "The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 20, pp. 7607–7612, 2006.

[77] S. T. Ohnishi, K. Shinzawa-Itoh, K. Ohta, S. Yoshikawa, and T. Ohnishi, "New insights into the superoxide generation sites in bovine heart NADH-ubiquinone oxidoreductase (Complex I): the significance of protein-associated ubiquinone and the dynamic shifting of generation sites between semiflavin and semiquinone radicals," *Biochimica et Biophysica Acta*, vol. 1797, no. 12, pp. 1901–1909, 2010.

[78] M. L. Genova, B. Ventura, G. Giuliano et al., "The site of production of superoxide radical in mitochondrial Complex I is not a bound ubisemiquinone but presumably iron-sulfur cluster N2," *FEBS Letters*, vol. 505, no. 3, pp. 364–368, 2001.

[79] P. T. Kang, L. Zhang, C. L. Chen, J. Chen, K. B. Green, and Y. R. Chen, "Protein thiyl radical mediates S-glutathionylation of complex I," *Free Radical Biology and Medicine*, vol. 53, no. 4, pp. 962–973, 2012.

[80] J. Chen, C.-L. Chen, S. Rawale et al., "Peptide-based antibodies against glutathione-binding domains suppress superoxide production mediated by mitochondrial complex," *The Journal of Biological Chemistry*, vol. 285, no. 5, pp. 3168–3180, 2010.

[81] C.-L. Chen, L. Zhang, A. Yeh et al., "Site-specific S-glutathiolation of mitochondrial NADH ubiquinone reductase," *Biochemistry*, vol. 46, no. 19, pp. 5754–5765, 2007.

[82] Y.-R. Chen, C.-L. Chen, L. Zhang, K. B. Green-Church, and J. L. Zweier, "Superoxide generation from mitochondrial NADH dehydrogenase induces self-inactivation with specific protein radical formation," *The Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37339–37348, 2005.

[83] K. R. Pryde and J. Hirst, "Superoxide is produced by the reduced flavin in mitochondrial complex I: a single, unified mechanism that applies during both forward and reverse electron transfer," *The Journal of Biological Chemistry*, vol. 286, no. 20, pp. 18056–18065, 2011.

[84] J. St-Pierre, J. A. Buckingham, S. J. Roebuck, and M. D. Brand, "Topology of superoxide production from different sites in the mitochondrial electron transport chain," *The Journal of Biological Chemistry*, vol. 277, no. 47, pp. 44784–44790, 2002.

[85] S. T. Ohnishi, T. Ohnishi, S. Muranaka et al., "A possible site of superoxide generation in the complex I segment of rat heart mitochondria," *Journal of Bioenergetics and Biomembranes*, vol. 37, no. 1, pp. 1–15, 2005.

[86] Y. Kushnareva, A. N. Murphy, and A. Andreyev, "Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state," *The Biochemical Journal*, vol. 368, no. 2, pp. 545–553, 2002.

[87] A. P. Kudin, N. Y.-B. Bimpong-Buta, S. Vielhaber, C. E. Elger, and W. S. Kunz, "Characterization of superoxide-producing sites in isolated brain mitochondria," *The Journal of Biological Chemistry*, vol. 279, no. 6, pp. 4127–4135, 2004.

[88] J. F. Turrens, B. A. Freeman, J. G. Levitt, and J. D. Crapo, "The effect of hyperoxia on superoxide production by lung submitochondrial particles," *Archives of Biochemistry and Biophysics*, vol. 217, no. 2, pp. 401–410, 1982.

[89] A. J. Lambert and M. D. Brand, "Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane," *Biochemical Journal*, vol. 382, no. 2, pp. 511–517, 2004.

[90] A. J. Lambert and M. D. Brand, "Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I)," *The Journal of Biological Chemistry*, vol. 279, no. 38, pp. 39414–39420, 2004.

[91] J. R. Treberg, C. L. Quinlan, and M. D. Brand, "Evidence for two sites of superoxide production by mitochondrial NADH-ubiquinone oxidoreductase (complex I)," *The Journal of Biological Chemistry*, vol. 286, no. 31, pp. 27103–27110, 2011.

[92] Y.-R. Chen, C.-L. Chen, D. R. Pfeiffer, and J. L. Zweier, "Mitochondrial complex II in the post-ischemic heart: oxidative

injury and the role of protein S-glutathionylation," *The Journal of Biological Chemistry*, vol. 282, no. 45, pp. 32640–32654, 2007.

[93] R. G. Hansford, B. A. Hogue, and V. Mildaziene, "Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age," *Journal of Bioenergetics and Biomembranes*, vol. 29, no. 1, pp. 89–95, 1997.

[94] I. Siebels and S. Dröse, "Q-site inhibitor induced ROS production of mitochondrial complex II is attenuated by TCA cycle dicarboxylates," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1827, no. 10, pp. 1156–1164, 2013.

[95] Y.-R. Chen, C.-L. Chen, A. Yeh, X. Liu, and J. L. Zweier, "Direct and indirect roles of cytochrome b in the mediation of superoxide generation and NO catabolism by mitochondrial succinate-cytochrome c reductase," *The Journal of Biological Chemistry*, vol. 281, no. 19, pp. 13159–13168, 2006.

[96] L. Zhang, L. Yu, and C.-A. Yu, "Generation of superoxide anion by succinate-cytochrome c reductase from bovine heart mitochondria," *Journal of Biological Chemistry*, vol. 273, no. 51, pp. 33972–33976, 1998.

[97] T. V. Votyakova and I. J. Reynolds, "DeltaPsi(m)-dependent and -independent production of reactive oxygen species by rat brain mitochondria," *Journal of Neurochemistry*, vol. 79, no. 2, pp. 266–277, 2001.

[98] C. L. Quinlan, A. L. Orr, I. V. Perevoshchikova, J. R. Treberg, B. A. Ackrell, and M. D. Brand, "Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions," *The Journal of Biological Chemistry*, vol. 287, no. 32, pp. 27255–27264, 2012.

[99] J. Guo and B. D. Lemire, "The ubiquinone-binding site of the Saccharomyces cerevisiae succinate-ubiquinone oxidoreductase is a source of superoxide," *The Journal of Biological Chemistry*, vol. 278, no. 48, pp. 47629–47635, 2003.

[100] V. Yankovskaya, R. Horsefield, S. Törnroth et al., "Architecture of succinate dehydrogenase and reactive oxygen species generation," *Science*, vol. 299, no. 5607, pp. 700–704, 2003.

[101] A. Boveris, E. Cadenas, and A. O. M. Stoppani, "Role of ubiquinone in the mitochondrial generation of hydrogen peroxide," *Biochemical Journal*, vol. 156, no. 2, pp. 435–444, 1976.

[102] E. Cadenas, A. Boveris, C. I. Ragan, and A. O. M. Stoppani, "Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria," *Archives of Biochemistry and Biophysics*, vol. 180, no. 2, pp. 248–257, 1977.

[103] J. F. Turrens, A. Alexandre, and A. L. Lehninger, "Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria," *Archives of Biochemistry and Biophysics*, vol. 237, no. 2, pp. 408–414, 1985.

[104] A. A. Starkov and G. Fiskum, "Myxothiazol induces H_2O_2 production from mitochondrial respiratory chain," *Biochemical and Biophysical Research Communications*, vol. 281, no. 3, pp. 645–650, 2001.

[105] D. Han, E. Williams, and E. Cadenas, "Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space," *The Biochemical Journal*, vol. 353, no. 2, pp. 411–416, 2001.

[106] F. L. Muller, Y. Liu, and H. Van Remmen, "Complex III releases superoxide to both sides of the inner mitochondrial membrane," *The Journal of Biological Chemistry*, vol. 279, no. 47, pp. 49064–49073, 2004.

[107] J. L. Cape, M. K. Bowman, and D. M. Kramer, "A semiquinone intermediate generated at the Qo site of the cytochrome bc1 complex: importance for the Q-cycle and superoxide production," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 19, pp. 7887–7892, 2007.

[108] S. K. Prabu, H. K. Anandatheerthavarada, H. Raza, S. Srinivasan, J. F. Spear, and N. G. Avadhani, "Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury," *The Journal of Biological Chemistry*, vol. 281, no. 4, pp. 2061–2070, 2006.

[109] L. K. Kwong and R. S. Sohal, "Substrate and site specificity of hydrogen peroxide generation in mouse Mitochondria," *Archives of Biochemistry and Biophysics*, vol. 350, no. 1, pp. 118–126, 1998.

[110] Z. Drahota, S. K. R. Chowdhury, D. Floryk et al., "Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide," *Journal of Bioenergetics and Biomembranes*, vol. 34, no. 2, pp. 105–113, 2002.

[111] S. Miwa, J. St-Pierre, L. Partridge, and M. D. Brand, "Superoxide and hydrogen peroxide production by *Drosophila* mitochondria," *Free Radical Biology and Medicine*, vol. 35, no. 8, pp. 938–948, 2003.

[112] H. J. Forman and J. Kennedy, "Dihydroorotate-dependent superoxide producton in rat brain and liver: a function of the primary dehydrogenase," *Archives of Biochemistry and Biophysics*, vol. 173, no. 1, pp. 219–224, 1976.

[113] M. Hey-Mogensen, R. L. S. Goncalves, A. L. Orr, and M. D. Brand, "Production of superoxide/H_2O_2 by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria," *Free Radical Biology and Medicine*, vol. 72, pp. 149–155, 2014.

[114] M. Löffler, C. Becker, E. Wegerle, and G. Schuster, "Catalytic enzyme histochemistry and biochemical analysis of dihydroorotate dehydrogenase/oxidase and succinate dehydrogenase in mammalian tissues, cells and mitochondria," *Histochemistry and Cell Biology*, vol. 105, no. 2, pp. 119–128, 1996.

[115] N. Hail, P. Chen, J. J. Kepa, L. R. Bushman, and C. Shearn, "Dihydroorotate dehydrogenase is required for N-(4-hydroxyphenyl)retinamide-induced reactive oxygen species production and apoptosis," *Free Radical Biology and Medicine*, vol. 49, no. 1, pp. 109–116, 2010.

[116] M. Giorgio, E. Migliaccio, F. Orsini et al., "Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis," *Cell*, vol. 122, no. 2, pp. 221–233, 2005.

[117] G. G. Camici, M. Schiavoni, P. Francia et al., "Genetic deletion of p66Shc adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 12, pp. 5217–5222, 2007.

[118] J. Vásquez-Vivar, B. Kalyanaraman, and M. C. Kennedy, "Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation," *The Journal of Biological Chemistry*, vol. 275, no. 19, pp. 14064–14069, 2000.

[119] L. Tretter and V. Adam-Vizi, "Generation of reactive oxygen species in the reaction catalyzed by α-ketoglutarate dehydrogenase," *The Journal of Neuroscience*, vol. 24, no. 36, pp. 7771–7778, 2004.

[120] A. A. Starkov, G. Fiskum, C. Chinopoulos et al., "Mitochondrial α-ketoglutarate dehydrogenase complex generates reactive oxygen species," *The Journal of Neuroscience*, vol. 24, no. 36, pp. 7779–7788, 2004.

[121] S. A. Whatley, D. Curti, F. Das Gupta et al., "Superoxide, neuroleptics and the ubiquinone and cytochrome b5 reductases in brain and lymphocytes from normals and schizophrenic patients," *Molecular Psychiatry*, vol. 3, no. 3, pp. 227–237, 1998.

[122] N. Hauptmann, J. Grimsby, J. C. Shih, and E. Cadenas, "The metabolism of tyramine by monoamine oxidase A/B causes oxidative damage to mitochondrial DNA," *Archives of Biochemistry and Biophysics*, vol. 335, no. 2, pp. 295–304, 1996.

[123] A. Maurel, C. Hernandez, O. Kunduzova et al., "Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 284, no. 4, pp. H1460–H1467, 2003.

[124] F. Carvalho, J. A. Duarte, M. J. Neuparth et al., "Hydrogen peroxide production in mouse tissues after acute *d*-amphetamine administration. Influence of monoamine oxidase inhibition," *Archives of Toxicology*, vol. 75, no. 8, pp. 465–469, 2001.

[125] C. Villeneuve, C. Guilbeau-Frugier, P. Sicard et al., "P53-PGC-1α pathway mediates oxidative mitochondrial damage and cardiomyocyte necrosis induced by monoamine oxidase-a upregulation: role in chronic left ventricular dysfunction in mice," *Antioxidants & Redox Signaling*, vol. 18, no. 1, pp. 5–18, 2013.

[126] N. Kaludercic, A. Carpi, T. Nagayama et al., "Monoamine Oxidase B prompts mitochondrial and cardiac dysfunction in pressure overloaded hearts," *Antioxidants and Redox Signaling*, vol. 20, no. 2, pp. 267–280, 2014.

[127] A. Sturza, M. S. Leisegang, A. Babelova et al., "Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta," *Hypertension*, vol. 62, no. 1, pp. 140–146, 2013.

[128] A. Sturza, O. M. Duicu, A. Vaduva et al., "Monoamine oxidases are novel sources of cardiovascular oxidative stress in experimental diabetes," *Canadian Journal of Physiology and Pharmacology*, vol. 93, no. 7, pp. 555–561, 2015.

[129] M. J. Kumar, D. G. Nicholls, and J. K. Andersen, "Oxidative α-ketoglutarate dehydrogenase inhibition via subtle elevations in monoamine oxidase B levels results in loss of spare respiratory capacity: implications for Parkinson's disease," *The Journal of Biological Chemistry*, vol. 278, no. 47, pp. 46432–46439, 2003.

[130] E. J. Anderson, J. T. Efird, S. W. Davies et al., "Monoamine oxidase is a major determinant of redox balance in human atrial myocardium and is associated with postoperative atrial fibrillation," *Journal of the American Heart Association*, vol. 3, no. 1, Article ID e000713, 2014.

[131] K.-U. Lee, I. K. Lee, J. Han et al., "Effects of recombinant adenovirus-mediated uncoupling protein 2 overexpression on endothelial function and apoptosis," *Circulation Research*, vol. 96, no. 11, pp. 1200–1207, 2005.

[132] Y. Teshima, M. Akao, S. P. Jones, and E. Marbán, "Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes," *Circulation Research*, vol. 93, no. 3, pp. 192–200, 2003.

[133] F. Valsecchi, S. Grefte, P. Roestenberg et al., "Primary fibroblasts of NDUFS4-/- mice display increased ROS levels and aberrant mitochondrial morphology," *Mitochondrion*, vol. 13, no. 5, pp. 436–443, 2013.

[134] L. Blanchet, S. Grefte, J. A. M. Smeitink, P. H. G. M. Willems, and W. J. H. Koopman, "Photo-induction and automated quantification of reversible mitochondrial permeability transition pore opening in primary mouse myotubes," *PLoS ONE*, vol. 9, no. 11, Article ID e114090, 2014.

[135] C. Brenner and M. Moulin, "Physiological roles of the permeability transition pore," *Circulation Research*, vol. 111, no. 9, pp. 1237–1247, 2012.

[136] F. Ichas and J.-P. Mazat, "From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state," *Biochimica et Biophysica Acta*, vol. 1366, no. 1-2, pp. 33–50, 1998.

[137] L. Sun, S. Shukair, T. J. Naik, F. Moazed, and H. Ardehali, "Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II," *Molecular and Cellular Biology*, vol. 28, no. 3, pp. 1007–1017, 2008.

[138] C.-L. Chen, J. Chen, S. Rawale et al., "Protein tyrosine nitration of the flavin subunit is associated with oxidative modification of mitochondrial complex II in the post-ischemic myocardium," *Journal of Biological Chemistry*, vol. 283, no. 41, pp. 27991–28003, 2008.

[139] J. Garcia, D. Han, H. Sancheti, L.-P. Yap, N. Kaplowitz, and E. Cadenas, "Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates," *Journal of Biological Chemistry*, vol. 285, no. 51, pp. 39646–39654, 2010.

[140] S. M. Beer, E. R. Taylor, S. E. Brown et al., "Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant defense," *Journal of Biological Chemistry*, vol. 279, no. 46, pp. 47939–47951, 2004.

[141] T. R. Hurd, R. Requejo, A. Filipovska et al., "Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: potential role of Cys residues in decreasing oxidative damage," *The Journal of Biological Chemistry*, vol. 283, no. 36, pp. 24801–24815, 2008.

[142] E. R. Taylor, F. Hurrell, R. J. Shannon, T.-K. Lin, J. Hirst, and M. P. Murphy, "Reversible glutathionylation of complex I increases mitochondrial superoxide formation," *Journal of Biological Chemistry*, vol. 278, no. 22, pp. 19603–19610, 2003.

[143] D. C. Liemburg-Apers, P. H. G. M. Willems, W. J. H. Koopman, and S. Grefte, "Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism," *Archives of Toxicology*, vol. 89, no. 8, pp. 1209–1226, 2015.

[144] K. A. Brand and U. Hermfisse, "Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species," *The FASEB Journal*, vol. 11, no. 5, pp. 388–395, 1997.

[145] G. Debska, A. Kicinska, J. Skalska et al., "Opening of potassium channels modulates mitochondrial function in rat skeletal muscle," *Biochimica et Biophysica Acta*, vol. 1556, no. 2-3, pp. 97–105, 2002.

[146] A. Petruş, O. M. Duicu, A. Sturza et al., "Modulation of mitochondrial respiratory function and ROS production by novel benzopyran analogues," *Canadian Journal of Physiology and Pharmacology*, vol. 93, no. 9, pp. 811–818, 2015.

[147] P. P. Dzeja, P. Bast, C. Ozcan et al., "Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 284, no. 4, pp. H1048–H1056, 2003.

[148] T. R. Cung, O. Morel, G. Cayla et al., "Cyclosporine before PCI in patients with acute myocardial infarction," *The New England Journal of Medicine*, vol. 373, no. 11, pp. 1021–1031, 2015.

[149] D. Atar, H. Arheden, A. Berdeaux et al., "Effect of intravenous TRO40303 as an adjunct to primary percutaneous coronary intervention for acute ST-elevation myocardial infarction: MITOCARE study results," *European Heart Journal*, vol. 36, no. 2, pp. 112–119, 2015.

[150] C. M. Gibson, R. P. Giugliano, R. A. Kloner et al., "EMBRACE STEMI study: a phase 2a trial to evaluate the safety, tolerability, and efficacy of intravenous MTP-131 on reperfusion injury in patients undergoing primary percutaneous coronary intervention," *European Heart Journal*, 2015.

[151] D. A. Jones, C. Pellaton, S. Velmurugan et al., "Randomized phase 2 trial of intracoronary nitrite during acute myocardial infarction," *Circulation Research*, vol. 116, no. 3, pp. 437–447, 2015.

[152] N. Siddiqi, C. Neil, M. Bruce et al., "Intravenous sodium nitrite in acute ST-elevation myocardial infarction: a randomized controlled trial (NIAMI)," *European Heart Journal*, vol. 35, no. 19, pp. 1255–1262, 2014.

[153] M. Forkink, F. Basit, J. Teixeira, H. G. Swarts, W. J. H. Koopman, and P. H. G. M. Willems, "Complex I and complex III inhibition specifically increase cytosolic hydrogen peroxide levels without inducing oxidative stress in HEK293 cells," *Redox Biology*, vol. 6, pp. 607–616, 2015.

[154] J. R. Burgoyne, H. Mongue-Din, P. Eaton, and A. M. Shah, "Redox signaling in cardiac physiology and pathology," *Circulation Research*, vol. 111, no. 8, pp. 1091–1106, 2012.

[155] S. F. Steinberg, "Oxidative stress and sarcomeric proteins," *Circulation Research*, vol. 112, no. 2, pp. 393–405, 2013.

[156] D. B. Zorov, C. R. Filburn, L.-O. Klotz, J. L. Zweier, and S. J. Sollott, "Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes," *The Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1001–1014, 2000.

[157] A. Daiber, "Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1797, no. 6-7, pp. 897–906, 2010.

[158] S. Karbach, P. Wenzel, A. Waisman, T. Münzel, and A. Daiber, "eNOS uncoupling in cardiovascular diseases-the role of oxidative stress and inflammation," *Current Pharmaceutical Design*, vol. 20, no. 22, pp. 3579–3594, 2014.

[159] J.-M. Li and A. M. Shah, "Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 287, no. 5, pp. R1014–R1030, 2004.

[160] Y. Liu, H. Zhao, H. Li, B. Kalyanaraman, A. C. Nicolosi, and D. D. Gutterman, "Mitochondrial sources of H_2O_2 generation play a key role in flow-mediated dilation in human coronary resistance arteries," *Circulation Research*, vol. 93, no. 6, pp. 573–580, 2003.

[161] D. X. Zhang, L. Borbouse, D. Gebremedhin et al., "H_2O_2-induced dilation in human coronary arterioles: role of protein kinase G dimerization and large-conductance Ca^{2+}-activated K^+ channel activation," *Circulation Research*, vol. 110, no. 3, pp. 471–480, 2012.

[162] Y. F. Pung, W. J. Sam, K. Stevanov et al., "Mitochondrial oxidative stress corrupts coronary collateral growth by activating adenosine monophosphate activated kinase-α signaling," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 8, pp. 1911–1919, 2013.

[163] S.-I. Saitoh, T. Kiyooka, P. Rocic et al., "Redox-dependent coronary metabolic dilation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 6, pp. H3720–H3725, 2007.

[164] P. A. Rogers, W. M. Chilian, I. N. Bratz, R. M. Bryan Jr., and G. M. Dick, "H_2O_2 activates redox- and 4-aminopyridine-sensitive Kv channels in coronary vascular smooth muscle," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 3, pp. H1404–H1411, 2007.

[165] R. D. Guzy and P. T. Schumacker, "Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia," *Experimental Physiology*, vol. 91, no. 5, pp. 807–819, 2006.

[166] B. Liu, A. K. Tewari, L. Zhang et al., "Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target," *Biochimica et Biophysica Acta—Proteins and Proteomics*, vol. 1794, no. 3, pp. 476–485, 2009.

[167] J. F. Spear, S. K. Prabu, D. Galati, H. Raza, H. K. Anandatheerthavarada, and N. G. Avadhani, "β_1-Adrenoreceptor activation contributes to ischemia-reperfusion damage as well as playing a role in ischemic preconditioning," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 5, pp. H2459–H2466, 2007.

[168] H.-L. Lee, C.-L. Chen, S. T. Yeh, J. L. Zweier, and Y.-R. Chen, "Biphasic modulation of the mitochondrial electron transport chain in myocardial ischemia and reperfusion," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 302, no. 7, pp. H1410–H1422, 2012.

[169] G. Paradies, G. Petrosillo, M. Pistolese, N. Di Venosa, A. Federici, and F. M. Ruggiero, "Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin," *Circulation Research*, vol. 94, no. 1, pp. 53–59, 2004.

[170] A. K. Gadicherla, D. F. Stowe, W. E. Antholine, M. Yang, and A. K. S. Camara, "Damage to mitochondrial complex i during cardiac ischemia reperfusion injury is reduced indirectly by anti-anginal drug ranolazine," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1817, no. 3, pp. 419–429, 2012.

[171] J. L. Zweier, J. T. Flaherty, and M. L. Weisfeldt, "Direct measurement of free radical generation following reperfusion of ischemic myocardium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 5, pp. 1404–1407, 1987.

[172] G. Petrosillo, F. M. Ruggiero, N. Di Venosa, and G. Paradies, "Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin," *The FASEB Journal*, vol. 17, no. 6, pp. 714–716, 2003.

[173] S. Dröse, P. J. Hanley, and U. Brandt, "Ambivalent effects of diazoxide on mitochondrial ROS production at respiratory chain complexes I and III," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1790, no. 6, pp. 558–565, 2009.

[174] P. Bianchi, O. Kunduzova, E. Masini et al., "Oxidative stress by monoamine oxidase mediates receptor-independent cardiomyocyte apoptosis by serotonin and postischemic myocardial injury," *Circulation*, vol. 112, no. 21, pp. 3297–3305, 2005.

[175] N. Kaludercic, A. Carpi, R. Menabò, F. Di Lisa, and N. Paolocci, "Monoamine oxidases (MAO) in the pathogenesis of heart failure and ischemia/reperfusion injury," *Biochimica et Biophysica Acta*, vol. 1813, no. 7, pp. 1323–1332, 2011.

[176] P. Bianchi, D. R. Pimentel, M. P. Murphy, W. S. Colucci, and A. Parini, "A new hypertrophic mechanism of serotonin in cardiac myocytes: receptor-independent ROS generation," *The FASEB Journal*, vol. 19, no. 6, pp. 641–643, 2005.

[177] A. Carpi, R. Menabò, N. Kaludercic, P. Pelicci, F. Di Lisa, and M. Giorgio, "The cardioprotective effects elicited by p66Shc ablation demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury," *Biochimica et Biophysica Acta*, vol. 1787, no. 7, pp. 774–780, 2009.

[178] N. Kaludercic, E. Takimoto, T. Nagayama et al., "Monoamine oxidase a-mediated enhanced catabolism of norepinephrine contributes to adverse remodeling and pump failure in hearts with pressure overload," *Circulation Research*, vol. 106, no. 1, pp. 193–202, 2010.

[179] S. D. Sivasubramaniam, C. C. Finch, M. J. Rodriguez, N. Mahy, and E. E. Billett, "A comparative study of the expression of monoamine oxidase-A and -B mRNA and protein in non-CNS human tissues," *Cell and Tissue Research*, vol. 313, no. 3, pp. 291–300, 2003.

[180] R. Vogel, H. Wiesinger, B. Hamprecht, and R. Dringen, "The regeneration of reduced glutathione in rat forebrain mitochondria identifies metabolic pathways providing the NADPH required," *Neuroscience Letters*, vol. 275, no. 2, pp. 97–100, 1999.

[181] I. Andreadou, E. K. Iliodromitis, D. Farmakis, and D. T. Kremastinos, "To prevent, protect and save the ischemic heart: antioxidants revisited," *Expert Opinion on Therapeutic Targets*, vol. 13, no. 8, pp. 945–956, 2009.

[182] P. M. W. Bath, L. J. Gray, A. J. G. Bath, A. Buchan, T. Miyata, and A. R. Green, "Effects of NXY-059 in experimental stroke: an individual animal meta-analysis," *British Journal of Pharmacology*, vol. 157, no. 7, pp. 1157–1171, 2009.

[183] T. Jaxa-Chamiec, B. Bednarz, K. Herbaczynska-Cedro, P. Maciejewski, and L. Ceremuzynski, "Effects of vitamins C and E on the outcome after acute myocardial infarction in diabetics: a retrospective, hypothesis-generating analysis from the MIVIT Study," *Cardiology*, vol. 112, no. 3, pp. 219–223, 2009.

[184] D. P. Vivekananthan, M. S. Penn, S. K. Sapp, A. Hsu, and E. J. Topol, "Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials," *The Lancet*, vol. 361, no. 9374, pp. 2017–2023, 2003.

[185] S. R. Steinhubl, "Why have antioxidants failed in clinical trials?" *The American Journal of Cardiology A*, vol. 101, no. 10, pp. S14–S19, 2008.

[186] A. Dhanasekaran, S. Kotamraju, S. V. Kalivendi et al., "Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis," *The Journal of Biological Chemistry*, vol. 279, no. 36, pp. 37575–37587, 2004.

[187] M. V. Covey, M. P. Murphy, C. E. Hobbs, R. A. J. Smith, and D. E. Oorschot, "Effect of the mitochondrial antioxidant, Mito Vitamin E, on hypoxic-ischemic striatal injury in neonatal rats: a dose-response and stereological study," *Experimental Neurology*, vol. 199, no. 2, pp. 513–519, 2006.

[188] S. M. Davidson and M. R. Duchen, "Endothelial mitochondria: contributing to vascular function and disease," *Circulation Research*, vol. 100, no. 8, pp. 1128–1141, 2007.

[189] V. J. Adlam, J. C. Harrison, C. M. Porteous et al., "Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury," *The FASEB Journal*, vol. 19, no. 9, pp. 1088–1095, 2005.

[190] L. E. Bakeeva, I. V. Barskov, M. V. Egorov et al., "Mitochondria-targeted plastoquinone derivatives as tools to interrupt execution of the aging program. 2. Treatment of some ROS- and age-related diseases (heart arrhythmia, heart infarctions, kidney ischemia, and stroke)," *Biochemistry*, vol. 73, no. 12, pp. 1288–1299, 2008.

[191] J. Navarro-Yepes, L. Zavala-Flores, A. Anandhan et al., "Antioxidant gene therapy against neuronal cell death," *Pharmacology and Therapeutics*, vol. 142, no. 2, pp. 206–230, 2014.

[192] Y.-B. Ouyang, C. M. Stary, R. E. White, and R. G. Giffard, "The use of microRNAs to modulate redox and immune response to stroke," *Antioxidants & Redox Signaling*, vol. 22, no. 2, pp. 187–202, 2015.

[193] D. J. Hearse, S. M. Humphrey, and E. B. Chain, "Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release," *Journal of Molecular and Cellular Cardiology*, vol. 5, no. 4, pp. 395–407, 1973.

[194] K. A. Reimer, C. E. Murry, and V. J. Richard, "The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and controversy?" *Journal of Molecular and Cellular Cardiology*, vol. 21, no. 12, pp. 1225–1239, 1989.

[195] C. E. Murry, R. B. Jennings, and K. A. Reimer, "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium," *Circulation*, vol. 74, no. 5, pp. 1124–1136, 1986.

[196] Z. Q. Zhao, J. S. Corvera, M. E. Halkos et al., "Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning," *American Journal of Physiology Heart and Circulatory Physiology*, vol. 285, no. 2, pp. H579–H588, 2003.

[197] G. Heusch, "Postconditioning: old wine in a new bottle?" *Journal of the American College of Cardiology*, vol. 44, no. 5, pp. 1111–1112, 2004.

[198] S. Y. Lim and D. J. Hausenloy, "Remote ischemic conditioning: from bench to bedside," *Frontiers in Physiology*, vol. 3, article 27, 2012.

[199] M. V. Cohen and J. M. Downey, "Signalling pathways and mechanisms of protection in pre- and postconditioning: historical perspective and lessons for the future," *British Journal of Pharmacology*, vol. 172, no. 8, pp. 1913–1932, 2015.

[200] G. Heusch, "Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning," *Circulation Research*, vol. 116, no. 4, pp. 674–699, 2015.

[201] T. L. Vanden Hoek, L. B. Becker, Z. Shao, C. Li, and P. T. Schumacker, "Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes," *The Journal of Biological Chemistry*, vol. 273, no. 29, pp. 18092–18098, 1998.

[202] R. Quarrie, B. M. Cramer, D. S. Lee et al., "Ischemic preconditioning decreases mitochondrial proton leak and reactive oxygen species production in the postischemic heart," *Journal of Surgical Research*, vol. 165, no. 1, pp. 5–14, 2011.

[203] I. Tritto, D. D'Andrea, N. Eramo et al., "Oxygen radicals can induce preconditioning in rabbit hearts," *Circulation Research*, vol. 80, no. 5, pp. 743–748, 1997.

[204] W. Chen, S. Gabel, C. Steenbergen, and E. Murphy, "A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart," *Circulation Research*, vol. 77, no. 2, pp. 424–429, 1995.

[205] R. A. Forbes, C. Steenbergen, and E. Murphy, "Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism," *Circulation Research*, vol. 88, no. 8, pp. 802–809, 2001.

[206] H. Otani, "Reactive oxygen species as mediators of signal transduction in ischemic preconditioning," *Antioxidants and Redox Signaling*, vol. 6, no. 2, pp. 449–469, 2004.

[207] A. D. T. Costa, R. Jakob, C. L. Costa, K. Andrukhiv, I. C. West, and K. D. Garlid, "The mechanism by which the mitochondrial ATP-sensitive K^+ channel opening and H_2O_2 inhibit the

mitochondrial permeability transition," *The Journal of Biological Chemistry*, vol. 281, no. 30, pp. 20801–20808, 2006.

[208] E. Murphy and C. Steenbergen, "Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury," *Physiological Reviews*, vol. 88, no. 2, pp. 581–609, 2008.

[209] V. Petronilli, D. Penzo, L. Scorrano, P. Bernardi, and F. Di Lisa, "The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings in situ," *The Journal of Biological Chemistry*, vol. 276, no. 15, pp. 12030–12034, 2001.

[210] D. J. Hausenloy, S. Y. Lim, S.-G. Ong, S. M. Davidson, and D. M. Yellon, "Mitochondrial cyclophilin-D as a critical mediator of ischaemic preconditioning," *Cardiovascular Research*, vol. 88, no. 1, pp. 67–74, 2010.

[211] N. Yamashita, S. Hoshida, N. Taniguchi, T. Kuzuya, and M. Hori, "Whole-body hyperthermia provides biphasic cardioprotection against Ischemia/reperfusion injury in the rat," *Circulation*, vol. 98, no. 14, pp. 1414–1421, 1998.

[212] Y. Yaguchi, H. Satoh, N. Wakahara et al., "Protective effects of hydrogen peroxide against ischemia/reperfusion injury in perfused rat hearts," *Circulation Journal*, vol. 67, no. 3, pp. 253–258, 2003.

[213] C. Penna, D. Mancardi, R. Rastaldo, and P. Pagliaro, "Cardioprotection: a radical view: free radicals in pre and postconditioning," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1787, no. 7, pp. 781–793, 2009.

[214] M. D. Dănilă, A. I. Privistirescu, S. N. Mirica et al., "Acute inhibition of monoamine oxidase and ischemic preconditioning in isolated rat hearts: interference with postischemic functional recovery but no effect on infarct size reduction," *Canadian Journal of Physiology and Pharmacology*, vol. 93, no. 9, pp. 819–825, 2015.

[215] C. Penna, R. Rastaldo, D. Mancardi et al., "Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K^+ channel and protein kinase C activation," *Basic Research in Cardiology*, vol. 101, no. 2, pp. 180–189, 2006.

[216] Y. M. Tsutsumi, T. Yokoyama, Y. Horikawa, D. M. Roth, and H. H. Patel, "Reactive oxygen species trigger ischemic and pharmacological postconditioning: In vivo and in vitro characterization," *Life Sciences*, vol. 81, no. 15, pp. 1223–1227, 2007.

[217] P. Sörensson, N. Saleh, F. Bouvier et al., "Effect of postconditioning on infarct size in patients with ST elevation myocardial infarction," *Heart*, vol. 96, no. 21, pp. 1710–1715, 2010.

[218] F. Thuny, O. Lairez, F. Roubille et al., "Post-conditioning reduces infarct size and edema in patients with ST-segment elevation myocardial infarction," *Journal of the American College of Cardiology*, vol. 59, no. 24, pp. 2175–2181, 2012.

[219] X. Freixa, N. Bellera, J. T. Ortiz-Pérez et al., "Ischaemic postconditioning revisited: lack of effects on infarct size following primary percutaneous coronary intervention," *European Heart Journal*, vol. 33, no. 1, pp. 103–112, 2012.

[220] J.-Y. Hahn, Y. B. Song, E. K. Kim et al., "Ischemic postconditioning during primary percutaneous coronary intervention: the effects of postconditioning on myocardial reperfusion in patients with st-segment elevation myocardial infarction (POST) randomized trial," *Circulation*, vol. 128, no. 17, pp. 1889–1896, 2013.

[221] J. T. Lønborg, "Targeting reperfusion injury in the era of primary percutaneous coronary intervention: hope or hype?" *Heart*, vol. 101, no. 20, pp. 1612–1618, 2015.

[222] K. Przyklenk, B. Bauer, M. Ovize, R. A. Kloner, and P. Whittaker, "Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion," *Circulation*, vol. 87, no. 3, pp. 893–899, 1993.

[223] D. J. Hausenloy and D. M. Yellon, "Remote ischaemic preconditioning: underlying mechanisms and clinical application," *Cardiovascular Research*, vol. 79, no. 3, pp. 377–386, 2008.

[224] C. Weinbrenner, F. Schulze, L. Sárváry, and R. H. Strasser, "Remote preconditioning by infrarenal aortic occlusion is operative via δ1-opioid receptors and free radicals in vivo in the rat heart," *Cardiovascular Research*, vol. 61, no. 3, pp. 591–599, 2004.

[225] T. Rassaf, M. Totzeck, U. B. Hendgen-Cotta, S. Shiva, G. Heusch, and M. Kelm, "Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning," *Circulation Research*, vol. 114, no. 10, pp. 1601–1610, 2014.

[226] P. Dromparis and E. D. Michelakis, "Mitochondria in vascular health and disease," *Annual Review of Physiology*, vol. 75, pp. 95–126, 2013.

[227] M. A. Kluge, J. L. Fetterman, and J. A. Vita, "Mitochondria and endothelial function," *Circulation Research*, vol. 112, no. 8, pp. 1171–1188, 2013.

[228] O. Culic, M. L. H. Gruwel, and J. Schrader, "Energy turnover of vascular endothelial cells," *American Journal of Physiology-Cell Physiology*, vol. 273, no. 1, part 1, pp. C205–C213, 1997.

[229] Y. Liu, H. Li, A. H. Bubolz, D. X. Zhang, and D. D. Gutterman, "Endothelial cytoskeletal elements are critical for flow-mediated dilation in human coronary arterioles," *Medical & Biological Engineering & Computing*, vol. 46, no. 5, pp. 469–478, 2008.

[230] A.-B. Al-Mehdi, V. M. Pastukh, B. M. Swiger et al., "Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription," *Science Signaling*, vol. 5, no. 231, article ra47, 2012.

[231] S.-B. Ong, S. Subrayan, S. Y. Lim, D. M. Yellon, S. M. Davidson, and D. J. Hausenloy, "Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury," *Circulation*, vol. 121, no. 18, pp. 2012–2022, 2010.

[232] S. M. Shenouda, M. E. Widlansky, K. Chen et al., "Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus," *Circulation*, vol. 124, no. 4, pp. 444–453, 2011.

[233] X. Tang, Y. X. Luo, H. Z. Chen, and D. P. Liu, "Mitochondria, endothelial cell function, and vascular diseases," *Frontiers in Physiology*, vol. 5, article 175, 2014.

[234] M. F. Algahim, S. Sen, and H. Taegtmeyer, "Bariatric surgery to unload the stressed heart: a metabolic hypothesis," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 302, no. 8, pp. H1539–H1545, 2012.

[235] B. G. Hill, G. A. Benavides, J. J. R. Lancaster Jr. et al., "Integration of cellular bioenergetics with mitochondrial quality control and autophagy," *Biological Chemistry*, vol. 393, no. 12, pp. 1485–1512, 2012.

[236] S. W. Ballinger, C. Patterson, C.-N. Yan et al., "Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells," *Circulation Research*, vol. 86, no. 9, pp. 960–966, 2000.

[237] F. Chen, S. Haigh, S. Barman, and D. J. Fulton, "From form to function: the role of Nox4 in the cardiovascular system," *Frontiers in Physiology*, vol. 3, article 412, 2012.

[238] B. Lassegue, A. San Martin, and K. K. Griendling, "Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system," *Circulation Research*, vol. 110, no. 10, pp. 1364–1390, 2012.

[239] S. I. Dikalov, A. E. Dikalova, A. T. Bikineyeva, H. H. H. W. Schmidt, D. G. Harrison, and K. K. Griendling, "Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production," *Free Radical Biology & Medicine*, vol. 45, no. 9, pp. 1340–1351, 2008.

[240] K. Schröder, M. Zhang, S. Benkhoff et al., "Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase," *Circulation Research*, vol. 110, no. 9, pp. 1217–1225, 2012.

[241] R. P. Brandes, I. Takac, and K. Schröder, "No superoxide-no stress?: Nox4, the good NADPH oxidase!," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 6, pp. 1255–1257, 2011.

[242] F. Veit, O. Pak, B. Egemnazarov et al., "Function of NADPH oxidase 1 in pulmonary arterial smooth muscle cells after monocrotaline-induced pulmonary vascular remodeling," *Antioxidants & Redox Signaling*, vol. 19, no. 18, pp. 2213–2231, 2013.

[243] F. J. Sánchez-Gómez, E. Calvo, R. Bretón-Romero et al., "NOX4-dependent Hydrogen peroxide promotes shear stress-induced SHP2 sulfenylation and eNOS activation," *Free Radical Biology & Medicine*, vol. 89, pp. 419–430, 2015.

[244] C. Schürmann, F. Rezende, C. Kruse et al., "The NADPH oxidase Nox4 has anti-atherosclerotic functions," *European Heart Journal*, vol. 36, no. 48, pp. 3447–3456, 2015.

[245] F. Paneni, P. Mocharla, A. Akhmedov et al., "Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes," *Circulation Research*, vol. 111, no. 3, pp. 278–289, 2012.

[246] C. Napoli, I. Martin-Padura, F. De Nigris et al., "Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 2112–2116, 2003.

[247] J. Feng and Z. Zuo, "Isoflurane preconditioning increases endothelial cell tolerance to in-vitro simulated ischaemia," *Journal of Pharmacy and Pharmacology*, vol. 63, no. 1, pp. 106–110, 2011.

[248] A. Beresewicz, M. Maczewski, and M. Duda, "Effect of classic preconditioning and diazoxide on endothelial function and O_2^- and NO generation in the post-ischemic guinea-pig heart," *Cardiovascular Research*, vol. 63, no. 1, pp. 118–129, 2004.

[249] M. W. Broadhead, R. K. Kharbanda, M. J. Peters, and R. J. MacAllister, "KATP channel activation induces ischemic preconditioning of the endothelium in humans in vivo," *Circulation*, vol. 110, no. 15, pp. 2077–2082, 2004.

[250] S. P. Loukogeorgakis, R. Williams, A. T. Panagiotidou et al., "Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a KATP channel-dependent mechanism," *Circulation*, vol. 116, no. 12, pp. 1386–1395, 2007.

[251] D. B. Zorov, M. Juhaszova, and S. J. Sollott, "Mitochondrial ROS-induced ROS release: an update and review," *Biochimica et Biophysica Acta*, vol. 1757, no. 5-6, pp. 509–517, 2006.

[252] A. K. Doughan, D. G. Harrison, and S. I. Dikalov, "Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction," *Circulation Research*, vol. 102, no. 4, pp. 488–496, 2008.

[253] C. C. Poon, S. W. Seto, A. L. Au et al., "Mitochondrial monoamine oxidase-A-mediated hydrogen peroxide generation enhances 5-hydroxytryptamine-induced contraction of rat basilar artery," *British Journal of Pharmacology*, vol. 161, no. 5, pp. 1086–1098, 2010.

[254] S. Meresse, M.-P. Dehouck, P. Delorme et al., "Bovine brain endothelial cells express tight junctions and monoamine oxidase activity in long-term culture," *Journal of Neurochemistry*, vol. 53, no. 5, pp. 1363–1371, 1989.

[255] P. Pagliaro, A. Chiribiri, D. Mancardi, R. Rastaldo, D. Gattullo, and G. Losano, "Coronary endothelial dysfunction after ischemia and reperfusion and its prevention by ischemic preconditioning," *Italian Heart Journal*, vol. 4, no. 6, pp. 383–394, 2003.

[256] M. N. Sack, "Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance," *Cardiovascular Research*, vol. 72, no. 2, pp. 210–219, 2006.

[257] R. S. Richardson, A. J. Donato, A. Uberoi et al., "Exercise-induced brachial artery vasodilation: role of free radicals," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 3, pp. H1516–H1522, 2007.

[258] N. P. Andrews, A. Prasad, and A. A. Quyyumi, "N-acetylcysteine improves coronary and peripheral vascular function," *Journal of the American College of Cardiology*, vol. 37, no. 1, pp. 117–123, 2001.

[259] T. J. Kizhakekuttu, J. Wang, K. Dharmashankar et al., "Adverse alterations in mitochondrial function contribute to type 2 diabetes mellitus-related endothelial dysfunction in humans," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 10, pp. 2531–2539, 2012.

[260] A. Szewczyk, W. Jarmuszkiewicz, A. Koziel et al., "Mitochondrial mechanisms of endothelial dysfunction," *Pharmacological Reports*, vol. 67, no. 4, pp. 704–710, 2015.

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Research Article

Role of TFEB Mediated Autophagy, Oxidative Stress, Inflammation, and Cell Death in Endotoxin Induced Myocardial Toxicity of Young and Aged Mice

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Elderly patients are susceptible to sepsis. LPS induced myocardial injury is a widely used animal model to assess sepsis induced cardiac dysfunction. The age dependent mechanisms behind sepsis susceptibility were not studied. We analyzed age associated changes to cardiac function, cell death, inflammation, oxidative stress, and autophagy in LPS induced myocardial injury. Both young and aged C57BL/6 mice were used for LPS administration. The results demonstrated that LPS induced more cardiac injury (creatine kinase, lactate dehydrogenase, troponin I, and cardiac myosin-light chains 1), cardiac dysfunction (left ventricular inner dimension, LVID, and ejection fraction (EF)), cell death, inflammation, and oxidative stress in aged mice compared to young mice. However, a significant age dependent decline in autophagy was observed. Translocation of Transcription Factor EB (TFEB) to nucleus and formation of LC3-II were significantly reduced in LPS administered aged mice compared to young ones. In addition to that, downstream effector of TFEB, LAMP-1, was induced in response to LPS challenge in young mice. The present study newly demonstrates that TFEB mediated autophagy is crucial for protection against LPS induced myocardial injury particularly in aging senescent heart. Targeting this autophagy-oxidative stress-inflammation-cell death axis may provide a novel therapeutic strategy for cardioprotection in the elderly.

1. Introduction

Sepsis is a leading cause of death among critically ill patients and elderly patients are most vulnerable to it [1, 2]. The elderly population will grow more rapidly and the world's elderly population will cross that of the young by 2050 when sepsis in those patients will be priority [3, 4]. Heart failure is a well-known complication of sepsis and also known as septic cardiomyopathy. The mechanism of septic cardiomyopathy has been studied well and a series of molecular mechanisms such as apoptosis, cytokines, immune regulation, toxin, mitochondria, and energy metabolism has been implicated. However, the precise mechanisms and their role in the pathogenesis of septic cardiomyopathy in aging remain incompletely understood.

Autophagy is an intracellular process of protein degradation and recycling. Autophagic deregulation leads to many diseases (neurodegenerative disorder, cancer, etc.) and protects against oxidative damage and inflammation [5]. Reduced autophagic potential leads to aging and increased autophagy delays aging [6, 7]. Autophagy is crucial to maintaining homeostasis in the heart, and a decline is associated with accelerated cardiac aging [8]. However, the role of autophagy in sepsis and associated cardiac dysfunction are not clearly understandable till date.

Transcription factor EB (TFEB) is one of the regulators of autophagy. TFEB translocates to nucleus and regulates hundreds of genes which consist of Coordinated Lysosomal Expression and Regulation (CLEAR) network [9, 10]. Those CLEAR networks genes are involved in autophagosomes

formation (such as LAMP-1, VPS11), vesicle formation and elongation (such as MAP1LC3), and cargo recognition and degradation (such as SQSTM1 or p62).

Cardiac aging leads to structural, functional changes in addition to cellular and molecular changes [11]. Oxidative stress is key contributor of cardiovascular aging at the molecular level [11]. In aging heart, the majority of ROS are derived from NOX (NADPH oxidase) and mitochondrial electron transport chain [12]. There is a close link between oxidation and inflammation and, as aging occurs, more oxidative/nitrative damaged biomolecules accumulated in the heart, which lead to more inflammation [13].

Here, we examined the mechanism of sepsis associated cardiac dysfunction in aging and autophagy-oxidative stress-inflammation axis played critical role in LPS induced cardiac dysfunction in aged mice.

2. Methods

2.1. Animal Treatments. Male C57BL/6 mice that are 4–6-week-old (young) and 22–24-week-old (aged) were obtained from the Experimental Animal Center of Shandong University (Jinan, Shandong, China). LPS was purchased from Sigma in China (Beijing, China). LPS was dissolved in normal saline and administered intraperitoneally (i.p.) at a volume of 10 μL/gram for each mouse. The mice were given 4 mg/kg of LPS for 18 hours.

Mice experimental protocols were approved by Institutional Animal Care and Use Committee of Shandong University and were in compliance with Health Ministry of the People's Republic of China. Mice were sacrificed under isoflurane (5%) deep anesthesia after completion of echocardiography.

2.2. Echocardiography. Mice were anesthetized with isoflurane (1%) mixed with oxygen. Echocardiographic cardiac parameters were determined by VisualSonics Vevo770 system (VisualSonics, Inc., Toronto, Canada) as described earlier [14, 15].

2.3. Real-Time PCR. mRNA level of TNFα (tumor necrosis factor), IL1β (interleukin 1 beta), MIP1α (macrophage inflammatory protein-1 alpha), MCP1 (CD46), MAP1lc3 (microtubule-associated protein 1 light chain 3), VPS11 (vacuolar protein sorting-associated protein 11), or β actin was detected by reverse transcription and real-time PCR. Total RNA was isolated by Trizol method as described earlier [8]. All predesigned primers were purchased from Qiagen. The fold changes were calculated based on relative quantification method [16].

2.4. Western Blot. Heart tissues were homogenized in lysis buffer and protein concentration was determined as described earlier [14].

Western transfer in PVDF membrane was performed after running equal amount of proteins in SDS-PAGE. Membranes were probed with LC3 antibody, GAPDH antibody (1 : 200, Cell Signaling Technology), anti-TFEB antibody (1 : 200, Santa Cruz Biotechnology), anti-LAMP1 antibody (1 : 200, Sigma-Aldrich), and Histone H3 (1 : 200, Santa Cruz Biotechnology) overnight at 4°C. The membranes were probed with HRP-conjugated secondary antibody (1 : 2000, Rockland, Gilbertsville) for 1 h at room temperature. The chemiluminescence in the membranes was analyzed on X-ray film.

2.5. Immunohistochemistry. Histological analyses were performed on paraffin embedded section. After deparaffinization and antigen retrieval process, sections were stained with anti-nitrotyrosine antibody overnight and developed with VECTASTAIN Elite ABC Kit Rabbit IgG and ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories) according to manufacturer's instruction.

2.6. DNA Fragmentation. DNA fragmentation was measured by ELISA based kit (Roche) according to manufacturer's instruction.

2.7. PARP Activity. PARP activity, we used the HT Universal Colorimetric PARP assay kit from Trevigen as described earlier [17].

2.8. Protein Nitrotyrosine Nitration. Protein nitrotyrosine nitration was determined using OxiSelect™ Nitrotyrosine ELISA Kit (Cell Biolabs) according to manufacturer's instruction.

2.9. Protein Carbonyl Content. Carbonyl content in protein from tissue lysate was determined by Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical) according to manufacturer's recommendation.

2.10. Statistical Analysis. Data were expressed as mean ± standard deviation (SD), and statistical analysis was done by using GraphPad Prism software. Paired t-test or one-way analysis of variance followed by Tukey's Post Test were performed. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Aged Mice Are Prone to LPS Induced Cardiac Dysfunction. To examine whether young mice confer more cardiac protective effects in vivo than aged mice, we used both young and aged C57BL/6 mice. LPS was administered intraperitoneally at 4 mg/kg overnight for about 18 hours, which caused marked myocardial tissue damage in young and aged mice as evidenced by elevated plasma lactate dehydrogenase (LDH) and creatine kinase (CK) in Figure 1. The tissue damage was more significant in aged mice compared to young mice. Cardiac troponin I (cTnI) and cardiac myosin-light chains 1 (cMLC1) testing are an essential component of acute heart disorders in particular as a highly specific marker for myocardial infarction or heart muscle cell death. In young group of mice cTnI level was increased from 0.36 to 6.46 (ng/mL) whereas in aged mice plasma of cTnI was increased from 1.29 to the level of 15.5 (ng/mL) when LPS was

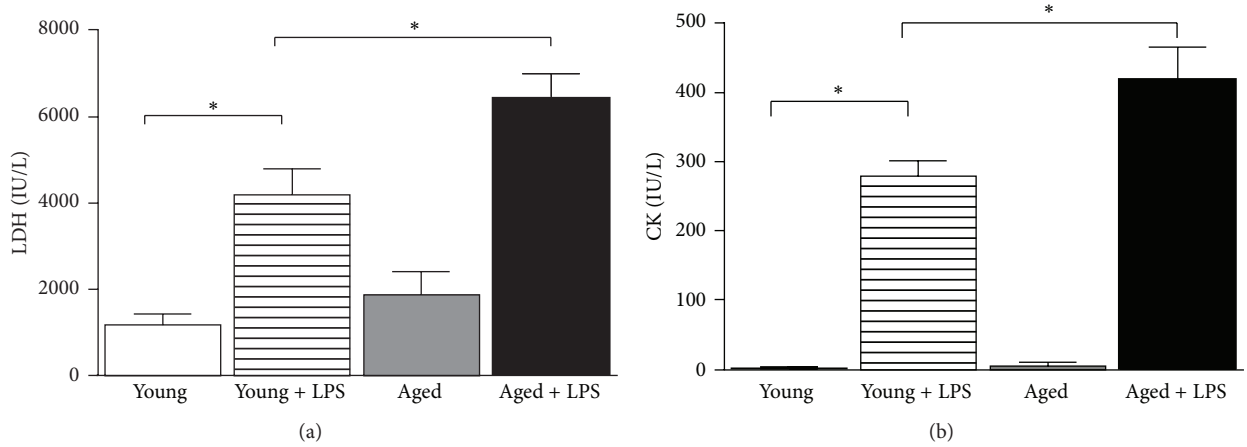

Figure 1: Effect of LPS in young and aged mice on cardiac injury. Cardiac injury was measured by plasma LDH (a) and CK (b). Both enzymes were significantly increased in LPS treated mice. Increase of LPS induced cardiac injury in aged mice was significantly higher than young ones. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

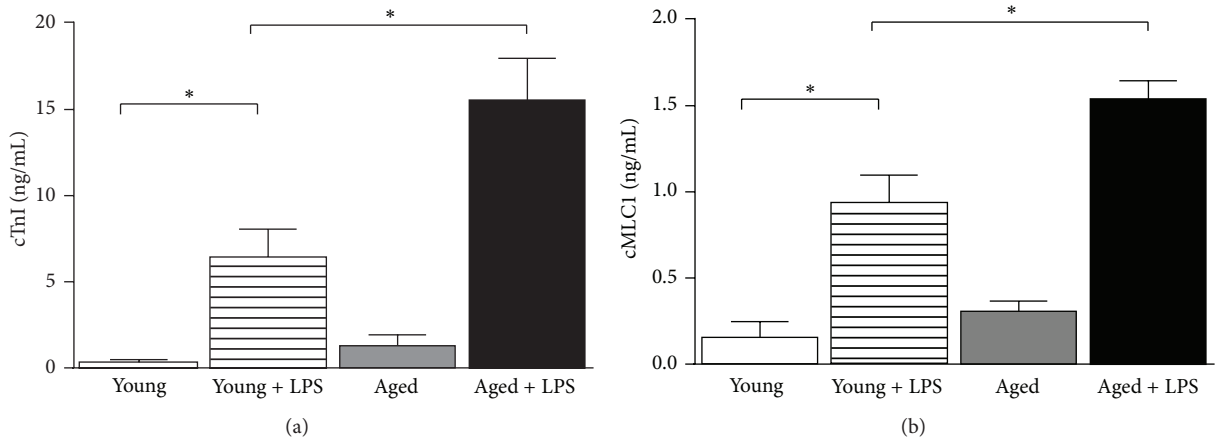

Figure 2: Effect of LPS in young and aged mice on cardiac damage. Cardiac damage was measured by plasma cTnI (a) and cMLC1 (b), which were secreted by damaged heart. Both markers were significantly increased in LPS treated mice. Increase of LPS induced cardiac damage in aged mice was significantly higher than young ones. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

administered (Figure 2(a)). Similarly, in young group of mice, cMLC1 level was increased from 0.15 to 0.92 (ng/mL) whereas in aged mice plasma of the same was increased from 0.30 to 1.54 (ng/mL) (Figure 2(b)). Similar pattern was observed earlier in other cardiac injury models [18].

Left ventricular (LV) structure and function were assessed by echocardiography. As shown in Figures 3(a) and 3(b), LPS caused an increase in end-diastolic left ventricular inner dimension (LVID) and a decrease in ejection fraction (EF) in both young and old mice. However cardiac dysfunction was more significant in older mice compared to young mice.

We have demonstrated earlier that LPS induced significant cardiac dysfunction [14]. The structural and functional alterations in aging hearts are indications of failing heart, which thus may increase the vulnerability of the aging heart to develop heart failure [19, 20]. In patients with septic shock dilation of left ventricles is reported [21]. Other hemodynamic profile of septic shock includes elevated cardiac index and reduced systemic vascular resistance [22]. In this study, we observed that aging heart was more susceptible to LPS induced myocardial toxicity. Notably cardiac diastolic dysfunction is associated with aging [23, 24]. We demonstrated that two key parameters, LVID and EF, of diastolic function were modulated with aging and significantly altered in LPS induced myocardial toxicity. It is reported in studies with elderly and younger individuals of similar physical status that the end-diastolic volume and ejection fraction are increased during exercise [25, 26]. Major components of cardiovascular aging are decrease in elasticity and an increase in stiffness of the arterial system that leads to systolic blood pressure and left ventricular hypertrophy and alteration in the left ventricular wall [27]. However, in mice model, we observed difference in diastolic function, which was altered by endotoxin.

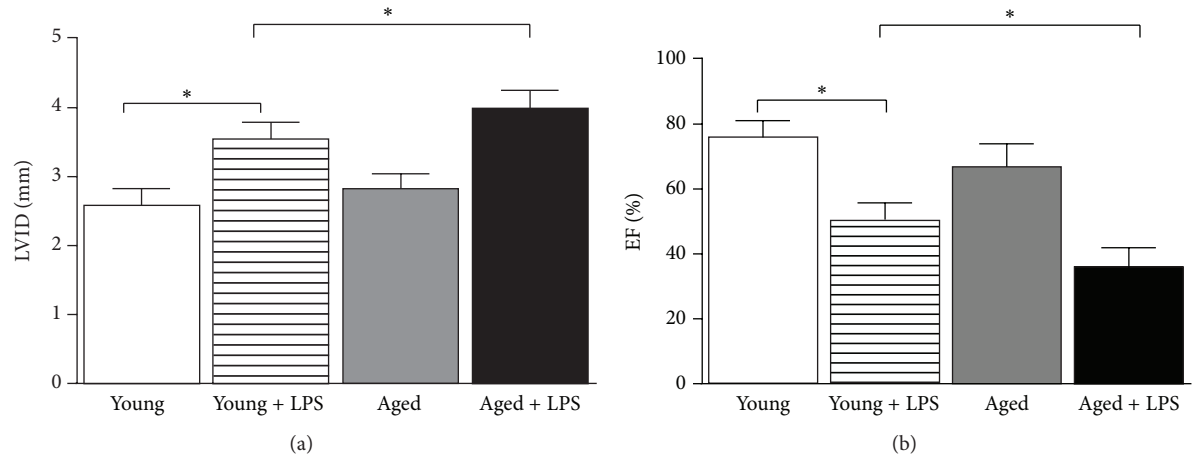

Figure 3: Effect of LPS in young and aged mice on cardiac function. Cardiac function parameters left ventricular internal dimension (LVID, (a)) and ejection fraction (EF, (b)) were measured by echocardiography. LVID was significantly increased whereas EF was decreased in LPS treated mice. The difference of LPS induced cardiac dysfunction in aged mice was significantly higher than young ones. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

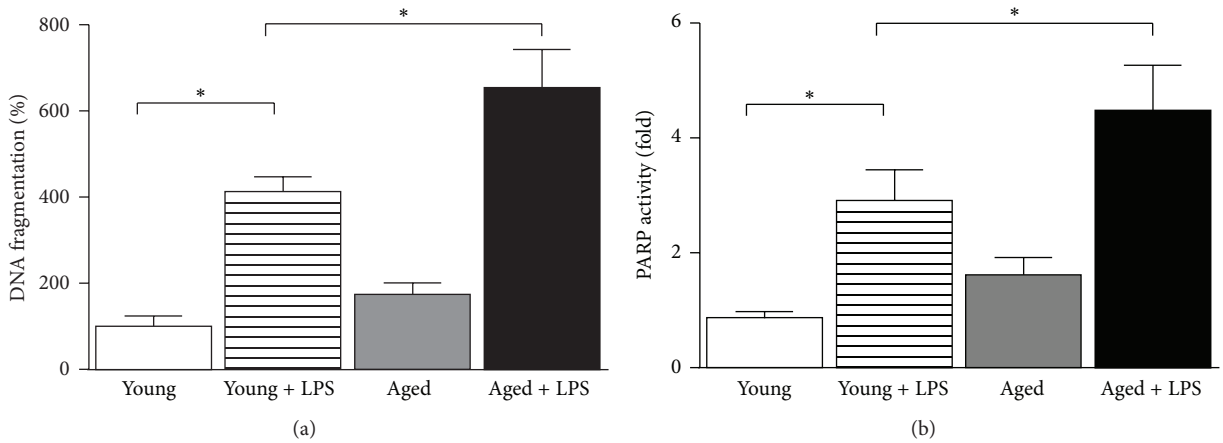

Figure 4: Effect of LPS in young and aged mice on cardiac cell death. Cardiac cell death markers DNA fragmentation (a) by quantitative ELISA and PARP activity assay (b). Both markers were significantly increased in LPS treated mice. Increase of LPS induced cardiac damage in aged mice was significantly higher than young ones. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

3.2. Cardiac Cell Death Increases with Aging in LPS Induced Cardiac Dysfunction. As we and others have shown earlier that cardiac cell death leads to cardiac dysfunction, we compared cell death between young and aged mice upon LPS administration [14]. LPS induced 412% and 654% increase of DNA fragmentation in young and aged mice, respectively (Figure 4). We also examine PARP activity, which also is marker for cell death. PARP activity was increased 2.9-fold in young mice upon LPS administration whereas it increased 4.5-fold in aged mice.

One of the key factors of cardiac dysfunction is cardiomyocytes cell death [28]. Two types of cell death (apoptosis and regulated necrosis) were associated with cardiac dysfunction [28]. We used two distinctive markers such as DNA fragmentation and PARP activity as representation of these types of cell death. Cardiac dysfunction was correlated with cell death data in both young and aged mice. The endotoxin induced cardiomyocytes cell death is primarily apoptotic in nature [14]. However, the process of endotoxin induced cell death is much more complex involving apoptosis, necrosis, pyroptosis, and oncosis [29]. The level of endotoxin and its timing play critical role in determining the prevalence of one pathway over the other.

3.3. Increase of Inflammatory Pathway with Aging in LPS Induced Cardiac Dysfunction. The expression of four proinflammatory cytokines in heart was examined by real-time PCR. As shown in Figure 5, LPS induced all four cytokines in both young and aged mice. The effect of LPS on aged mice was more significant than younger mice. We also observed that basal expression of inflammatory cytokines in aged heart is higher compared to young heart. TNFα mRNA increased to 4.2-, 1.7-, and 8.3-fold in the hearts of young with LPS,

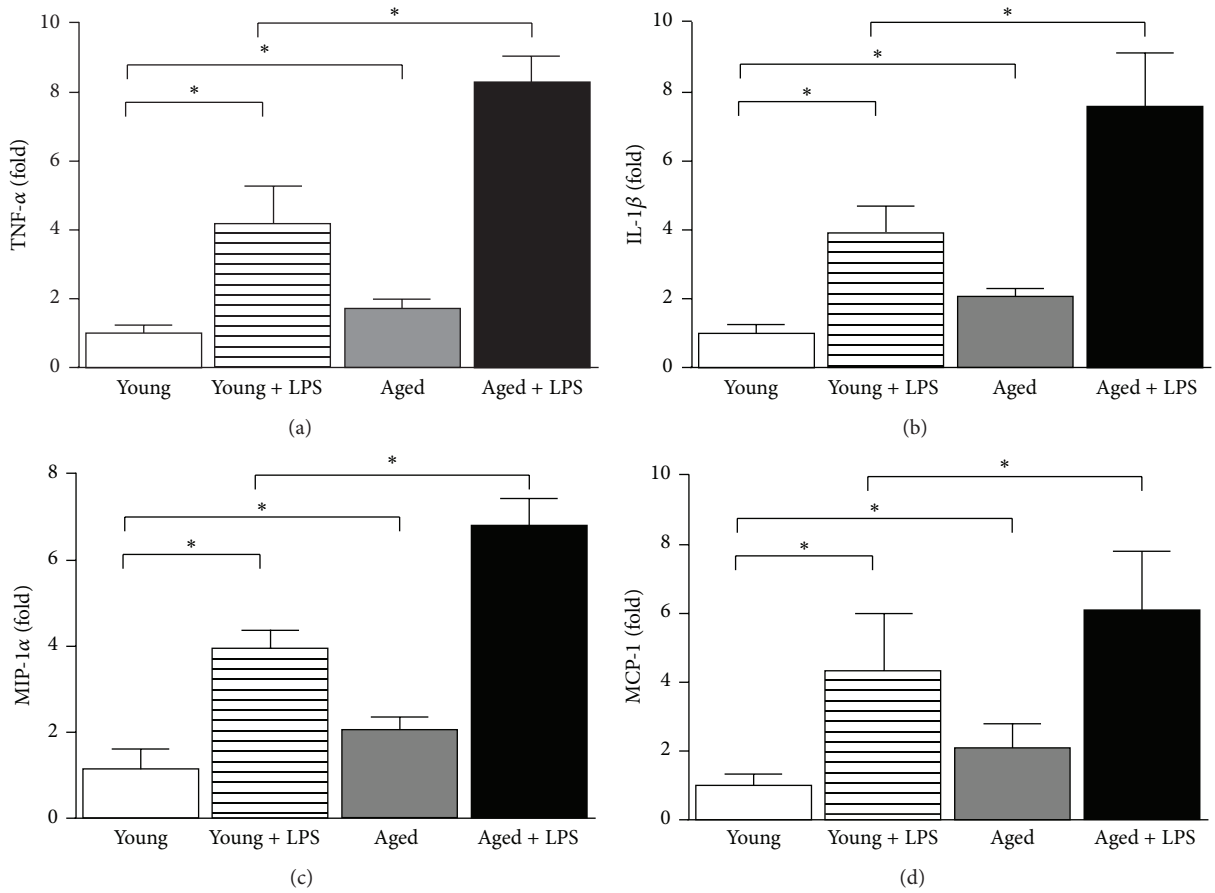

Figure 5: Effect of LPS in young and aged mice on cardiac inflammation. Cardiac inflammation markers TNF-α (a), IL-1β (b), MIP-1α (c), and MCP (d) were measured by real-time PCR. All markers were significantly increased in LPS treated mice. Increase of LPS induced cardiac inflammation in aged mice was significantly higher than young ones. Values represented as means ± SD; $^*P < 0.05$ and $n = 6$/group.

aged, and aged with LPS group, respectively (Figure 5(a)). IL-1β mRNA increased to 3.9-, 2.0- and 7.6-fold in the hearts of young with LPS, aged, and aged with LPS group, respectively (Figure 5(b)). MIP-1α mRNA increased to 3.8-, 2.1-, and 6.8-fold in the hearts of young with LPS, aged, and aged with LPS group, respectively (Figure 5(c)). MCP mRNA increased to 4.34-, 2.09-, and 6.1-fold in the hearts of young with LPS, aged, and aged with LPS group, respectively (Figure 5(d)).

Inflammatory pathways are responsible for cell death associated cardiac dysfunction in aging heart [30, 31]. Our study also demonstrated that a significant increase of inflammation is correlated with cell death in aging heart. Aging association with chronic inflammation in heart has been reported earlier in addition to low level systemic inflammation [11]. Our data is consistent with earlier publications. In cardiovascular aging, inflammation is associated with other cardiovascular disease and induced by many stimulus [32]. One of such key pathways is TNFα signaling and associated NF-κB (nuclear factor kappa-B) activation [33]. Inflammation contributes to pathogenesis in a range of cardiac conditions but interventional approach with anti-inflammatory is not promising [32]. Excessive inflammation is a major cause of heart failure in sepsis and mitochondria play significant role [34]. In response to endotoxin, mitochondria produce large bust of reactive oxygen species (ROS), which target protein, lipid, and DNA in the cell [14]. Heart is specifically prone to mitochondrial stress as mitochondria consist of one-third volume and age dependent mitochondrial damage is mainly caused by its ROS [34].

3.4. Increase of Oxidative/Nitrative Stress with Aging in LPS Induced Cardiac Dysfunction. LPS administration led to increased reactive oxygen species (ROS) production in both young and aged mice as evidenced by protein nitration and carbonyl content. Protein nitration or protein nitrotyrosine nitration is well-known marker for ROS production [35]. Histological staining of paraffin embedded section of hearts demonstrated distinct pattern in both LPS treated young and aged mice (Figure 6(a)). The level of staining in aged mice is significantly stronger than young mice upon LPS administration. Quantitative determination of same protein nitrotyrosine marker by ELISA demonstrated that LPS induced more protein nitrotyrosine nitration in old mice compared to young mice and there is basal level increase of protein nitrotyrosine in aged mice.

The major contributors of oxidative/nitrative stress in heart are the reactive oxygen species (ROS) family of molecules including superoxide anion, lipid radicals, nitric oxide,

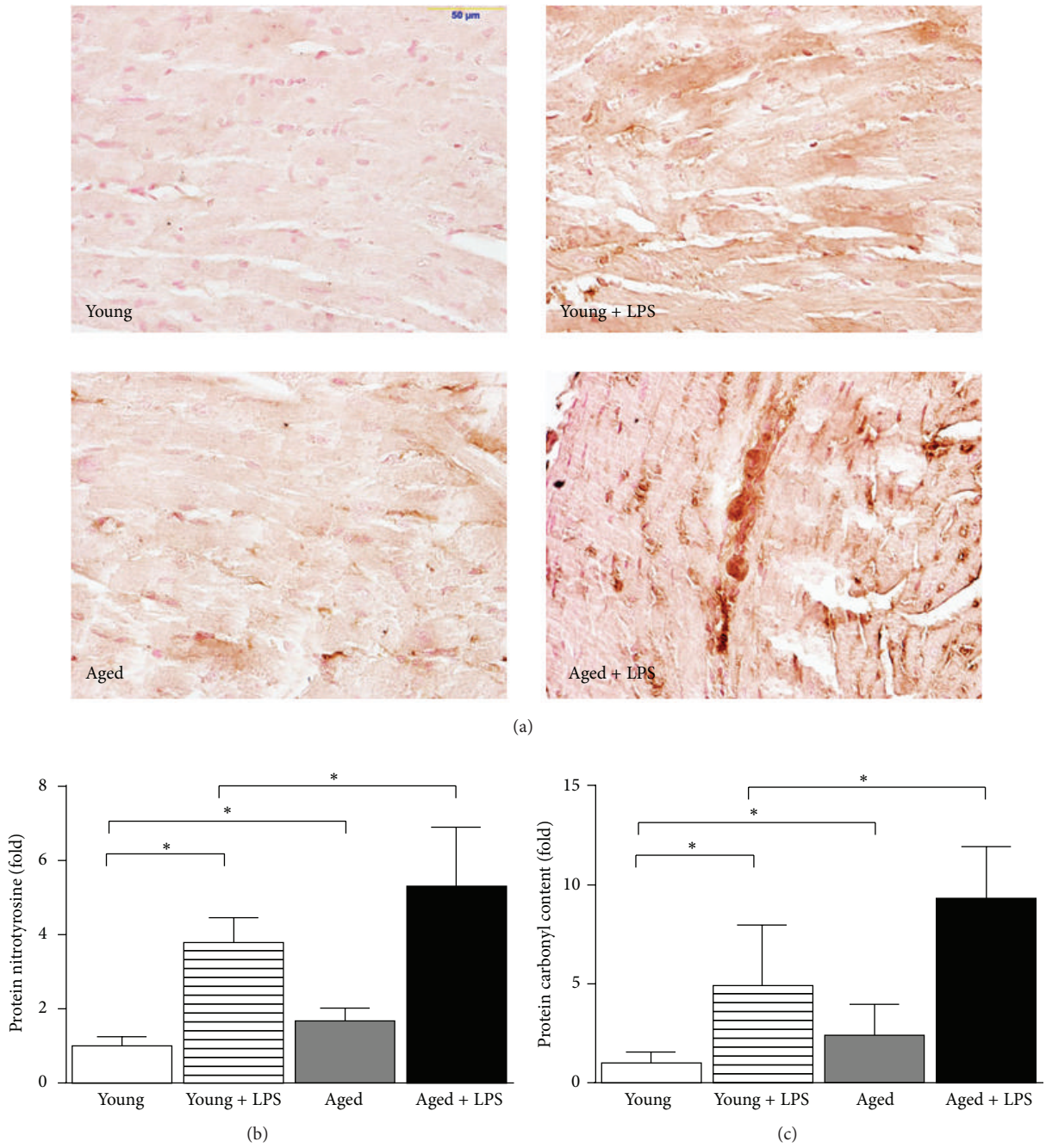

FIGURE 6: Effect of LPS in young and aged mice on cardiac oxidative damage. Cardiac oxidative markers protein nitration (a) by histology and protein nitrotyrosine (b) and carbonyl (c) content measured by quantitative ELISA. All markers were significantly increased in LPS treated mice. Increase of LPS induced cardiac inflammation in aged mice was significantly higher than young ones. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

hydrogen peroxide, and peroxynitrite [36]. The method of detection of ROS is technically difficult in live animals; therefore most studies were focused on oxidative footprints such as protein nitrotyrosine nitration and protein carbonyl modification [37]. Here, we demonstrated by two independent methods that LPS induced oxidative stress in heart and such effect was significantly enhanced in aging.

3.5. Inefficient Autophagy in Aging Correlates to Increase of LPS Induced Cardiac Cell Death. Autophagy maintains cell homeostasis in heart under starvation, remodeling, and aging [38]. We have observed that LPS administration led to induction of autophagy (using LC3II marker) in both young and aged mice (Figure 7). However, level of LCII is much less in aged mice compared to young mice upon LPS administration.

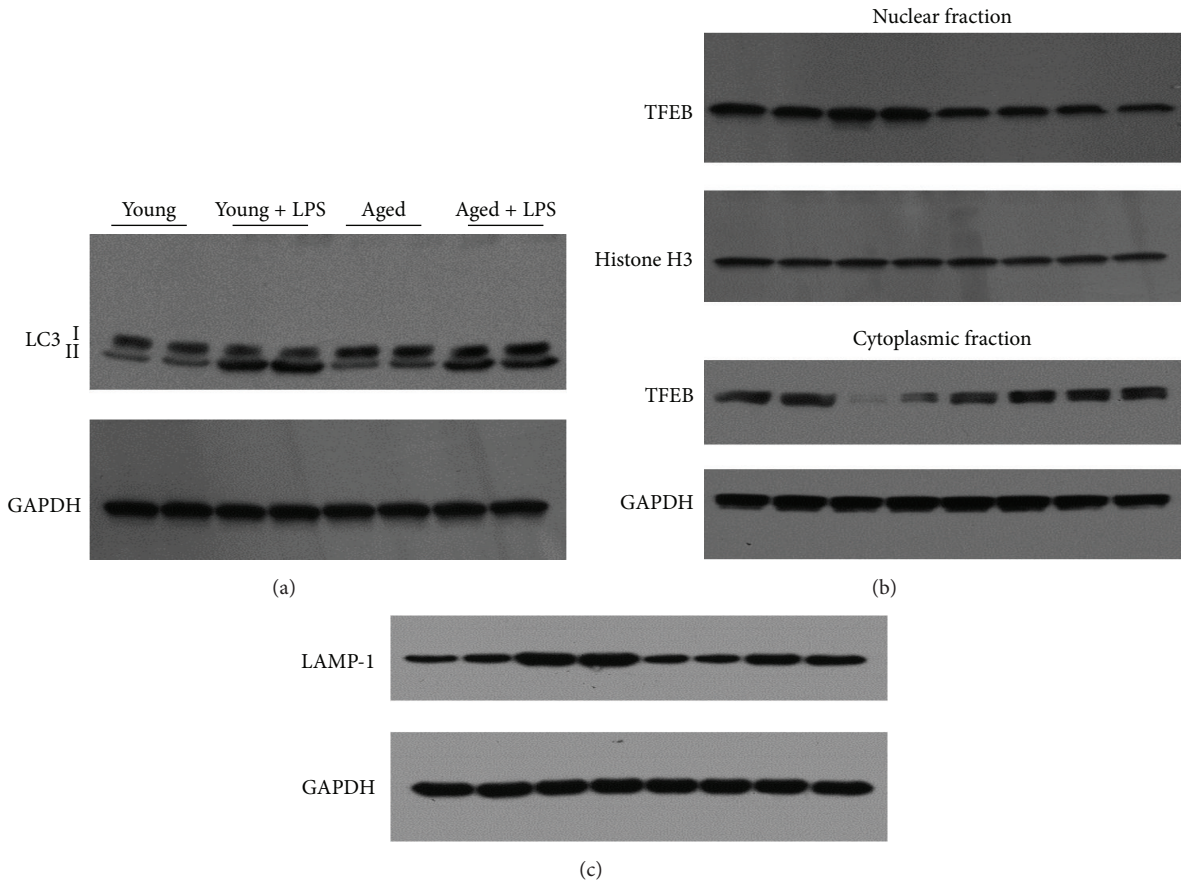

Figure 7: Effect of LPS in young and aged mice on cardiac autophagy. Markers of autophagy LC3 with loading control GAPDH were determined by western blot analyses (a). Both nuclear and cytoplasmic fractions of TFEB, along with nuclear specific marker Histone H3 and cytoplasmic marker GAPDH, were determined by western blot experiments (b). LAMP-1, downstream effector of TFEB and involved in lysosomal biogenesis, was also analyzed (c). Significant autophagy response in LPS treated mice was observed. However, LPS induced cardiac autophagic response in aged mice was significantly lower than young ones.

These results demonstrated that autophagy machinery might be inefficient in heart of aging mice compared to young ones. The transcription factor EB (TFEB), a regulator of autophagy and its nuclear localization, leads to positive regulation of CLEAR (Coordinated Lysosomal Expression and Regulation) network [39]. We also examined whether TFEB has any role in LPS induced myocardial toxicity particularly in aging. We found that LPS induced significant nuclear localization of TFEB in young mice whereas such increase is clearly absent in aged mice. In cytoplasmic fraction a corresponding decrease in TFEB content was observed in LPS treated young mice and such distinct pattern was absent in aged mice. We also examined the downstream effector of TFEB and key protein in lysosomal biogenesis, LAMP-1, and it was induced significantly in young mice when administered with LPS (Figure 7(c)). However, there was little increase in LAMP-1 in aged mice when administered with LPS and the changes were lower than that of young ones. We further analyzed Map1lc3 and Vps11, well-known targets of TFEB, by real-time PCR (Figure 8). LPS induced those gene expressions in young mice whereas such induction was absent in old mice.

Oxidative stress in cardiomyocytes has been reported to serve as important stimuli of autophagy in response to cell stress [40]. LPS induced oxidative stress might lead to autophagy. Autophagy is a protection and remodeling mechanism to protect cardiomyocytes against cell stress [41]. We have observed that autophagy is significantly impaired in aged mice with LPS compared to young ones whereas cardiomyocytes cell death and cardiac dysfunction are highly pronounced in aging heart with LPS. Our data suggested that inefficient autophagy machinery in aging is a key determining factor in protection against LPS induced myocardial toxicity. Autophagy also plays cardioprotective role in other cardiac injury models [42, 43]. To understand further the autophagic machinery, we examined the role of TFEB in the process. TFEB is master regulator of CLEAR gene network consisting of over 400 genes, which encoded proteins for lysosomal biogenesis and function. These are important part of autophagic machinery [10]. We also observed TFEB mediated regulation of LAMP-1 protein and gene expression of Map1lc3 and Vps11. All these markers are also key members of CLEAR network. In response to cellular stress, TFEB translocates to nucleus

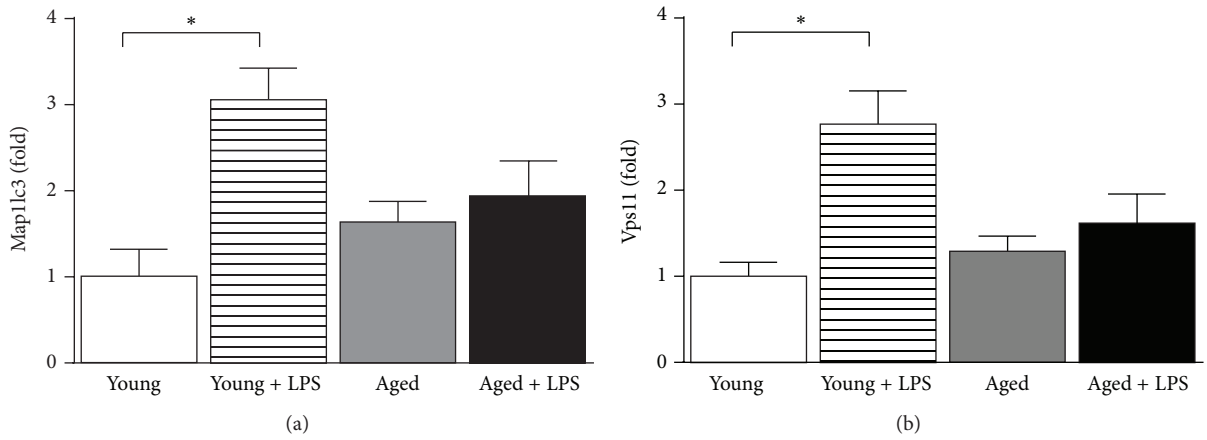

FIGURE 8: Effect of LPS in young and aged mice in downstream effector genes of TFEB. TFEB regulated genes Map1lc3 and Vps11 were examined at mRNA level by real-time PCR. LPS induced MRNA level in both genes in young mice whereas such pattern is absent in old mice. Values represented as means ± SD; $^{*}P < 0.05$ and $n = 6$/group.

and activates lysosomal biogenesis genes [44, 45]. Here we observed that, despite cellular stress with LPS, TFEB did not translocate to nucleus in aged mice, thus preventing the network for switching to autophagy machinery.

Autophagy is important for repair of cellular and also involved in self-killing of irreversibly injured cells. Programmed cell death is also classified into PCD1 (apoptosis) and PCD2 (autophagic cell death) [46]. Both pathways have overlapping mechanisms and are involved in cardiomyocytes cell death [47].

Autophagy leads to cardioprotection by removing misfolded protein or dysfunctional mitochondria or maintaining energy homeostasis [41]. In sepsis, a burst of ROS leads to oxidative damage which leads to inflammation and cell death followed by acute cardiac injury. Oxidative damage also leads to induction of autophagy machinery and autophagy leads to protection against oxidative damage by removing damaged mitochondria (Figure 9). In aging, a higher basal level of oxidative stress and inflammation exists and LPS further exacerbated those stimuli which lead to profound cell death, chronic injury, and associated cardiac dysfunction (Figure 9).

4. Conclusion

We demonstrated for the first time that TFEB mediated autophagy played critical role in age dependent LPS induced myocardial toxicity which led to cardiac dysfunction. In aging heart, the translocation of TFEB to nucleus was greatly impaired and the autophagy machinery was impaired. Due to impaired autophagy, the basal level of inflammation and oxidative stress is higher in aging heart, which was further escalated in response to LPS.

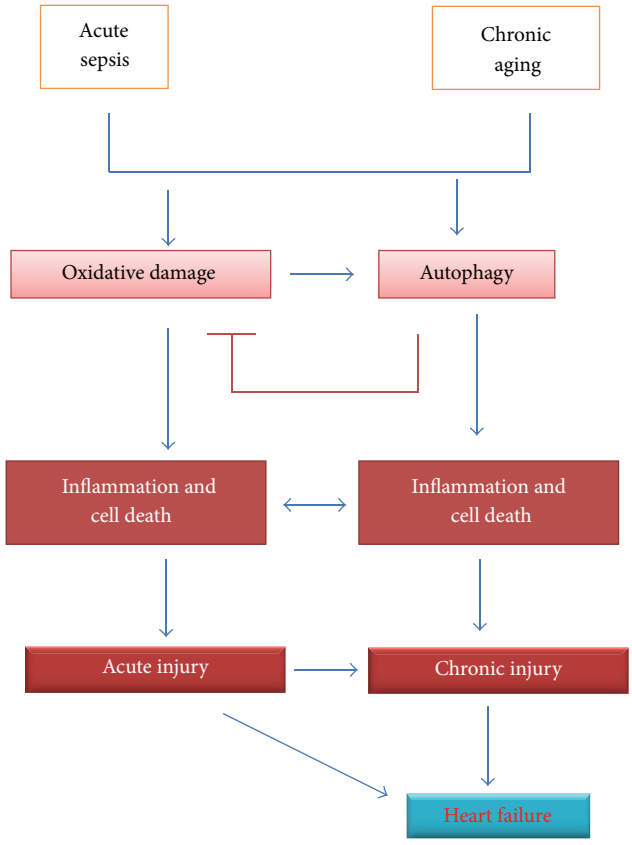

FIGURE 9: Schematic diagram of LPS induced heart failure in young and aged mice. Aging led to oxidative damage which may induce autophagy, where autophagic response leads to less oxidative damage. Oxidative damage leads to inflammation and cell death and is more pronounced in aging. This leads to chronic injury and associated cardiac dysfunction or heart failure.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Fang Li and Fangfang Lang contributed equally to this work.

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References

[1] R. Umberger, B. Callen, and M. L. Brown, "Severe sepsis in older adults," *Critical Care Nursing Quarterly*, vol. 38, no. 3, pp. 259–270, 2015.

[2] J. Carbajal-Guerrero, A. Cayuela-Domínguez, E. Fernández-García et al., "Epidemiology and long-term outcome of sepsis in elderly patients," *Medicina Intensiva*, vol. 38, no. 1, pp. 21–32, 2014.

[3] P. Nasa, D. Juneja, and O. Singh, "Severe sepsis and septic shock in the elderly: an overview," *World Journal of Critical Care Medicine*, vol. 1, no. 1, pp. 23–30, 2012.

[4] H. Palomba, T. D. Corrêa, E. Silva, A. Pardini, and M. S. de Assuncao, "Comparative analysis of survival between elderly and non-elderly severe sepsis and septic shock resuscitated patients," *Einstein*, vol. 13, no. 3, pp. 357–363, 2015.

[5] D. Heymann, "Autophagy: a protective mechanism in response to stress and inflammation," *Current Opinion in Investigational Drugs*, vol. 7, no. 5, pp. 443–450, 2006.

[6] D. C. Rubinsztein, G. Mariño, and G. Kroemer, "Autophagy and aging," *Cell*, vol. 146, no. 5, pp. 682–695, 2011.

[7] L. Knuppertz and H. D. Osiewacz, "Orchestrating the network of molecular pathways affecting aging: role of nonselective autophagy and mitophagy," *Mechanisms of Ageing and Development*, vol. 153, pp. 30–40, 2016.

[8] L. J. Leon and Å. B. Gustafsson, "Staying young at heart: autophagy and adaptation to cardiac aging," *Journal of Molecular and Cellular Cardiology*, 2015.

[9] C. Settembre and A. Ballabio, "TFEB regulates autophagy: an integrated coordination of cellular degradation and recycling processes," *Autophagy*, vol. 7, no. 11, pp. 1379–1381, 2011.

[10] M. Palmieri, S. Impey, H. Kang et al., "Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways," *Human Molecular Genetics*, vol. 20, no. 19, Article ID ddr306, pp. 3852–3866, 2011.

[11] J. Wu, S. Xia, B. Kalionis, W. Wan, and T. Sun, "The role of oxidative stress and inflammation in cardiovascular aging," *BioMed Research International*, vol. 2014, Article ID 615312, 13 pages, 2014.

[12] D.-F. Dai and P. S. Rabinovitch, "Cardiac aging in mice and humans: the role of mitochondrial oxidative stress," *Trends in Cardiovascular Medicine*, vol. 19, no. 7, pp. 213–220, 2009.

[13] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[14] E. Hao, F. Lang, Y. Chen et al., "Resveratrol alleviates endotoxin-induced myocardial toxicity via the Nrf2 transcription factor," *PLoS ONE*, vol. 8, no. 7, Article ID e69452, 2013.

[15] E. Hao, P. Mukhopadhyay, Z. Cao et al., "Cannabidiol protects against doxorubicin-induced cardiomyopathy by modulating mitochondrial function and biogenesis," *Molecular Medicine*, vol. 21, pp. 38–45, 2015.

[16] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[17] F. Lang, Z. Qin, F. Li, H. Zhang, Z. Fang, and E. Hao, "Apoptotic cell death induced by resveratrol is partially mediated by the autophagy pathway in human ovarian cancer cells," *PLoS ONE*, vol. 10, no. 6, Article ID e0129196, 2015.

[18] M. K. ElZarrad, P. Mukhopadhyay, N. Mohan et al., "Trastuzumab alters the expression of genes essential for cardiac function and induces ultrastructural changes of cardiomyocytes in mice," *PLoS ONE*, vol. 8, no. 11, Article ID e79543, 2013.

[19] S. Bátkai, M. Rajesh, P. Mukhopadhyay et al., "Decreased age-related cardiac dysfunction, myocardial nitrative stress, inflammatory gene expression, and apoptosis in mice lacking fatty acid amide hydrolase," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 2, pp. H909–H918, 2007.

[20] K. Boengler, A. Buechert, Y. Heinen et al., "Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice," *Circulation Research*, vol. 102, no. 1, pp. 131–135, 2008.

[21] R. M. Grocott-Mason and A. M. Shah, "Cardiac dysfunction in sepsis: new theories and clinical implications," *Intensive Care Medicine*, vol. 24, no. 4, pp. 286–295, 1998.

[22] A. F. Suffredini, R. E. Fromm, M. M. Parker et al., "The cardiovascular response of normal humans to the administration of endotoxin," *The New England Journal of Medicine*, vol. 321, no. 5, pp. 280–287, 1989.

[23] S.-Y. Li, M. Du, E. K. Dolence et al., "Aging induces cardiac diastolic dysfunction, oxidative stress, accumulation of advanced glycation endproducts and protein modification," *Aging Cell*, vol. 4, no. 2, pp. 57–64, 2005.

[24] J. M. Capasso, T. Palackal, G. Olivetti, and P. Anversa, "Severe myocardial dysfunction induced by ventricular remodeling in aging rat hearts," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 259, no. 4, pp. H1086–H1096, 1990.

[25] J. L. Fleg, "Normative aging changes in cardiovascular structure and function," *The American Journal of Geriatric Cardiology*, vol. 5, pp. 7–15, 1996.

[26] E. G. Lakatta, "Changes in cardiovascular function with aging," *European Heart Journal*, vol. 11, pp. 22–29, 1990.

[27] M. D. Cheitlin, "Cardiovascular physiology—changes with aging," *The American Journal of Geriatric Cardiology*, vol. 12, no. 1, pp. 9–13, 2003.

[28] R. S. Whelan, V. Kaplinskiy, and R. N. Kitsis, "Cell death in the pathogenesis of heart disease: mechanisms and significance," *Annual Review of Physiology*, vol. 72, pp. 19–44, 2009.

[29] S. L. Fink and B. T. Cookson, "Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells," *Infection and Immunity*, vol. 73, no. 4, pp. 1907–1916, 2005.

[30] R. S. Vasan, L. M. Sullivan, R. Roubenoff et al., "Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: the Framingham Heart Study," *Circulation*, vol. 107, no. 11, pp. 1486–1491, 2003.

[31] J. Baynes and D. B. Murray, "Cardiac and renal function are progressively impaired with aging in Zucker diabetic fatty type II diabetic rats," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 328–334, 2009.

[32] M. Coggins and A. Rosenzweig, "The fire within: cardiac inflammatory signaling in health and disease," *Circulation Research*, vol. 110, no. 1, pp. 116–125, 2012.

[33] J. W. Gordon, J. A. Shaw, and L. A. Kirshenbaum, "Multiple facets of NF-κB in the heart: to be or not to NF-κB," *Circulation Research*, vol. 108, no. 9, pp. 1122–1132, 2011.

[34] Q. S. Zang, S. E. Wolf, and J. P. Minei, "Sepsis-induced cardiac mitochondrial damage and potential therapeutic interventions in the elderly," *Aging and Disease*, vol. 5, no. 2, pp. 137–149, 2014.

[35] R. Radi, "Nitric oxide, oxidants, and protein tyrosine nitration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 12, pp. 4003–4008, 2004.

[36] M. M. Elahi, Y. X. Kong, and B. M. Matata, "Oxidative stress as a mediator of cardiovascular disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 259–269, 2009.

[37] P. Mukhopadhyay, M. Rajesh, S. Bátkai et al., "Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 5, pp. H1466–H1483, 2009.

[38] K. Nishida, M. Taneike, and K. Otsu, "The role of autophagic degradation in the heart," *Journal of Molecular and Cellular Cardiology*, vol. 78, pp. 73–79, 2015.

[39] C. Settembre, C. Di Malta, V. A. Polito et al., "TFEB links autophagy to lysosomal biogenesis," *Science*, vol. 332, no. 6036, pp. 1429–1433, 2011.

[40] E. E. Essick and F. Sam, "Oxidative stress and autophagy in cardiac disease, neurological disorders, aging and cancer," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 3, pp. 168–177, 2010.

[41] Å. B. Gustafsson and R. A. Gottlieb, "Recycle or die: the role of autophagy in cardioprotection," *Journal of Molecular and Cellular Cardiology*, vol. 44, no. 4, pp. 654–661, 2008.

[42] C. Huang, S. Yitzhaki, C. N. Perry et al., "Autophagy induced by ischemic preconditioning is essential for cardioprotection," *Journal of Cardiovascular Translational Research*, vol. 3, no. 4, pp. 365–373, 2010.

[43] A. Nakai, O. Yamaguchi, T. Takeda et al., "The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress," *Nature Medicine*, vol. 13, no. 5, pp. 619–624, 2007.

[44] E. J. Feeney, C. Spampanato, R. Puertollano, A. Ballabio, G. Parenti, and N. Raben, "What else is in store for autophagy? Exocytosis of autolysosomes as a mechanism of TFEB-mediated cellular clearance in Pompe disease," *Autophagy*, vol. 9, no. 7, pp. 1117–1118, 2013.

[45] M. Decressac, B. Mattsson, P. Weikop, M. Lundblad, J. Jakobsson, and A. Björklund, "TFEB-mediated autophagy rescues midbrain dopamine neurons from α-synuclein toxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 19, pp. E1817–E1826, 2013.

[46] A. Terman and U. T. Brunk, "Autophagy in cardiac myocyte homeostasis, aging, and pathology," *Cardiovascular Research*, vol. 68, no. 3, pp. 355–365, 2005.

[47] S. Kostin, L. Pool, A. Elsässer et al., "Myocytes die by multiple mechanisms in failing human hearts," *Circulation Research*, vol. 92, no. 7, pp. 715–724, 2003.

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Research Article

Neuroprotective Effects of Isosteviol Sodium Injection on Acute Focal Cerebral Ischemia in Rats

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Previous report has indicated that isosteviol has neuroprotective effects. However, isosteviol was administered preventively before ischemia and the inclusion criteria were limited. In the present study, a more soluble and injectable form of isosteviol sodium (STVNA) was administered intravenously hours after transient or permanent middle cerebral artery occlusion (tMCAO or pMCAO) to investigate its neuroprotective effects in rats. The rats were assessed for neurobehavioral deficits 24 hours after ischemia and sacrificed for infarct volume quantification and histology evaluation. STVNA 10 $mg \cdot kg^{-1}$ can significantly reduce the infarct volumes compared with vehicle in animals subjected to tMCAO and is twice as potent as previously reported. Additionally, the therapeutic window study showed that STVNA could reduce the infarct volume compared with the vehicle group when administered 4 hours after reperfusion. A similar effect was also observed in animals treated 4 hours after pMCAO. Assessment of neurobehavioral deficits after 24 hours showed that STVNA treatment significantly reduced neurobehavioral impairments. The number of restored NeuN-labeled neurons was increased and the number of TUNEL positive cells was reduced in animals that received STVNA treatment compared with vehicle group. All of these findings suggest that STVNA might provide therapeutic benefits against cerebral ischemia-induced injury.

1. Introduction

Stroke is the second leading cause of death worldwide in people aged over 60 years [1]. Ischemic stroke accounts for 70% of all strokes [2]. Ischemic stroke is the result of a transient or permanent fall in cerebral blood flow, which is restricted to the territory of a major brain artery [3]. Though timely recanalization of the occluded vessel is an effective treatment, it can result in brain injuries, such as cerebral edema, parenchymal hemorrhage, and neuronal death. Moreover, there are no FDA approved neuroprotectants to treat ischemic stroke.

Isosteviol sodium, the sodium salt of isosteviol, is a beyerane diterpene obtained by the acid hydrolysis of stevioside [4, 5]. Stevioside, a major component of the *Stevia rebaudiana* leaf, is used as a conventional noncaloric sweetener and has been used in traditional medicine for several hundred years [6, 7]. Several studies indicate that isosteviol possesses a variety of biological activities including antihypertension [8], antihyperglycemia [9], antioxidant [10], anti-inflammatory [11], and antitumor effects [12] and relieves ischemia-reperfusion injury in the rat brain [13, 14].

In previous studies, isosteviol was administered before ischemia and these studies were not designed according to the suggestions of the Stroke Therapy Academic Industry Roundtable [15]. Isosteviol is also only slightly soluble in water and this low solubility affects its bioavailability. Thus, it is difficult to use isosteviol as an aqueous injection, which limits its applications in emergency treatment. STVNA is an injectable formulation of the isosteviol sodium salt dissolved in a mixture containing water and organic solvents and has increased bioavailability compared with isosteviol alone. Therefore, the present study was designed to demonstrate the possible therapeutic effects of STVNA in focal acute ischemia/reperfusion (IR) injury in rats.

2. Materials and Methods

2.1. Animals. Adult male Sprague-Dawley rats weighing 250–280 g were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The rats were housed four per cage in a room under controlled temperature, humidity, and 12-hour light/12-hour dark cycles with free access to food and water. The rats were allowed to acclimatize for at least three days before the experiment. All of the experimental procedures complied with current national and international laws and recommendations, and the study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

2.2. Middle Cerebral Artery Occlusion Model. The middle cerebral artery occlusion (MCAO) was performed through an intraluminal suture as previously described [16]. Occlusion of the right MCA was induced either transiently for 2 hours (tMCAO) followed by a reperfusion period of 22 hours or permanently for 24 hours (pMCAO). Briefly, anesthesia was induced in rats with 3% isoflurane and was maintained with 2% isoflurane in a gas mixture of 5% CO_2 and 95% O_2. The temperature was maintained constant at $(37.0 \pm 0.5)^{\circ}C$ by a thermostatically controlled heating blanket throughout the surgical procedure. The right common carotid artery and the internal carotid artery were exposed under an operative microscope through a neck midline incision. The pterygopalatine artery was ligated close to its origin. A 3-0 nylon filament suture coated at the tip with 5 mm of silicone was inserted into the common carotid artery and up to the internal carotid artery for a distance of 19 to 21 mm from the common carotid artery bifurcation. After 2 hours of ischemia, the nylon suture was removed to allow reperfusion period for 22 hours in the case of the tMCAO or the occlusion was maintained permanently for 24 hours in the case of the pMCAO. The incision was closed, and the rats were returned to the cages after they awakened from anesthesia. The middle cerebral artery blood flow was monitored in real time by laser-Doppler flowmetry (PeriFlux System 5000, Perimed AB, Stockholm, Sweden) 2.5 mm lateral and 2.0 mm posterior to the bregma during preischemia, postischemia, and reperfusion periods to verify the success of the cerebral ischemia/reperfusion procedure. Physiological parameters including heart rate, breathing rate, and SpO_2 (Pulse Oximeter Oxygen Saturation) were monitored during the surgical procedure by a MouseOx Plus Pulse Oximeter (STARR Life Sciences Corp., Oakmont, USA). Sham animals were subjected to the same surgical procedures except that the suture was not advanced into the middle cerebral artery.

2.3. Drugs and Treatment Schedule. STVNA was provided by the Chemical Development Laboratories of Key Biological Pharmaceutical Company (Dongguan, China). Edaravone was purchased from Simcere Pharmaceutical Co., Ltd. (Nanjing, China).

In all of the experiments, the drugs were administered by an i.v. drip at a rate of 0.25 mL/min for 1 min (loading infusion) followed by a rate of 0.25 mL/h for 3 hours (maintaining infusion) with a micro-perfusion pump (LSP04-1A, Longer Precision Pump Co., Ltd., Baoding, China). First, the femoral vein was carefully exposed and two silk ligatures were placed around the vein by blunt dissection. A small incision was then made in the femoral vein and a cannula (PE-10) was inserted to a depth of 3.0 cm. The cannula was then securely held in place by tying two ligatures around the cannulated femoral vein. A hard tube was used to sheathe the vein cannula to prevent the animal from biting it. The animal was limited into a Plexiglas box for 3 hours, and when the infusion was finished, they were placed back in the cages.

The STVNA dose-response relationship was investigated in the transient ischemia model (tMCAO). STVNA (1, 5, 10, and 20 mg/kg, $N = 11$ per group), edaravone (3 mg/kg, $N = 11$), and vehicle ($N = 11$) were administered to the respective groups of rats subjected to tMCAO starting 1 hour before reperfusion. Solvent was administered to sham animals 1 hour before reperfusion ($N = 11$). The STVNA therapeutic window was investigated at a single dose of 10 mg/kg in both transient (tMCAO) and permanent (pMCAO) ischemia models. In the tMCAO experiments, STVNA was administered at 0, 2, 4, or 6 hours after reperfusion ($N = 8$ per group). Vehicle was delivered 2 hours after MCAO ($N = 8$). In the pMCAO experiments, STVNA was administered at 0, 2, 4, or 6 hours after the middle cerebral artery occlusion ($N = 8$ per group). Vehicle was delivered 2 hours after MCAO ($N = 8$). Moreover, in the measurement of brain edema experiments, STVNA (10 mg/kg) was administered 1 hour before reperfusion ($N = 6$). Sham and vehicle were delivered 1 hour after MCAO ($N = 6$ per group). Rats were randomly assigned to each experimental group before MCAO.

2.4. Evaluation of Neurological Deficits. Twenty-four hours after MCAO, rats were tested for neurological deficits according to Bederson et al.'s test [17]. The scores were assigned using the following scale: 0, no neurological deficits; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling to the right; and 4, absence of spontaneous walking and depressed levels of consciousness. The investigator applying the behavior test did not know the identity of the experimental treatment groups.

2.5. Measurement of Cerebral Infarction. Rats were euthanized by intraperitoneal injection of 100 mg/kg sodium pentobarbital 24 hours after MCAO. Then, the brain tissue was removed and sliced into 2.0 mm thick sections using a brain matrix (J&K Seiko Electronic, Dongguan, China). The brain slices were incubated for 10 min at $37^{\circ}C$ in 2% triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Saint Louis, USA) dissolved in saline solution. The TTC-stained coronal sections from each individual were scanned with a color flatbed scanner (Scanjet G3100, Hewlett-Packard, Shanghai, China). Infarction volume was measured using the ImageJ v1.48 software (US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/) and calculated as previously described [18].

2.6. Measurement of Edema. Twenty-four hours after stroke, cerebral edema was determined by comparing the wet-to-dry tissue weight ratios as previously described [19]. Briefly, the

brain was quickly removed after the animal was sacrificed. Then, the brain was blotted to remove residual moisture and dissected through the interhemispheric fissure into right and left hemispheres. Samples were immediately weighed to obtain the wet weight. The dry weight was obtained after drying for 2 days at 120°C. The water content of both hemispheres was determined using the following equation: brain water content (%) = (wet weight − dry weight) × 100/wet weight.

2.7. Tissue Preparation and HE Staining. To evaluate the histological damage, three animals in each group from the dose-response study were sacrificed after 22 hours of reperfusion and were perfused with 100 mL of saline solution, followed by 100 mL of freshly prepared 4% (v/v) paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.4). The brain was then removed and fixed in 10% (w/v) paraformaldehyde in 0.01 M PBS for 1 week. Then, a 4 mm coronal section of the brain was cut 2.0 mm anterior and posterior to the bregma and the block was embedded in paraffin. The block was then cut into 5 μm coronal sections that were stained with hematoxylin-eosin (HE) using standard methods.

2.8. Immunohistochemistry Assays. Coronal brain sections (5 μm thick) were randomly selected between 0 and 2.0 mm posterior to the bregma. After deparaffinization and rehydration, the sections were incubated for 10 min at 95–98°C in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. The sections were allowed to cool down to room temperature (RT) and were then incubated for 15 min in H_2O_2 (1% in 0.01 M PBS, v/v) and 0.5 hours in 5% bovine serum albumin (Sigma, St. Louis, USA) blocking solution (5% in 0.01 M PBS, w/v) at RT. Then, the sections were incubated overnight at 4°C in a solution of anti-NeuN antibody (1 : 500, v/v, clone A60, MAB377, Merck Millipore, Darmstadt, Germany). The sections were washed with 0.01 M PBS and then incubated with the Mouse EnVision+ System-HRP (K4006, DAKO, Glostrup, Denmark) for 1 hour at RT. Positive staining was visualized with 3,3′-diaminobenzidine (DAB) using a DAB-enhanced liquid substrate system (DAKO, Glostrup, Denmark). Finally, the sections were counterstained with hematoxylin, dehydrated, mounted, and observed under a microscope (Leica DM4000 BLED, Wetzlar, Germany). The number of immunopositive cells was counted in five randomly selected fields from the peri-infarct area per slice and presented as the number of cells/mm^2. All of the positively stained cells (brown color) were included in the count regardless of their morphology.

2.9. TUNEL Assays. Coronal sections between 0 and 2.0 mm posterior to the bregma were chosen. For the detection of neuronal cell death, in situ nick end labeling was performed using a commercially available kit (ApopTag^5 Plus Peroxidase In Situ Apoptosis Detection Kit S7101, Millipore, Darmstadt, Germany) according to product's specifications. Briefly, after dewaxing in water, tissue sections were washed in PBS (0.01 M, pH 7.4) for 5 min, permeabilized by using proteinase K (20 μg/mL in 0.01 M PBS) for 10 min, and quenched for 5 min in H_2O_2 (3% in 0.01 M PBS, v/v) at RT. Then, the sections were incubated in equilibration buffer for 20 min and labeled overnight at 4°C; the reaction was stopped by the addition of stop buffer. Sections were rinsed with PBS (0.01 M, pH 7.4) and incubated in peroxidase streptavidin conjugate for 45 min. TUNEL positive cells were visualized with a DAB kit. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. TUNEL positive cells were observed at an effective magnification of 400x in the measured areas and photographed using a digital camera (Leica DM4000 BLED, Wetzlar, Germany). The number of TUNEL positive cells throughout the ipsilateral hemisphere was counted in five fields from random peri-infarct areas per slice and presented as the number of cells/mm^2. All of the positively stained cells (brown color) were included in the count regardless of their morphology.

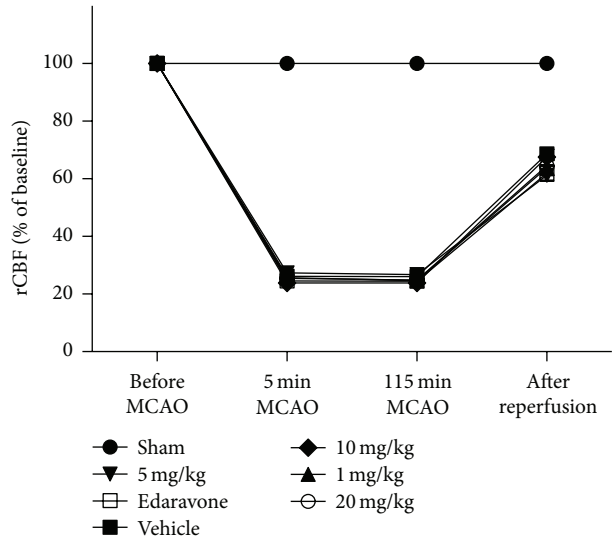

Figure 1: Regional cerebral blood flow (rCBF) before and during middle cerebral artery occlusion (MCAO) and after reperfusion. The rCBF was monitored 5 min before the transient middle cerebral artery occlusion (MCAO) and at 5 and 115 min during MCAO and 5 min after reperfusion. Monitoring of rCBF insured that the MCAO model was successful. Sham = sham group; vehicle = model group. The 1 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg represent different treatment dosage groups. All of the data are shown as the mean ± the SEM (n = 8).

2.10. Statistical Analyses. Data were presented as the mean ± the SEM. Differences between groups were examined using the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. A p value less than 0.05 was often reported as statistically significant.

3. Results

3.1. Cerebral Blood Flow. The regional cerebral blood flow (rCBF) value during ischemia decreased to less than 30% of the baseline value in all of the groups except for the sham group in the dose-response study (Figure 1) and remained constant during the ischemic period in rats. The rCBF of the vehicle group was not significantly different compared with the STVNA-treated groups at the same time points.

Table 1: Physiologic variables in the dose-response study.

	Sham	Vehicle	STVNA, mg/kg				Edaravone
			1	5	10	20	
Before Ischemia (10 minutes)							
BR	63.5 ± 4.5	59.6 ± 5.1	63.5 ± 3.2	64.9 ± 1.4	63.3 ± 2.7	63.7 ± 4.0	62.8 ± 4.2
HR	363 ± 14	376.4 ± 7.6	379.4 ± 8.4	375.7 ± 7.8	380.8 ± 10.5	378.4 ± 11.3	374.2 ± 11
SpO_2	99.3 ± 0.2	99.3 ± 0.2	99.1 ± 0.3	99.1 ± 0.1	99.2 ± 0.2	99.7 ± 0.1	98.1 ± 0.3
T	36.2 ± 0.2	36.2 ± 0.3	36.6 ± 0.2	36.0 ± 0.3	36.4 ± 0.3	36.8 ± 0.2	36.4 ± 0.3
After Ischemia (10 minutes)							
BR	62.7 ± 5.3	69.4 ± 2.3	63 ± 5.1	70.5 ± 3.4	65.4 ± 1.6	68.4 ± 1.5	63.5 ± 2.6
HR	369 ± 10	378.9 ± 4.5	385.3 ± 5.6	376.7 ± 10	378.7 ± 7.8	390.2 ± 7.5	375 ± 8.4
SpO_2	99.3 ± 0.1	99.3 ± 0.1	99.1 ± 0.2	98.9 ± 0.1	98.9 ± 0.2	99.4 ± 0.2	98.8 ± 0.3
T	36.4 ± 0.2	36.7 ± 0.1	36.3 ± 0.3	36.9 ± 0.3	36.5 ± 0.2	37.3 ± 0.1	36.2 ± 0.3
Before reperfusion (10 minutes)							
BR	72.5 ± 2.5	70 ± 1.5	69.5 ± 1.0	71.8 ± 1.5	70.2 ± 2.7	71.3 ± 1.6	67.8 ± 2.3
HR	377 ± 10	390 ± 5.8	400 ± 6.9	405 ± 8.5	400.2 ± 6.6	398.2 ± 9.6	402.5 ± 5.6
SpO_2	99.4 ± 0.1	99.4 ± 0.1	99.5 ± 0.1	99.4 ± 0.1	99.5 ± 0.1	99.0 ± 0.2	99.4 ± 0.1
T	36.2 ± 0.2	37.9 ± 0.2	38.2 ± 0.1	37.9 ± 0.3	38.0 ± 0.1	38.1 ± 0.2	38.3 ± 0.1
After reperfusion (10 minutes)							
BR	73.2 ± 3.2	68.6 ± 2.4	72.6 ± 1.4	72.3 ± 1.9	72.1 ± 3.1	70.5 ± 1.5	69.8 ± 3.2
HR	387 ± 9	401.4 ± 8.4	401.7 ± 2.9	397.1 ± 5.5	396.7 ± 7.8	408.7 ± 4.1	394 ± 10
SpO_2	99.3 ± 0.1	98.3 ± 0.1	98.7 ± 0.4	99.4 ± 0.1	99.5 ± 0.1	99.7 ± 0.1	99.4 ± 0.1
T	37.5 ± 0.1	37.6 ± 0.3	37.9 ± 0.2	37.8 ± 0.1	37.6 ± 0.1	37.0 ± 0.1	37.8 ± 0.2

All of the data were shown as the mean ± the SEM ($n = 8$). BR: breathing rate (breaths/min); HR: heart rate (beats/min); T: temperature (°C); SpO_2: Pulse Oximeter Oxygen Saturation (%).

3.2. Physiologic Variables. All of the animals in this study showed similar values for heart rate, breathing rate, and SpO_2 (Table 1). Although tMCAO caused transient and mild hyperthermia approximately 120 minutes after the occlusion, that effect was not changed by STVNA.

3.3. STVNA Alleviated Brain Edema in the Focal Ischemia Rats. As shown in Figure 2, 2 hours of MCAO and 22 hours of reperfusion resulted in an evident increase in brain water content in the ipsilateral hemisphere compared with the sham-operated rat brain ($p < 0.01$). The brain water in the ipsilateral hemisphere observed in the vehicle group (82.9 ± 0.9%) was significantly reduced to 80.8 ± 1.2% in the STVNA-treated group, while the water content in the contralateral hemisphere did not show significant differences between the two groups. Briefly, STVNA treatment (10 mg/kg) significantly reduced the brain water content compared with the vehicle group.

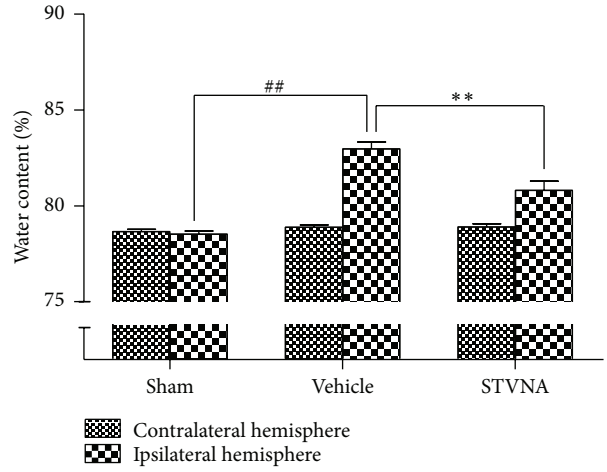

Figure 2: Hemispheric water content. Water content in the ischemic and nonischemic contralateral brain hemispheres studied 24 hours after 2 hours of MCAO in rats with and without STVNA administration. STVNA significantly inhibited edema formation in the ischemic hemisphere. Histograms represent the mean ± the SEM ($n = 6$). $^{\#\#}p < 0.01$ versus sham group; $^{**}p < 0.01$ versus vehicle group by 1-way analysis of variance with Tukey's multiple comparison test.

3.4. Dose-Response Study. Figure 3 shows the dose-response features of the histological and functional amelioration induced by STVNA. Infarct volume was calculated after 24 hours using TTC staining (Figure 3(a)). A significant reduction in infarct size was already observed at 5 mg/kg (30.6 ± 1.6%), and the highest reduction was obtained at 10 mg/kg (22.7 ± 1.5%) compared with the vehicle group (41.3 ± 2.1%), whereas the 1 mg/kg (38.2 ± 1.7%) dose was ineffective and the 20 mg/kg (29.1 ± 1.9%) dose did not contribute to further alleviation of the infarct volume (Figure 3(b)). Edaravone, a reactive oxygen species scavenger approved as a neuroprotectant in Japan for the treatment of cerebral infarction, was used as a positive control. The results indicate

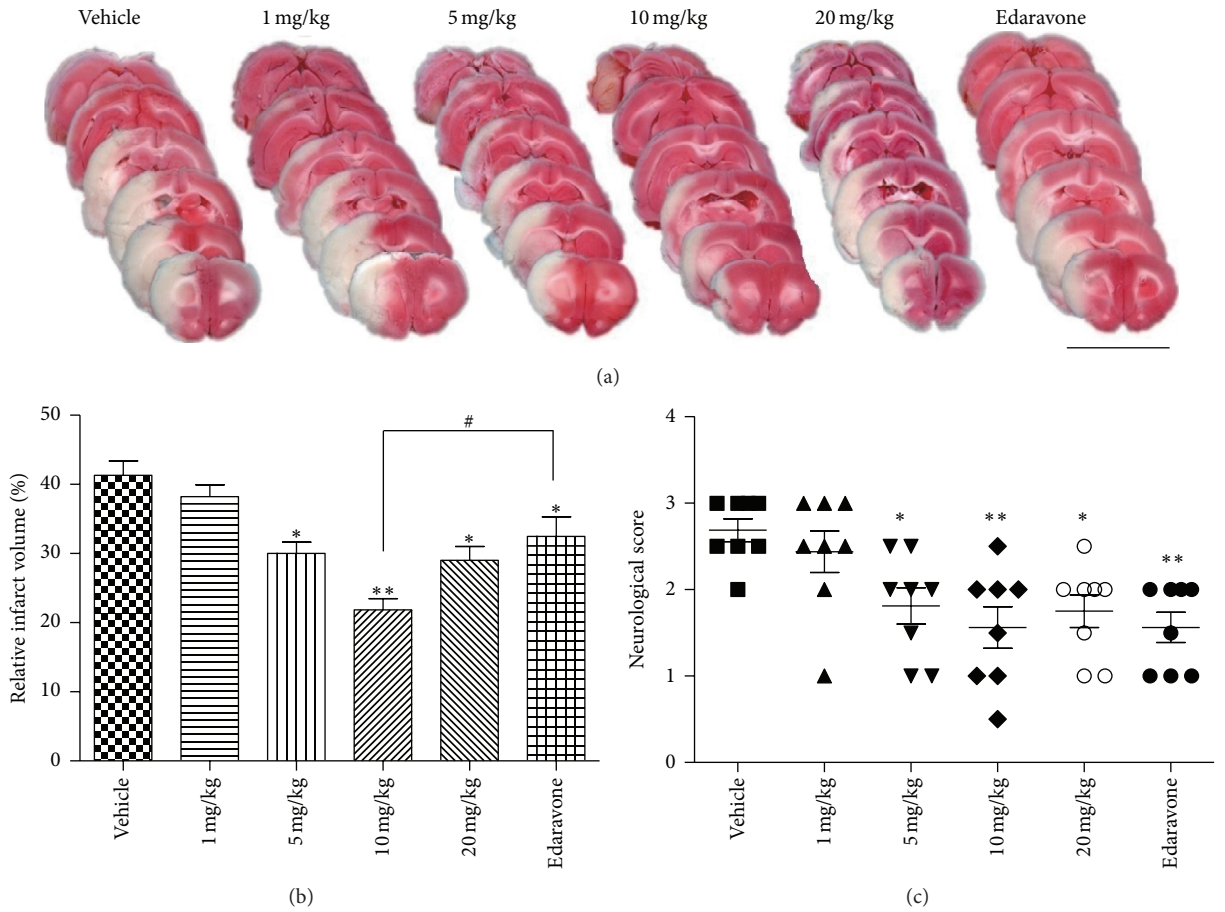

Figure 3: Effects of STVNA on various parameters measured 24 hours after tMCAO in rats. (a) Infarction in serial brain sections stained by TTC (magenta: healthy tissue; white: damaged tissue). Scale bar = 0.5 cm. (b) Statistical analysis of the percentage of infarct volume was determined for each group. (c) Neurological scores after transient middle cerebral artery occlusion (tMCAO) in the vehicle and STVNA treatment groups and edaravone group. Data were expressed as the mean ± the SEM ($n = 8$ per group). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle group; $^{\#}p < 0.05$ versus edaravone group by 1-way analysis of variance with Tukey's multiple comparison test.

that 10 mg/kg STVNA was significantly more effective than edaravone (22.7 ± 1.5% versus 32.4 ± 2.8%, $p < 0.05$) in reducing the infarct size.

To determine whether the histological improvement offered by STVNA had effects on ischemic behavioral patterns, we evaluated neurological deficits 24 hours after transient ischemia. The scores obtained in the groups treated with 5 mg/kg and 10 mg/kg of STVNA were significantly better than those in the animals treated with vehicle (1.8 ± 0.2 and 1.5 ± 0.2 versus 2.6 ± 0.1, resp.) (Figure 3(c)).

3.5. Therapeutic Window Study. A systematic study was performed to examine the time interval after the induction of transient ischemia at which STVNA was still capable of protecting the brain. The vehicle was immediately administered after reperfusion and 10 mg/kg STVNA was administered at 0, 2, 4, or 6 hours after reperfusion. A significant reduction of the infarct volumes was observed when STVNA was administered 2 hours (27 ± 3.5%) or 4 hours (31 ± 1.2%) after reperfusion compared with the vehicle group (41.3 ± 2.1%), whereas no significant effect was observed when the administration time was delayed to 6 hours (40.8 ± 0.9%) (Figure 4(a)). Consistently, a remarkable improvement in neurological scores was detected in the STVNA-treated group (Figure 4(b)).

3.6. Permanent Ischemia Study. In permanent MCAO study, STVNA (10 mg/kg) was administered to rats 0, 2, 4, or 6 hours after ischemia induction. In the permanent MCAO model, the tissue damage observed in the contralateral hemisphere was greater than that seen in the transient MCAO (50 ± 0.8% versus 41.3 ± 2.1%) 24 hours after vehicle treatment. Treatment with STVNA showed a statistically significant reduction of ischemic injury when applied up to 4 hours after MCAO (Figure 5(a)). In particular, the treatment with STVNA 4 hours after the occlusion still significantly reduced the infarct volume compared with the vehicle group (29 ± 1.7% versus 50 ± 0.8%, $p < 0.05$); likewise the administration after 6 hours resulted in decreased damage, although this difference was not statistically significant. The neurological deficit was significantly improved in rats treated with STVNA compared with those treated with the vehicle (Figure 5(b)).

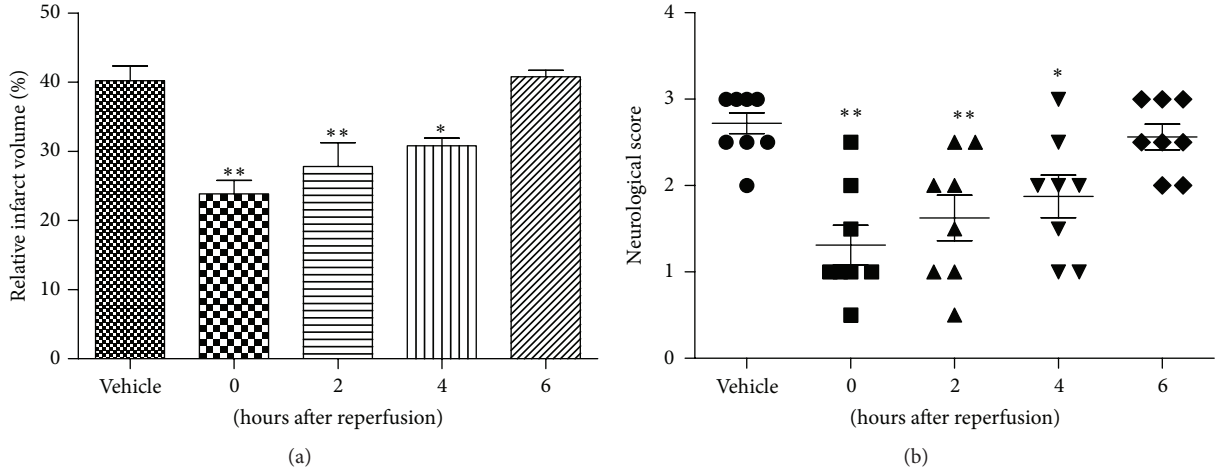

Figure 4: Therapeutic window characteristics of STVNA treatment in tMCAO. (a) Quantitative analyses of infarct volumes 24 hours after reperfusion. A high infarction volume was seen in the group without STVNA treatment, but the volume was greatly decreased in rats with STVNA administration in a time-dependent manner. (b) Neurologic scores after transient middle cerebral artery occlusion (MCAO) in different groups with STVNA administered at 0, 2, 4, and 6 hours after reperfusion. Neurological deficit scores 24 hours after reperfusion. A high score was seen in the group without STVNA treatment, but the scores were greatly lowered in animals with STVNA administration. Data represent mean ± SEM ($n = 8$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$ versus vehicle group by 1-way analysis of variance with Tukey's multiple comparison test.

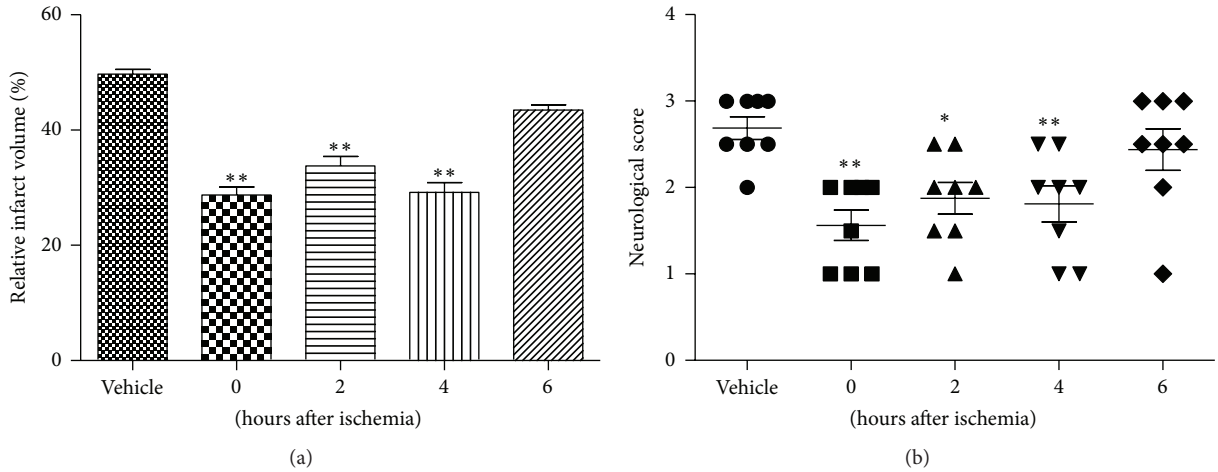

Figure 5: Effects of STVNA treatment in pMCAO. (a) The infarct volume of each group 24 hours after pMCAO. The volume was greatly decreased in rats treated with STVNA administered 4 hours after ischemia. (b) Neurological scores after permanent middle cerebral artery occlusion (pMCAO) in different groups with STVNA administered at 0, 2, 4, and 6 hours after ischemia. The neurological scores were greatly lowered in animals to which STVNA was administered 4 h after ischemia. The data represent the mean ± the SEM ($n = 8$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle group by 1-way analysis of variance with Tukey's multiple comparison test.

3.7. Histopathology Analyses. The results from the HE staining are shown in Figure 6(b). In the sham group, the brain tissues were undamaged, cortical neurons were normomorphic, and the nuclei were centered and displayed clear staining. In the model group, most of the neurons from the peri-infarct area appeared shrunken with eosinophilic cytoplasm accompanied by a decreased optical density. However, when treated with STVNA (10 mg/kg) and edaravone, the number of normal neurons significantly increased and the extent of the damage was significantly diminished.

The number of neurons in the peri-infarction area was detected by anti-NeuN immunohistochemistry (Figure 6(c)). The ischemic injury model (tMCAO) showed a clear decrease in NeuN staining compared with the sham group. Treatment

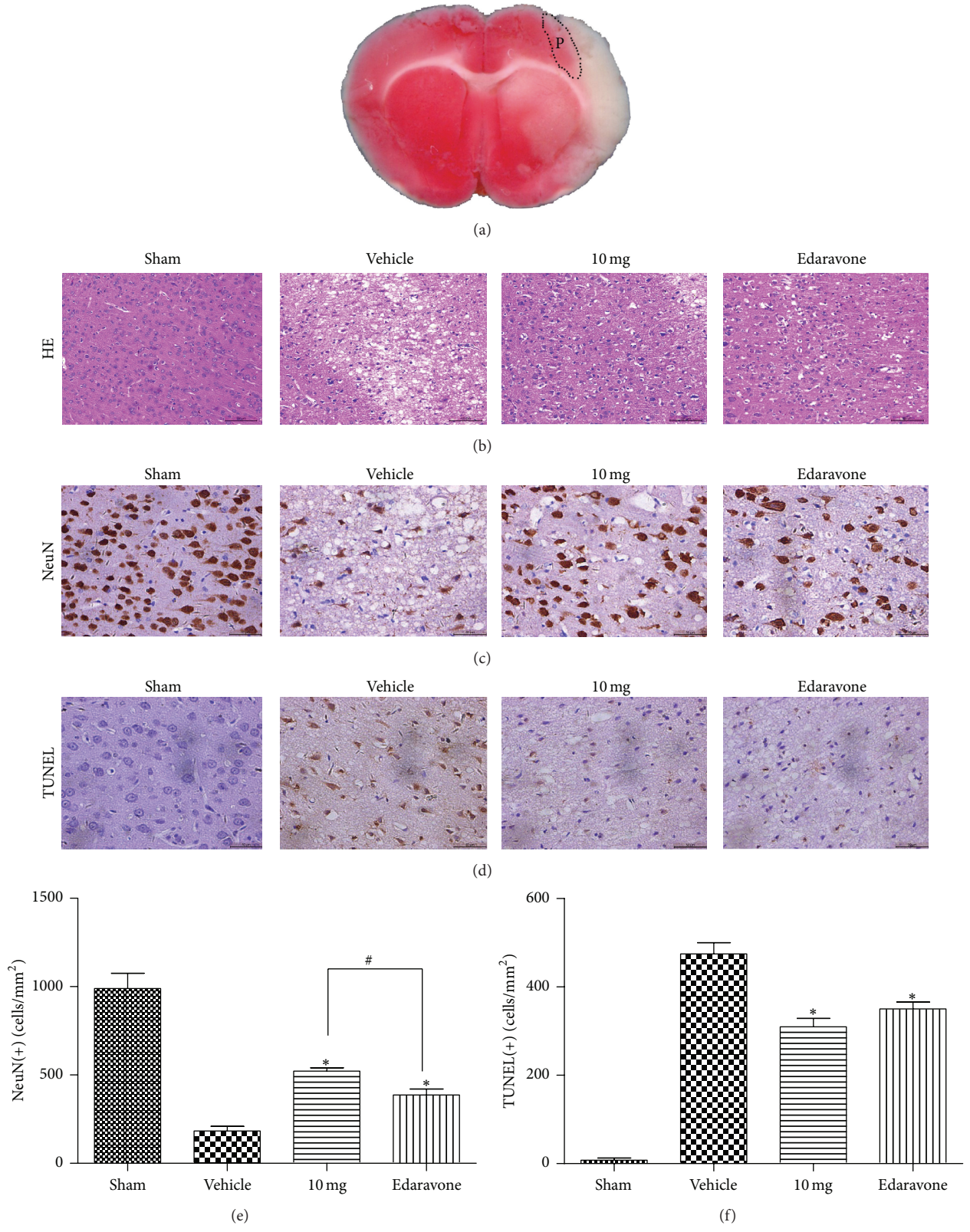

Figure 6: Effects of STVNA on the histopathology. (a) Brain sections stained by TTC; the P represents the peri-infarct area. (b) Hematoxylin-eosin stains of coronal sections of the brain after 24 hours of reperfusion in the dose-response study groups ($n = 3$ per group). 10 mg: 10 mg/kg STVNA. Scale bar = 100 μm. (c) Effects of STVNA on neuronal immunoreactivity in tMCAO rats ($n = 3$ per group). Scale bar = 50 μm. (d) Effect of STVNA on apoptosis in the rat ischemic brain ($n = 3$ per group). Scale bar = 50 μm. (e) Number of NeuN-immunopositive cells/mm^2 of brain section. Histograms represent the mean ± the SEM. $^*p < 0.05$ versus vehicle group and $^\#p < 0.05$ versus edaravone group by 1-way analysis of variance with Tukey's multiple comparison test. (f) Number of TUNEL positive cells/mm^2 in the brain sections. Histograms represent the mean ± the SEM. $^*p < 0.05$ versus vehicle group by 1-way analysis of variance with Tukey's multiple comparison test.

with STVNA showed a clear increase in NeuN immunoreactivity and a significant decrease in the extent of damage, even when compared with edaravone (Figure 6(e)).

Furthermore, we performed TUNEL assays to verify whether STVNA treatment reduced apoptosis. The brain sections obtained from the sham group showed only a slight background staining (Figure 6(d)) with no TUNEL positive cells. The brain sections obtained from the vehicle groups showed an increase in the number of TUNEL positive cells, which appeared dark brown. The increase in TUNEL positive cells was inhibited by STVNA treatment. In particular, the reduction of TUNEL positive cells was found to be 34.9% and 26.2% in the STVNA- (10 mg/kg) and edaravone-treated groups, respectively (Figure 6(f)).

4. Discussion

In the current study, we demonstrated the dose-response relationship and defined the therapeutic window for the neuroprotective effects of STVNA in tMCAO and pMCAO. The presented data show that moderate doses of STVNA (5, 10, and 20 mg/kg) have a protective effect against neuronal damage induced by cerebral ischemia and that this protection is associated with an improvement in neurological functions. Furthermore, the data showed that STVNA was still effective even if the treatment was delayed up to 4 hours after recirculation in temporary focal ischemia or up to 4 hours after artery occlusion in permanent ischemia.

In the first part of this investigation, the results showed that STVNA posttreatment at 10 mg/kg dramatically reduced the infarct size and neurological deficits. These data are consistent with a previously published study that demonstrated the neuroprotective efficacy of isosteviol after ischemia/reperfusion injury when administered before ischemia at doses of 5 to 20 mg/kg. The rCBF of the animals was not monitored, which was very important for producing a stable model in the previous study. The rCBF was monitored in our study. Furthermore the drug we used is more water soluble than isosteviol; thus, 10 mg/kg STVNA in our study was as effective as 20 mg/kg of isosteviol in Xu et al.'s study [14]. Additionally, we changed the method of STVNA administration to continuous intravenous injection instead of bolus, and the time of drug administration was delayed up to 1 hour after ischemia, which caters to the requirements of clinical therapy. Moreover, the best dosage of the STVNA was reduced by half compared with isosteviol (20 mg/kg) with more security, and the efficacy of STVNA is better than that of isosteviol in reducing the infarct volume percentage (45% versus 33%).

Another significant observation made in our study was the beneficial therapeutic window of STVNA using a clinically relevant route in a transient ischemia model. Generally, it is difficult to get patients to the hospital, evaluate them, and enroll them in a clinical trial within 3 hours of symptom onset. Thus, whether a candidate drug has an advantageous therapeutic window is crucially important in preclinical studies, and rodent studies appear to be relevant for determining a therapeutic window for neuroprotective drugs [20, 21]. Here we found that the favorable effects of STVNA were still present even when the compound was administered 4 hours after the recirculation in transient ischemia or 4 hours after artery occlusion in permanent ischemia. This indicates that STVNA is effective in rodents in a time window that is relevant to clinical practice. We also found that when STVNA was administered at the onset of reperfusion, the infarct volume was not significantly different compared to when STVNA was administered 1 hour before reperfusion ($23.8 \pm 1.9\%$ versus $22.7 \pm 1.5\%$, $p > 0.05$). As we know that reperfusion may be more harmful to tissues than ischemia [22, 23], our results suggest that STVNA may be more effective in preventing reperfusion injury than ischemia injury.

Stroke is an extremely variable clinical condition; the time until the restoration of blood flow by thrombolysis and subsequent delivery of oxygen and nutrients to the ischemic brain varies. Therefore, it is vital that potential therapeutic compounds are able to ameliorate the consequences of permanent occlusion in an animal model [24]. In contrast to transient ischemia, the infarct area was more apparent after permanent MCAO. However, STVNA at a dose of 10 mg/kg, the most effective dose in transient ischemia, still produced a modest effect in the permanent occlusion model (46.3% reduction in infarct size in transient MCAO versus 40% reduction in permanent MCAO). These results indicate that STVNA is active in a model of ischemia without reperfusion and that this effect is not dependent on reperfusion time, which reinforces the interest in STVNA for stroke.

The mechanisms of the protective effects of STVNA have been recently investigated. Some studies had previously reported that STV protects against heart ischemia [25] and cerebral ischemia [14]. It has also been demonstrated that pretreatment with isosteviol enhances the expression of the antiapoptosis factor Bcl-2 and inhibits the expression of NF-kB and COX-2. It also increases SOD and GSH-PX activity and decreases MDA content of the myocardium induced by ischemia-reperfusion in anesthetized rats. It had examined the anti-inflammatory activity of isosteviol using the mouse ear inflammatory test induced by 12-O-tetradecanoylphorbol-13-acetate. The pretreatment with isosteviol resulted in a marked reduction in ear disk edema, with an inhibitory effect of 53% [12]. Oxidation stress and the inflammatory response play very important roles in the cascade after cerebral ischemia, and they can also induce the apoptosis of neurons. As shown in Figure 2, our study demonstrated that edema was inhibited in the ischemia/reperfusion injury acute response period. Apoptosis of neurons was also reduced after STVNA treatment (Figure 6(d)). Therefore, STVNA is a potential neuroprotectant in in vivo models of cerebral ischemia and can act on multiple pathways in the ischemic cascade, suggesting that STVNA might have a greater chance of success in interrupting ischemic injury in acute stroke.

Although many compounds with neuroprotective action are in various stages of the drug discovery, STVNA shows individual advantages. Stevioside (the origin of STVNA) has been extensively used as a sweetener and demonstrated its safety for human [26]. Secondly, isosteviol is lipophilic and can easily diffuse across biological membranes and the

BBB. Thirdly, STVNA provides substantial neuroprotection through cerebral perfusion-independent effects and in permanent MCAO models of stroke, unlike tirilazad (a perfusion-dependent neuroprotective agent) [27] and anti-intercellular adhesion molecule-1 antibody [28], which have only been proven effective in tMCAO. Moreover, we compared the neuroprotective potency of STVNA with other established antioxidants with neuroprotective abilities. STVNA resulted in better histological and functional improvement than edaravone (Figures 6 and 3(b)), a ROS scavenger approved in Japan for the treatment of cerebral infarction.

In summary, the present study demonstrated that STVNA can reduce the damage induced by focal cerebral ischemia and was still effective even if the treatment was delayed up to 4 hours after recirculation in temporary focal ischemia or up to 4 hours after artery occlusion in permanent ischemia. Meanwhile, the neuroprotective effectiveness of STVNA is better than edaravone. Therefore, STVNA may be a potent alternative treatment for acute ischemic stroke.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] S. C. Johnston, S. Mendis, and C. D. Mathers, "Global variation in stroke burden and mortality: estimates from monitoring, surveillance, and modelling," *The Lancet Neurology*, vol. 8, no. 4, pp. 345–354, 2009.

[2] G. Silvestrelli, F. Corea, M. Paciaroni et al., "The Perugia hospital-based stroke registry: report of the 2nd year," *Clinical and Experimental Hypertension*, vol. 24, no. 7-8, pp. 485–491, 2002.

[3] W.-H. Shin, S.-J. Park, and E.-J. Kim, "Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats," *Life Sciences*, vol. 79, no. 2, pp. 130–137, 2006.

[4] A. G. Avent, J. R. Hanson, and B. H. De Oliveira, "Hydrolysis of the diterpenoid glycoside, stevioside," *Phytochemistry*, vol. 29, no. 8, pp. 2712–2715, 1990.

[5] E. Mosettig, U. Beglinger, F. Dolder, H. Lichti, P. Quitt, and J. A. Waters, "The absolute configuration of steviol and isosteviol," *Journal of the American Chemical Society*, vol. 85, no. 15, pp. 2305–2309, 1963.

[6] E. L. Ghisalberti, "The biological activity of naturally occurring kaurane diterpenes," *Fitoterapia*, vol. 68, no. 4, pp. 303–325, 1997.

[7] A. D. Kinghorn and D. D. Soejarto, "Discovery of terpenoid and phenolic sweeteners from plants," *Pure and Applied Chemistry*, vol. 74, no. 7, pp. 1169–1179, 2002.

[8] J.-C. Liu, P.-F. Kao, M.-H. Hsieh, Y.-J. Chen, and P. Chan, "The antihypertensive effect of stevioside derivative isosteviol in spontaneously hypertensive rats," *Acta Cardiologica Sinica*, vol. 17, no. 3, pp. 133–140, 2001.

[9] J. Ma, Z. Ma, J. Wang et al., "Isosteviol reduces plasma glucose levels in the intravenous glucose tolerance test in Zucker diabetic fatty rats," *Diabetes, Obesity and Metabolism*, vol. 9, no. 4, pp. 597–599, 2007.

[10] K. L. Wong, K. C. Wu, E. C. So, R. S. Wu, and T. H. Cheng, "The anti-oxidative effect of isosteviol on angiotensin-II-induced reactive oxygen species generation in hypertensive injury of aortic smooth muscle cells: 9AP8-1," *European Journal of Anaesthesiology*, vol. 24, p. 125, 2007.

[11] K. Yasukawa, S. Kitanaka, and S. Seo, "Inhibitory effect of stevioside on tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin," *Biological and Pharmaceutical Bulletin*, vol. 25, no. 11, pp. 1488–1490, 2002.

[12] Y. Mizushina, T. Akihisa, M. Ukiya et al., "Structural analysis of isosteviol and related compounds as DNA polymerase and DNA topoisomerase inhibitors," *Life Sciences*, vol. 77, no. 17, pp. 2127–2140, 2005.

[13] W. Tan, "The use fo kauranes compounds in the manufacture medicament," World Intellectual Property Organization, PCT Patent, WO/2005/110383, 2005.

[14] D. Xu, W. Du, L. Zhao, A. K. Davey, and J. Wang, "The neuroprotective effects of isosteviol against focal cerebral ischemia injury induced by middle cerebral artery occlusion in rats," *Planta Medica*, vol. 74, no. 8, pp. 816–821, 2008.

[15] M. Fisher, "New approaches to neuroprotective drug development," *Stroke*, vol. 42, no. 1, pp. S24–S27, 2011.

[16] E. Z. Longa, P. R. Weinstein, S. Carlson, and R. Cummins, "Reversible middle cerebral artery occlusion without craniectomy in rats," *Stroke*, vol. 20, no. 1, pp. 84–91, 1989.

[17] J. B. Bederson, L. H. Pitts, M. Tsuji, M. C. Nishimura, R. L. Davis, and H. Bartkowski, "Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination," *Stroke*, vol. 17, no. 3, pp. 472–476, 1986.

[18] R. A. Swanson, M. T. Morton, G. Tsao-Wu, R. A. Savalos, C. Davidson, and F. R. Sharp, "A semiautomated method for measuring brain infarct volume," *Journal of Cerebral Blood Flow and Metabolism*, vol. 10, no. 2, pp. 290–293, 1990.

[19] H. Nishi, T. Watanabe, H. Sakurai, S. Yuki, and A. Ishibashi, "Effect of MCI-186 on brain edema in rats," *Stroke*, vol. 20, no. 9, pp. 1236–1240, 1989.

[20] Stroke Therapy Academic Industry Roundtable (STAIR), "Recommendations for standards regarding preclinical neuroprotective and restorative drug development," *Stroke*, vol. 30, no. 12, pp. 2752–2758, 1999.

[21] Stroke Therapy Academic Industry Roundtable II (STAIR-II), "Recommendations for clinical trial evaluation of acute stroke therapies," *Stroke*, vol. 32, no. 7, pp. 1598–1606, 2001.

[22] J. Aronowski and L. A. Labiche, "Perspectives on reperfusion-induced damage in rodent models of experimental focal ischemia and role of γ-protein kinase C," *ILAR Journal*, vol. 44, no. 2, pp. 105–109, 2003.

[23] J. Aronowski, R. Strong, and J. C. Grotta, "Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats," *Journal of Cerebral Blood Flow and Metabolism*, vol. 17, no. 10, pp. 1048–1056, 1997.

[24] M. Fisher, G. Feuerstein, D. W. Howells et al., "Update of the stroke therapy academic industry roundtable preclinical recommendations," *Stroke*, vol. 40, no. 6, pp. 2244–2250, 2009.

[25] D. Xu, Y. Li, J. Wang, A. K. Davey, S. Zhang, and A. M. Evans, "The cardioprotective effect of isosteviol on rats with heart ischemia-reperfusion injury," *Life Sciences*, vol. 80, no. 4, pp. 269–274, 2007.

[26] J. M. C. Geuns and J. G. Buyse, "The safety of Stevia," in *Proceedings of the 1st Symposium*, Katholieke Universiteit Leuven, Heverlee, Belgium, April 2004.

[27] D. Xue, A. Slivka, and A. M. Buchan, "Tirilazad reduces cortical infarction after transient but not permanent focal cerebral ischemia in rats," *Stroke*, vol. 23, no. 6, pp. 894–899, 1992.

[28] M. Lou, C. C. Eschenfelder, T. Herdegen, S. Brecht, and G. Deuschl, "Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats," *Stroke*, vol. 35, no. 2, pp. 578–583, 2004.

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Research Article

Isosteviol Sensitizes sarcK_{ATP} Channels towards Pinacidil and Potentiates Mitochondrial Uncoupling of Diazoxide in Guinea Pig Ventricular Myocytes

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K_{ATP} channel is an important mediator or factor in physiological and pathological metabolic pathway. Activation of K_{ATP} channel has been identified to be a critical step in the cardioprotective mechanism against IR injury. On the other hand, desensitization of the channel to its opener or the metabolic ligand ATP in pathological conditions, like cardiac hypertrophy, would decrease the adaption of myocardium to metabolic stress and is a disadvantage for drug therapy. Isosteviol, obtained by acid hydrolysis of stevioside, has been demonstrated to play a cardioprotective role against diseases of cardiovascular system, like anti-IR injury, antihypertension, antihyperglycemia, and so forth. The present study investigated the effect of isosteviol (STV) on sarcK_{ATP} channel current induced by pinacidil and mitochondrial flavoprotein oxidation induced by diazoxide. Our results showed that preincubating cells with STV not only increased the current amplitude and activating rate of sarcK_{ATP} channels induced by pinacidil but also potentiated diazoxide-elicited oxidation of flavoprotein in mitochondria.

1. Introduction

The ATP-sensitive potassium (K_{ATP}) channel was first discovered by Noma in 1983 in isolated membrane patches prepared from guinea pig ventricular myocytes and subsequently identified in many other tissues, including brain, smooth muscle, skeletal muscle, and pancreas [1]. These channels are inhibited by intracellular ATP and activated by intracellular nucleoside diphosphates, thereby coupling cell metabolic condition to membrane electrical activity in various cell types [2, 3]. Structurally, K_{ATP} channels are heterooctamers composed of four Kir6.x and four sulfonylurea receptor (SUR) subunits [4]. Kir6.x subunits form the K^+ transporting channel pore, whereas SURs serve as regulatory subunits and endow the channel with sensitivity to sulfonylureas, nucleotides, and the K_{ATP} channel openers such as pinacidil and diazoxide [5, 6]. Functionally, K_{ATP} channels have been demonstrated to be involved in many physiology activities like insulin secretion, smooth muscle contraction, and so forth [7–9].

Opening of K_{ATP} channel has been shown to be an endogenous protective mechanism in response to various stresses under altered metabolic states, including hyperglycemia, hypertension, ischemia, and hypoxia. For example, prepharmacological opening of K_{ATP} channels has been demonstrated to play a cardioprotective role against IR injury and K_{ATP} channel has been identified to be a key component in the phenomenon termed ischemic preconditioning (IPC), in which single or multiple brief periods of ischemia have been shown to protect the heart against a subsequent prolonged ischemic insult [10]. Both sarcolemmal (sarcK_{ATP}) and mitochondrial K_{ATP} (mitoK_{ATP}) channels have been proposed to be involved in the cardioprotective role against IR injury through distinct mechanism [11–14]. ATP-sensitive K channels are essential for maintaining the cellular homeostasis

against various metabolic stresses. Disease induced structural remodeling of cardiomyocytes may decrease or diminish the sensitivity of K_{ATP} channel to ATP and/or its openers and alter this adaptive response to such stresses [15–18]. The dysfunctional K_{ATP} channels in these conditions may fail to protect the myocardium from metabolic stresses or make the pharmacological therapy targeted to K_{ATP} channels ineffective. So, finding a drug or substance which would increase the sensitivity but not induce direct activation of K_{ATP} channel is necessary.

Isosteviol is a derivative of stevioside, which has been used commercially as a sugar substitute for years [19]. Studies indicated that both stevioside and isosteviol may possess a variety of biological activities including antihypertension, antihyperglycemia, anti-inflammatory, and potential antitumor effects [20–26]. Besides, the myocardium protective effects of isosteviol against ischemia-reperfusion injury have been reported by Tan and other groups [27–29]. According these studies, isosteviol could reduce the infraction area and restore the contractility in cardiac IR in vivo and in isolated hearts without introducing or even improving arrhythmia [27–29], which as an adverse effect limits the usage of classical KCOs in clinic [30]. The protective effects of isosteviol could be partially blocked by 5-HD, a mitoK_{ATP} channel blocker, which suggested a potential involvement of mitoK_{ATP} in the protective mechanism of isosteviol [27–29]. Despite that, the underlying mechanism of isosteviol against ischemia and other stresses at molecular level is not clear so far and needs to be further investigated.

The present study investigated the possible effects of isosteviol sodium (the sodium salt of isosteviol, STV) on sarcK_{ATP} currents induced by pinacidil and flavoprotein fluorescence elicited by diazoxide in isolated guinea pig cardiomyocytes. We found that isosteviol potentiated both the pinacidil-induced sarcK_{ATP} channel current and diazoxide-elicited flavoprotein oxidation, but surprisingly isosteviol sodium alone played no effects on either of them. In addition, the potentiation effects of STV were completely blocked by N-acetyl-cysteine (NAC)—a ROS scavenger. Since the flavoprotein oxidation induced by diazoxide is often used to investigate the activity of mitoK_{ATP} channel as an indirect approach on intact cell [31], we infer from the results that isosteviol may act as a sensitizer or modulator but not a direct opener for both sarc- and mitoK_{ATP} channels and the sensitization effects of STV on both channels are ROS dependent.

2. Materials and Methods

2.1. Isolation of Guinea Pig Ventricular Myocytes. Single ventricular myocytes were isolated from guinea pig hearts using a standard enzymatic technique. The protocols were approved by our institutional ethics committee. Briefly, adult guinea pigs (250–350 g) were anesthetized with an injection of 5% pentobarbital sodium (0.2 mL/100 g). Heparin (500 U/Kg) was administered to prevent coagulation during heart removal. Then, hearts were rapidly removed, mounted on a Langendorff perfusion apparatus, and retrogradely perfused with Ca^{2+}-free tyrode solution composed of (in mM) NaCl 120, KCl 5.4, $MgCl_2$ 0.5, HEPES 25, and glucose 10 (pH 7.4 with NaOH) at 37°C. After 5 min, the perfusion solution was changed to the tyrode solution containing type II collagenase (0.6 mg/mL, Sigma) and Ca^{2+} (50 μM) for more than 30 mins. After perfusion, the ventricular tissue was cut into small pieces in a petri dish with the same solution and was shaken gently for the dispersion of dissociated cardiac myocytes. A 250 μM mesh screen was used to separate the isolated cardiac myocytes from cardiac tissue. Then, cells were collected by centrifugation at 500 rpm for 45 s. Finally, the Ca^{2+} concentration was gradually restored to 1 mM. Cells were stored in M199 medium at a 37°C CO_2 incubator until use.

2.2. Electrophysiological Recordings. Whole-cell patch-clamp recordings were performed at room temperature with an EPC-10 amplifier (HEKA, Lambrecht, Germany). Cells were placed in an experimental chamber mounted on a stage of an inverted microscope (Nikon, Japan). Pipettes were pulled from borosilicate glass (Sutter, Novato, CA) and had a resistance of between 2 and 4 MΩ. The bath perfusate for sarcK_{ATP} channel and AP recording was the Ca^{2+} containing tyrode's solution consisting of (in mM) NaCl 137, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1, NaH_2PO_4 1.2, HEPES 20, and glucose 10, with the pH controlled to 7.4 with NaOH. The pipette solution was (in mM) KCl 140, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, Na_2ATP 1, and HEPES 10 (pH 7.2, with KOH). K_{ATP} channel opener pinacidil and antagonist glibenclamide were added to the bath with a perfusion drug delivery system (Scientific Instruments, Cambridgeshire, UK). The pipette solution for I_{Kr} was (in mM) KCl 135, $MgCl_2$ 2, HEPES 10, EGTA 10, and Mg-ATP 5 (pH 7.2 with KOH). Nifedipine (10 μM) and chromanol B (10 μM) were added to the bath solution to block L-type calcium and I_{Ks} currents, respectively. For L-type calcium channel recording, the bath solution was (in mM) TEA-Cl 135, $CaCl_2$ 2, glucose 10, HEPES 10, and $MgCl_2$ 1. The internal solution was (in mM) CsCl 110, Mg-ATP 5, EGTA 10, and TEA-Cl 30 (pH 7.2 with CsOH). STV was delivered by preincubating cells in the drug containing culture solution for record of currents or by a drug delivery system for AP recording. For investigating the role of ROS on the potentiation effects of STV, 100 μM reactive oxygen species scavenger NAC was coadministered with STV.

K_{ATP} channel currents were elicited by applying the opener pinacidil at a concentration of 100 μM and the protocol used was a depolarizing voltage step to 0 mV for 500 ms from a holding potential of −40 mV applied every 30 s. *I-V* curves of K_{ATP} currents were obtained by applying the voltage steps between −80 and +60 mV with a 10 mV increment. In order to compensate for variations in cell size, currents were normalized to cell capacitance and reported as current densities (pA/pF). APs were measured at the current-clamp mode and evoked by applying a 5-ms depolarizing square pulse with the amplitude of 800 pA. Action potential duration was measured at 90% repolarization (APD_{90}). I_{Kr} currents were elicited by applying the voltage steps from −40 to +40 mV for 1 s and then repolarized to −40 mV for 1 s for tail current recording. The L-type calcium currents

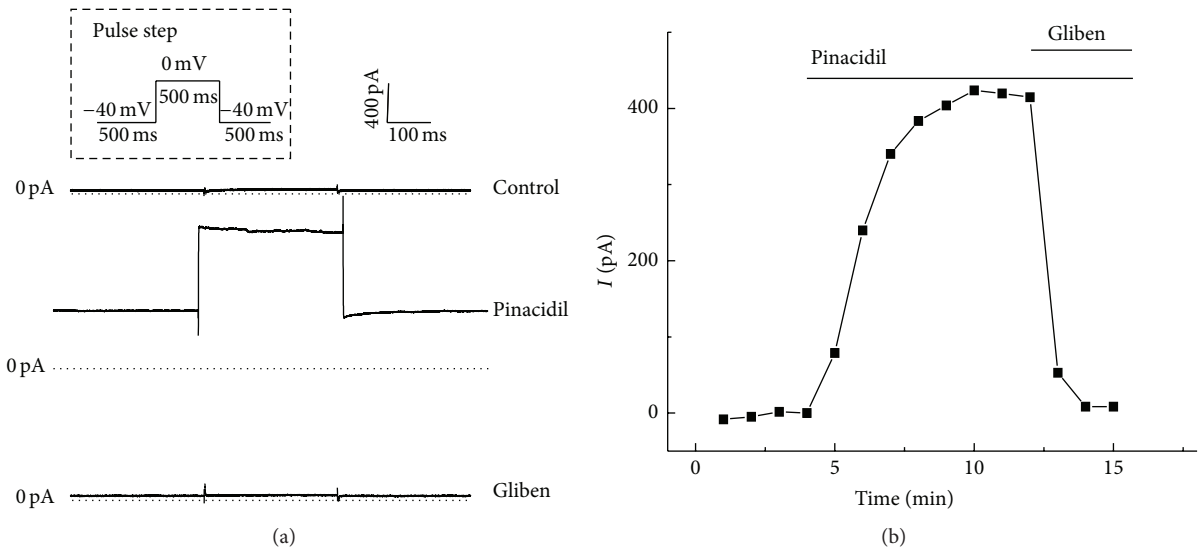

FIGURE 1: SarcK$_{ATP}$ channel current was elicited by pinacidil and blocked by glibenclamide. (a) Representative whole-cell current traces of sarcK$_{ATP}$ channel without pinacidil (up), with 100 μM pinacidil (middle) and with 10 μM glibenclamide (bottom). (b) The representative time course of K$_{ATP}$ channel activation by pinacidil and inhibition by glibenclamide.

were evoked by step depolarization to test potentials between −40 mV and +60 mV for 500 ms. Data were sampled at 10 kHz and filtered at 2.9 kHz. Cell capacitance (C_m) and series resistance (R_s) were electrically compensated in the voltage clamp experiments. The PatchMaster (HEKA, Lambrecht, Germany) and origin 8 software (OriginLab, Northampton, MA) were used for data acquisition and analysis, respectively.

2.3. Flavoprotein Fluorescence Measurements. Opening of mitoK$_{ATP}$ channels dissipates the inner mitochondrial membrane potential established by the proton pump, which accelerates electron transfer by the respiratory chain and leads to net oxidation of the mitochondria. Mitochondrial redox state can be monitored by recording the fluorescence of flavoprotein in the mitochondria as described by Liu et al. [31]. Exposure to dinitrophenol (DNP) uncouples respiration from ATP synthesis, collapses the mitochondrial potential, and induces maximal oxidation. The values of flavoprotein fluorescence were expressed as a percentage of the DNP-induced fluorescence. The fluorescence intensity with the test substances was calculated according to the following equation:

$$\text{Percentage } I_f = \frac{I_f - I_{\text{bas}}}{I_{\text{DNP}} - I_{\text{bas}}} * 100\%, \quad (1)$$

where I_f, I_{DNP}, and I_{bas} were the fluorescence intensity during exposure to the test agent (diazoxide or isosteviol), DNP, and baseline, respectively. Images of endogenous flavoprotein fluorescence were obtained with a Zeiss 710 confocal laser-scanning microscope (ZEISS, Germany). Fluorescence was excited by the 488-nm line of an argon laser, and the emission at 530 nm was recorded. A time series of images were collected at intervals of 10 seconds. Images were analyzed on a personal computer with the software ZEN2011 (ZEISS, Germany). The extracellular solution was Hanks' balanced salts solution (HBSS, Sigma). STV was delivered after diazoxide or by incubating cells with it previously. The incubation period was more than 1 hr, which is consistent with that needed to observe the maximal effect of STV for sarcK$_{ATP}$ current. For investigating the effect of ROS, 100 μM NAC was coadministered with STV. All the recordings were performed at 37°C.

2.4. Chemicals and Drugs. The sodium salt of isosteviol was provided by Key Pharma Biomedical Inc. All chemicals for tyrode's solution and external and internal solution of patch-clamp recording were bought from Sigma Chemical Co. Collagenase was purchased from Worthington. Diazoxide, pinacidil, glibenclamide, and DNP were also purchased from Sigma Chemical Co. and dissolved in DMSO before being added to experimental solutions. The final concentration of DMSO was ≤0.1%. Pentobarbital sodium was obtained from Sangon Biotech (Shanghai) and heparin was obtained from Aladdin Reagent.

2.5. Statistical Analysis. The data were expressed as mean ± SEM (standard error of the mean), n = number of independent experiments. Statistical analysis was performed by one-way ANOVA using a Bonferroni test. Significant differences between groups were defined at $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. SarcK$_{ATP}$ Channel Was Elicited by Pinacidil and Blocked by Glibenclamide. SarcK$_{ATP}$ channel current was elicited by a specific potassium channel opener, pinacidil (100 μM), measuring at a 500 ms test pulse of 0 mV from a holding potential of −40 mV. Figure 1(a) shows the representative

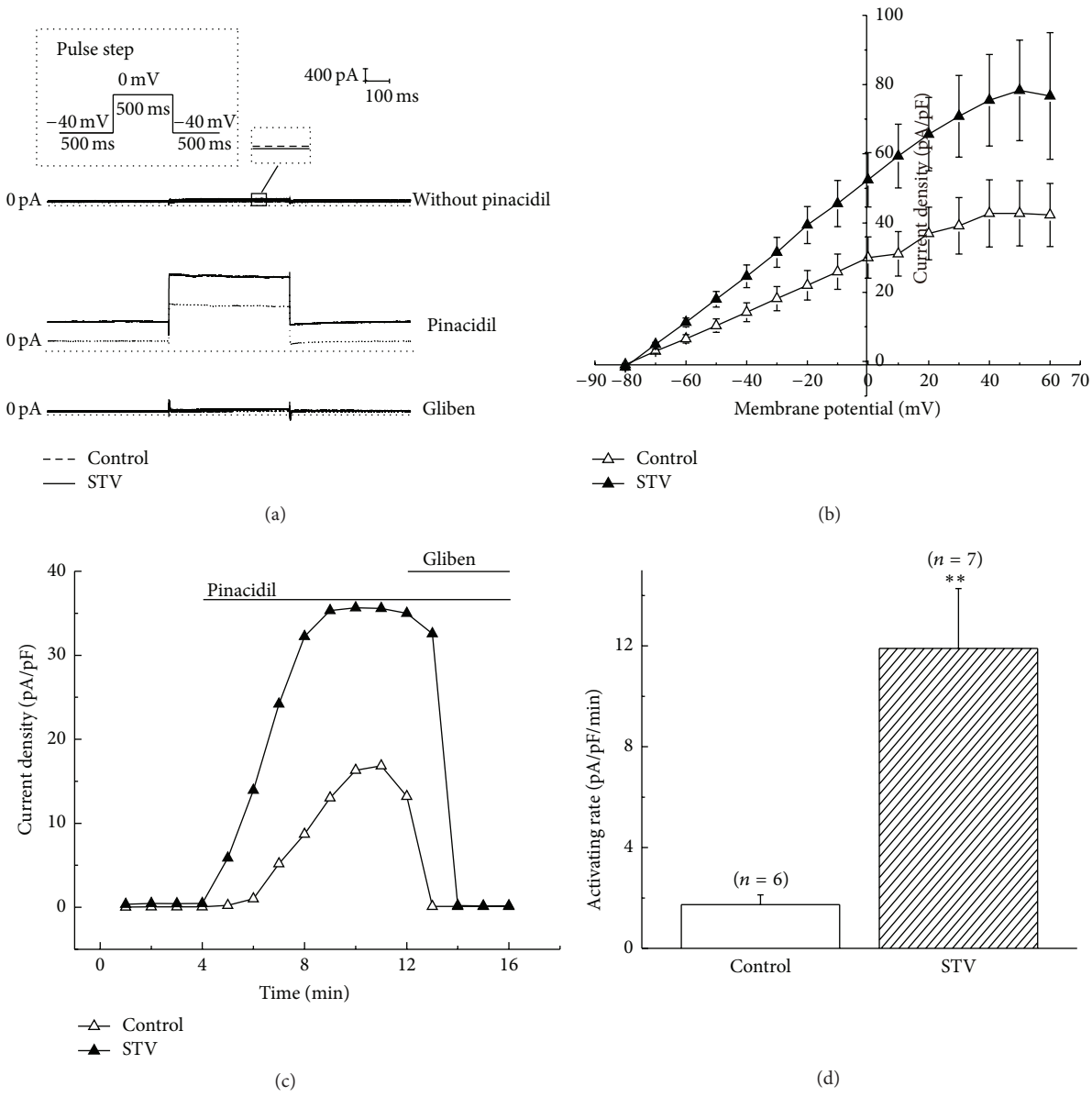

FIGURE 2: STV increased the amplitude and activating rate of pinacidil-induced K_{ATP} current. (a) Representative pinacidil-induced K_{ATP} currents of STV-preincubated (10 μM) and control group. (b) Effect of 10 μM STV on current-voltage (*IV*) relationship of pinacidil-induced K_{ATP} channel current. Currents were normalized to cell capacitance. (c) The time course of K_{ATP} channel activation by pinacidil for STV-preincubated and control group. (d) Comparison of activation rates of K_{ATP} channel between STV-preincubated and control group. ** means $p < 0.01$.

trace of whole-cell $I_{K_{ATP}}$ activated by pinacidil (Figure 1(a) middle). Spontaneous activation of $I_{K_{ATP}}$ was not observed in the absence of pinacidil during control condition (Figure 1(a) top). The outward current was completely suppressed by the antagonist glibenclamide (1 μM, Figure 1(a) bottom), which indicates that the pinacidil-elicited current is caused by the activation of sarcolemmal K_{ATP} channels. The time course of K_{ATP} channel activation by pinacidil is shown in Figure 1(b). The current was usually activated within 5 minutes after pinacidil application. Current amplitude was measured at the end of the test pulse to 0 mV.

3.2. STV-Preincubation Increased the Current Density and Activating Rate of SarcK_{ATP} Channel Elicited by Pinacidil. Next, we investigated the effect of isosteviol sodium (STV) on sarcK_{ATP} channel current by preincubating the isolated myocytes with the drug. We found that STV alone did not elicit K_{ATP} channel current at the concentration of 10 μM (Figure 2(a) up), whereas preincubating cells with STV of the same concentration significantly increased K_{ATP} channel current elicited by 100 μM pinacidil compared with control group (Figure 2(a) middle). 1 μM glibenclamide blocked both the currents of STV-preincubation and control group

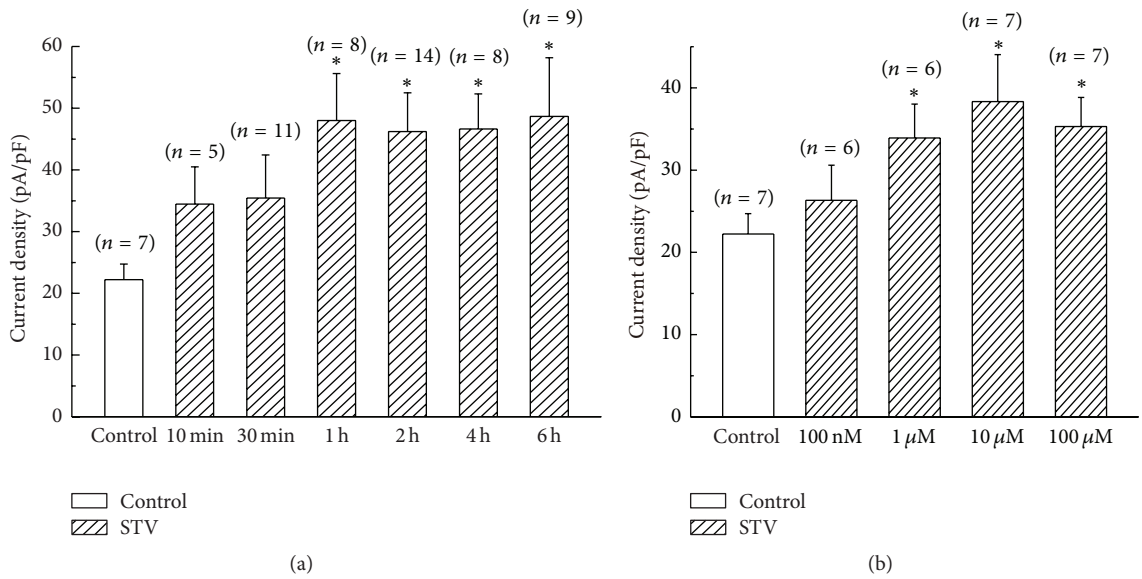

Figure 3: Time and concentration dependence of STV on pinacidil-activated K_{ATP} current. (a) The time dependence of STV-preincubation (10 μM) on pinacidil-induced K_{ATP} channel current. (b) The concentration dependence of STV on K_{ATP} current with incubation period of more than 1 hr. $*$ means $p < 0.05$.

(Figure 2(a) bottom). At 0 mV, the current density of K_{ATP} channels was increased to 52.5 ± 7.8 pA/pF ($n = 7$, $p < 0.05$) by preincubating cells with 10 μM STV from 30.0 ± 6.0 pA/pF ($n = 6$) of control condition. To investigate the effect of STV on current-voltage relationship of the K_{ATP} current induced by pinacidil, command voltage pulses of 500 ms in duration to various membrane potentials (from −80 mV to +60 mV) were applied to the ventricular myocytes. Data shows that STV increased the current density of pinacidil-elicited K_{ATP} channel at all test potentials above −70 mV (Figure 2(b)). The reversal potential of K_{ATP} channel was not changed by STV. Both had a reversal potential of about −80 mV, which is close to the estimated equilibrium potential for K^+ under the experimental conditions. Besides current amplitude, the activating rate of pinacidil-induced sarcK_{ATP} current was also examined. Activating rate was calculated by dividing the pinacidil-elicited current density changes by the time consumed. Figure 2(c) represents the activation course of sarcK_{ATP} channel induced by pinacidil of control group and drug-preincubated group. Data shows that the activating rate of STV-preincubated group was significantly increased compared with control group. The activating rates were 11.9 ± 2.4 ($n = 7$) and 1.7 ± 0.4 ($n = 6$) pA/pF/min for drug-preincubated and control group, respectively (Figure 2(d)).

3.3. The Time and Concentration Dependence of STV on Pinacidil-Activated K_{ATP} Current. To investigate the time dependence of STV on pinacidil-induced sarcK_{ATP} currents, we incubated the isolated myocytes in STV-containing tyrode solution with different time at the concentration of 10 μM. Figure 3(a) indicates that the current density of $I_{K_{ATP}}$ was gradually increased with the incubation time prolonged and got saturated after 1 hour. The current densities of $I_{K_{ATP}}$ were 48.0 ± 7.6 ($n = 8$), 46.2 ± 6.3 ($n = 14$), 46.6 ± 5.7 ($n = 8$), and 48.7 ± 9.5 ($n = 9$) pA/pF at 1, 2, 4, and 6 hrs, respectively, which were significantly larger than that of 22.2 ± 2.5 pA/pF ($n = 7$) for control group. The concentration dependence of STV on pinacidil-activated K_{ATP} current was also investigated by preincubating cells with STV for more than 1 hour with different concentration. Figure 3(b) shows that the concentration of 1 μM or more of STV significantly increased the current density of $I_{K_{ATP}}$. The current densities of K_{ATP} channel elicited by pinacidil were 33.9 ± 4.1 ($n = 6$) and 38.3 ± 5.7 ($n = 7$) pA/pF at the concentration of 1 μM and 10 μM, respectively, both of which were significantly larger than that of control group (22.2 ± 2.5 pA/pF, $p < 0.05$). 100 μM STV did not further enlarge the current density of $I_{K_{ATP}}$ compared with 10 μM.

3.4. Effect of STV on I_{Kr} and L-Type Ca^{2+} Current. Besides K_{ATP} channel, we also examined the effect of STV on the rapidly activated delayed rectifier K^+ (I_{Kr}) and L-type Ca^{2+} channel (I_{CaL}) currents. Cells were incubated with STV for no less than 1 hour before recording. Figures 4(a) and 4(b) show that the tail current of I_{Kr} at +40 mV was not affected significantly by preincubating cells with 10 μM STV. The mean current densities of I_{Kr} tail currents at +40 mV for drug-preincubated and control group were 1.2 ± 0.1 and 1.3 ± 0.1 pA/pF, respectively. Figures 4(c) and 4(d) show that the current amplitude of L-type calcium channel at +10 mV was not affected by STV either. The current densities of calcium channel at +10 mV were 4.2 ± 0.2 and 4.1 ± 0.2 pA/pF for drug-preincubated and control group, respectively. Neither the amplitude of I_{Kr} nor I_{CaL} was affected by STV-preincubation.

3.5. Effect of STV on Action Potential of Guinea Pig Ventricular Myocytes. Besides the effects of STV on membrane currents,

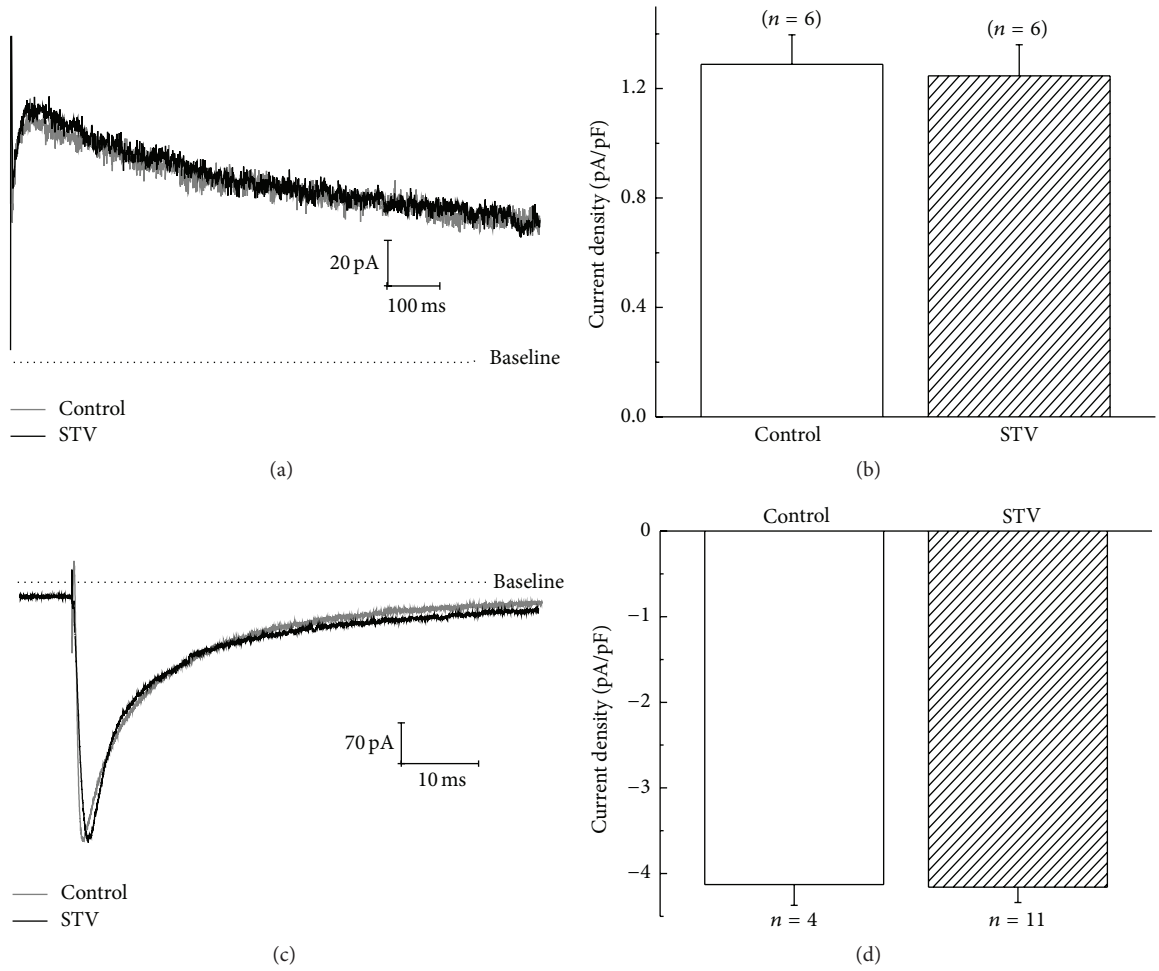

FIGURE 4: Effects of STV on I_{Kr} and L-type calcium current of guinea pig ventricular myocytes. (a) and (b) show the representative currents and the mean current densities of I_{Kr} tail current at +40 mV for STV-preincubated and control group. (c) and (d) show the representative currents and mean current density of L-type calcium current at +10 mV for STV-preincubated and control group, respectively. Cells were incubated with STV for no less than 1 hour before I_{Kr} and I_{CaL} recordings.

we also investigated its effect on action potential of guinea pig ventricular myocytes. STV was applied by a perfusion drug delivery system. We observed the change of the action potential duration (APD) and the resting membrane potential (RMP) after 5 mins of drug application, when the action potential of cardiomyocytes becomes steady. Data show that 10 μM STV changed neither the action potential duration (APD_{90}) nor the resting membrane potential (RMP) of ventricular myocytes significantly (Figures 5(a), 5(b), and 5(c)). The APD and RMP for control group were 495.5 ± 60.5 ms ($n = 5$) and −71.2 ± 0.6 mV ($n = 5$), which were not significantly different from the value of 412.4 ± 25.6 ms and −72.6 ± 0.2 mV for STV group. These results are consistent with the above results that STV alone had no effect on $sarcK_{ATP}$, I_{Kr}, and L-type calcium currents. For comparison, we also examined the effect of pinacidil on action potential of myocytes. Figures 5(d), 5(e), and 5(f) show that both the APD_{90} and RMP of cardiomyocytes were significantly affected by pinacidil. The APD_{90} was shortened from 460.3 ± 42.9 ms to 83.2 ± 33.9 ms ($n = 8$, $p < 0.01$), whereas the RMP was hyperpolarized from −68.3 ± 1.2 mV to −73 ± 0.8 mV ($n = 8$, $p < 0.05$) in about 3 mins by pinacidil's application. We also investigated the preincubation effect of STV on pinacidil-induced shortage of AP. The results showed that STV-preincubation made the shortening of AP by pinacidil faster, although the change did not reach a significant level (data are not shown). We probably could attribute this unexpected result to the extremely rapid influence of pinacidil on AP and the state of cardiomyocytes, which often causes cells to shrink and die in several minutes.

3.6. STV-Preincubation Enhanced the Flavoprotein Fluorescence Induced by Diazoxide. Diazoxide, as a specific $mitoK_{ATP}$ channel opener, is usually used to investigate $mitoK_{ATP}$ channel activity pharmacologically. Mitochondrial flavoprotein fluorescence was measured during exposure to diazoxide (200 μM) and the percentage to DNP (200 μM) induced fluorescence was calculated for comparison. Figure 6 showed that preincubating cells with 10 μM STV significantly

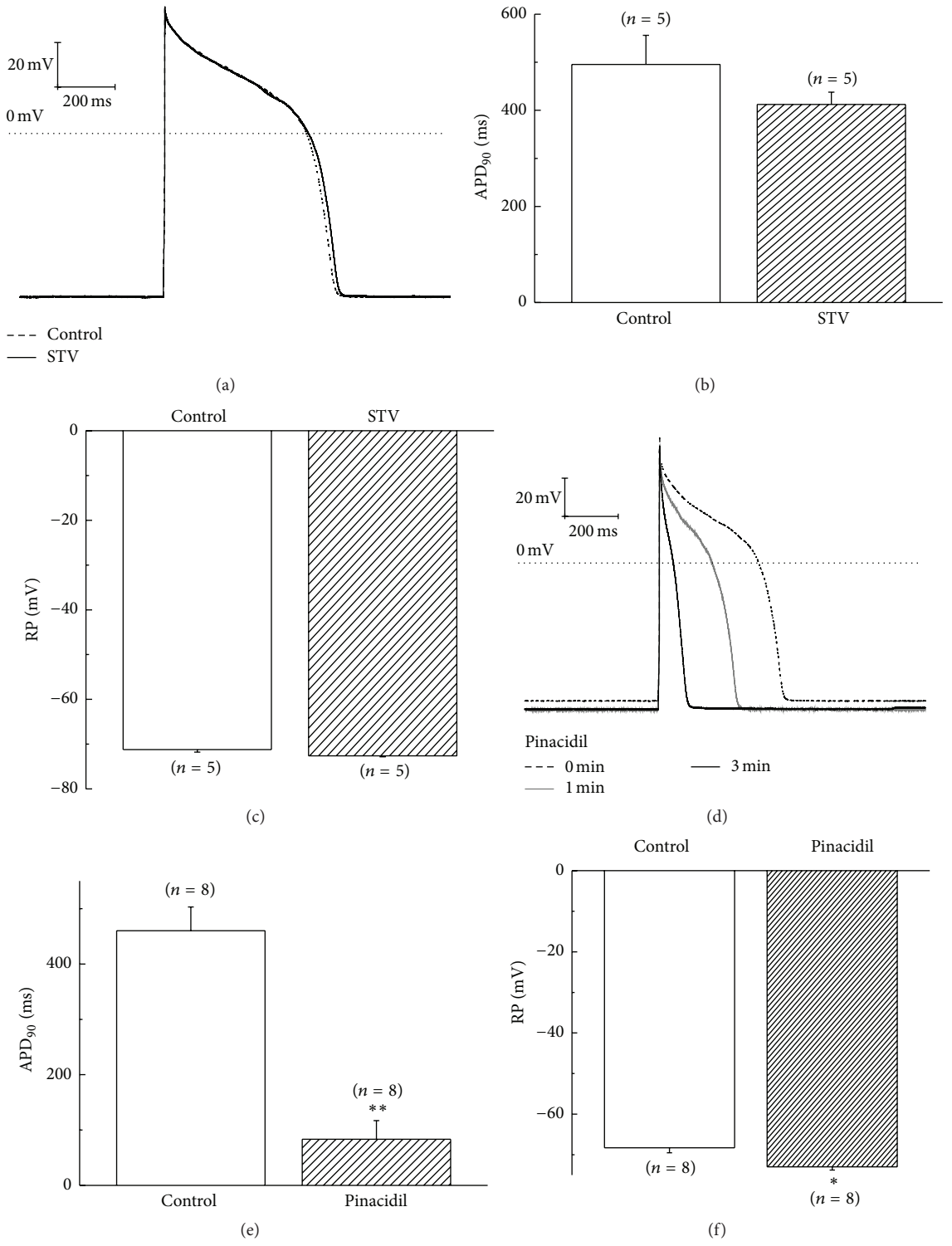

Figure 5: Effects of STV and pinacidil on action potential of guinea pig ventricular myocytes. (a) Representative AP curve of STV-preincubated and control ventricular myocytes. (b) and (c) Effects of STV on action potential duration at 90% of repolarization (APD_{90}) and resting membrane potential (RMP), respectively. (d) Effect of pinacidil on action potential of ventricular myocytes. (e) and (f) Effects of pinacidil on action potential duration at APD_{90} and RMP, respectively. $*$ means $p < 0.05$ and $**$ means $p < 0.01$.

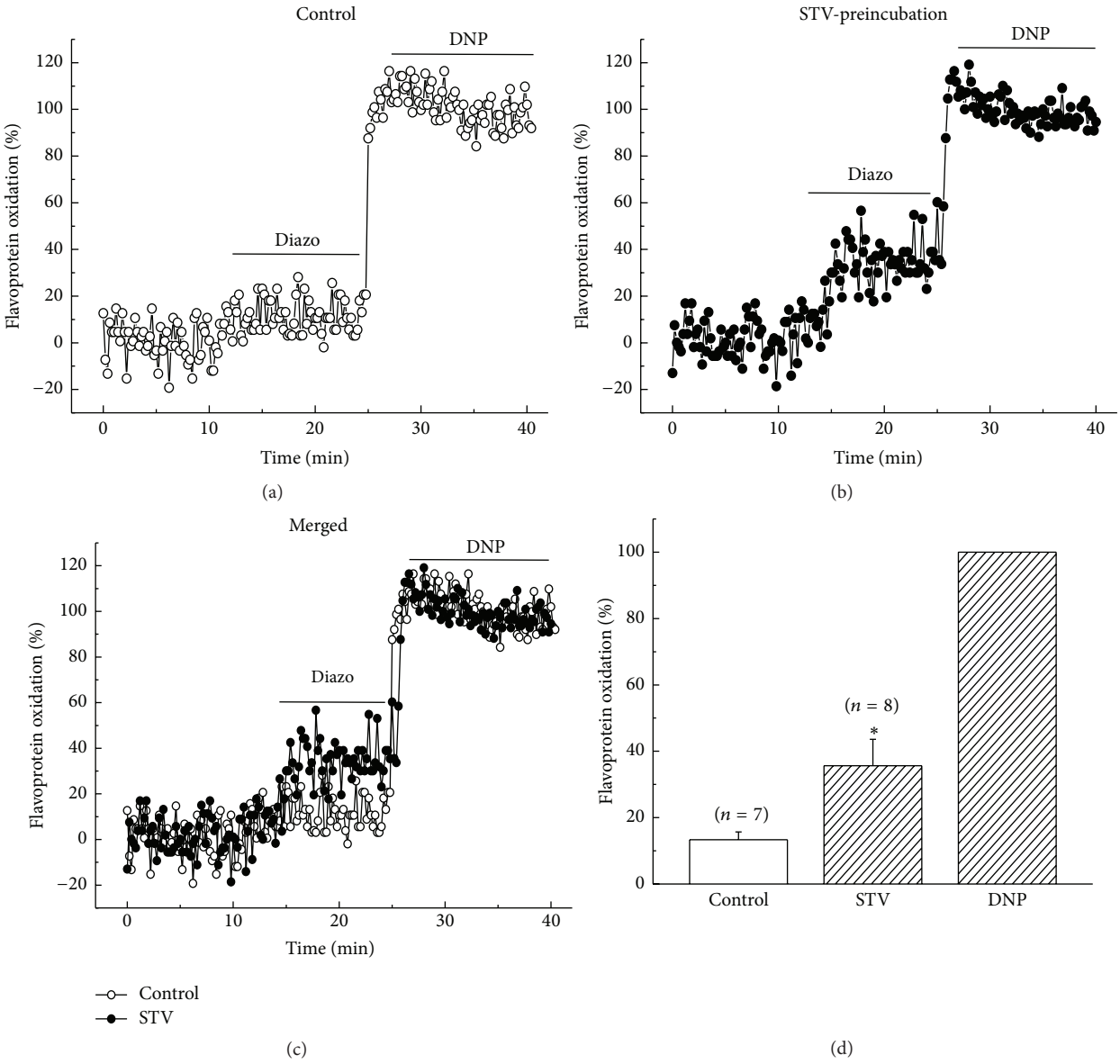

Figure 6: Effect of STV-preincubation on fluorescence intensity of flavoprotein induced by diazoxide. (a) and (b) Time course of flavoprotein fluorescence induced by diazoxide for control and STV-preincubation group. (c) A merged figure from (a) and (b). (d) The mean values of flavoprotein fluorescence normalized by DNP of control and STV-preincubation group. The preincubation time was no less than 1 hour.

enhanced the intensity of diazoxide-induced flavoprotein fluorescence. The normalized fluorescence intensity was 35.6 ± 8.0% for STV-preincubated group, which was about 2.5 times larger than control group (13.4 ± 2.4%) ($n = 6$, $p < 0.05$, Figure 6(c)). STV alone did not induce an obvious flavoprotein fluorescence compared with baseline (Figure 6(b), 0–10 min).

3.7. STV Had No Effect on Flavoprotein Oxidation When Applied after Diazoxide. In addition, we also investigated the effect of STV-application after diazoxide. Figures 7(a) and 7(b) showed that 10 μM STV-application after diazoxide did not induce a significant change in the fluorescence intensity of flavoprotein either. The percentage oxidations induced by diazoxide and diazoxide + STV were 11.8 ± 1.4% and 8.5 ± 1.1%, respectively ($n = 6$). These results suggest that STV had no influences on the uncoupling effect of diazoxide when applied after diazoxide.

3.8. The Potentiation Effects of STV on SarcK$_{ATP}$ Current and Flavoprotein Oxidation Were ROS Dependent. To examine if the potentiation effects exerted by STV on sarcK$_{ATP}$ channels and mitochondrial flavoprotein oxidation were mediated by ROS, the ROS scavenger NAC was coadministered. Figure 8(a) shows that preincubation of cardiomyocytes with 10 μM STV significantly increased the current density elicited by 50 μM pinacidil (22.5 ± 2.3 pA/pF ($n = 7$) for STV group versus 12.8 ± 1.0 pA/pF ($n = 8$)

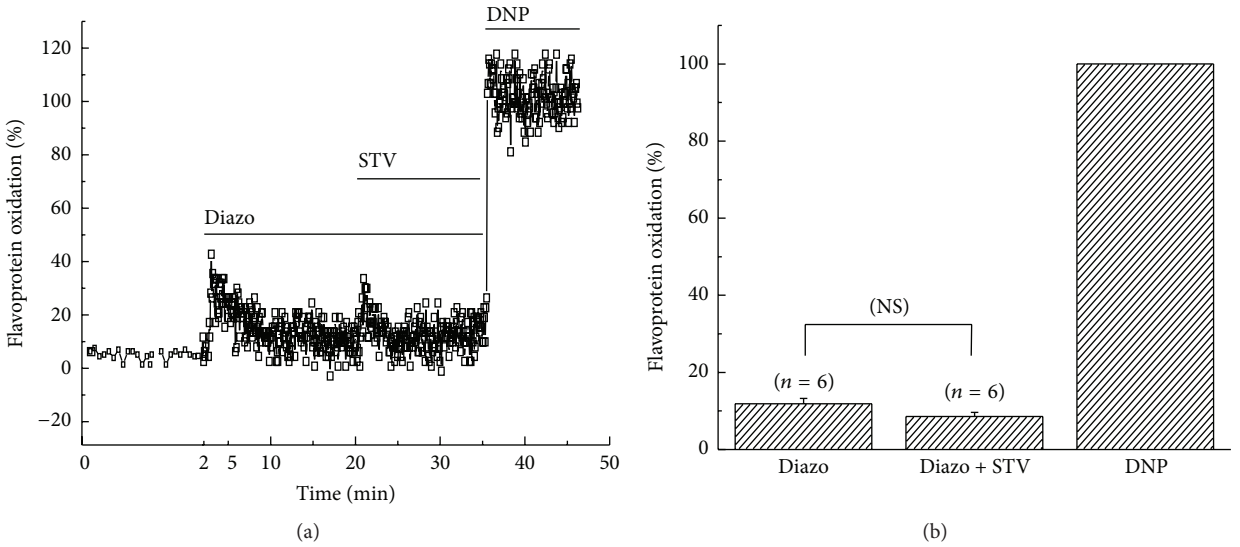

Figure 7: Effect of STV on flavoprotein oxidation applied after diazoxide. (a) Time course of flavoprotein fluorescence induced by diazoxide (Diazo) and Diazo + STV subsequently. The first 2 minutes-duration was elongated to get a good view of baseline. (b) Normalized mean values of fluorescence intensity caused by Diazo and Diazo + STV, respectively.

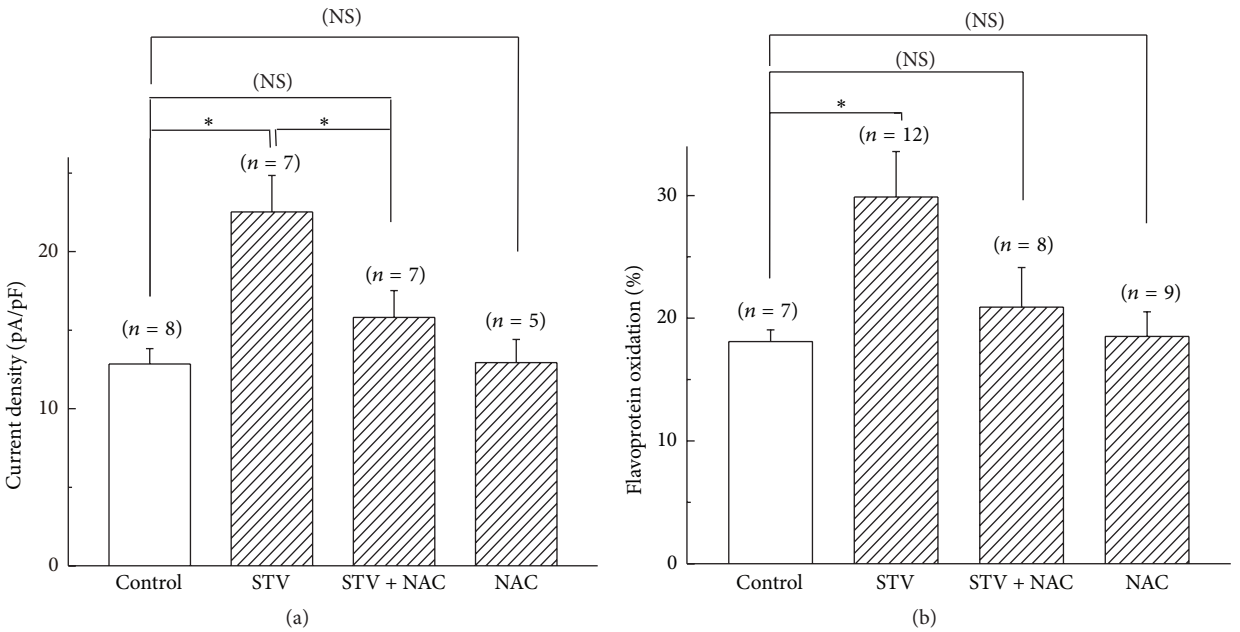

Figure 8: NAC blocked the potentiation effects of STV on sarc- and mitoK_{ATP} channels. (a) Current density of $I_{sarcK_{ATP}}$ elicited by pinacidil for control, STV-preincubation, STV + NAC, and NAC group. (b) The DNP normalized fluorescence intensity induced by diazoxide for control, STV-preincubation, STV + NAC, and NAC group. $*$ means $p < 0.05$ and NS means no significant difference.

for control group, $p < 0.05$), whereas coadministering with 100 μM NAC blocked the enhancing effect of STV (15.8 ± 1.7 pA/pF ($n = 7$) for STV + NAC group). NAC (100 μM) alone had no effects on pinacidil-elicited sarcK_{ATP} channel current. For mitochondria, NAC also blocked the potentiation effect of STV on diazoxide-elicited flavoprotein fluorescence. Figure 8(b) shows that the percentage oxidations to DNP induced by 200 μM diazoxide were 18.1 ± 1.0%, 29.9 ± 3.7%, and 20.9 ± 3.2% for control, STV, and STV + NAC group, respectively. Similarly, NAC (100 μM) alone had no effect on the fluorescence intensity of flavoprotein elicited by diazoxide (18.5 ± 2.0% for NAC alone group).

4. Discussion

The present study investigated the cardioprotective mechanism of isosteviol by examining the effect of isosteviol

sodium on sarcolemmal K_{ATP} channel and mitochondrial flavoprotein oxidation on guinea pig ventricular myocytes. For sarcK_{ATP}, isosteviol enhanced both pinacidil-activated $I_{K_{ATP}}$ current amplitude and the rate of activation. The effect of isosteviol on sarcK_{ATP} channel currents was time and dose dependent. The longer period or larger dose of incubation with isosteviol resulted in stronger enhancement of K_{ATP} currents. Similarly, in mitochondria, preincubating cells with isosteviol sodium significantly enhanced diazoxide-induced mitochondrial uncoupling indicated by mitochondrial flavoprotein fluorescence, which was not elicited by isosteviol alone. The ROS scavenger NAC prevented STV induced potentiation of both sarcK_{ATP} activity and mitochondrial oxidation. If the diazoxide-induced flavoprotein oxidation could be attributed to mitoK_{ATP} channel activity, then these results indicate that isosteviol, as a modulator but not a direct opener, sensitizes both sarc- and mitoK_{ATP} channels to their openers and the effects of STV on K_{ATP} channels are ROS dependent. Furthermore, the result that STV applied after diazoxide induced no effect on mitoK_{ATP} channel activity suggests that mitoK_{ATP} channels may not respond to isosteviol during activation.

ATP-sensitive potassium channels are thought to provide mechanisms for adaptation of cardiac myocytes to various stresses, including hypoxia, ischemia, hypertensive, and hypertrophy. The cardioprotective role of K_{ATP} channel opening against IR injury by pharmacological method or IPC has been extensively studied. Both sarc- and mitoK_{ATP} channels are demonstrated to be involved. Opening of sarcK_{ATP} channels produced by hypoxia, ischemia, or pharmacological K_{ATP} openers would hyperpolarize cell membrane, shorten action potential duration, inhibit calcium influx, and finally lead to a cardioprotective effect by depression of contractility [11–13]. Opening of mitoK_{ATP} channels could also protect the heart against ischemia-reperfusion (IR) injury by regulating mitochondria matrix volume and maintaining or enhancing ATP synthesis [14]. However, in metabolic stress-induced pathological conditions, the sensitivity of K_{ATP} channel to the metabolic ligand ATP or its pharmacological openers is dramatically diminished. For example, in a model of heart failure induced by transgenic expression of the cytokine tumor factor alpha (TNFα), the recognition of the ligand ATP for K_{ATP} channels was significantly impaired [15]. Another study showed that the response of K_{ATP} channel to its opener was markedly diminished for hypertensive rats [16]. Myocardial pathological hypertrophy not only reduces the responsiveness of K_{ATP} channel to ATP but also makes K_{ATP} channel fail to sensitize the opener cromakalim [17, 18]. The impaired responsiveness of K_{ATP} channel to the metabolic ligand or KCOs disrupts K_{ATP} channel mediated cellular stress tolerance. The results of this study showed that isosteviol elevated the sensitivity of both sarc- and mitoK_{ATP} channels to their respective openers, which suggests a potential value of STV in treating stress-induced diseases like hypertrophy or heart failure. In another hand, isosteviol itself did not activate these channels directly like other K_{ATP} channel openers. This is an advantage over classic KCOs which could open K_{ATP} channel directly and are often arrhythmogenic as a consequence. The result that isosteviol did not induce action potential shortening as pinacidil did further confirmed the nonarrhythmogenic property of isosteviol.

Reactive oxygen species (ROS) are a kind of free radical in the living organisms and the main source of them is assumed to be mitochondria [32–34]. Originally, ROS were considered to be the by-products of normal physiological processes and have only destructive roles to the organism metabolism. Recent studies tend to support that moderate increase in ROS plays a critical second messenger role in a variety of physiological functions. In cardiomyocytes, it has been demonstrated that a small increase of ROS level is essential for activating the signaling pathways in IPC and leads to cardioprotection against IR injury [35, 36]. Studies have demonstrated that in the mechanism of IP protection, opening of mitoK_{ATP} channels increased the ROS production, which phosphorylation dependently feed back to K_{ATP} channel again and make the channel open persistently [37]. The results of this study consistently showed that the sensitization effects of STV on sarc- and mitoK_{ATP} channels are ROS dependent. However, the specific molecular mechanism and signaling pathway still remain unclear. For signal transduction of STV to mitochondria, cGMP and PKG may serve as critical factors, considering that almost all signaling cascades for ischemic or pharmacological conditions start from GPCR of sarcolemma and use cGMP as a second messenger and PKG as the terminal kinase interacting with mitochondria [37]. PKC may function as a vital signaling molecular for the signal transduction in mitochondria. Studies demonstrated that in the IP protection cascade mitoK_{ATP} channel opening causes an increase in ROS production, which activates protein kinase C (PKCε), which prolongs the phosphorylation-dependent open state of mitoK_{ATP} by forming a positive feedback loop [37]. For sarcK_{ATP} channels, the mechanism by which STV potentiates the channel activity via a ROS dependent manner is also not well known. Sukhodub et al. in their study identified that AMP-activated kinase (AMPK) is essential for preconditioning-induced recruitment of sarcolemmal K_{ATP} channels [38]. Besides, several investigations have suggested that PKC is involved in controlling the distribution and maintaining an elevated sarcK_{ATP} channel concentration [39, 40]. We speculate, in this study, that AMPK and PKC may be also involved in the ROS dependent potentiation effect of STV on sarcK_{ATP} channels by regulating the trafficking and expression of the channel on sarcolemma.

Since the isolated mitochondria or mitoplasts are unavoidably contaminated with plasma membranes, many studies on mitoK_{ATP} channels tend to use indirect approaches on intact cells by measuring flavoprotein fluorescence, swelling-induced light scatter, or changes of mitochondrial membrane potential. In this study, we investigate the change of flavoprotein fluorescence induced by diazoxide and/or STV and attributed it to the activity of mitoK_{ATP} channels. However, this is a little arbitrary. In fact, the exact molecular composition of mitoK_{ATP} channel remains elusive and the mitochondrial pathways of cardioprotective mechanism have not been clearly understood. Evidence for the existence of a mitochondrial K_{ATP} channel and its

cardioprotective role is largely pharmacological. Diazoxide and 5-HD, which are used as selective mitoK$_{ATP}$ opener and blocker, have been criticized due to the lack of specificity and several off-target effects. For example, diazoxide has been identified to inhibit mitochondrial respiratory complex II (succinate dehydrogenase) [41]. Furthermore, the specificity of diazoxide has been questioned, since it could activate sarcK$_{ATP}$ channel at higher concentration [42]. However, these evidences could not absolutely exclude the existence of mitoK$_{ATP}$ channel. Unlike their counterparts in the sarcolemma, which are composed of Kir6.xs and SURs, mitoK$_{ATP}$ channel may be a multiprotein complex containing complex II (succinate dehydrogenase) as an important regulator or component. Inhibition of complex II by diazoxide or other inhibitors may activate mitoK$_{ATP}$ channels [43, 44]. As a supplement, we measured the effect of diazoxide with different concentrations on mitochondria membrane potential. The results showed that although 100 μM diazoxide did not cause a change, concentration of 200 and 300 μM did induce mitochondrial membrane depolarization (data are not shown). This complementary experiment may indicate that the diazoxide-induced flavoprotein oxidation is caused by mitochondrial membrane potential depolarization through probable potassium ion transport. However, this conclusion may be also arbitrary considering that diazoxide could also act as a protonophore and depolarize mitochondria by facilitating transmembrane proton translocation [45].

From this study we still cannot get a clue about how isosteviol sensitizes sarc- or mitoK$_{ATP}$ channels. It is possible that STV may slightly compromise mitochondrial functions and disturb overall cellular energies, which increased the susceptibility of mitochondrial to uncoupling (induced by diazoxide) and elevated the sensitivity of sarcK$_{ATP}$ channels towards KCOs (pinacidil) due to a decreased intracellular ATP/ADP ratio. This speculation would be consistent with the effects of prolonged incubation of cardiomyocytes with STV (Figure 3) and the absence of an acute effect of this drug on flavoprotein fluorescence following diazoxide application (Figure 7). Bienengraeber and his coworkers suggested in their study that SUR of K$_{ATP}$ channel has the intrinsic ATPase activity and KCOs could increase the open probability of K$_{ATP}$ channel by binding to SUR and promoting its ATPase activity [46]. So, the STV may also sensitize K$_{ATP}$ channels by playing a role in the ATPase activity of SURs.

In conclusion, the present study demonstrates that isosteviol sodium could modulate sarcK$_{ATP}$ channel by increasing the pinacidil-elicited transmembrane current. It may also, although not absolutely, potentiate the mitoK$_{ATP}$ channel activity. These potentiation effects of STV on sarc- and mitoK$_{ATP}$ channels are ROS dependent. However, the signal pathway in which isosteviol modulates sarc- or mitoK$_{ATP}$ channel is not clear from this study and merits further investigation. Furthermore, we only studied the sensitization effects of STV on cardiomyocytes in physiological state. Whether or not STV would increase the sensitivity of K$_{ATP}$ channel impaired in pathological conditions needs to be further investigated.

Abbreviations

SUR: Sulfonylurea receptor
IPC: Ischemic preconditioning
IR: Ischemia-reperfusion
KCO: Potassium channel opener
5-HD: 5-Hydroxydecanoate
ROS: Reactive oxygen species
STV: Isosteviol
NAC: N-Acetyl-cysteine
APD: Action potential duration
DNP: Dinitrophenol
RMP: Resting membrane potential
PKC: Protein kinase C
AMPA: AMP-activated kinase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] A. Noma, "ATP-regulated K^+ channels in cardiac muscle," *Nature*, vol. 305, no. 5930, pp. 147–148, 1983.

[2] F. M. Ashcroft, "Adenosine 5′-triphosphate-sensitive potassium channels," *Annual Review of Neuroscience*, vol. 11, pp. 97–118, 1988.

[3] A. Terzic, A. Jahangir, and Y. Kurachi, "Cardiac ATP-sensitive K^+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs," *The American Journal of Physiology—Cell Physiology*, vol. 269, no. 3, pp. C525–C545, 1995.

[4] N. Inagaki, T. Gonoi, J. P. Clement et al., "Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor," *Science*, vol. 270, no. 5239, pp. 1166–1170, 1995.

[5] S. J. Tucker, F. M. Gribble, C. Zhao, S. Trapp, and F. M. Ashcroft, "Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor," *Nature*, vol. 387, no. 6629, pp. 179–183, 1997.

[6] M. A. Burke, R. K. Mutharasan, and H. Ardehali, "The sulfonylurea receptor, an atypical ATP-binding cassette protein, and its regulation of the KATP channel," *Circulation Research*, vol. 102, no. 2, pp. 164–176, 2008.

[7] T. Miki, K. Nagashima, F. Tashiro et al., "Defective insulin secretion and enhanced insulin action in K$_{ATP}$ channel-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 18, pp. 10402–10406, 1998.

[8] F. M. Ashcroft, "ATP-sensitive potassium channelopathies: focus on insulin secretion," *Journal of Clinical Investigation*, vol. 115, no. 8, pp. 2047–2058, 2005.

[9] G. C. Kane, X.-K. Liu, S. Yamada, T. M. Olson, and A. Terzic, "Cardiac KATP channels in health and disease," *Journal of Molecular and Cellular Cardiology*, vol. 38, no. 6, pp. 937–943, 2005.

[10] M. V. Cohen, C. P. Baines, and J. M. Downey, "Ischemic preconditioning: from adenosine receptor to KATP channel," *Annual Review of Physiology*, vol. 62, no. 1, pp. 79–109, 2000.

[11] W. C. Cole, C. D. McPherson, and D. Sontag, "ATP-regulated K+ channels protect the myocardium against ischemia/reperfusion damage," *Circulation Research*, vol. 69, no. 3, pp. 571–581, 1991.

[12] G. J. Gross and J. A. Auchampach, "Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs," *Circulation Research*, vol. 70, no. 2, pp. 223–233, 1992.

[13] T. Mizumura, K. Nithipatikom, and G. J. Gross, "Bimakalim, an ATP-sensitive potassium channel opener, mimics the effects of ischemic preconditioning to reduce infarct size, adenosine release, and neutrophil function in dogs," *Circulation*, vol. 92, no. 5, pp. 1236–1245, 1995.

[14] K. D. Garlid, P. Dos Santos, Z.-J. Xie, A. D. T. Costa, and P. Paucek, "Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K^+ channel in cardiac function and cardioprotection," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1606, no. 1–3, pp. 1–21, 2003.

[15] D. M. Hodgson, L. V. Zingman, G. C. Kane et al., "Cellular remodeling in heart failure disrupts K_{ATP} channel-dependent stress tolerance," *The EMBO Journal*, vol. 22, no. 8, pp. 1732–1742, 2003.

[16] H. Takaba, T. Nagao, S. Ibayashi, T. Kitazono, K. Fujii, and M. Fujishima, "Altered cerebrovascular response to a potassium channel opener in hypertensive rats," *Hypertension*, vol. 28, no. 1, pp. 143–146, 1996.

[17] F. Yuan, N. R. Brandt, J. M. Pinto, B. J. Wasserlauf, R. J. Myerburg, and A. L. Bassett, "Hypertrophy decreases cardiac K_{ATP} channel responsiveness to exogenous and locally generated (Glycolytic) ATP," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 10, pp. 2837–2848, 1997.

[18] Z. V. Alvin, R. M. Millis, W. Hajj-Mousssa, and G. E. Haddad, "ATP-sensitive potassium channel currents in eccentrically hypertrophied cardiac myocytes of volume-overloaded rats," *International Journal of Cell Biology*, vol. 2011, Article ID 838951, 6 pages, 2011.

[19] J. M. C. Geuns, "Stevioside," *Phytochemistry*, vol. 64, no. 5, pp. 913–921, 2003.

[20] M. S. Melis and A. R. Sainati, "Participation of prostaglandins in the effect of stevioside on rat renal function and arterial pressure," *Brazilian Journal of Medical and Biological Research*, vol. 24, no. 12, pp. 1269–1276, 1991.

[21] P. Chan, D.-Y. Xu, J.-C. Liu et al., "The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats," *Life Sciences*, vol. 63, no. 19, pp. 1679–1684, 1998.

[22] Y.-H. Hsu, J.-C. Liu, P.-F. Kao et al., "Antihypertensive effect of stevioside in different strains of hypertensive rats," *Chinese Medical Journal*, vol. 65, no. 1, pp. 1–6, 2002.

[23] M.-H. Hsieh, P. Chan, Y.-M. Sue et al., "Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study," *Clinical Therapeutics*, vol. 25, no. 11, pp. 2797–2808, 2003.

[24] K. Yasukawa, S. Kitanaka, and S. Seo, "Inhibitory effect of stevioside on tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin," *Biological and Pharmaceutical Bulletin*, vol. 25, no. 11, pp. 1488–1490, 2002.

[25] J.-C. Liu, P.-F. Kao, M.-H. Hsieh, Y.-J. Chen, and P. Chan, "The antihypertensive effect of stevioside derivative isosteviol in spontaneously hypertensive rats," *Acta Cardiologica Sinica*, vol. 17, no. 3, pp. 133–140, 2001.

[26] J. Ma, Z. Ma, J. Wang et al., "Isosteviol reduces plasma glucose levels in the intravenous glucose tolerance test in Zucker diabetic fatty rats," *Diabetes, Obesity and Metabolism*, vol. 9, no. 4, pp. 597–599, 2007.

[27] W. Tan, "The use of kauranes compounds in the manufacture medicament," PCT Patent WO/110383, World Intelleture Property Organization, 2005.

[28] D. Xu, S. Zhang, D. Foster Jr., and J. Wang, "The effects of isosteviol against myocardium injury induced by ischaemia-reperfusion in the isolated guinea pig heart," *Clinical and Experimental Pharmacology and Physiology*, vol. 34, no. 5-6, pp. 488–493, 2007.

[29] D. Xu, Y. Li, J. Wang, A. K. Davey, S. Zhang, and A. M. Evans, "The cardioprotective effect of isosteviol on rats with heart ischemia-reperfusion injury," *Life Sciences*, vol. 80, no. 4, pp. 269–274, 2007.

[30] R. Padrini, S. Bova, G. Cargnelli, D. Piovan, and M. Ferrari, "Effects of pinacidil on guinea-pig isolated perfused heart with particular reference to the proarrhythmic effect," *British Journal of Pharmacology*, vol. 105, no. 3, pp. 715–719, 1992.

[31] Y. Liu, T. Sato, B. O'Rourke, and E. Marban, "Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection?" *Circulation*, vol. 97, no. 24, pp. 2463–2469, 1998.

[32] P. Ježek and L. Hlavatá, "Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism," *The International Journal of Biochemistry & Cell Biology*, vol. 37, no. 12, pp. 2478–2503, 2005.

[33] A. J. Lambert and M. D. Brand, "Reactive oxygen species production by mitochondria," *Methods in Molecular Biology*, vol. 554, pp. 165–181, 2009.

[34] J. F. Turrens, "Mitochondrial formation of reactive oxygen species," *Journal of Physiology*, vol. 552, no. 2, pp. 335–344, 2003.

[35] T. Pain, X.-M. Yang, S. D. Critz et al., "Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals," *Circulation Research*, vol. 87, no. 6, pp. 460–466, 2000.

[36] M. V. Cohen, X.-M. Yang, G. S. Liu, G. Heusch, and J. M. Downey, "Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels," *Circulation Research*, vol. 89, no. 3, pp. 273–278, 2001.

[37] K. D. Garlid, A. D. T. Costa, C. L. Quinlan, S. V. Pierre, and P. Dos Santos, "Cardioprotective signaling to mitochondria," *Journal of Molecular and Cellular Cardiology*, vol. 46, no. 6, pp. 858–866, 2009.

[38] A. Sukhodub, S. Jovanović, D. U. Qingyou et al., "AMP-activated protein kinase mediates preconditioning in cardiomyocytes by regulating activity and trafficking of sarcolemmal ATP-sensitive K^+ channels," *Journal of Cellular Physiology*, vol. 210, no. 1, pp. 224–236, 2007.

[39] K. Hu, C. S. Huang, Y. N. Jan, and L. Y. Jan, "ATP-sensitive potassium channel traffic regulation by adenosine and protein kinase C," *Neuron*, vol. 38, no. 3, pp. 417–432, 2003.

[40] A. G. Edwards, M. L. Rees, R. A. Gioscia et al., "PKC-permitted elevation of sarcolemmal KATP concentration may explain female-specific resistance to myocardial infarction," *The Journal of Physiology*, vol. 587, no. 23, pp. 5723–5737, 2009.

[41] P. J. Hanley, M. Mickel, M. Löffler, U. Brandt, and J. Daut, "KATP channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart," *Journal of Physiology*, vol. 542, part 3, pp. 735–741, 2002.

[42] N. D'hahan, C. Moreau, A. L. Prost et al., "Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 21, pp. 12162–12167, 1999.

[43] A. P. Wojtovich and P. S. Brookes, "The complex II inhibitor atpenin A5 protects against cardiac ischemia-reperfusion injury via activation of mitochondrial KATP channels," *Basic Research in Cardiology*, vol. 104, no. 2, pp. 121–129, 2009.

[44] A. P. Wojtovich, K. W. Nehrke, and P. S. Brookes, "The mitochondrial complex II and ATP-sensitive potassium channel interaction: quantitation of the channel in heart mitochondria," *Acta Biochimica Polonica*, vol. 57, no. 4, pp. 431–434, 2010.

[45] E. L. Holmuhamedov, A. Jahangir, A. Oberlin, A. Komarov, M. Colombini, and A. Terzic, "Potassium channel openers are uncoupling protonophores: implication in cardioprotection," *FEBS Letters*, vol. 568, no. 1–3, pp. 167–170, 2004.

[46] M. Bienengraeber, A. E. Alekseev, M. R. Abraham et al., "ATPase activity of the sulfonylurea receptor: a catalytic function for the K_{ATP} channel complex," *The FASEB Journal*, vol. 14, no. 13, pp. 1943–1952, 2000.

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Research Article

Notch1 Pathway Protects against Burn-Induced Myocardial Injury by Repressing Reactive Oxygen Species Production through JAK2/STAT3 Signaling

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Oxidative stress plays an important role in burn-induced myocardial injury, but the cellular mechanisms that control reactive oxygen species (ROS) production and scavenging are not fully understood. This study demonstrated that blockade of Notch signaling via knockout of the transcription factor RBP-J or a pharmacological inhibitor aggravated postburn myocardial injury, which manifested as deteriorated serum CK, CK-MB, and LDH levels and increased apoptosis *in vitro* and *in vivo*. Interruption of Notch signaling increased intracellular ROS production, and a ROS scavenger reversed the exacerbated myocardial injury after Notch signaling blockade. These results suggest that Notch signaling deficiency aggravated postburn myocardial injury through increased ROS levels. Notch signaling blockade also decreased MnSOD expression *in vitro* and *in vivo*. Notably, Notch signaling blockade downregulated p-JAK2 and p-STAT3 expression. Inhibition of JAK2/STAT3 signaling with AG490 markedly decreased MnSOD expression, increased ROS production, and aggravated myocardial injury. AG490 plus GSI exerted no additional effects. These results demonstrate that Notch signaling protects against burn-induced myocardial injury through JAK2/STAT3 signaling, which activates the expression of MnSOD and leads to decreased ROS levels.

1. Introduction

Severe burn injury results in multiple organ dysfunction, which is the leading cause of death in intensive care units (ICUs) [1, 2]. Myocardial injury is a major contributor to mortality, particularly in individuals with preexisting cardiac pathology [3, 4]. Numerous experimental studies have investigated the molecular mechanisms involved in burn-induced myocardial injury to create novel therapeutic interventions and agents to reduce the incidence of life-threatening complications. However, there is still a lack of effective therapies that increase myocardial resistance to burn injury despite decades of laboratory studies and clinical practice.

Mounting evidence demonstrates that oxidative stress plays an important role in burn-induced myocardial injury [5–7]. However, the cellular mechanisms that control ROS production and scavenging are not fully understood. The Notch pathway is an evolutionarily conserved signaling system that plays a crucial role in cell fate decisions, differentiation, proliferation, and apoptosis. Four Notch receptors (Notch1–4) and five Notch ligands (Delta-like1, 3, 4, and Jagged1, 2) have been identified in mammals. The binding of a Notch ligand to its receptor triggers γ-secretase-mediated proteolytic cleavage of the Notch intracellular domain (NICD), which translocates to the nucleus to form a transcription-activating complex. This complex mediates the transcription of downstream target genes such as Hes1, Hey1, and cyclin D [8].

Jagged1 and Notch1 are expressed in the adult heart [9], and these proteins protect cardiac tissue under various

pathophysiological conditions [10], including alcoholic cardiomyopathy, myocardial infarction [11], cardiac hypertrophy [12], and ischemia-reperfusion injury [13]. More recent studies have revealed that Notch1 suppresses oxidative stress in hepatocytes [14] and endothelial cells [15]. Notably, we recently found that Notch1 protected against MI/R injury via the reduction of oxidative/nitrative stress [13]. However, whether Notch1 signaling plays a role in burn-induced myocardial injury has yet to be determined. This study demonstrated that the Notch1 pathway protected against postburn myocardial injury via the repression of reactive oxygen species (ROS) production through JAK2/STAT3 signaling.

2. Materials and Methods

2.1. Animals. Healthy adult male Sprague-Dawley (SD) rats (weighing 200~250 g) or newborn SD rats (1–3 days old) were obtained from the Experimental Animal Center of Fourth Military Medical University. All animal experiments were performed in accordance with the guidelines from the Administration of Animal Experiments for Medical Research Purposes issued by the Ministry of Health of China. The animal ethics number was XJYYLL-2014177. Animals were fed ad libitum standard diet and water throughout the study. All animals were housed separately and kept under standard conditions at room temperature (22~24°C) in a 12 h light/12 h dark cycle.

Conditional RBP-J allele (RBP-J floxed) mice were a generous gift from Professor Hua Han, M.D., Ph.D. (Department of Medical Genetics and Developmental Biology, Fourth Military Medical University). Myh6-Cre mice were obtained from Model Animal Research Center of Nanjing University. Cardio-specific RBP-J knockout mice were generated from RBP-J floxed mice and Myh6-Cre mice. Tamoxifen was administered to each mouse, when mouse reached 6 weeks after birth, at 50 mg/kg by intraperitoneal injections once a day for 5 days.

2.2. Burn Procedure. Animals were anesthetized lightly with pentobarbital sodium (50 mg/kg). Rats were placed in a prefabricated template with a rectangular opening that exposed the dorsal skin surface but protected the remaining skin from burn exposure. This template limits the burn area to a predetermined 30% TBSA. The exposed skin surface was immersed in 95°C water for 18 s as previously described [16, 17]. Sham-burn control animals were treated identically to animals in the burn group, except that the skin was immersed in 37°C water. Mice (18–22 g) were then placed in a template estimating 30% total body surface area and subjected to a steam burn for 8 s as previously described [18]. Animals were immediately infused with Ringer's lactate solution according to Parkland's formula and received a subcutaneous injection of normal saline with 0.1 mg/kg of buprenorphine (Sigma, St. Louis, MO) for pain control. Samples were collected from burn and sham animals after the burn procedure at various time points.

2.3. Serum Collection. Animals were killed under anesthesia at each endpoint after burn injury. Rats blood samples were taken from the aortaventralis. Mice blood samples were taken by eyeball extirpating. The collected blood was centrifuged at 1500 g for 10 minutes, and the serum was gathered and stored at −80°C for further use. Serums from rats 12 h after burn were used to challenge cardiomyocytes.

2.4. Cardiomyocyte Culture and Stimulation with Burn Serum. Neonatal rat cardiomyocytes were isolated from the ventricles of one to 3-day-old Sprague-Dawley rats using a previously described procedure [19]. Cells enriched for cardiomyocytes were placed on 6 plates and maintained in Cardiac Myocyte Medium (CMM, ScienCell, USA), which included cardiac myocyte growth supplement (CMGS) and a low concentration of fetal bovine serum (5%) in a CO_2 incubator at 37°C at the indicated time points. A γ-secretase inhibitor (GSI; Calbiochem, La Jolla, CA) was used at the concentration of 75 μM, with dimethyl sulfoxide (DMSO) as a control. Burn serum was added at 15% (v/v).

2.5. Evaluation of Myocardial Injury and Apoptosis. Myocardial injury was evaluated using mice serum creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), and lactate dehydrogenase (LDH). All assays were performed using a chemistry autoanalyzer (Vitros 750, Johnson & Johnson, Rochester, USA). Cardiomyocyte injury was assessed via measurement of LDH release into the culture medium. Briefly, the incubation medium was stored at 4°C at the end of treatment, and the same volume of cold buffer (10 mM Tris–HCl [pH 7.4], 1 mM EDTA) was added to the cells. The cells were scraped and lysed using trituration. Lysates were centrifuged at 4°C, and the supernatant was stored at 4°C. LDH levels in the medium (released LDH) and cell lysate (retained LDH) were measured using a spectrophotometric assay. The results are expressed as the percent released LDH compared with total (released plus retained) LDH.

Myocardial apoptosis was analyzed using TUNEL assays and an in situ cell death detection kit according to the manufacturers' protocol. Cardiomyocyte apoptosis was evaluated using acridine orange (AO)/ethidium bromide (EB). Cells were harvested at 70–80% confluency, seeded in 48-well plates at 2×10^5 cells/well, and incubated overnight for the cells to attach. The AO/EB solution was prepared with 100 μg/mL of each reagent. Cells were treated for 12 h with Burn serum, and each sample was stained with 100 μL of AO/EB solution just prior to microscopy and quantification. At least 300 cells were counted for each treatment, and the percentages of apoptotic (red-orange nucleus) and live (green nucleus) cells were calculated.

2.6. ROS Measurement. Cardiomyocytes were labeled with 2′, 7′-dichlorofluorescein (DCFH-DA) (S0033, Bryotime, Shanghai, China) for the measurement of intracellular ROS generation following recommended protocols. Intracellular ROS levels were determined via measurement of the oxidative conversion of cell-permeable DCFH-DA to fluorescent dichlorofluorescein (DCF). Cells were incubated with DCFH-DA at 37°C for 20 min. The DCF fluorescence

distribution of 200,000 cells was detected using flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The levels of intracellular ROS were quantified using mean fluorescent intensity (MFI), and the results were statistically compared between groups, as described.

Myocardial superoxide anion content was determined using lucigenin-enhanced luminescence [13]. Samples were weighed, cut into uniform cubes ($0.5\,mm^3$), and transferred into polypropylene tubes containing 1 mL of PBS and lucigenin (Sigma, 0.25 mmol/L). Tubes were placed in an FB12-Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany). The RLU emitted was recorded and integrated over 30 s intervals for 5 min. Activity was normalized to dry tissue weights.

2.7. Western Blot Analysis. Myocardial and cardiomyocyte samples were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 1% DTT, and 1% of a protease inhibitor cocktail. Lysates were centrifuged at 12,000 ×g for 15 min, and the resulting supernatants were transferred to a new tube and stored at −70°C. Protein concentrations were determined using a Bradford protein assay kit. Equal amounts of proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline and Tween 20 (TBST, pH 7.6) containing 5% nonfat dry milk and incubated overnight at 4°C with antibodies against Notch1 ICD and Hes1 (Abcam, Cambridge, MA), MnSOD (Santa Cruz Biotechnology), JAK2/phospho-JAK2 (Abcam, Cambridge, MA), STAT3/phospho-STAT3, and GAPDH (Cell Signaling Technology, Danvers, MA), followed by washes with TBST. The membranes were probed with appropriate secondary antibodies (1 : 3000 dilution) at room temperature for 90 min and washed with TBST. Protein bands were detected using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using the Quantity One software package (West Berkeley, CA, USA).

2.8. Statistical Analysis. All values in the text and figures are presented as means ± SEM. Data (except for Western blot density) were subjected to ANOVA followed by the Bonferroni correction for post hoc t-tests. Western blot densities were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. p values < 0.05 were considered statistically significant.

3. Results

3.1. Notch1 Pathway Responds to Myocardial Injury after Burn. Normal mice were subjected to burn injury, and the protein expression of Notch1 and Hes1 in myocardium tissue was examined at various time points from 0 to 24 h. Notch1 ICD protein levels, which are a marker of Notch1 activation, were significantly increased at an early time point (3 and 6 h) after burn application. The maximum Notch1 ICD protein levels occurred 12 h after burn application and decreased at 24 and 48 h (Figure 1(a)). Protein levels of Hes1, which is a downstream effector of Notch1 signaling, reached their peak at 12 h and were downregulated at 24 and 48 h (Figure 1(c)). A similar trend was observed in cultured rat cardiomyocytes after exposure to burn serum (Figures 1(b) and 1(d)). Notch1 ICD and Hes1 protein expression increased at early time points (6, 12 , and 24 h) and decreased at a later time point (48 h). These results demonstrated that cardiac Notch1 signaling was activated during burn injury.

3.2. Notch Signal Blockade Aggravates Burn-Induced Cardiomyocyte Apoptosis In Vitro. Rat cardiomyocytes were pretreated with GSI or vehicle, followed by the addition of burn serum to investigate the role of Notch1 signaling in cardiomyocytes postburn injury. AO/EB staining revealed significantly elevated apoptosis in cells challenged with burn serum. Notably, burn serum induced remarkably increased apoptosis when Notch signaling was blocked by GSI (Figures 2(a) and 2(b)). Burn serum also caused a significant increase in caspase-3 expression, and blockade of Notch1 signaling by GSI further aggravated caspase-3 expression (Figure 2(c)). GSI also significantly aggravated cardiomyocyte injury, as evidenced by increased LDH levels in the supernatants (Figure 2(d)).

3.3. Notch Signal Deficiency Exacerbates Burn-Induced Myocardial Injury In Vivo. We used a conditional RBP-J-knockout approach to further elucidate the role of the Notch1 signaling pathway in the postburn myocardium. Significantly higher levels of serum CK, CK-MB, and LDH were detected in RBP-J KO mice at 12 h and 24 h postburn injury compared to normal mice subjected to burn injury (Figures 3(a)–3(c)). TUNEL staining revealed that burn injury induced more apoptotic cells in RBP-J KO mice than in normal mice (Figures 3(d) and 3(e)). These data suggest that disruption of Notch1 signaling aggravated postburn myocardial injury.

3.4. Notch Blockade Leads to Increased ROS Production. We examined ROS production in rat cardiomyocytes treated with burn serum in the absence of Notch signaling. Figures 4(a) and 4(b) show that burn serum markedly increased ROS levels in cardiomyocytes as determined by FACS, and blockade of Notch signaling with GSI remarkably increased ROS levels after burn serum challenge. In the *in vivo* experiments, mice were subjected to burn injury for 12 h. The same phenomena were detected in RBP-J KO mice (Figure 4(c)). Superoxide production in the RBP-J KO mice was significantly increased in KO mice compared to control mice. These results suggested that the exacerbated myocardial injury resulting from Notch blockade was mediated by an increase in ROS production. We pretreated cardiomyocytes with GSI and exposed the cells to burn serum, followed by treatment with the ROS scavenger NAC to further investigate this hypothesis. GSI remarkably increased ROS levels in rat cardiomyocytes after burn serum challenge. In sharp contrast, NAC effectively decreased ROS in the GSI-treated and vehicle groups (Figures 5(a) and 5(b)). The GSI-treated group exhibited aggravated apoptosis and LDH levels compared to the vehicle group. Notably, NAC significantly reduced cardiomyocyte apoptosis and LDH levels (Figures 5(c), 5(d), and 5(e)). These findings

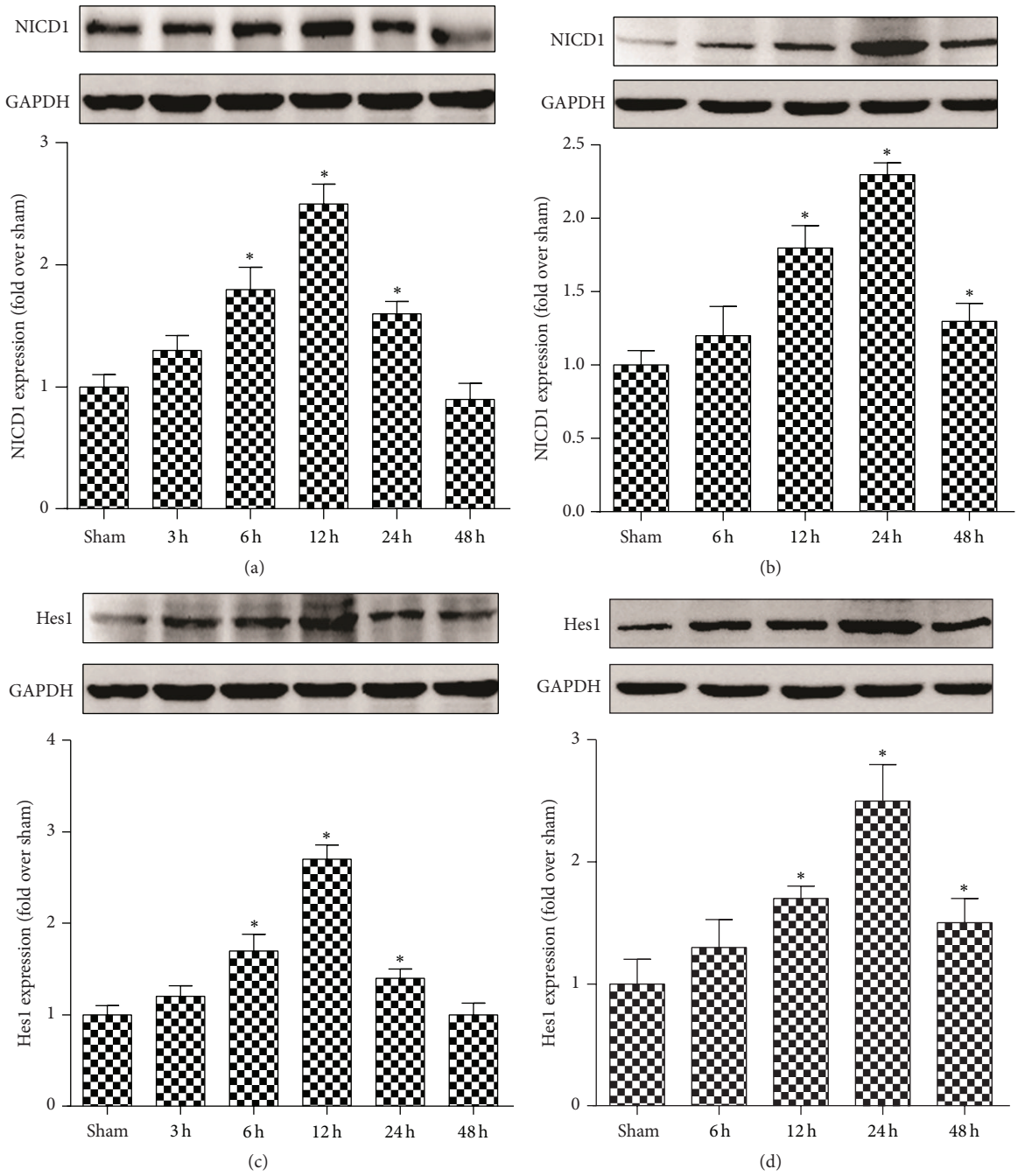


Figure 1: Notch1 pathway responds to myocardial injury after burn. Mice were subjected to burn injury. (a, c) Protein expression of Notch1 intracellular domain (NICD) and Hes1 in mouse myocardial tissue after burn injury over time. Rat cardiomyocytes were challenged with burn serum *in vitro*. (b, d) showed the NICD1 and Hes1 protein expression in cardiomyocytes. The values presented are the mean ± SEM ($n = 8$ per group). $^{*}P < 0.05$ compared to the value in sham groups.

suggest that blockade of Notch signaling aggravated postburn myocardial injury through increased ROS production.

3.5. Disruption of Notch Signal Leads to Downregulation of MnSOD. Mitochondrial respiration provides more than 90% of intracellular ROS, which is scavenged by MnSOD. The expression of MnSOD in cardiomyocytes treated with burn serum in the presence of GSI was downregulated significantly (Figure 6(a)). In the *in vivo* experiments, RBP-J KO mice subjected to burn injury also exhibited a significant downregulation of MnSOD expression in the myocardium (Figure 6(b)). These data suggest that blockade of Notch signaling downregulated MnSOD expression, which increased ROS scavenging and aggravated myocardial injury.

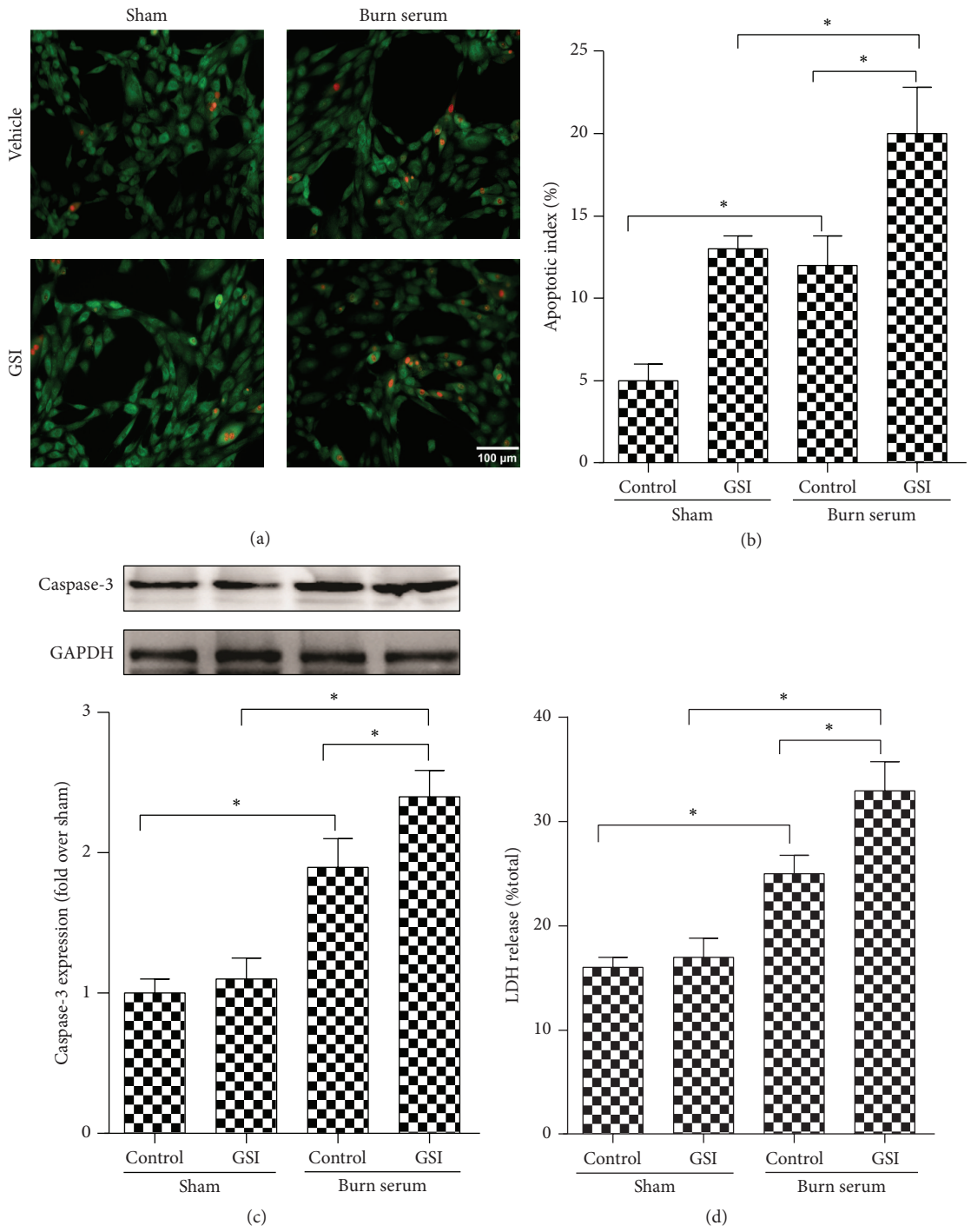


FIGURE 2: Notch signal blockade aggravates burn-induced cardiomyocyte apoptosis *in vitro*. Rat cardiomyocytes were challenged with burn serum *in vitro* in the presence of DMSO or GSI. Apoptotic cells were stained by AO/EB staining 12 hours after challenge (a, magnification ×200) and were quantified (b). Cells were collected to determine the expression of caspase-3 protein expression (c). Cell supernatants in (c) were collected and LDH production (d) was assessed. The values presented are the mean ± SEM ($n = 8$ per group). $^{*}P < 0.05$.

3.6. Notch Signal Blockade Attenuates STAT3 Activation during Burn Injury. JAK2/STAT3 signaling transactivates MnSOD, which suggests that the inhibition of Notch1 signaling downregulates the transcription of MnSOD through decreased STAT3 activation and leads to increased ROS and aggravated oxidative stress injury. Figures 6(c) and 6(d) show that burn serum significantly decreased p-JAK2 and p-STAT3 expression, and the inhibition of Notch1 signaling by GSI further attenuated p-JAK2 and p-STAT3 expression. RBP-J KO mice subjected to burn injury also exhibited a remarkable

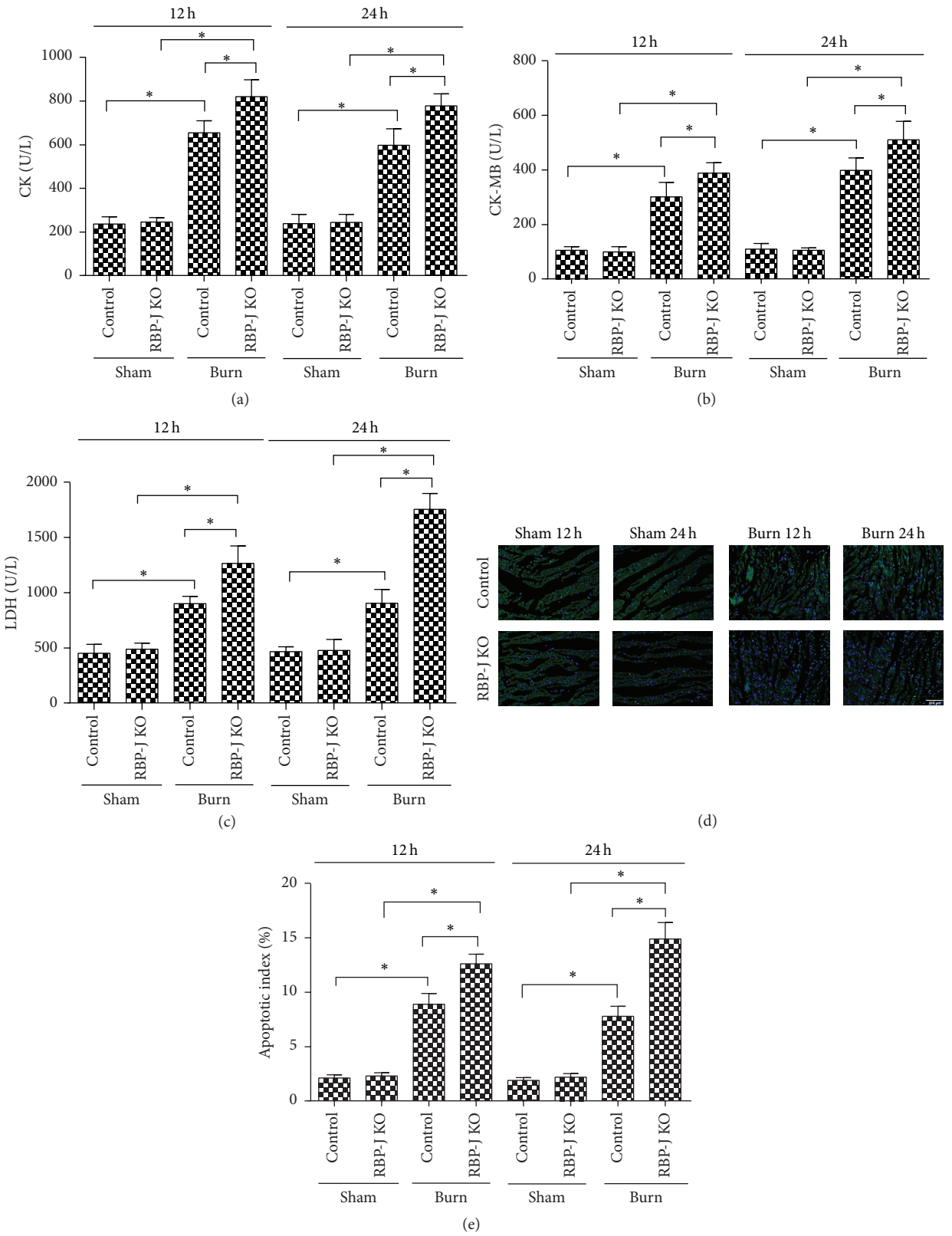


Figure 3: Notch signal deficiency exacerbates burn-induced myocardial injury *in vivo*. RBP-J KO or control mice were subjected to burn injury and were examined 12 hours or 24 hours after injury. Serum CK (a), CK-MB (b), and LDH (c). (d) Apoptotic cells were stained by TUNEL staining in myocardial tissues. (e) Quantitative comparison of apoptotic cells upon TUNEL staining in (d). The values presented are the mean ± SEM ($n = 8$ per group). $^{*}p < 0.05$.

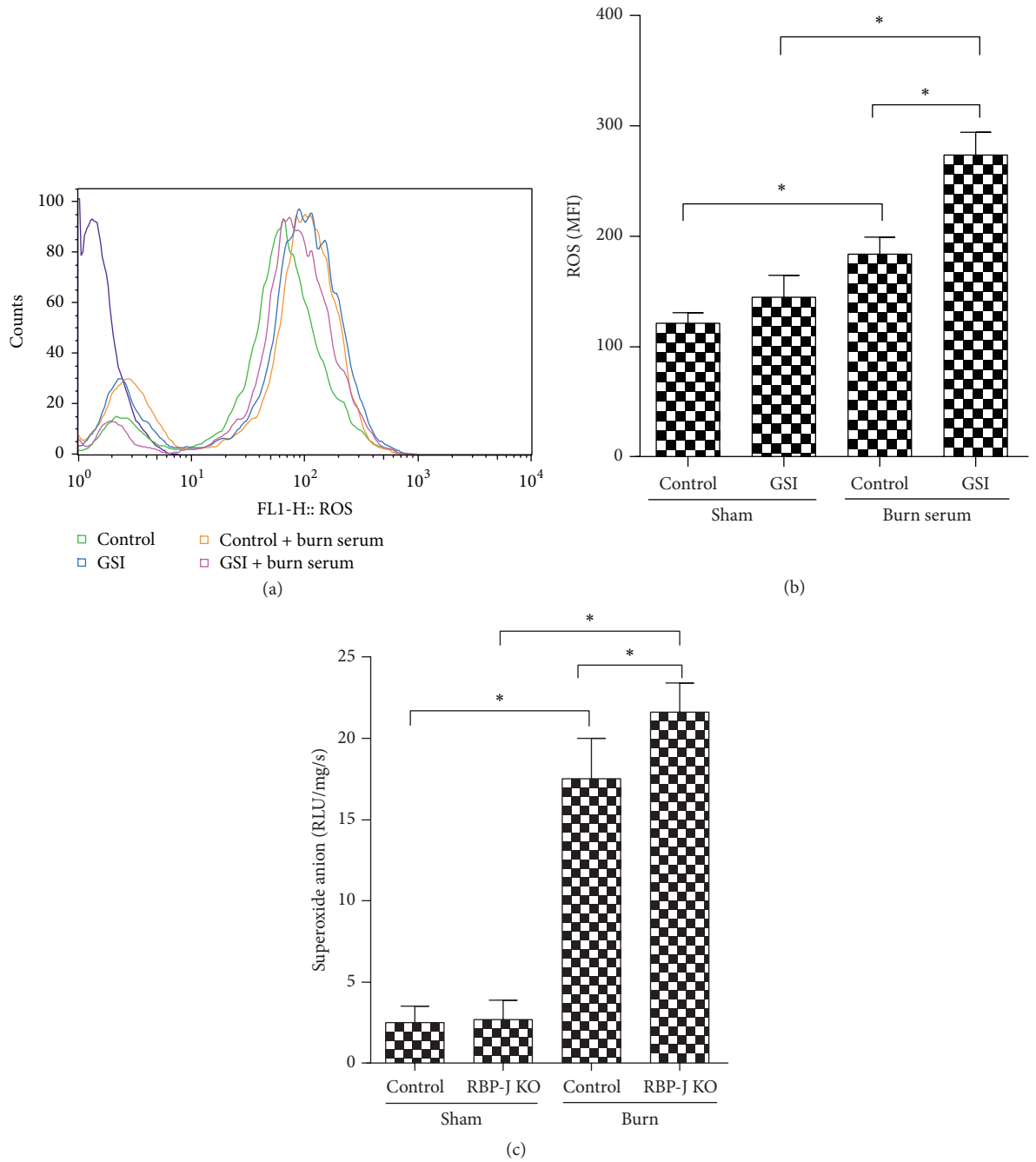


Figure 4: Notch blockade leads to increased ROS production. Rat cardiomyocytes were challenged with burn serum *in vitro* in the presence of DMSO or GSI. ROS were examined by way of FACS (a) and were quantified by way of mean fluorescence intensity (b). RBP-J KO or control mice were subjected to burn injury and were examined 12 hours after injury. (c) Myocardium tissues were isolated and examined for superoxide anions content by way of lucigenin-enhanced luminescence. The values presented are the mean ± SEM ($n = 8$ per group). $^{*}p < 0.05$.

further downregulation of p-JAK2 and p-STAT3 expression in the myocardium (Figures 6(e) and 6(f)).

3.7. Inhibition of JAK2/STAT3 Signaling Preferentially Aggravated Myocardial Injury after Burn. Our results suggest that the inhibition of Notch1 aggravates ROS production and myocardial injury via the inhibition of JAK2/STAT3 signaling. An additional series of experiments was performed to obtain more evidence to support this hypothesis. Rat cardiomyocytes were subjected to burn serum as described above and treated with either AG490 (a JAK2/STAT3 inhibitor, 2 μM) or AG490 plus GSI. Figures 7(a) and 7(b) show that treatment with AG490 significantly reduced p-JAK2 and p-STAT3 expression. Treatment with GSI plus

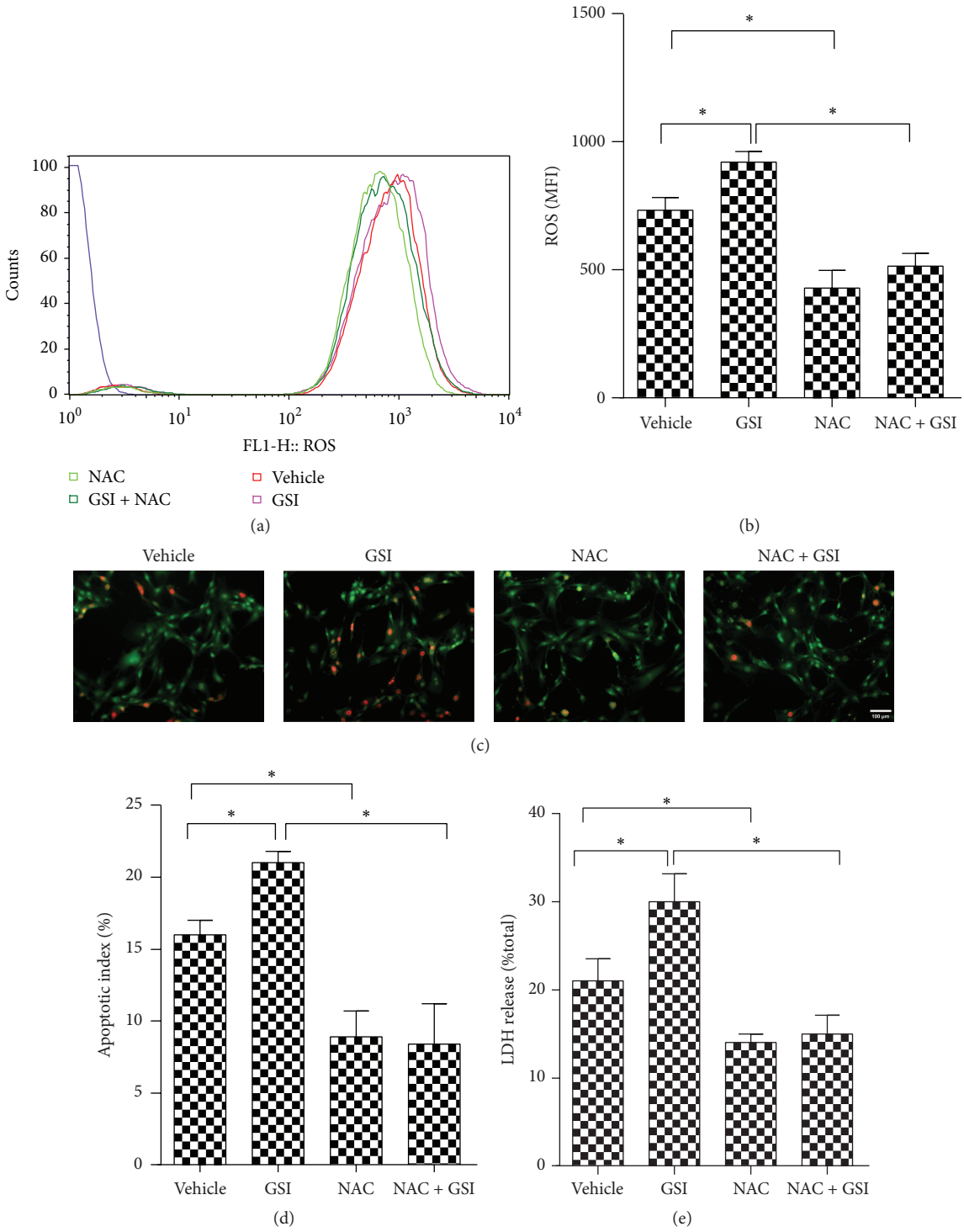
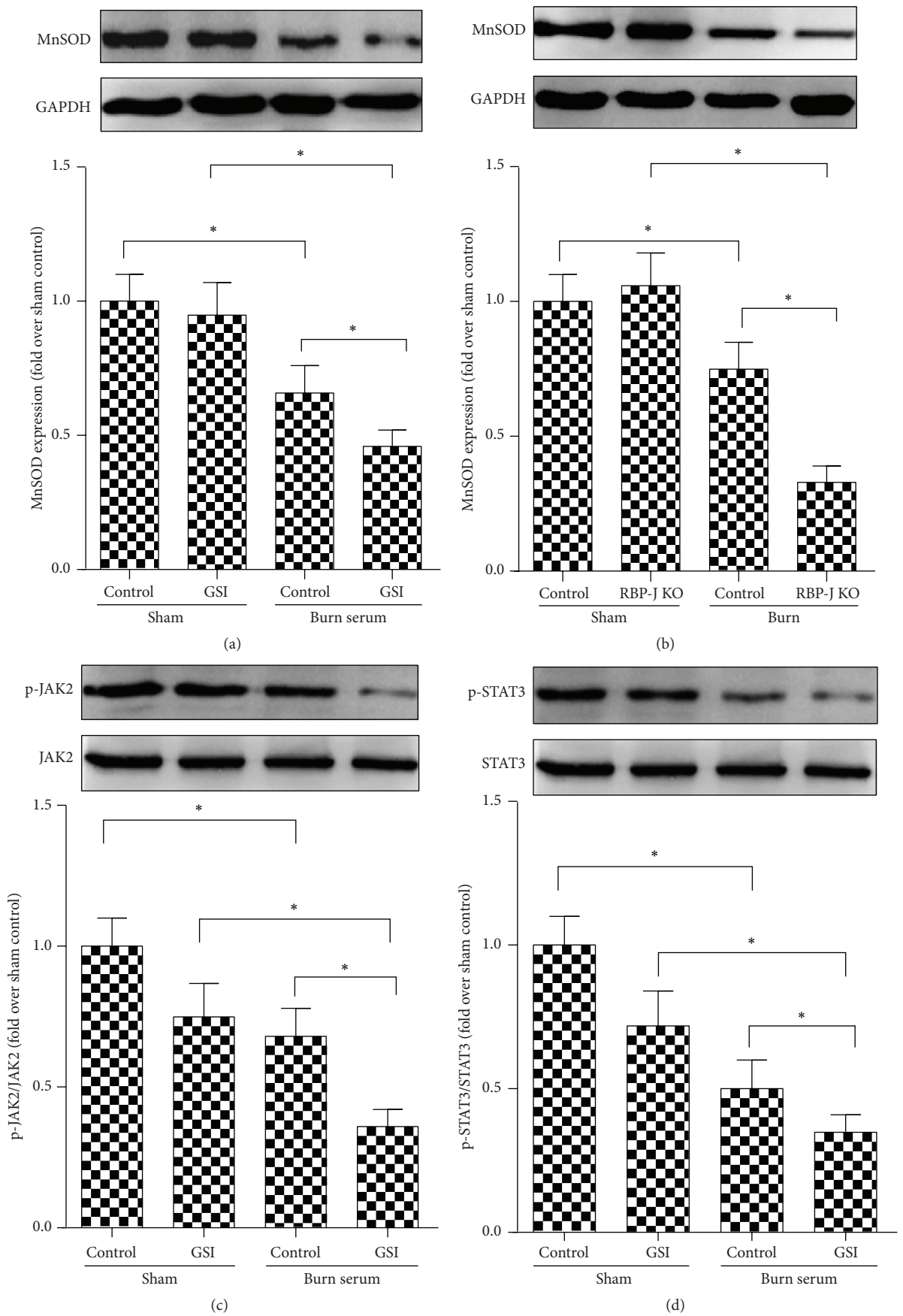


Figure 5: ROS scavenger alleviates burn-induced myocardial injury aggravated by Notch signal deficiency. Rat cardiomyocytes were challenged with burn serum *in vitro* in the presence of DMSO or GSI, with or without the ROS scavenger, NAC. ROS were examined by way of FACS (a) and were quantified by way of mean fluorescence intensity (b). (c) Apoptotic cells were stained by AO/EB staining. (d) Quantitative comparison of apoptotic cells upon AO/EB staining in (c). Cell supernatants were collected and LDH production (e) was assessed. The values presented are the mean ± SEM ($n = 8$ per group). $^{*}p < 0.05$.



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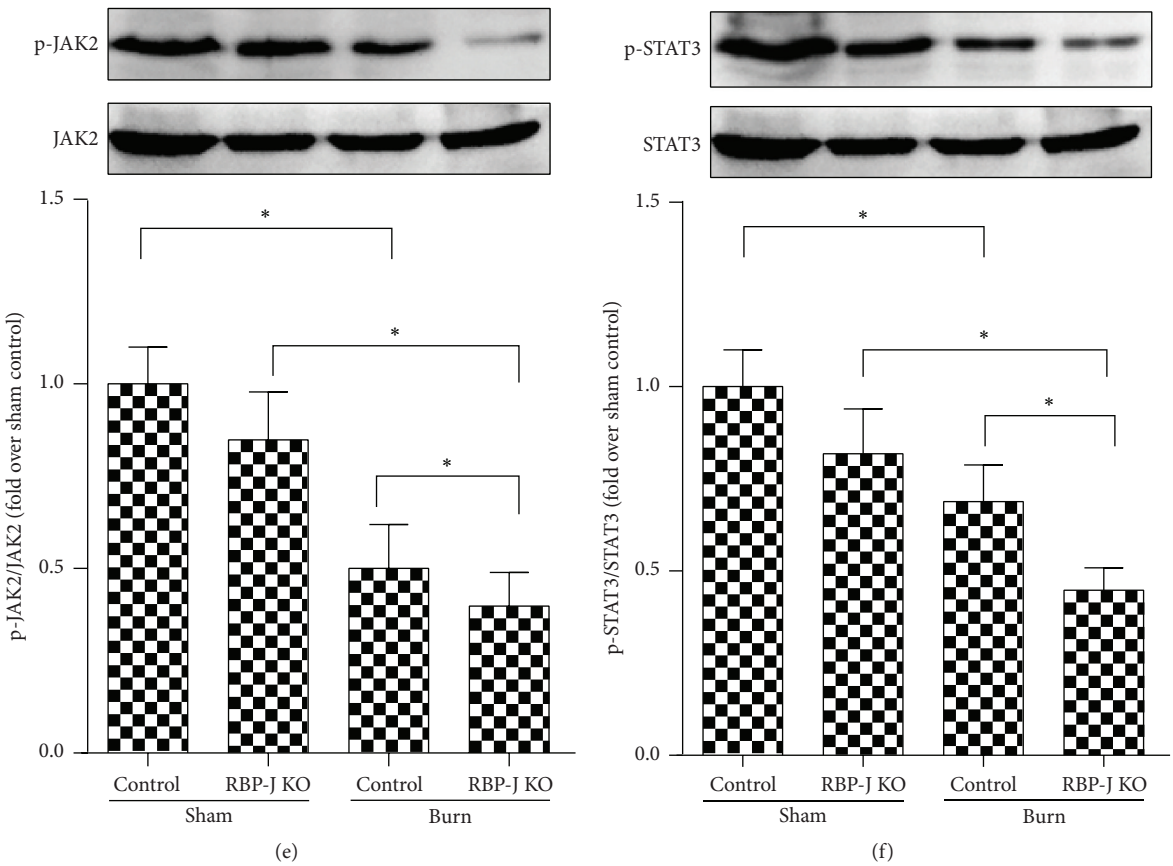


Figure 6: Notch signal blockade attenuates MnSOD and JAK2/STAT3 activation during burn injury. Rat cardiomyocytes were challenged with burn serum *in vitro* in the presence of DMSO or GSI. MnSOD (a) and JAK2/STAT3 (c, d) expression was determined by western blot in cardiomyocytes. MnSOD (b) and JAK2/STAT3 (e, f) expression in myocardial tissues of RBP-JKO and control mice subjected to burn injury for 12 hours. The values presented are the mean ± SEM ($n = 8$ per group). $^*p < 0.05$.

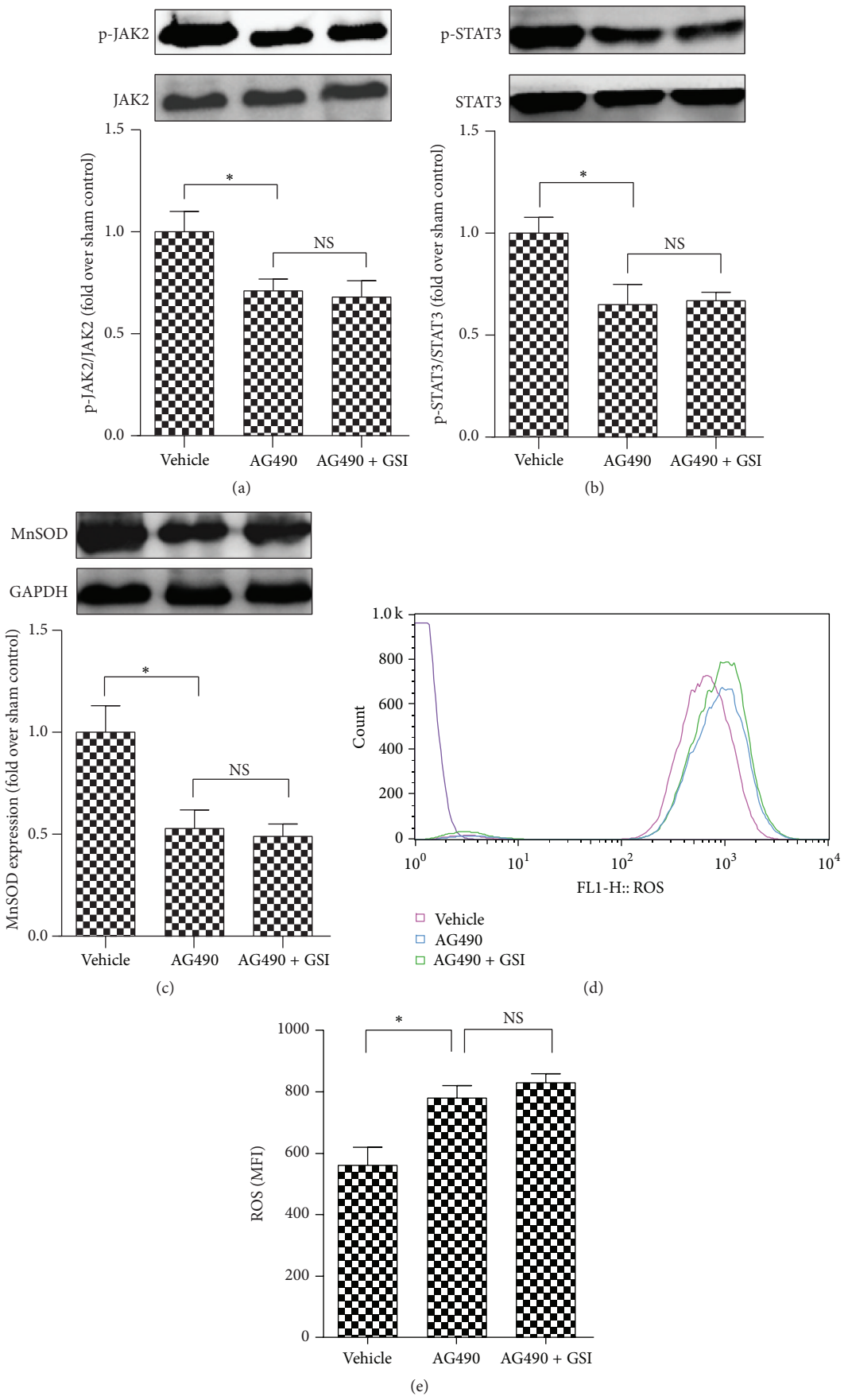
AG490 induced no additional effects. The expression of MnSOD had a consistent trend (Figure 7(c)). AG490 treatment also markedly increased oxidative stress and aggravated myocardial injury. Treatment with GSI plus AG490 induced no additional cardioprotective effects (Figures 7(d)–7(g)). These results demonstrated that the inhibition of JAK2/STAT3 signaling preferentially aggravated postburn myocardial injury, and JAK2/STAT3 signaling played a critical role in the cardioprotection of Notch1.

4. Discussion

This study produced the following major findings. First, genetic knockout or pharmacological inhibition of Notch1 significantly aggravated myocardial injury *in vitro* and *in vivo*. Second, we demonstrated for the first time that Notch signal blockade aggravated postburn myocardial injury via JAK2/STAT3 signaling, which downregulated MnSOD expression and increased ROS levels. These results indicate that endogenous Notch1 signaling is critical for burn-induced myocardial injury, and this signaling pathway may serve as a new therapeutic target.

Notch signaling is highly relevant for proper myocardial function and response to injury. Our previous study found that Notch1 knockdown significantly aggravated MI/R injury, as evidenced by an enlarged infarct size, depressed cardiac function, and increased myocardial apoptosis [13]. Activation of Notch1 by Jagged1 also attenuated MI/R injury. The present study examined the role of the Notch1 pathway in burn-induced myocardial injury for the first time and further confirmed the cardioprotective effects of the Notch1 pathway. These data are similar to the previous study in other experiments in which Notch signaling exerts protection in several pathophysiological conditions, including alcoholic cardiomyopathy [10], myocardial infarction [11], and cardiac hypertrophy [12].

ROS causes oxidative stress and acts as the major mediator of postburn myocardial injury [7, 20, 21]. Mounting evidence has demonstrated that the Notch1 pathway plays a key role in ROS production [22–24]. Our previous study also demonstrated that activation of Notch1 signaling inhibited ROS production in hepatic ischemia/reperfusion (I/R) injury and MI/R injury [14]. Our present data demonstrated that complete Notch deficiency increased burn-induced ROS production in Notch RBP-J knockout mice, which suggests



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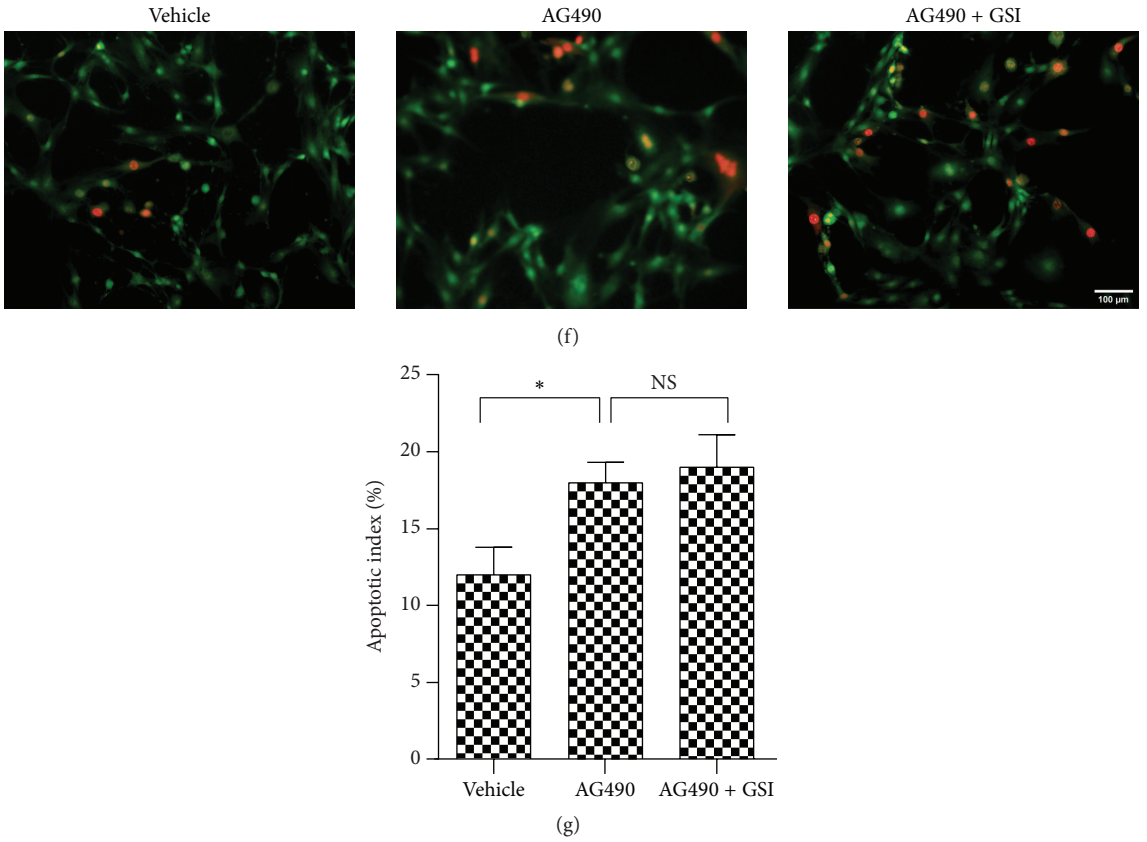

Figure 7: Inhibition of JAK2/STAT3 signaling preferentially aggravated myocardial injury after burn. Rat cardiomyocytes were challenged with burn serum *in vitro* in the presence of AG490, with or without GSI. JAK2/STAT3 (a, b) and MnSOD (c) expression was determined by western blot in cardiomyocytes. ROS were examined by way of FACS (d) and were quantified by way of mean fluorescence intensity (e). (f) Apoptotic cells were stained by AO/EB staining. (g) Quantitative comparison of apoptotic cells upon AO/EB staining in (f). The values presented are the mean ± SEM ($n = 8$ per group). $^{*}P < 0.05$. NS, not significant.

that the cardioprotective effects of the Notch1 pathway are involved in the regulation of ROS.

Oxidative stress is an imbalance between the production and elimination of ROS [25]. Under normal condition, the body has a potent antioxidant defense system, fighting against excessive generation of ROS. However, during some pathological conditions, these natural antioxidant defenses are damaged or the excessive ROS is generated, and oxidative stress occurs, leading to structural and functional injury [26]. The antioxidant defense system in living organism is complex. Among them, MnSOD is the most crucial enzyme in the cellular antioxidant system [27].

In the present study, our results reported here indicate that MnSOD, which is a critical ROS scavenger in mitochondria, was markedly downregulated after Notch deficiency. These observations suggest that a reduction in SOD activity is responsible for the suppression of ROS afforded by Notch deficiency in the postburn myocardium.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway controls multiple biological processes in metazoan development and tissue homoeostasis [28]. Four mammalian JAKs (JAK1, 2, 3, and Tyk2) and seven mammalian STATs (STAT1, 2, 3, 4, 5a, 5b, and 6) have been identified [29]. The JAK2/STAT3 signaling pathway is a highly evolutionarily conserved pathway that is involved in cell proliferation, differentiation, apoptosis, and inflammation [30–32]. Recent studies found that the JAK2/STAT3 signaling pathway plays an important role in myocardial I/R injury [33–35]. Certain cardioprotective agents, including hydrogen sulfide and fasudil, protect the myocardium against I/R injury via activation of the JAK2/STAT3 survival pathway [36, 37]. Mounting evidence has confirmed that the JAK2/STAT3 signaling plays a critical role in the regulation of oxidative stress responses [35, 38]. Similarly, our present study found that inhibition of JAK2/STAT3 signaling by AG490 markedly increased oxidative stress and aggravated postburn myocardial injury, which supports the critical role of JAK2/STAT3 signaling in burn-induced myocardial injury.

JAK2/STAT3 signaling regulates the transcription of MnSOD [14]. Our present data demonstrated that blockade of Notch signaling markedly attenuated p-JAK2 and p-STAT3 expression, which suggests that the inhibition of Notch1 signaling downregulates MnSOD transcription via decreased STAT3 activation and leads to increased ROS and aggravated I/R injury. Inhibition of JAK2/STAT3 signaling

also preferentially aggravated oxidative stress and postburn myocardial injury, and pretreatment with GSI had no effect. These results further support the notion that Notch signaling regulates oxidative stress and postburn myocardial injury via JAK2/STAT3 signaling. These data are similar to our previous study that demonstrated that canonical Notch signaling protects hepatocytes from I/R injury via the activation of JAK2/STAT3 signaling, which activates the expression of MnSOD and leads to ROS scavenging.

However, some limitations in this study need to be noted. The mechanism by which ROS is influenced after down-regulation of Notch1 is quite complex. The Notch1 pathway has been reported to enhance Akt activity in myocardium [39]. Results from our previous study and others showed that PI3K/Akt signaling played a critical role in burn-induced cardiomyocyte apoptosis [40–42]. Interestingly, several studies indicated that Akt signaling regulated ROS production in several models [43–45]. These facts suggest that PI3K-Akt signaling may play an important role in the regulation of oxidative stress by Notch1 during burn injury. Further study is needed to further investigate the possible mechanism of oxidative stress by Notch1.

In conclusion, we used genetic knockout and pharmacological inhibition of Notch1 to demonstrate novel roles of Notch1 signaling in burn-induced myocardial injury. Notch signaling also protects against burn-induced myocardial injury through JAK2/STAT3 signaling, which activates MnSOD expression and inhibits gp91phox expression and leads to decreased ROS levels. These findings suggest new therapeutic targets to limit burn-associated myocardial injury.

Conflict of Interests

The authors declare no competing financial interests.

Authors' Contribution

Weixia Cai, Xuekang Yang, Shichao Han, and Haitao Guo contributed equally to this work.

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References

[1] O. Kallinen, K. Maisniemi, T. Böhling, E. Tukiainen, and V. Koljonen, "Multiple organ failure as a cause of death in patients with severe burns," *Journal of Burn Care & Research*, vol. 33, no. 2, pp. 206–211, 2012.

[2] J. Rocha, M. Eduardo-Figueira, A. Barateiro et al., "Erythropoietin reduces acute lung injury and multiple organ failure/dysfunction associated to a scald-burn inflammatory injury in the rat," *Inflammation*, vol. 38, no. 1, pp. 312–326, 2015.

[3] J. W. Horton, N. M. Garcia, D. J. White, and J. Keffer, "Postburn cardiac contractile function and biochemical markers of postburn cardiac injury," *Journal of the American College of Surgeons*, vol. 181, no. 4, pp. 289–298, 1995.

[4] Y. Zhang, S.-J. Lv, H. Yan et al., "Effects of glycine supplementation on myocardial damage and cardiac function after severe burn," *Burns*, vol. 39, no. 4, pp. 729–735, 2013.

[5] X. Jiang, W. Liu, J. Deng et al., "Polydatin protects cardiac function against burn injury by inhibiting sarcoplasmic reticulum Ca^{2+} leak by reducing oxidative modification of ryanodine receptors," *Free Radical Biology and Medicine*, vol. 60, pp. 292–299, 2013.

[6] S. Liu, H. B. Ren, X. L. Chen et al., "Puerarin attenuates severe burn-induced acute myocardial injury in rats," *Burns*, 2015.

[7] D.-X. Zhang, H. Yan, J.-Y. Hu et al., "Identification of mitochondria translation elongation factor Tu as a contributor to oxidative damage of postburn myocardium," *Journal of Proteomics*, vol. 77, pp. 469–479, 2012.

[8] F. Geisler and M. Strazzabosco, "Emerging roles of Notch signaling in liver disease," *Hepatology*, vol. 61, no. 1, pp. 382–392, 2015.

[9] N. Gude, E. Joyo, H. Toko et al., "Notch activation enhances lineage commitment and protective signaling in cardiac progenitor cells," *Basic Research in Cardiology*, vol. 110, no. 3, article 29, 2015.

[10] T. Quillard and B. Charreau, "Impact of Notch signaling on inflammatory responses in cardiovascular disorders," *International Journal of Molecular Sciences*, vol. 14, no. 4, pp. 6863–6888, 2013.

[11] G. Felician, C. Collesi, M. Lusic et al., "Epigenetic modification at Notch responsive promoters blunts efficacy of inducing notch pathway reactivation after myocardial infarction," *Circulation Research*, vol. 115, no. 7, pp. 636–649, 2014.

[12] M. Nemir, M. Metrich, I. Plaisance et al., "The Notch pathway controls fibrotic and regenerative repair in the adult heart," *European Heart Journal*, vol. 35, no. 32, pp. 2174–2185, 2014.

[13] H. Pei, Q. Yu, Q. Xue et al., "Notch1 cardioprotection in myocardial ischemia/reperfusion involves reduction of oxidative/nitrative stress," *Basic Research in Cardiology*, vol. 108, no. 5, article 373, 2013.

[14] H.-C. Yu, H.-Y. Qin, F. He et al., "Canonical notch pathway protects hepatocytes from ischemia/reperfusion injury in mice by repressing reactive oxygen species production through JAK2/STAT3 signaling," *Hepatology*, vol. 54, no. 3, pp. 979–988, 2011.

[15] P. Wang, H. Du, C.-C. Zhou et al., "Intracellular NAMPT–NAD^+–SIRT1 cascade improves post-ischaemic vascular repair by modulating Notch signalling in endothelial progenitors," *Cardiovascular Research*, vol. 104, no. 3, pp. 477–488, 2014.

[16] X. Bai, L. Fan, T. He et al., "SIRT1 protects rat lung tissue against severe burn-induced remote ALI by attenuating the apoptosis of PMVECs via p38 MAPK signaling," *Scientific Reports*, vol. 5, article 10277, 2015.

[17] W.-F. Zhang, X.-X. Zhu, D.-H. Hu, C.-F. Xu, Y.-C. Wang, and G.-F. Lv, "Intensive insulin treatment attenuates burn-initiated acute lung injury in rats: role of the protective endothelium," *Journal of Burn Care & Research*, vol. 32, no. 3, pp. e51–e58, 2011.

[18] M. Krzyzaniak, G. Cheadle, C. Peterson et al., "Burn-induced acute lung injury requires a functional toll-like receptor 4," *Shock*, vol. 36, no. 1, pp. 24–29, 2011.

[19] Y. Liu, K. Lian, L. Zhang et al., "TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury," *Basic Research in Cardiology*, vol. 109, article 415, 2014.

[20] R. Xiao, M. Teng, Q. Zhang, X.-H. Shi, and Y.-S. Huang, "Myocardial autophagy after severe burn in rats," *PLoS ONE*, vol. 7, no. 6, Article ID e39488, 2012.

[21] X. Yao, J. G. Wigginton, D. L. Maass et al., "Estrogen-provided cardiac protection following burn trauma is mediated through a reduction in mitochondria-derived DAMPs," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 306, no. 6, pp. H882–H894, 2014.

[22] W. X. Cai, L. Liang, L. Wang et al., "Inhibition of Notch signaling leads to increased intracellular ROS by up-regulating Nox4 expression in primary HUVECs," *Cellular Immunology*, vol. 287, no. 2, pp. 129–135, 2014.

[23] C. Caliceti, P. Nigro, P. Rizzo, and R. Ferrari, "ROS, Notch, and Wnt signaling pathways: crosstalk between three major regulators of cardiovascular biology," *BioMed Research International*, vol. 2014, Article ID 318714, 8 pages, 2014.

[24] H.-C. Yu, L. Bai, S.-Q. Yue et al., "Notch signal protects non-parenchymal cells from ischemia/reperfusion injury in vitro by repressing ROS," *Annals of Hepatology*, vol. 12, no. 5, pp. 815–821, 2013.

[25] O. S. Kornfeld, S. Hwang, M. H. Disatnik, C. H. Chen, N. Qvit, and D. Mochly-Rosen, "Mitochondrial reactive oxygen species at the heart of the matter: new therapeutic approaches for cardiovascular diseases," *Circulation Research*, vol. 116, no. 11, pp. 1783–1799, 2015.

[26] H. Ha, J. Park, Y. S. Kim, and H. Endou, "Oxidative stress and chronic allograft nephropathy," *Yonsei Medical Journal*, vol. 45, no. 6, pp. 1049–1052, 2004.

[27] X. Wang, C. X. Hai, X. Liang, S. X. Yu, W. Zhang, and Y. L. Li, "The protective effects of *Acanthopanax senticosus* Harms aqueous extracts against oxidative stress: role of Nrf2 and antioxidant enzymes," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 424–432, 2010.

[28] K. Takeda, K. Noguchi, W. Shi et al., "Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 8, pp. 3801–3804, 1997.

[29] K. Boengler, D. Hilfiker-Kleiner, H. Drexler, G. Heusch, and R. Schulz, "The myocardial JAK/STAT pathway: from protection to failure," *Pharmacology and Therapeutics*, vol. 120, no. 2, pp. 172–185, 2008.

[30] S.-K. Park, M. K. Dahmer, and M. W. Quasney, "MAPK and JAK-STAT signaling pathways are involved in the oxidative stress-induced decrease in expression of surfactant protein genes," *Cellular Physiology and Biochemistry*, vol. 30, no. 2, pp. 334–346, 2012.

[31] A. M. Terrell, P. R. Crisostomo, G. M. Wairiuko, M. Wang, E. D. Morrell, and D. R. Meldrum, "JAK/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation and hypertrophy in the heart," *Shock*, vol. 26, no. 3, pp. 226–234, 2006.

[32] S.-H. Wen, Y. Li, C. Li et al., "Ischemic postconditioning during reperfusion attenuates intestinal injury and mucosal cell apoptosis by inhibiting JAK/STAT signaling activation," *Shock*, vol. 38, no. 4, pp. 411–419, 2012.

[33] S. Qiao, X. Mao, Y. Wang et al., "Remifentanil preconditioning reduces postischemic myocardial infarction and improves left ventricular performance via activation of the janus activated kinase-2/signal transducers and activators of transcription-3 signal pathway and subsequent inhibition of glycogen synthase kinase-3β in rats," *Critical Care Medicine*, 2015.

[34] L. Wu, J. L. Tan, Z. H. Wang et al., "ROS generated during early reperfusion contribute to intermittent hypobaric hypoxia-afforded cardioprotection against postischemia-induced Ca^{2+} overload and contractile dysfunction via the JAK2/STAT3 pathway," *Journal of Molecular and Cellular Cardiology*, vol. 81, pp. 150–161, 2015.

[35] Y. Yang, W. Duan, Z. Jin et al., "JAK2/STAT3 activation by melatonin attenuates the mitochondrial oxidative damage induced by myocardial ischemia/reperfusion injury," *Journal of Pineal Research*, vol. 55, no. 3, pp. 275–286, 2013.

[36] Y. Li, W. Zhu, J. Tao et al., "Fasudil protects the heart against ischemia-reperfusion injury by attenuating endoplasmic reticulum stress and modulating SERCA activity: the differential role for PI3K/Akt and JAK2/STAT3 signaling pathways," *PLoS ONE*, vol. 7, no. 10, Article ID e48115, 2012.

[37] H. F. Luan, Z. B. Zhao, Q. H. Zhao, P. Zhu, M. Y. Xiu, and Y. Ji, "Hydrogen sulfide postconditioning protects isolated rat hearts against ischemia and reperfusion injury mediated by the JAK2/STAT3 survival pathway," *Brazilian Journal of Medical and Biological Research*, vol. 45, no. 10, pp. 898–905, 2012.

[38] W. Duan, Y. Yang, W. Yi et al., "New role of JAK2/STAT3 signaling in endothelial cell oxidative stress injury and protective effect of melatonin," *PLoS ONE*, vol. 8, no. 3, Article ID e57941, 2013.

[39] N. A. Gude, G. Emmanuel, W. Wu et al., "Activation of Notch-mediated protective signaling in the myocardium," *Circulation Research*, vol. 102, no. 9, pp. 1025–1035, 2008.

[40] W. Cao, Y.-H. Xie, X.-Q. Li et al., "Burn-induced apoptosis of cardiomyocytes is survivin dependent and regulated by PI3K/Akt, p38 MAPK and ERK pathways," *Basic Research in Cardiology*, vol. 106, no. 6, pp. 1207–1220, 2011.

[41] X. Lu, T. Costantini, N. E. Lopez et al., "Vagal nerve stimulation protects cardiac injury by attenuating mitochondrial dysfunction in a murine burn injury model," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 5, pp. 664–671, 2013.

[42] G. F. Lv, M. L. Dong, D. H. Hu et al., "Insulin-mediated inhibition of p38 mitogen-activated protein kinase protects cardiomyocytes in severe burns," *Journal of Burn Care & Research*, vol. 32, no. 6, pp. 591–599, 2011.

[43] G. Chang, D. Zhang, H. Yu et al., "Cardioprotective effects of exenatide against oxidative stress-induced injury," *International Journal of Molecular Medicine*, vol. 32, no. 5, pp. 1011–1020, 2013.

[44] A. Rotte, V. Pasham, W. Yang et al., "Phosphoinositide 3-kinase-dependent regulation of Na^+/H^+ exchanger in dendritic cells," *Pflügers Archiv—European Journal of Physiology*, vol. 460, no. 6, pp. 1087–1096, 2010.

[45] A. M. Yeves, M. C. Villa-Abrille, N. G. Pérez, A. J. Medina, E. M. Escudero, and I. L. Ennis, "Physiological cardiac hypertrophy: critical role of AKT in the prevention of NHE-1 hyperactivity," *Journal of Molecular and Cellular Cardiology*, vol. 76, pp. 186–195, 2014.